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

(First Edition)

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FOREWORD

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

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

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

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

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

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

PREFACE

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

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

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

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

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

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

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

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

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

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

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

ABSTRACT

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

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

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

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SECTION 1

INTRODUCTION

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

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

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

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

1.5 Guidance for the implementation of toxicity tests in the NPDES program is provided in the Technical Support Document for Water Quality-Based Toxics Control (USEPA, l991a).

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

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

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

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

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

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

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

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

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

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

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

SECTION 2

SHORT-TERM METHODS FOR ESTIMATING CHRONIC TOXICITY

2.1 INTRODUCTION

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

2.2 **TYPES OF TESTS**

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

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

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

2.2.4 Receiving (ambient) water toxicity tests commonly employ two treatments, a control and the undiluted receiving water, but may also consist of a series of receiving water dilutions.

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

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

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

2.3 **STATIC TESTS**

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

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

2.4 **ADVANTAGES AND DISADVANTAGES OF TOXICITY TEST TYPES**

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

Advantages:

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

Disadvantages:

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

2.4.2 STATIC RENEWAL, SHORT-TERM TOXICITY TESTS:

Advantages:

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

Disadvantages:

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

SECTION 3

HEALTH AND SAFETY

3.1 GENERAL PRECAUTIONS

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

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

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

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

3.2 SAFETY EQUIPMENT

3.2.1 PERSONAL SAFETY GEAR

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

3.2.2 LABORATORY SAFETY EQUIPMENT

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

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

3.3 **GENERAL LABORATORY AND FIELD OPERATIONS**

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

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

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

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

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

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

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

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

3.4 **DISEASE PREVENTION**

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

3.5 **SAFETY MANUALS**

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

3.6 **WASTE DISPOSAL**

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

SECTION 4

QUALITY ASSURANCE

4.1 INTRODUCTION

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

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

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

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

4.2 FACILITIES, EQUIPMENT, AND TEST CHAMBERS

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

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

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

4.3 **TEST ORGANISMS**

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

4.4 **LABORATORY WATER USED FOR CULTURING AND TEST DILUTION WATER**

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

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

4.5 **EFFLUENT AND RECEIVING WATER SAMPLING AND HANDLING**

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

4.6 **TEST CONDITIONS**

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

4.7 **QUALITY OF TEST ORGANISMS**

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

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

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

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

4.8 **FOOD QUALITY**

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

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

4.8.3 New batches of food used in culturing and testing should be analyzed for toxic organics and metals or whenever difficulty is encountered in meeting minimum acceptability criteria for control survival, reproduction, development or growth. If the concentration of total organochlorine pesticides exceeds 0.15 µg/g wet weight, or the concentration of total organochlorine pesticides plus PCBs exceeds 0.30 µg/g wet weight, or toxic metals (Al, As, C, Co, Cu, Pb, Ni, Zn, expressed as total metal) exceed 20 µg/g wet weight, the food should not be used (for analytical methods, see AOAC, 1990; and USDA, 1989). For foods (e.g., YCT) which are used to culture and test organisms, the quality of the food should meet the requirements for the laboratory water used for culturing and test dilution water as described in Section 4.4 above.

4.9 **ACCEPTABILITY OF CHRONIC TOXICITY TESTS**

4.9.1 Each test method contain specific test acceptability criteria defining minimum acceptable control performance for each endpoint (e.g., the mean larval development must be at least 80% in the controls), statistical resolution (e.g., minimum significant difference), and test conditions (e.g., salinity 34 ± 2‰). If these criteria are not met, the test must be repeated. Test acceptability criteria are used to demonstrate the sensitivity of the test organisms and the laboratory performance with a routinue reference toxicant.

4.9.2 An individual test may be conditionally acceptable if temperature, DO, and other specified conditions fall outside specifications, depending on the degree of the departure and the objectives of the tests (see test conditions and test acceptability criteria summaries). The acceptability of the test will depend on the experience and professional judgment of the laboratory investigator and the reviewing staff of the regulatory authority. Any deviation from test specifications must be noted when reporting data from a test.

4.10 **ANALYTICAL METHODS**

4.10.1 Routine chemical and physical analyses for culture and dilution water, food, and test solutions must include established quality assurance practices outlined in USEPA methods manuals (USEPA, 1979a and USEPA, 1979b).

4.10.2 Reagent containers should be dated and catalogued when received from the supplier, and the shelf life should not be exceeded. Also, working solutions should be dated when prepared, and the recommended shelf life should be observed.

4.11 **CALIBRATION AND STANDARDIZATION**

4.11.1 Instruments used for routine measurements of chemical and physical parameters, such as pH, DO, temperature, conductivity, and salinity, must be calibrated and standardized according to instrument manufacturers procedures as indicated in the general section on quality assurance (see USEPA Methods 150.1, 360.1, 170.1, and 120.1 in USEPA, 1979b). Calibration data are recorded in a permanent log book.

4.11.2 Wet chemical methods used to measure hardness, alkalinity, and total residual chlorine, must be standardized prior to use each day according to the procedures for those specific USEPA methods (see USEPA Methods 130.2 and 310.1 in USEPA, 1979b).

4.12 **REPLICATION AND TEST SENSITIVITY**

4.12.1 The sensitivity of the tests will depend in part on the number of replicates per concentration, the significance level selected, and the type of statistical analysis. If the variability remains constant, the sensitivity of the test will increase as the number of replicates is increased. The minimum recommended number of replicates varies with the objectives of the test and the statistical method used for analysis of the data.

4.13 **VARIABILITY IN TOXICITY TEST RESULTS**

4.13.1 Factors which can affect test success and precision include: (1) the experience and skill of the laboratory analyst; (2) test organism age, condition, and sensitivity; (3) dilution water quality; (4) temperature control; (5) and the quality and quantity of food provided. The results will depend upon the species used and the strain or source of the test organisms, and test conditions, such as temperature, DO, food, and water quality. The repeatability or precision of toxicity tests is also a function of the number of test organisms used at each toxicant concentration. Jensen (1972) discussed the relationship between sample size (number of fish) and the standard error of the test, and considered 20 fish per concentration as optimum for Probit Analysis.

4.14 **TEST PRECISION**

4.14.1 The ability of the laboratory personnel to obtain consistent, precise results must be demonstrated with reference toxicants before they attempt to measure effluent toxicity. The single-laboratory precision of each type of test to be used in a laboratory should be determined by performing at least five or more tests with a reference toxicant.

4.14.2 Test precision can be estimated by using the same strain of organisms under the same test conditions, and employing a known toxicant, such as a reference toxicant.

4.14.3 Precision data for each of the tests described in this manual are presented in the sections describing the individual test methods.

4.14.4 Additional information on toxicity test precision is provided in the Technical Support Document for Water Quality-based Toxic Control (see pp. 2-4, and pp. 11-15 in USEPA, 1991a).

4.14.5 In cases where the test data are used in Probit Analysis or other point estimation techniques (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis), precision can be described by the mean, standard deviation, and relative standard deviation (percent coefficient of variation, or CV) of the calculated endpoints from the replicated tests. In cases where the test data are used in the Linear Interpolation Method, precision can be estimated by empirical confidence intervals derived by using the ICPIN Method (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). However, in cases where the results are reported in terms of the No-Observed-Effect-Concentration (NOEC) and Lowest-Observed-Effect-Concentration (LOEC) (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis), precision can only be described by listing the NOEC-LOEC interval for each test. It is not possible to express precision in terms of a commonly used statistic. However, when all tests of the same toxicant yield the same NOEC-LOEC interval, maximum precision has been attained. The "true" no effect concentration could fall anywhere within the interval, NOEC ± (LOEC minus NOEC).

4.14.6 It should be noted here that the dilution factor selected for a test determines the width of the NOEC-LOEC interval and the inherent maximum precision of the test. As the absolute value of the dilution factor decreases, the width of the NOEC-LOEC interval increases, and the inherent maximum precision of the test decreases. When a dilution factor of 0.3 is used, the NOEC

could be considered to have a relative uncertainty as high as ± 300%. With a dilution factor of 0.5, the NOEC could be considered to have a relative variability of ± 100%. As a result of the variability of different dilution factors, **USEPA recommends the use of a $0.5 dilution factor**. Other factors which can affect test precision include: test organism age, condition, and sensitivity; temperature control; and feeding.

4.15 DEMONSTRATING ACCEPTABLE LABORATORY PERFORMANCE

4.15.1 It is a laboratory's responsibility to demonstrate its ability to obtain consistent, precise results with reference toxicants before it performs toxicity tests with effluents for permit compliance purposes. To meet this requirement, the intralaboratory precision, expressed as percent coefficient of variation (CV%), of each type of test to be used in a laboratory should be determined by performing five or more tests with different batches of test organisms, using the same reference toxicant, at the same concentrations, with the same test conditions (i.e., the same test duration, type of dilution water, age of test organisms, feeding, etc.), and same data analysis methods. A reference toxicant concentration series (0.5 or higher) should be selected that will consistently provide partial mortalities at two or more concentrations.

4.16 DOCUMENTING ONGOING LABORATORY PERFORMANCE

4.16.1 Satisfactory laboratory performance is demonstrated by performing at least one acceptable test per month with a reference toxicant for each toxicity test method commonly used in the laboratory. For a given test method, successive tests must be performed with the same reference toxicant, at the same concentrations, in the same dilution water, using the same data analysis methods. Precision may vary with the test species, reference toxicant, and type of test.

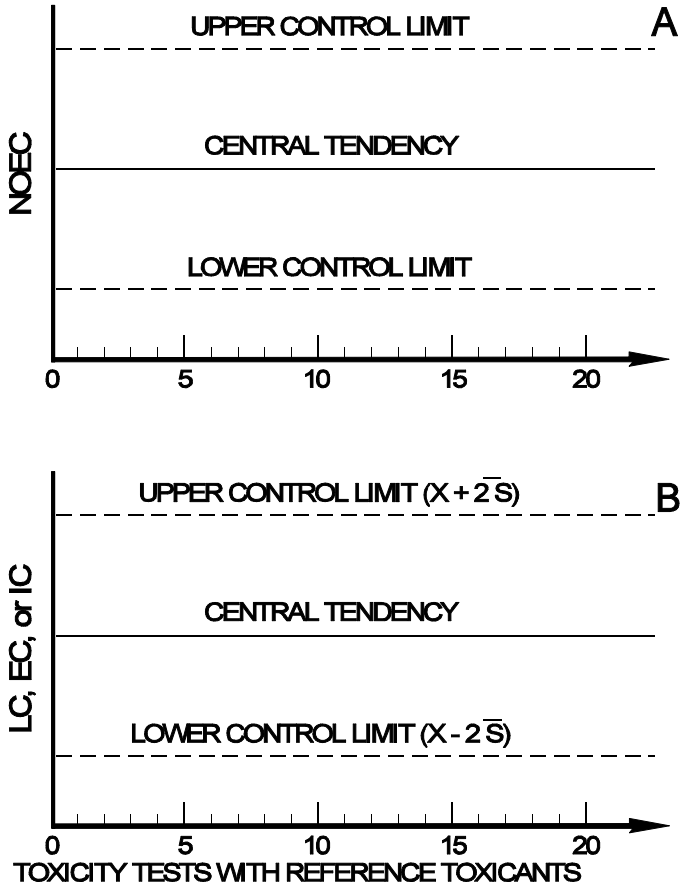
4.16.2 A control chart should be prepared for each combination of reference toxicant, test species, test conditions, and endpoints. Toxicity endpoints from five or six tests are adequate for establishing the control charts. Successive toxicity endpoints (NOECs, IC25s, LC50s, etc.) should be plotted and examined to determine if the results (X_1) are within prescribed limits (Figure 1). The types of control charts illustrated (see USEPA, 1979a) are used to evaluate the cumulative trend of results from a series of samples. For endpoints that are point estimates (LC50s and IC25s), the cumulative mean (X) and upper and lower control limits (±2S) are re-calculated with each successive test result. Endpoints from

hypothesis tests (NOEC, NOAEC) from each test are plotted directly on the control chart. The control limits would consist of one concentration interval above and below the concentration representing the central tendency. After two years of data collection, or a minimum of 20 data points, the control (cusum) chart should be maintained using only the 20 most recent data points.

4.16.3 The outliers, which are values falling outside the upper and lower control limits, and trends of increasing or decreasing sensitivity, are readily identified. In the case of endpoints that are point estimates (LC50s and IC25s), at the P = 0.05 probability level, one in 20 tests would be expected to fall outside of the control limits by chance alone. If more than one out of 20 reference toxicant tests fall outside the control limits, the effluent toxicity tests conducted during the month in which the second reference toxicant test failed are suspect, and should be considered as provisional and subject to careful review. Control limits for the NOECs will also be exceeded occasionally, regardless of how well a laboratory performs.

4.16.4 If the toxicity value from a given test with a reference toxicant fall <u>well</u> outside the expected range for the test organisms when using the standard dilution water and other test conditions, the sensitivity of the organisms and the overall credibility of the test system are suspect. In this case, the test procedure should be examined for defects and should be repeated with a different batch of test organisms.

4.16.5 Performance should improve with experience, and the control limits for endpoints that are point estimates should gradually narrow. However, control limits of ±2S will be exceeded 5% of the time by chance alone, regardless of how well a laboratory performs. Highly proficient laboratories which develop very narrow control limits may be unfairly penalized if a test result which falls just outside the control limits is rejected ***de facto***. For this reason, the width of the control limits should be considered by the permitting authority in determining whether the outliers should be rejected.



$$\bar{X} = \frac{\sum_{i=1}^{n} X_i}{n}$$

$$S = \sqrt{\frac{\sum_{i=1}^{n} X_i^2 - \frac{\left(\sum_{i=1}^{n} X_i\right)^2}{n}}{n-1}}$$

Where: X_i = Successive toxicity values from toxicity tests.
n = Number of tests.
$\bar{X}$ = Mean toxicity value.
S = Standard deviation.

Figure 1. Control (cusum) charts. (A) hypothesis testing results; (B) point estimates (LC, EC, or IC).

4.17 **REFERENCE TOXICANTS**

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

4.18 **RECORD KEEPING**

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

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

SECTION 5

FACILITIES, EQUIPMENT, AND SUPPLIES

5.1 GENERAL REQUIREMENTS

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

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

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

adsorbed toxicants from one test to another, if reused. However, these containers may be repeatedly reused for storing uncontaminated waters such as deionized or laboratory-prepared dilution waters and receiving waters. Glass or disposable polystyrene containers can be used as test chambers. The use of large ($ 20 L) glass carboys is discouraged for safety reasons.

5.1.4 New plastic products of a type not previously used should be tested for toxicity before initial use by exposing the test organisms in the test system where the material is used. Equipment (pumps, valves, etc.) which cannot be discarded after each use because of cost, must be decontaminated according to the cleaning procedures listed below (see Section 5, Facilities, Equipment, and Supplies, Subsection 5.3.2). Fiberglass, in addition to the previously mentioned materials, can be used for holding, acclimating, and dilution water storage tanks, and in the water delivery system, but once contaminated with pollutants the fiberglass should not be reused. All material should be flushed or rinsed thoroughly with the test media before using in the test.

5.1.5 Copper, galvanized material, rubber, brass, and lead must not come in contact with culturing, holding, acclimation, or dilution water, or with effluent samples and test solutions. Some materials, such as several types of neoprene rubber (commonly used for stoppers) may be toxic and should be tested before use.

5.1.6 Silicone adhesive used to construct glass test chambers absorbs some organochlorine and organophosphorus pesticides, which are difficult to remove. Therefore, as little of the adhesive as possible should be in contact with water. Extra beads of adhesive inside the containers should be removed.

5.2 **TEST CHAMBERS**

5.2.1 Test chamber size and shape are varied according to size of the test organism. Requirements are specified in each toxicity test method.

5.3 **CLEANING TEST CHAMBERS AND LABORATORY APPARATUS**

5.3.1 New plasticware used for sample collection or organism exposure vessels generally does not require thorough cleaning before use. It is sufficient to rinse new sample containers once with dilution water before use. New, disposable, plastic test chambers may have to be rinsed with dilution water before use. New glassware must be soaked overnight in 10% acid (see below) and also be rinsed well in deionized water and seawater.

5.3.2 All non-disposable sample containers, test vessels, pumps, tanks, and other equipment that has come in contact with effluent must be washed after use to remove surface contaminants, as described below.

1. Soak 15 minutes in tap water and scrub with detergent, or clean in an automatic dishwasher.
2. Rinse twice with tap water.
3. Carefully rinse once with fresh dilute (10% V:V) hydrochloric acid or nitric acid to remove scale, metals and bases. To prepare a 10% solution of acid, add 10 mL of concentrated acid to 90 mL of deionized water.
4. Rinse twice with deionized water.
5. Rinse once with full-strength, pesticide-grade acetone to remove organic compounds (use a fume hood or canopy).
6. Rinse three times with deionized water.

5.3.3 All test chambers and equipment must be thoroughly rinsed with the dilution water immediately prior to use in each test.

5.4 **APPARATUS AND EQUIPMENT FOR CULTURING AND TOXICITY TESTS**

5.4.1 Apparatus and equipment requirements for culturing and toxicity tests are specified in each toxicity test method. Also, see USEPA, 1993a.

5.4.2 WATER PURIFICATION SYSTEM

5.4.2.1 A good quality deionized water, providing 18 mega-ohm, laboratory grade water, should be available in the laboratory and with sufficient capacity for laboratory needs. Deionized water may be obtained from MILLIPORE® MILLI-Q®, MILLIPORE® QPAK™$_2$ or equivalent system. If large quantities of high quality deionized water are needed, it may be advisable to supply the laboratory grade water deionizer with preconditioned water from a Culligen®, Continental®, or equivalent.

5.5 **REAGENTS AND CONSUMABLE MATERIALS**

5.5.1 SOURCES OF FOOD FOR CULTURE AND TOXICITY TESTS

1. Brine Shrimp, ***Artemia*** sp. cysts -- A list of commercial sources is provided in Table 1.

TABLE 1. COMMERCIAL SUPPLIERS OF BRINE SHRIMP(*ARTEMIA*) CYSTS[1,2]

Aquafauna Biomarine
P.O. Box 5
Hawthorne, CA 90250
Tel. (213) 973-5275
Fax. (213) 676-9387
(Great Salt Lake North Arm, San Francisco Bay)

Aquarium Products
180L Penrod Court
Glen Burnie, MD 21061
Tel. (800) 368-2507
Tel. (301) 761-2100
(Columbia)

Argent Chemical
8702 152nd Ave. NE
Redmond, WA 98052
Tel. (800) 426-6258
Tel. (206) 855-3777
Fax. (206) 885-2112
(Platinum Label - San Francisco Bay; Label - San Francisco Bay, Gold Brazil; Silver Label - Great Australia; Bronze Label - China, Canada, other)

Artemia Systems
Wiedauwkaai 79
B-9000 Ghent, Belgium
Tel. 011-32-91-534142
Fax. 011-32-91-536893
(For marine species - AF grade)[small nauplii], UL grade [large nauplii], for freshwater species Salt Lake, -HI grade [small nauplii], EG [large nauplii]

Bonneville Artemia International, Inc.
P.O. Box 511113
Salt Lake City, UT 84151-1113
Tel. (801) 972-4704
Fax. (801) 972-4795

Golden West Artemia
411 East 100 South
Salt Lake City, UT 84111
Tel. (801) 532-1400
Fax. (801) 531-8160

Ocean Star International
P.O. Box 643
Snowville, UT 84336
Tel. (801) 872-8217
Fax (801) 872-8272
(Great Salt Lake)

Pennsylvania Pet Products
Box 191
Spring City, PA 19475
Tel. Not listed.
(Great Salt Lake)

Sanders Brine Shrimp Co.
3850 South 540 West
Ogden, UT 84405
Tel. (801) 393-5027
(Great Salt Lake)

San Francisco Bay Brand
8239 Enterprise Drive
Newark, CA 94560
Tel. (415) 792-7200
(Great Salt Lake, San Francisco Bay)

Sea Critters Inc.
P.O. Box 1508
Tavernier, FL 33070
Tel. (305) 367-2672

Western Brine Shrimp
957 West South Temple
Salt Lake City, UT 84104
Tel. (801) 364-3642
Fax. (801) 534-0211
(Great Salt Lake)

[1]List from David A. Bengtson, University of Rhode Island, Narragansett, RI.

[2]The geographic sources from which the vendors obtain the brine shrimp cysts are shown in parentheses.

2. Feeding requirements and other specific foods are indicated in the specific toxicity test method.

5.5.1.1 All food should be tested for nutritional suitability and chemically analyzed for organochlorine pesticides, PCBs, and toxic metals (see Section 4, Quality Assurance).

5.5.2 Reagents and consumable materials are specified in each toxicity test method. Also, see Section 4, Quality Assurance.

5.6 **TEST ORGANISMS**

5.6.1 Test organisms are obtained from inhouse cultures or commercial suppliers (see specific toxicity test method; Sections 4, Quality Assurance and 6, Test Organisms).

5.7 **SUPPLIES**

5.7.1 See toxicity test methods (see Sections 11-16) for specific supplies.

SECTION 6

TEST ORGANISMS

6.1 TEST SPECIES

6.1.1 The species used in characterizing the chronic toxicity of effluents and/or receiving waters will depend on the requirements of the regulatory authority and the objectives of the test. It is desirable that good quality test organisms be readily available throughout the year from inhouse or commercial sources to meet NPDES monitoring requirements. The organisms used in toxicity tests must be identified to species. If there is any doubt as to the identity of the test organisms, representative specimens should be sent to a taxonomic expert to confirm the identification.

6.1.2 Toxicity test conditions and culture methods for the species listed in Subsection 6.1.3 are provided in this manual (also, see USEPA, 1993c).

6.1.3 The organisms used in the short-term tests described in this manual are the topsmelt, ***Atherinops affinis***, the red abalone, ***Haliotis rufescens***; the Pacific oyster, ***Crassostrea gigas*** and mussel, ***Mytilus spp.***; the mysid, ***Holmesimysis costata***; the sea urchin, ***Strongylocentrotus purpuratus*** and the sand dollar, ***Dendraster excentricus***; and the giant kelp, ***Macrocystis pyrifera***.

6.1.4 Some states have developed culturing and testing methods for indigenous species that may be as sensitive or more sensitive, than the species recommended in Subsection 6.1.3. However, USEPA allows the use of indigenous species only where state regulations require their use or prohibit importation of the species in Section 6, Facilities, Equipment, and Supplies, Subsection 6.1.3. Where state regulations prohibit importation of non-native fishes or use of the recommended test species, permission must be requested from the appropriate state agency prior to their use.

6.1.5 Where states have developed culturing and testing methods for indigenous species other than those recommended in this manual, data comparing the sensitivity of the substitute species and one or more of the recommended species must be obtained in side-by-side toxicity tests with reference toxicants and/or effluents to ensure that the species selected are at least as sensitive as the recommended species. These data must be submitted to the permitting authority (State or Region) if required. USEPA acknowledges that reference toxicants prepared from pure chemicals may not always be representative of

effluents. However, because of the observed and/or potential variability in the quality and toxicity of effluents, it is not possible to specify a representative effluent.

6.1.6 Guidance for the selection of test organisms where the salinity of the effluent and/or receiving water requires special consideration is provided in the Technical Support Document for Water Quality-based Toxics Control (USEPA, 1991a).

1. Where the salinity of the receiving water is < 1‰, freshwater organisms are used regardless of the salinity of the effluent.
2. Where the salinity of the receiving water is $ 1‰, the choice of organisms depends on state water quality standards and/or permit requirements.

6.2 SOURCES OF TEST ORGANISMS

6.2.1 Some of the test organisms recommended in this manual can be obtained from broodstock cultured in the laboratory using culturing and handling methods for each organism described in the respective test method sections.

6.2.2 Inhouse broodstock cultures should be established wherever it is cost effective. If inhouse cultures cannot be maintained or it is not cost effective, test organisms should be purchased from experienced commercial suppliers.

6.2.3 Red abalone, oyster, mussels, topsmelt, mysids, sea urchins, sand dollars, and giant kelp blades may be purchased from commercial suppliers. However, some of these organisms (e.g., adult mysids or adult topsmelt) may not always be available from commercial suppliers and may have to be collected in the field and brought back to the laboratory for release of larvae or mysids.

6.2.4 If, because of their source, there is any uncertainty concerning the identity of the organisms, it is advisable to have them examined by a taxonomic specialist to confirm their identification. For guidance on identification, see the individual toxicity test methods.

6.2.5 FERAL (NATURAL OCCURRING, WILD CAUGHT) ORGANISMS

6.2.5.1 The use of test species taken from the receiving water has strong appeal, and would seem to be the logical approach. However, it is generally impractical and not recommended for the following reasons:

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

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

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

6.3 **LIFE STAGE**

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

6.4 **LABORATORY CULTURING**

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

6.5 **HOLDING AND HANDLING TEST ORGANISMS**

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

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

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

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

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

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

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

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

6.6 **TRANSPORTATION TO THE TEST SITE**

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

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

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

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

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

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

6.7 **TEST ORGANISM DISPOSAL**

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

SECTION 7

DILUTION WATER

7.1 TYPES OF DILUTION WATER

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

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

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

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

7.2 STANDARD, SYNTHETIC DILUTION WATER

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

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

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

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

7.2.3 STANDARD, SYNTHETIC SEAWATER

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

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

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

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

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

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

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

7.3 **USE OF RECEIVING WATER AS DILUTION WATER**

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

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

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

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

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

7.3.5 HYPERSALINE BRINE

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

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

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

7.3.5.4 Freeze Preparation of Brine

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

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

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

7.3.5.5 Heat Preparation of Brine

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

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

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

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

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

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

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

7.4 **USE OF TAP WATER AS DILUTION WATER**

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

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

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

7.5 **DILUTION WATER HOLDING**

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

SECTION 8

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

8.1 EFFLUENT SAMPLING

8.1.1 The effluent sampling point should be the same as that specified in the NPDES discharge permit (USEPA, l988b). Conditions for exception would be: (l) better access to a sampling point between the final treatment and the discharge outfall; (2) if the processed waste is chlorinated prior to discharge, it may also be desirable to take samples prior to contact with the chlorine to determine toxicity of the unchlorinated effluent; or (3) in the event there is a desire to evaluate the toxicity of the influent to municipal waste treatment plants or separate wastewater streams in industrial facilities prior to their being combined with other wastewater streams or non-contact cooling water, additional sampling points may be chosen.

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

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

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

8.2 EFFLUENT SAMPLE TYPES

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

8.2.1.1 GRAB SAMPLES

Advantages:

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

Disadvantages:

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

8.2.1.2 COMPOSITE SAMPLES:

Advantages:

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

Disadvantages:

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

8.3 **EFFLUENT SAMPLING RECOMMENDATIONS**

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

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

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

8.3.4 THE FOLLOWING EFFLUENT SAMPLING METHODS ARE RECOMMENDED:

8.3.4.1 Continuous Discharges

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

8.3.4.2 Intermittent Discharges

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

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

8.4 **RECEIVING WATER SAMPLING**

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

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

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

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

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

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

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

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

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

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

8.5.6 SAMPLES USED IN ON-SITE TESTS

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

8.5.7 SAMPLES SHIPPED TO OFF SITE FACILITIES

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

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

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

8.6 **SAMPLE RECEIVING**

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

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

8.7 **PERSISTENCE OF EFFLUENT TOXICITY DURING SAMPLE SHIPMENT AND HOLDING**

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

8.8 **PREPARATION OF EFFLUENT AND RECEIVING WATER SAMPLES FOR TOXICITY TESTS**

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

8.9 **PRELIMINARY TOXICITY RANGE-FINDING TESTS**

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

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

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

8.10 **MULTICONCENTRATION (DEFINITIVE) EFFLUENT TOXICITY TESTS**

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

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

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

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

8.11 **RECEIVING WATER TESTS**

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

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

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

SECTION 9

CHRONIC TOXICITY TEST ENDPOINTS AND DATA ANALYSIS

9.1 **ENDPOINTS**

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

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

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

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

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

the observable effect is death or immobility, the term, Lethal Concentration (LC), should be used (see Subsection 9.1.1.5). A certain EC or LC value might be judged from a biological standpoint to represent a threshold concentration, or lowest concentration that would cause an adverse effect on the observed response.

9.1.1.5 Lethal Concentration (LC) - The toxicant concentration that would cause death in a given percent of the test population. Identical to EC when the observable adverse effect is death. For example, the LC50 is the concentration of toxicant that would cause death in 50% of the test population.

9.1.1.6 Inhibition Concentration (IC) - The toxicant concentration that would cause a given percent reduction in a nonquantal biological measurement for the test population. For example, the IC25 is the concentration of toxicant that would cause a 25% reduction in growth for the test population, and the IC50 is the concentration of toxicant that would cause a 50% reduction.

9.2 **RELATIONSHIP BETWEEN ENDPOINTS DETERMINED BY HYPOTHESIS TESTING AND POINT ESTIMATION TECHNIQUES**

9.2.1 If the objective of chronic aquatic toxicity tests with effluents and pure compounds is to estimate the highest "safe or no-effect concentration" ofthese substances, it is imperative to understand how the statistical endpoints of these tests are related to the "safe" or "no-effect" concentration. NOECs and LOECs are determined by hypothesis testing (Dunnett's Test, a t test with the Bonferroni adjustment, Steel's Many-One Rank Test, or the Wilcoxon Rank Sum Test), whereas LCs, ICs, and ECs are determined by point estimation techniques (Probit Analysis, the Spearman-Karber Method, the Trimmed Spearman-Karber Method, the Graphical Method or Linear Interpolation Method). There are inherent differences between the use of a NOEC or LOEC derived from hypothesis testing to estimate a "safe" concentration, and the use of a LC, IC, EC, or other point estimates derived from curve fitting, interpolation, etc.

9.2.2 Most point estimates, such as the LC, IC, or EC are derived from a mathematical model that assumes a continuous dose-response relationship. By definition, any LC, IC, or EC value is an estimate of some amount of adverse effect. Thus the assessment of a "safe" concentration must be made from a biological standpoint rather than with a statistical test. In this instance, the biologist must determine some amount of adverse effect that is deemed to be "safe," in the sense that from a practical biological viewpoint it will not affect the normal propagation of fish and other aquatic life in receiving waters.

9.2.3 The use of NOECs and LOECs, on the other hand, assumes either (1) a continuous dose-response relationship, or (2) a non-continuous (threshold) model of the dose-response relationship.

9.2.3.1 In the case of a continuous dose-response relationship, it is also assumed that adverse effects that are not "statistically observable" are also not important from a biological standpoint, since they are not pronounced enough to test as statistically significant against some measure of the natural variability of the responses.

9.2.3.2 In the case of non-continuous dose-response relationships, it is assumed that there exists a true threshold, or concentration below which there is no adverse effect on aquatic life, and above which there is an adverse effect. The purpose of the statistical analysis in this case is to estimate as closely as possible where that threshold lies.

9.2.3.3 In either case, it is important to realize that the amount of adverse effect that is statistically observable (LOEC) or not observable (NOEC) is highly dependent on all aspects of the experimental design, such as the number of concentrations of toxicant, number of replicates per concentration, number of organisms per replicate, and use of randomization. Other factors that affect the sensitivity of the test include the choice of statistical analysis, the choice of an alpha level, and the amount of variability between responses at a given concentration.

9.2.3.4 Where the assumption of a continuous dose-response relationship is made, by definition some amount of adverse effect might be present at the NOEC, but is not great enough to be detected by hypothesis testing.

9.2.3.5 Where the assumption of a noncontinuous dose-response relationship is made, the NOEC would indeed be an estimate of a "safe" or "no-effect" concentration if the amount of adverse effect that appears at the threshold is great enough to test as statistically significantly different from the controls in the face of all aspects of the experimental design mentioned above. If, however, the amount of adverse effect at the threshold were not great enough to test as statistically different, some amount of adverse effect might be present at the NOEC. In any case, the estimate of the NOEC with hypothesis testing is always dependent on the aspects of the experimental design mentioned above. For this reason, the reporting and examination of some measure of the sensitivity of the test (either the minimum significant difference or the percent change from the control that this minimum difference represents) is extremely important.

9.2.4 In summary, the assessment of a "safe" or "no-effect" concentration cannot be made from the results of statistical

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

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

9.3 **PRECISION**

9.3.1 HYPOTHESIS TESTS

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

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

9.3.2 POINT ESTIMATION TECHNIQUES

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

9.4 **DATA ANALYSIS**

9.4.1 ROLE OF THE STATISTICIAN

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

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

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

9.4.2 PLOTTING THE DATA

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

9.4.3 DATA TRANSFORMATIONS

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

9.4.4 INDEPENDENCE, RANDOMIZATION, AND OUTLIERS

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

9.4.5 REPLICATION AND SENSITIVITY

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

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

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

9.4.6 RECOMMENDED ALPHA LEVELS

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

9.5 **CHOICE OF ANALYSIS**

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

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

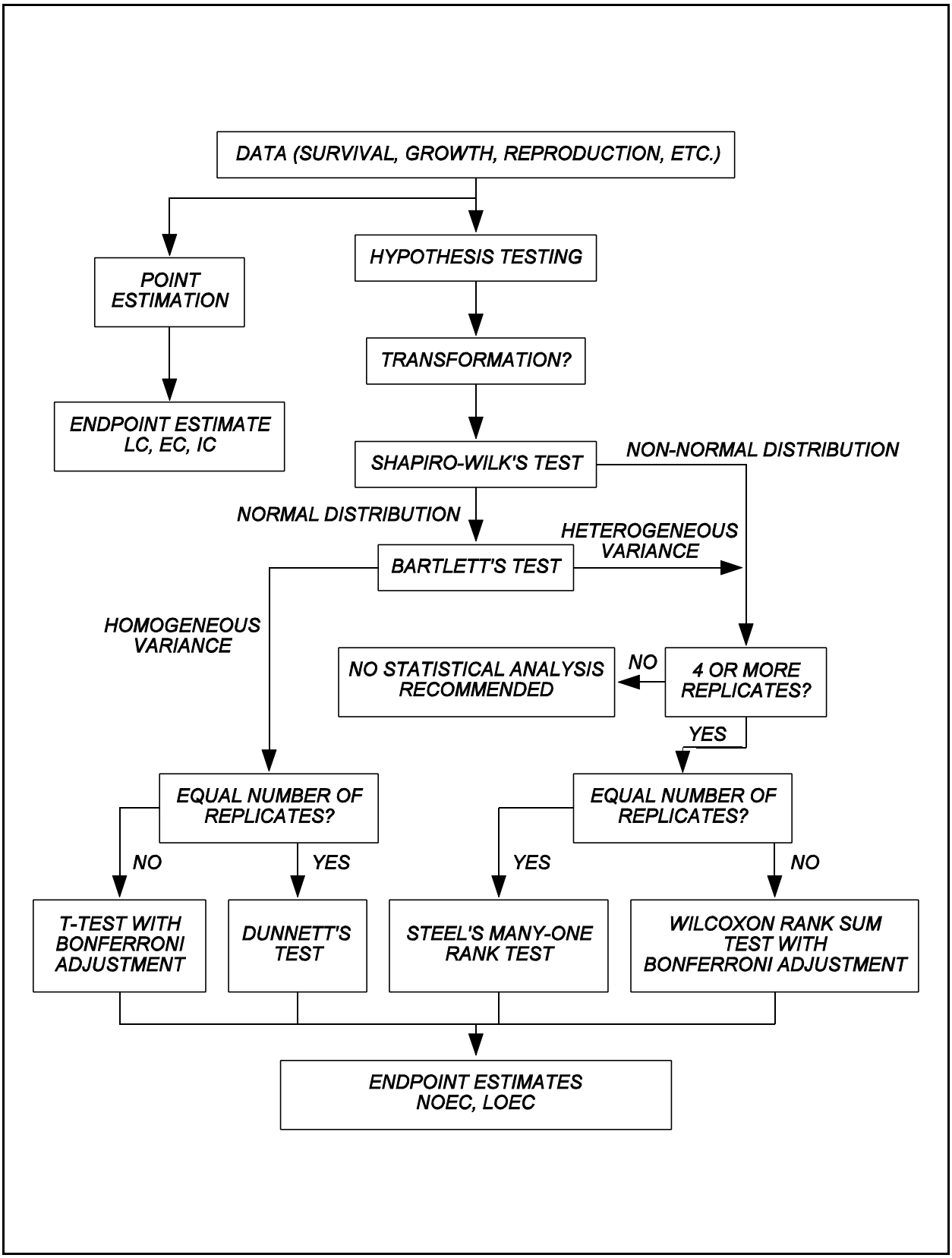


Figure 2. Flowchart for statistical analysis of test data.

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

9.5.3 ANALYSIS OF GROWTH DATA

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

9.5.4 ANALYSIS OF FERTILIZATION, GERMINATION AND DEVELOPMENT DATA

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

9.5.5 ANALYSIS OF MORTALITY DATA

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

9.6 **HYPOTHESIS TESTS**

9.6.1 DUNNETT'S PROCEDURE

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

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

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

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

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

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

9.6.2 T TEST WITH THE BONFERRONI ADJUSTMENT

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

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

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

Appendix D.

9.6.3 STEEL'S MANY-ONE RANK TEST

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

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

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

9.6.4 WILCOXON RANK SUM TEST

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

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

9.6.5 A CAUTION IN THE USE OF HYPOTHESIS TESTING

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

9.7 **POINT ESTIMATION TECHNIQUES**

9.7.1 PROBIT ANALYSIS

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

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

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

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

9.7.2 LINEAR INTERPOLATION METHOD

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

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

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

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

SECTION 10

REPORT PREPARATION

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

10.1 **INTRODUCTION**

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

10.2 **PLANT OPERATIONS**

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

10.3 **SOURCE OF EFFLUENT, RECEIVING WATER, AND DILUTION WATER**

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

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

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

10.4 **TEST METHODS**

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

10.5 **TEST ORGANISMS**

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

10.6 **QUALITY ASSURANCE**

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

10.7 **RESULTS**

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

10.8 **CONCLUSIONS AND RECOMMENDATIONS**

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

SECTION 11

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

Adapted from a method developed by
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Appendix I Step-by Step Summary

SECTION 11

TOPSMELT, *ATHERINOPS AFFINIS* LARVAL SURVIVAL AND GROWTH TEST

11.1 SCOPE AND APPLICATION

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

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

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

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

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

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

11.2 SUMMARY OF METHOD

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

1.3 **INTERFERENCES**

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

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

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

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

11.4 **SAFETY**

11.4.1 See Section 3, Health and Safety.

11.5 **APPARATUS AND EQUIPMENT**

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

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

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

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

11.5.5 Refractometer -- for determining salinity.

11.5.6 Hydrometer(s) -- for calibrating refractometer.

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

11.5.8 Thermometer, National Bureau of Standards Certified (see USEPA METHOD l70.1, USEPA, l979) -- to calibrate laboratory thermometers.

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

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

11.5.11 Winkler bottles -- for dissolved oxygen determinations.

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

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

11.5.14 Glass stirring rods -- for mixing test solutions.

11.5.15 Graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 50-l000 mL for making test solutions. (Note: not to be used interchangeably for gametes or embryos and test solutions).

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

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

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

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

11.5.20 Wash bottles -- for dilution water.

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

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

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

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

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

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

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

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

11.5.29 Light box -- for counting and observing larvae.

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

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

11.5.32 Desiccator -- for holding dried larvae.

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

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

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

11.6 **REAGENTS AND SUPPLIES**

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

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

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

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

11.6.5 Parafilm -- to cover graduated cylinders and vessels.

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

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

11.6.8 Pipet tips -- for automatic pipets.

11.6.9 Coverslips -- for microscope slides.

11.6.10 Lens paper -- for cleaning microscope optics.

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

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

11.6.13 pH buffers 4, 7, and l0 (or as per instructions of instrument manufacturer) -- for standards and calibration check (see USEPA Method l50.l, USEPA, l979).

11.6.14 Membranes and filling solutions -- for dissolved oxygen probe (see USEPA Method 360.l, USEPA, l979), or reagents for modified Winkler analysis.

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

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

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

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

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

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

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

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

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

11.6.24 HYPERSALINE BRINES

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

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

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

11.6.24.4 Freeze Preparation of Brine

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

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

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

11.6.24.5 Heat Preparation of Brine

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

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

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

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

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

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

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

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

11.6.24.6 Artificial Sea Salts

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

11.6.24.7 Dilution Water Preparation from Brine

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

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

11.6.24.8 Test Solution Salinity Adjustment

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

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

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

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

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

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

11.6.24.9 Preparing Test Solutions

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

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

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

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

SERIAL DILUTION:

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

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

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

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

INDIVIDUAL PREPARATION

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

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

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

11.6.24.10 Brine Controls

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

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

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

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

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

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

11.6.25.3 ***Artemia*** nauplii are obtained as follows:

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

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

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

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

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

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

11.6.26 TEST ORGANISMS

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

11.6.26.2 Species Identification

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

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

11.6.26.3 Obtaining Broodstock

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

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

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

11.6.26.4 Broodstock Culture and Handling

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

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

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

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

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

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

11.6.26.5 Culture Materials

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

11.6.26.6 Test Organisms

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

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

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

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

11.7 EFFLUENTS AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

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

11.8 CALIBRATION AND STANDARDIZATION

11.8.1 See Section 4, Quality Assurance

11.9 QUALITY CONTROL

11.9.1 See Section 4, Quality Assurance

11.10 TEST PROCEDURES

11.10.1 TEST DESIGN

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

11.10.1.2 Effluent concentrations are expressed as percent effluent.

11.10.2 TEST SOLUTIONS

11.10.2.1 Receiving waters

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

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

11.10.2.2 Effluents

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

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

11.10.2.2.3 The volume in each test chamber is 200 mL.

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

11.10.2.3 Dilution Water

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

11.10.2.4 Reference Toxicant Test

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

11.10.2.4.2 The preferred reference toxicant for topsmelt is copper chloride ($CuCl_2$º2H_2O). Reference toxicant tests provide an indication of the sensitivity of the test organisms and the suitability of the testing laboratory (see Section 4 Quality Assurance). Another toxicant may be specified by the appropriate regulatory agency. Prepare a 10,000 µg/L copper stock solution by adding 0.0268 g of copper chloride ($CuCl_2$º2H_2O) to one liter of reagent water in a polyethylene volumetric flask. Alternatively, certified standard solutions can be ordered from commercial companies.

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

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

11.10.3 START OF THE TEST

11.10.3.1 Prior to Beginning the Test

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

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

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

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

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

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

11.10.3.2 Randomized Placement of Larvae into Test Chambers

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

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

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

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

11.10.4 LIGHT, PHOTOPERIOD, SALINITY AND TEMPERATURE

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

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

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

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

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

11.10.5 DISSOLVED OXYGEN (DO) CONCENTRATION

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

11.10.6 FEEDING

11.10.6.1 ***Artemia*** nauplii are prepared as described below.

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

11.10.7 DAILY CLEANING OF TEST CHAMBERS

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

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

11.10.8 OBSERVATIONS DURING THE TEST

11.10.8.1 Routine Chemical and Physical Observations

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

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

11.10.8.1.3 Record all the measurements on the data sheet.

11.10.8.2 Routine Biological Observations

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

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

11.10.9 TEST SOLUTION RENEWAL

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

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

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

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

11.10.10 TERMINATION OF THE TEST

11.10.10.1 Ending the Test

11.10.10.1.1 Record the time the test is terminated.

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

11.10.10.2 Sample Preservation

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

11.10.10.3 Weighing

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

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

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

11.10.10.4 Endpoints

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

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

11.11 **SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA**

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

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

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

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

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

11.12 **ACCEPTABILITY OF TEST RESULTS**

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

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

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

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

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

11.13 **DATA ANALYSIS**

11.13.1 GENERAL

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

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

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

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

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

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

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

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

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

11.13.2.5 Example of Analysis of Survival Data

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

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

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

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

[1]Five replicates of 5 larvae each.

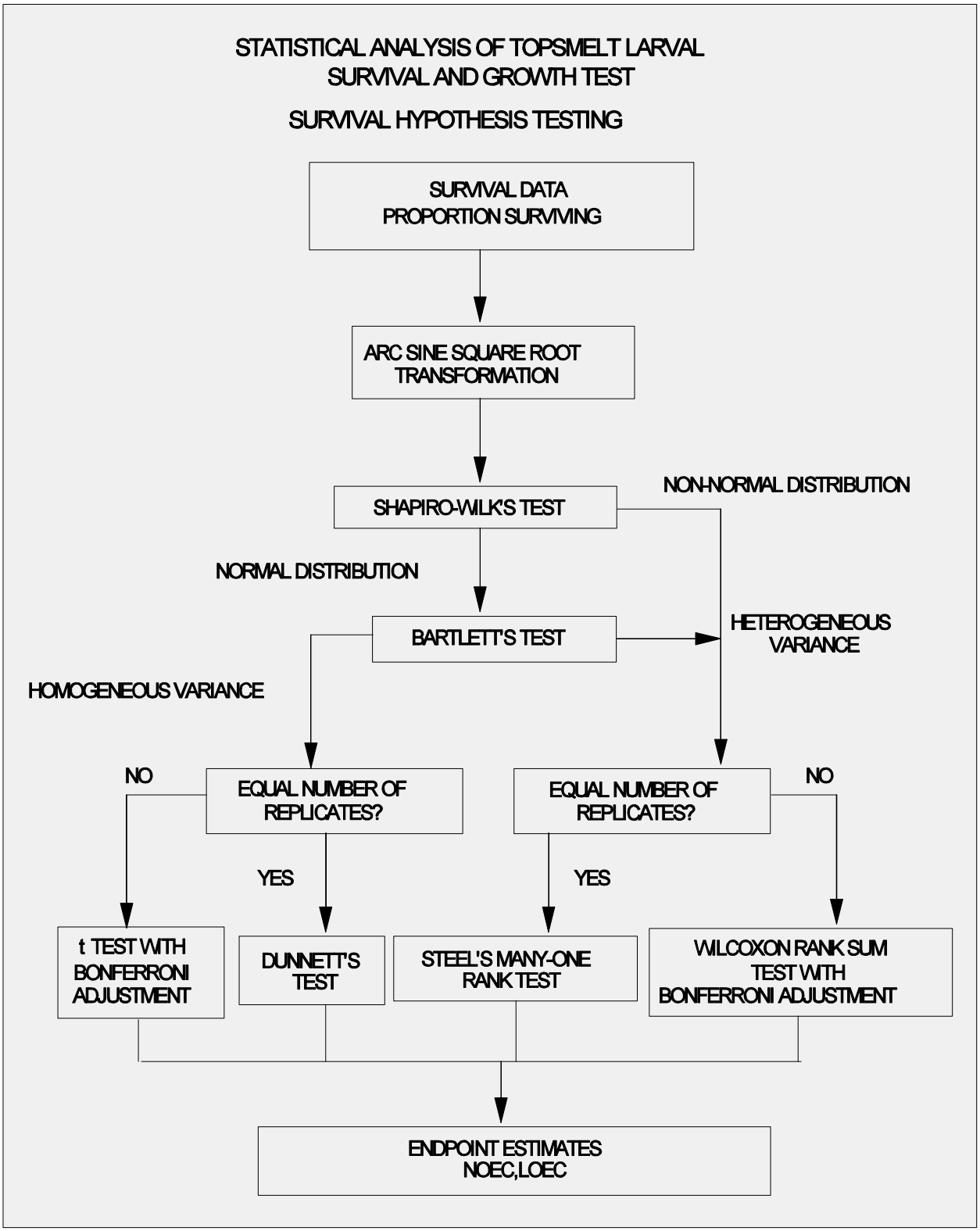

Figure 1. Flowchart for statistical analysis of the topsmelt, ***Atherinis affinis***, larval survival data by hypothesis testing.

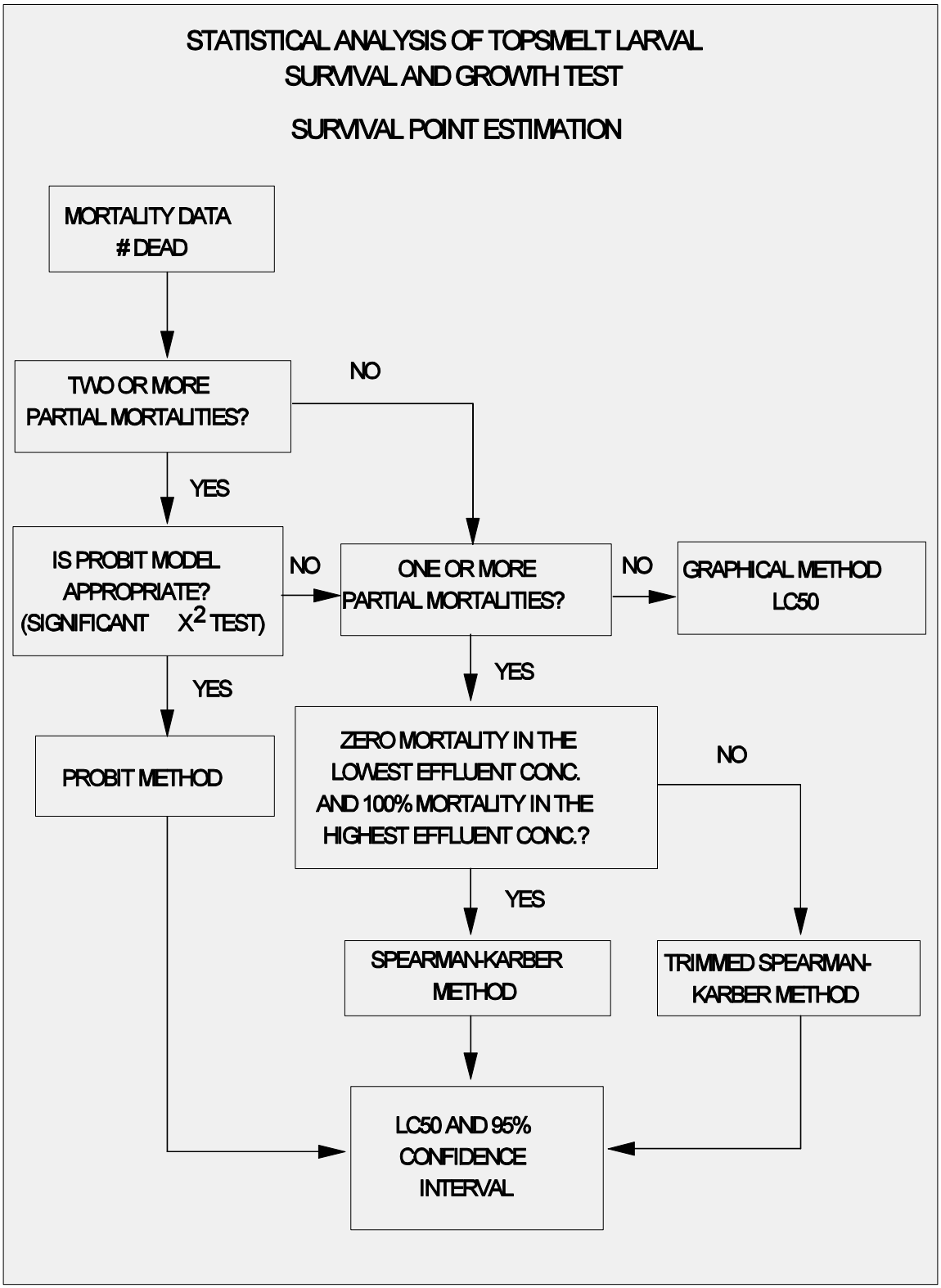

Figure 2. Flowchart for statistical analysis of the topsmelt, *Atherinis affinis*, larval survival data by point estimation.

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

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

11.13.2.6 Test for Normality

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

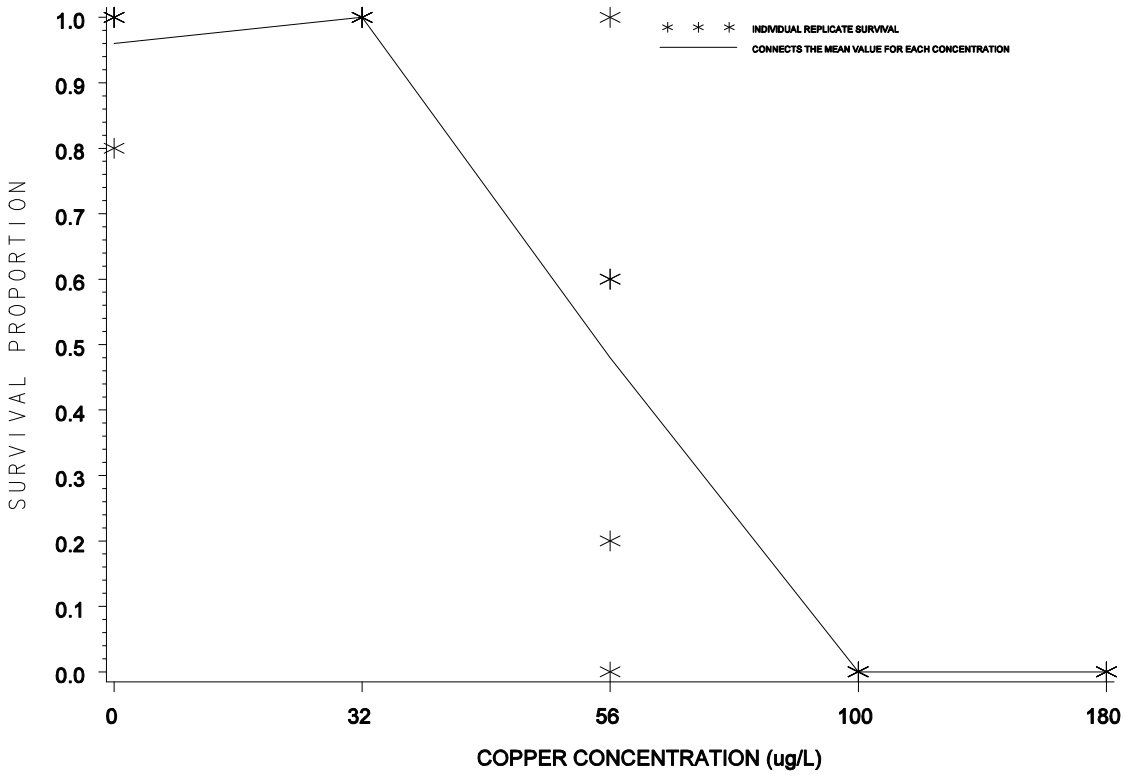

Figure 3 Plot of mean survival proportion data in Table 5.

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

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

11.13.2.6.2 Calculate the denominator, D, of the statistic:

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

Where: X_i = the ith centered observation

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

n = the total number of centered observations

11.13.2.6.3 For this set of data,

n = 15

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

D = 0.793

11.13.2.6.4 Order the centered observations from smallest to largest

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

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

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

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

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

11.13.2.6.6 Compute the test statistic, W, as follows:

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

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

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

11.13.2.6.7 The decision rule for this test is to compare W as calculated in Subsection 11.13.2.6.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For the data in this example, the critical value at a significance

level of 0.01 and n = 15 observations is 0.835. Since W = 0.842 is greater than the critical value, conclude that the data are normally distributed.

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

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

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

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

to analyze the survival data.

11.13.2.7 Steel's Many-one Rank Test

11.13.2.7.1 For each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign the ranks (1, 2, ..., 10) to the ordered observations with a rank of 1 assigned to the smallest observation, rank of 2 assigned to the next larger observation, etc. If ties occur when ranking, assign the average rank to each tied observation.

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

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

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

11.13.2.8 Calculation of the LC50

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

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

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

TABLE 10. TABLE OF RANKS

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

TABLE 11. RANK SUMS

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

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

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

$$p_0^s , p_1^s , \frac{0.040 \% 0.000}{2} , 0.020$$

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

= 1.7479

11.13.2.8.6 Calculate the estimated variance of m as follows:

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

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

n_i = the number of organisms tested at effluent concentration i

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

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

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

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

$$= 0.0006376$$

11.13.2.8.7 Calculate the 95% confidence interval for m: m ± 2.0 **%&&&&&&**

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

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

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

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

LC50 = antilog(m) = antilog(1.7479) = 56.0 µg/L.

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

lower limit: antilog(1.6974) = 49.8 µg/L

upper limit: antilog(1.7984) = 62.9 µg/L

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

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

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

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

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

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

11.13.3.5 Test for Normality

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

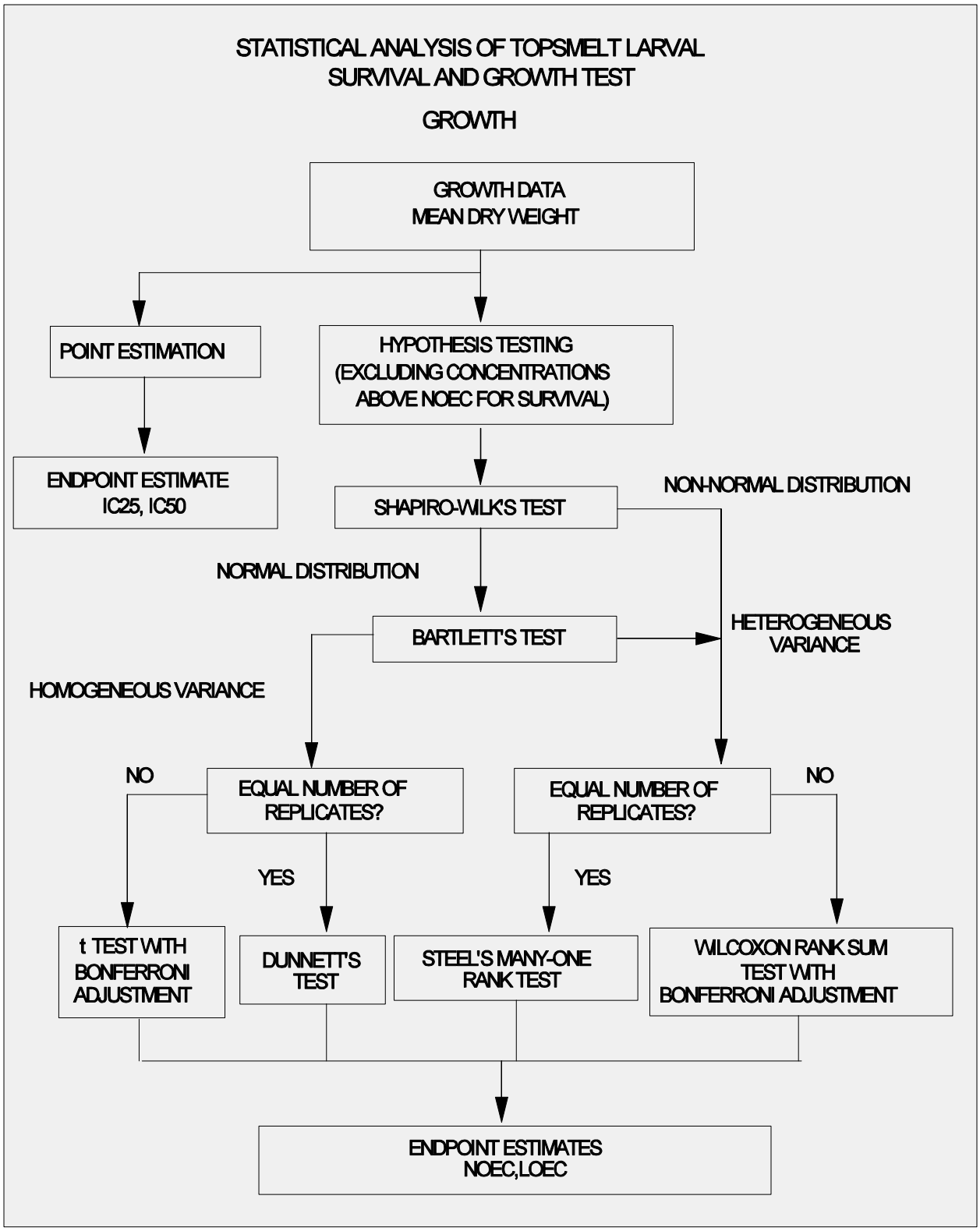

Figure 4. Flowchart for statistical analysis of the topsmelt, *Atherinops affinis*, larval growth data.

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

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

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

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

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

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

Where: X_i = the ith centered observation

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

n = the total number of centered observations.

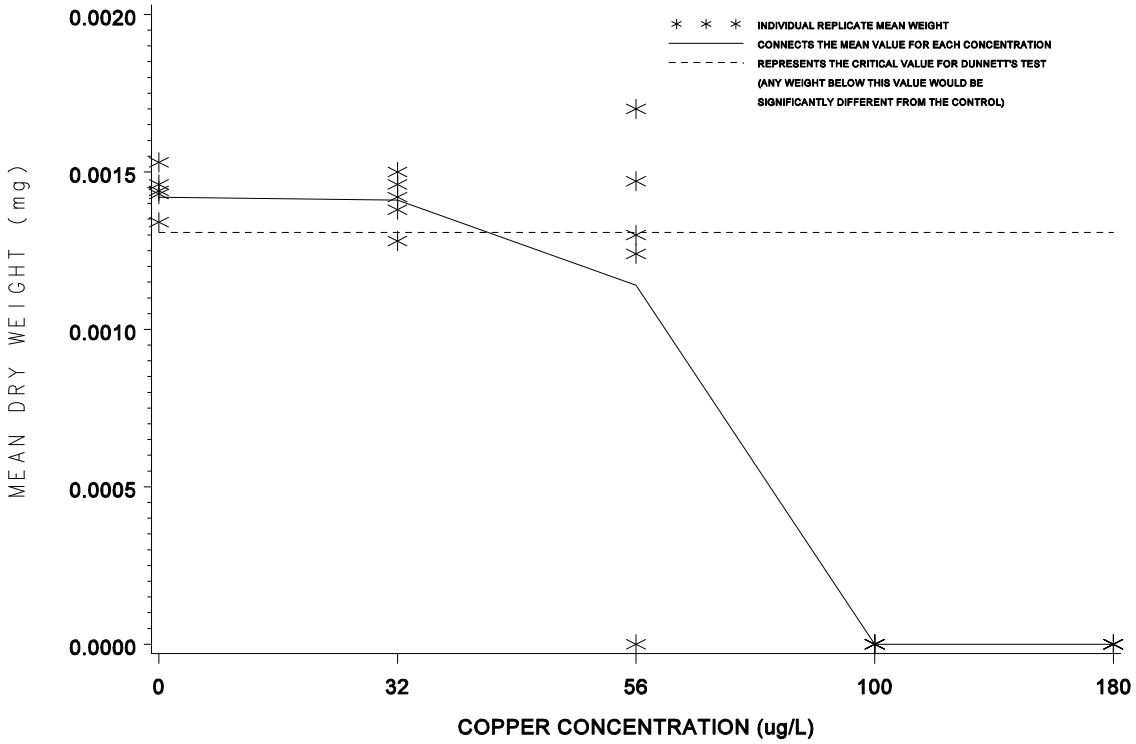

Figure 5. Plot of weight data from topsmelt, ***Atherinis affinis***, larval survival and growth test.

For this set of data, n = 10

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

$$D = 0.000000055$$

11.13.3.5.3 Order the centered observations from smallest to largest:

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

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

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

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

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

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

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

11.13.3.5.5 Compute the test statistic, W, as follows:

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

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

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

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

11.13.3.6 Test for Homogeneity of Variance

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

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

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

n_i = the number of replicates for concentration i

p = number of levels of effluent concentration including the control

ln = $\log_e$

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

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

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

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

11.13.3.6.3 Bartlett's statistic is therefore:

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

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

$$= 0.028/1.125$$

$$= 0.0249$$

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

11.13.3.7 Dunnett's Procedure

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

TABLE 17. ANOVA TABLE

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

Where: p = number of concentration levels including the control

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

n_i = number of observations in concentration i

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

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

$SSW = SST - SSB$ Within Sum of Squares

G = the grand total of all sample observations,

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

T_i = the total of the replicate measurements for concentration i

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

11.13.3.7.2 For the data in this example:

$n_1 = n_2 = 5$

$N = 10$

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

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

$G = T_1 + T_2 = 0.01415$

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

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

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

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

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

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

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

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

TABLE 18. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

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

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

$$t_i = \frac{(Y_1 - Y_i)}{S_W\sqrt{(1/n_1)+(1/n_i)}}$$

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

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

S_W = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i.

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

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

TABLE 19. CALCULATED t VALUES

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

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

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

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

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

S_W = the square root of the within mean square

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

n_1 = the number of replicates in the control.

11.13.3.7.8 In this example:

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

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

$$= 0.000116$$

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

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

11.13.3.8 Calculation of the ICp

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

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

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

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

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

$$ICp = C_j\%[M_1(1\&p/100)\&M_j]\frac{(C_{(j\%1)}\&C_j)}{(M_{(j\%1)}\&M_j)}$$

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

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

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

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

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

11.14.1 PRECISION

11.14.1.1 Single-Laboratory Precision

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

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

11.14.1.2 Multilaboratory Precision

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

11.11.2 ACCURACY
11.11.2.1 The accuracy of toxicity tests cannot be determined.

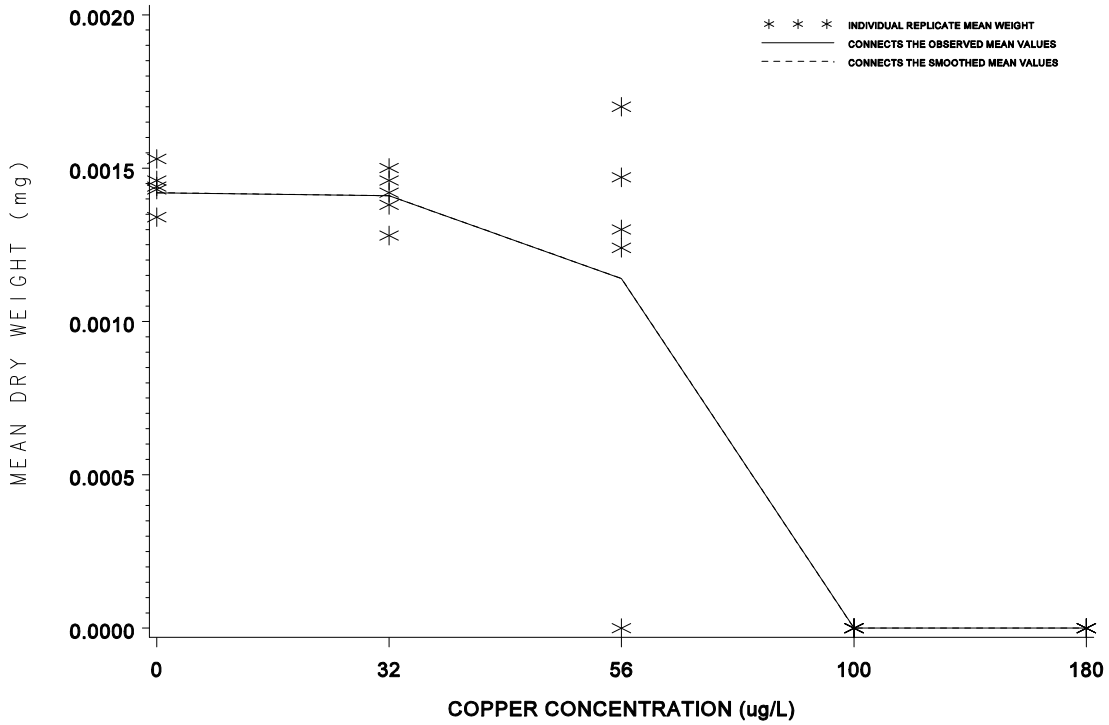

Figure 6. Plot of raw data, observed means, and smoothed means for topsmelt, ***Atherinis affinis***, growth data from Tables 4 and 21.

```
Conc. ID             1         2         3         4         5
-------------------------------------------------------------
Conc. Tested         0        32        56       100       180
-------------------------------------------------------------
Response  1     .00134    .00146         0         0         0
Response  2     .00153    .00142    .00147         0         0
Response  3     .00134    .00150    .00170         0         0
Response  4     .00146    .00138    .00124         0         0
Response  5     .00144    .00128    .00130         0         0
-------------------------------------------------------------
*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Copper
Test Start Date:    Test Ending Date:
Test Species: Atherinops affinis
Test Duration:          7 days
DATA FILE: wc_aa.icp
OUTPUT FILE: wc_aa.i25
-----------------------------------------------------------------------
Conc.     Number     Concentration     Response      Std.       Pooled
 ID     Replicates            ug/L       Means       Dev.   Response Means
-----------------------------------------------------------------------
  1          5              0.000          0.001      0.000      0.001
  2          5             32.000          0.001      0.000      0.001
  3          5             56.000          0.001      0.001      0.001
  4          5            100.000          0.000      0.000      0.000
  5          5            180.000          0.000      0.000      0.000
-----------------------------------------------------------------------
The Linear Interpolation Estimate:    58.9089   Entered P Value: 25
-----------------------------------------------------------------------
Number of Resamplings:   80
The Bootstrap Estimates Mean:  58.1571 Standard Deviation:     7.9299
Original Confidence Limits:   Lower:    44.2778 Upper:    67.0000
Expanded Confidence Limits:   Lower:    36.9622 Upper:    71.0455
Resampling time in Seconds:     0.11  Random_Seed: -498847050
```

Figure 7. ICPIN program output for the IC25

TABLE 21. SINGLE LABORATORY PRECISION OF THE TOPSMELT, ***ATHERINOPS AFFINIS*** SURVIVAL ENDP0INT WITH COPPER (CU FG/L) CHLORIDE AS A REFERENCE TOXICANT

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

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

TABLE 22. SINGLE LABORATORY PRECISION OF THE TOPSMELT, ***ATHERINOPS AFFINIS*** GROWTH ENDP0INT WITH COPPER (CU FG/L) CHLORIDE AS A REFERENCE TOXICANT

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

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

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

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

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

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

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

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

PREPARATION OF TEST SOLUTIONS

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

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

C. Prepare a copper reference toxicant stock solution (10,000 µg/L) by adding 0.0268 g of copper chloride ($CuCl_2$º2H_2O) to 1 liter of reagent water.

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

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

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

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

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

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

PREPARATION AND ANALYSIS OF TEST ORGANISMS

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

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

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

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

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

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

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

H. Analyze the data.

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

Data Sheet for Larval Fish Toxicity Test

Test Start Date: Start Time:
Fish Species:

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

Reference Toxicant:
Broodstock Source:

Fish Age at Start:

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

Data Sheet for Weighing Larval Fish

Test Start Date: Start Time: Fish Species :

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

Toxicant: Fish Age at Start:

Sample Source:

Sample Type: Sediment Elutriate Porewater Water

Test Container Number	Site Code or Concentration	Foil Number	Foil Weight (mg)	Total Weight (mg)	Weight of Larval Fish (mg)	Number of Fish Larvae	Weight per Larval Fish (mg)
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
14							
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30							
31							
32							
33							
34							
35							

Computer Data Storage
Disk:
File:

Notes

Note: See larval mortality data on separate sheet.

SECTION 12

MYSID, *Holmesimysis costata* SURVIVAL AND GROWTH TEST METHOD

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

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

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Appendix I Step-by Step Summary

SECTION 12

MYSID, *HOLMESIMYSIS COSTATA* SURVIVAL AND GROWTH TEST

12.1 SCOPE AND APPLICATION

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

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

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

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

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

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

12.2 SUMMARY OF METHOD

12.2.1 This method provides step-by-step instructions for

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

12.3 **INTERFERENCES**

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

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

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

12.4 **SAFETY**

12.4.1 See Section 3, Health and Safety.

12.5 **APPARATUS AND EQUIPMENT**

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

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

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

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

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

12.5.5 Refractometer -- for determining salinity.

12.5.6 Hydrometer(s) -- for calibrating refractometer.

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

12.5.8 Thermometer, National Bureau of Standards Certified (see USEPA METHOD l70.l, USEPA, l979) -- to calibrate laboratory thermometers.

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

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

12.5.11 Winkler bottles -- for dissolved oxygen determinations.

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

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

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

12.5.15 Glass stirring rods -- for mixing test solutions.

12.5.16 Graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 50-l000 mL for making test solutions.

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

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

delivery volumes from 0.010 to 100 mL.

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

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

12.5.21 Wash bottles -- for dilution water.

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

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

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

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

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

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

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

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

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

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

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

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

12.5.34 Light box -- for examining organisms.

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

12.5.36 Desiccator -- for holding dried organisms.

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

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

12.6 **REAGENTS AND SUPPLIES**

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

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

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

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

12.6.5 Parafilm -- to cover graduated cylinders and vessels.

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

12.6.7 Pipets, serological -- l-l0 mL, graduated.

12.6.8 Pipet tips -- for automatic pipets.

12.6.9 Coverslips -- for microscope slides.

12.6.10 Lens paper -- for cleaning microscope optics.

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

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

12.6.13 pH buffers 4, 7, and l0 (or as per instructions of instrument manufacturer) -- for standards and calibration check (see USEPA Method l50.l, USEPA, l979).

12.6.14 Membranes and filling solutions -- for dissolved oxygen probe (see USEPA Method 360.l, USEPA, l979), or reagents for modified Winkler analysis.

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

12.6.16 Test chambers -- 1000 mL, five chambers per concentration. The chambers should be borosilicate glass (for effluents) or nontoxic disposable plastic labware (for reference toxicants). To avoid contamination from the air and excessive evaporation of test solutions during the test, the chambers should be covered during the test with safety glass plates or a plastic sheet (6 mm thick).

12.6.17 Micro-weighing pans, aluminum -- to determine the dry weight of organisms. Weighting pan should be about 5 mg or less to minimize noise in measurement of the small mysids.

12.6.18 Fronds of kelp (***Macrocystis***) for habitat in culture.

12.6.19 Reference toxicant solutions (see Subsection 12.10.2.4 and see Section 4, Quality Assurance).

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

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

12.6.22 Dilution water and hypersaline brine -- see Section 7, Dilution Water and Section 12.6.24, Hypersaline Brines. The dilution water should be uncontaminated 1-µm-filtered natural seawater. Hypersaline brine should be prepared from dilution water.

12.6.23 HYPERSALINE BRINES

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

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

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

12.6.23.4 Freeze Preparation of Brine

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

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

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

12.6.23.5 Heat Preparation of Brine

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

12.6.23.5.2 Before adding seawater to the brine generator, thoroughly clean the generator, aeration supply tube, heater, and

any other materials that will be in direct contact with the brine. A good quality biodegradable detergent should be used, followed by several (at least three) thorough reagent water rinses.

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

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

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

12.6.23.5.4 After the required salinity is attained, the HSB

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

12.6.23.6 Artificial Sea Salts

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

12.6.23.7 Dilution Water Preparation from Brine

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

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

12.6.23.8 Test Solution Salinity Adjustment

12.6.23.8.1 Table 2 illustrates the preparation of test solutions (up to 50% effluent) at 34‰ by combining effluent, HSB, and dilution water. Note: if the highest effluent concentration does not exceed 50% effluent, it is convenient to prepare brine so that the sum of the effluent salinity and brine salinity equals 68‰; the required brine volume is then always equal to the effluent volume needed for each effluent concentration as in the example in Table 2.

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

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

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

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

12.6.23.9 Preparing Test Solutions

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

12.6.23.9.2 To prepare a test solution at higher effluent concentrations, hypersaline brine must usually be used. For example, to prepare 40% effluent, add 400 mL of effluent to a 1-liter volumetric flask. Then, assuming an effluent salinity of 2‰ and a brine salinity of 66‰, add 400 mL of brine (see

equation above and Table 2) and top off the flask with dilution water. Stopper the flask and shake well. Distribute equal volumes into the replicate test chambers.

12.6.23.10 Brine Controls

12.6.23.10.1 Use brine controls in all tests where brine is used. Brine controls contain the same volume of brine as does the highest effluent concentration using brine, plus the volume of reagent water needed to reproduce the hyposalinity of the effluent in the highest concentration, plus dilution water. Calculate the amount of reagent water to add to brine controls by rearranging the above equation, (See, 12.6.23.8.3) setting SE = 0, and solving for VE.

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

If effluent salinity is essentially 0‰, the reagent water volume needed in the brine control will equal the effluent volume at the highest test concentration. However, as effluent salinity and effluent concentration increase, less reagent water volume is needed.

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

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

12.6.24.2 Each new batch of ***Artemia*** cysts should be evaluated for size (Vanhaecke and Sorgeloos, 1980, and Vanhaecke et al., 1980) and nutritional suitability (Leger, et al., 1985, Leger, et al., 1986) against known suitable reference cysts by performing a side-by-side larval growth test using the "new" and "reference" cysts. The "reference" cysts used in the suitability test may be

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

12.6.24.3 ***Artemia*** nauplii are obtained as follows:

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

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

12.6.24.4.1 The primary criteria for acceptability of each new supply of brine shrimp cysts is adequate survival, and growth of the mysids. The mysids used to evaluate the acceptability of the brine shrimp nauplii must be the same geographical origin and stage of development (3 to 4 days old) as those used routinely in the toxicity tests. Two 7-day chronic tests are performed side-by-side, each consisting of five replicate test vessels containing five juveniles (25 organisms per test, total of 50

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

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

SERIAL DILUTION:

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

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

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

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

INDIVIDUAL PREPARATION

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

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

organisms). The juveniles in one set of test chambers is fed reference (acceptable) nauplii and the other set is fed nauplii from the "new" source of ***Artemia*** cysts.

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

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

12.6.25 TEST ORGANISMS

12.6.25.1 The test organisms for this method are juveniles of the mysid crustacean, ***Holmesimysis costata*** (Holmes 1900; previously referred to as ***Acanthomysis sculpta***). ***H. costata*** occurs in the surface canopy of the giant kelp ***Macrocystis pyrifera*** where it feeds on zooplankters, kelp, epiphytes, and detritus. There are few references to the ecology of this mysid species (Holmquist, 1979; Clutter, 1967, 1969; Green, 1970; Turpen et al., 1994). ***H. costata*** is numerically abundant in kelp forest habitats and is considered to be an important food source for kelp forest fish (Clark 1971, Mauchline 1980). Mysids are called opossum shrimp because females brood their young in an abdominal pouch, the marsupium. ***H. costata*** eggs develop for about 20 days in the marsupium before the young are released as juveniles; broods are released at night during molting. Females release their first brood at 55 to 70 days post-release (at 12EC), and may have multiple broods throughout their approximately 120-day life.

12.6.25.2 ***H. costata*** has been used in previous toxicity studies with a variety of toxicants (Tatem and Portzer, 1985; Davidson et al., 1986; Machuzac and Mikel, 1987; Reish and Lemay, 1988; Asato, 1988; Martin et al., 1989; Singer et al., 1990; 1991; Hunt et al., In Press). Mysids are useful as toxicity test organisms because of their widespread availability, ecological importance, sensitivity to toxicants, and amenability to laboratory culture

(Nimmo et al., 1977; Mauchline, 1980; Gentile et al., 1982; Lussier et al., 1985).

12.6.25.3 Species Identification

12.6.25.3.1 Laboratories unfamiliar with the test organism should collect preliminary samples to verify species identification. Refer to Holmquist (1979) or send samples of mysids and any similar co-occurring organisms to a qualified taxonomist. Request certification of species identification from any organism suppliers. Records of verification should be maintained along with a few preserved specimens.

12.6.25.3.2 There have been recent revisions to the taxonomy of ***H. costata.*** Previous authors have referred to this species as ***Acanthomysis sculpta.*** However, Holmquist's (1979) review considers previous references to ***Acanthomysis sculpta*** in California to be synonymous with ***Holmesimysis costata;*** we consider Holmquist's designation to be definitive.

12.6.25.4 Obtaining Broodstock

12.6.25.4.1 ***H. costata*** can be collected by sweeping a small-mesh (0.5 - 1 mm) hand net through the water just under the surface canopy blades of giant kelp ***Macrocystis pyrifera.*** Although this method collects mysids of all sizes, attention should be paid to the number of gravid females collected because these are used to produce the juvenile mysids used in toxicity testing. Mysids should be collected from waters remote from sources of pollution to minimize the possibility of physiological or genetic adaptation to toxicants.

12.6.25.4.2 Mysids can be transported for a short time (< 3 hours) in tightly covered 20 liter plastic buckets. The buckets should be filled to the top with seawater from the collection site, and should be gently aerated or oxygenated to maintain dissolved oxygen above 60% saturation. Transport temperatures should remain within 3EC of the temperature at the collection site.

12.6.25.4.3 For longer transport times of up to 36 hours, mysids can be shipped in sealed plastic bags filled with seawater. The following transport procedure has been used successfully: 1)

fill the plastic bag with one liter of dilution water seawater, 2) saturate the seawater with oxygen by bubbling pure oxygen for at least 10 minutes, 3) place 25-30 adult mysids, or up to 100 juvenile mysids in each bag, 4) for adults add about 20 ***Artemia*** nauplii per mysid, for 100 juveniles add a pinch (10 to 20 mg) of ground Tetramin® flake food and 200 newly-hatched ***Artemia*** nauplii, 5) seal the bag securely, eliminating any airspace, then 6) place it within a second sealed bag in an ice chest. Do not overfeed mysids in transport, as this may deplete dissolved oxygen, causing stress or mortality in transported mysids. A well insulated ice chest should be cooled to approximately 15EC by adding one 1-liter blue ice block for every five 1-liter bags of mysids (a temperature range of 12 to 16EC is tolerable). Wrap the ice in newspaper and a plastic bag to insulate it from the mysid bags. Pack the bags tightly to avoid shifting within the cooler.

12.6.25.5 Broodstock Culture and Handling

12.6.25.5.1 After collection, the mysids should be transported directly to the laboratory and placed in seawater tanks or aquaria equipped with flowing seawater or adequate aeration and filtration. Initial flow rates should be adjusted so that any temperature change occurs gradually (0.5EC per hour). The water temperature should be held at 15 ± 1EC. **Note:** Mysids collected north of Pt. Conception, California, should be held and tested at 13 ± 1EC.

12.6.25.5.2 Mysids can be cultured in tanks ranging from 4 to 1000 liters. Tanks should be equipped with gentle aeration and blades of ***Macrocystis*** to provide habitat. Static culture tanks can be used if there is constant aeration, temperature control, and frequent water changes (one half the water volume changed at least twice a week). Maintain culture density below 20 animals per liter by culling out adult males or juveniles.

12.6.25.5.3 Adult mysids should be fed 100 ***Artemia*** nauplii per mysid per day. Juveniles should be fed 5 to 10 newly released ***Artemia*** nauplii per juvenile per day and a pinch (10 to 20 mg) of ground Tetramin® flake food per 100 juveniles per day. Static chambers should be carefully monitored and rations adjusted to

prevent overfeeding and fouling of culture water. Refer to section 12.6.19 for details of ***Artemia*** culture and quality control.

12.6.25.6 Culture Materials

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

12.6.25.7 Test Organisms

12.6.25.7.1 Approximately 150 gravid female mysids should be isolated to provide approximately 400 juveniles for each set of toxicity tests (5 juveniles/chamber x 30 reference toxicant chambers and approximately 35 effluent chambers, plus additional mysids so that only healthy active juveniles are used in the test). Gravid females can be identified by their large, extended marsupia filled with (visible) eyed juveniles. Marsupia appear distended and gray when females are ready to release young, due to presence of the juveniles.

12.6.25.7.2 Gravid females are easily isolated from other mysids using the following technique: (1) use a small dip net to capture about 100 mysids from the culture tank, (2)transfer the mysids to a screen-bottomed plastic tube (150 µm-mesh, 25-cm diam.) partly immersed in a water bath or bucket, (3)lift the screen-tube out of the water to immobilize mysids on the damp screen, (4)gently draw the gravid females off the screen with a suction bulb and fire-polished glass tube (5-mm bore), (5) collect the gravid females in a separate screen tube. Re-immerse the screen continuously during the isolation process; mysids should not be exposed to air for more than a few seconds at a time.

12.6.25.7.3 Four or five days before a toxicity test begins, transfer gravid females into a removable, 2-mm-mesh screened cradle suspended within an aerated 80-liter aquarium. Before transfer, make sure there are no juveniles in with the adult females. Extraneous juveniles are excluded to avoid

inadvertently mixing them with the soon-to-be released juveniles used in testing. Provide the gravid females with newly hatched ***Artemia*** nauplii (approximately 200 per mysid) to help stimulate juvenile release. ***Artemia*** can be provided continuously throughout the night from an aerated reservoir holding approximately 75,000 ***Artemia***. Direct the flow from the feeder into the screened compartment with the females, and add a few blades of ***Macrocystis*** for habitat. The females are placed within the screened compartment so that as the juveniles are released, they can swim through the mesh into the bottom of the aquarium. Outflows on flow-through aquaria should be screened (150-µm-mesh) to retain juveniles and allow some ***Artemia*** to escape.

12.6.25.7.4 Juveniles are generally released at night, so it is important to turn off all lights at night to promote release. In the morning, the screened compartment containing the females should be removed and placed in a separate aquarium. Juveniles should be slowly siphoned through a wide-diameter hose into a 150-µm-mesh screen-bottom tube (25 cm diam.) immersed in a bucket filled with clean seawater. Once the release aquarium is emptied, it should be washed with hot fresh water to eliminate stray juveniles that might mix with the next cohort.

12.6.25.7.5 After collection, the number of juveniles should be estimated visually or by counting subsamples with a small beaker. If there are not enough juveniles to conduct the necessary tests, they can be mixed with juveniles from one previous or subsequent release so that the test is initiated with three and/or four-day old juveniles. Initial experiments indicate that mysids 2-days-old and younger survive poorly in toxicity tests and that mysids older than four days may vary in their toxicant sensitivity or survival rate (Hunt et al., 1989; Martin et al., 1989).

12.6.25.7.6 Test juveniles should be transferred to additional screen-tubes (or to 4-liter static beakers if flowing seawater is unavailable). The screen-tubes are suspended in a 15-liter bucket so that dilution water seawater (0.5 liter/min) can flow into the tube, through the screen, and overflow from the bucket. Check water flow rates (< one liter/min) to make sure that juveniles or ***Artemia*** nauplii are not forced down onto the screen. The height of the bucket determines the level of water in the screen tube. About 200 to 300 juveniles can be held in each screen-tube (200 juveniles per static 4-liter beaker). Juveniles

should be fed 40 newly hatched ***Artemia*** nauplii per mysid per day and a pinch (10 to 20 mg) of ground Tetramin® flake food per 100 juveniles per day. A blade of ***Macrocystis*** (well rinsed in seawater) should be added to each chamber. Chambers should be gently aerated and temperature controlled at 15 ± 1EC (or 13 ± 1EC if collected north of Pt. Conception). Half of the seawater in static chambers should be changed at least once between isolation and test initiation.

12.6.25.7.7 The day juveniles are isolated is designated day 0 (the morning after their nighttime release). The toxicity test should begin on day three or four. For example, if juveniles are isolated on Friday, the toxicity test should begin on the following Monday or Tuesday.

12.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

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

12.8 CALIBRATION AND STANDARIZATION

12.8.1 See Section 4, Quality Assurance.

12.9 QUALITY CONTROL

12.9.1 See Section 4, Quality Assurance.

12.10 TEST PROCEDURES

12.10.1 TEST DESIGN

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

12.10.1.2 Effluent concentrations are expressed as percent effluent.

12.10.2 TEST SOLUTIONS

12.10.2.1 Receiving waters

12.10.2.1.1 The sampling point is determined by the objectives of the test. At estuarine and marine sites, samples are usually collected at mid-depth. Receiving water toxicity is determined with samples used directly as collected or with samples passed through a 60 µm NITEX® filter and compared without dilution, against a control. Using five replicates chambers per test, each containing 200 mL would require approximately 1 L or more of sample per test per renewal.

12.10.2.2 Effluents

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

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

12.10.2.2.3 The volume of effluent required for a 75% renewal of five replicates per concentration for five concentrations of effluent and two controls, each containing 200 mL of test solution, is approximately 370 mL.

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

12.10.2.3 Dilution Water

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

12.10.2.4 Reference Toxicant Test

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

12.10.2.4.2 The preferred reference toxicant for mysids is zinc sulfate ($ZnSO_4 \cdot 7H_2O$). Reference toxicant tests provide an indication of the sensitivity of the test organisms and the suitability of the testing laboratory (see Section 4 Quality Assurance). Another toxicant may be specified by the appropriate regulatory agency. Prepare a 10,000 µg/L zinc stock solution by adding 0.0440 g of zinc sulfate ($ZnSO_4 \cdot 7H_2O$) to one liter of reagent water in a polyethylene volumetric flask. Alternatively, certified standard solutions can be ordered from commercial companies.

12.10.2.4.3 Reference toxicant solutions should be five replicates each of 0 (control), 10, 18, 32, and 56, and 100 µg/L total zinc. Prepare one liter of each concentration by adding 0, 1.0, 1.8, 3.2, 5.6, and 10.0 mL of stock solution, respectively, to one-liter volumetric flasks and fill with dilution water. Start with control solutions and progress to the highest concentration to minimize contamination.

12.10.2.4.4 If the effluent and reference toxicant tests are to be run concurrently, then the tests must use juvenile originating from or released from the same pool of gravid females. The tests must be handled in the same way and test solutions delivered to the test chambers at the same time. Reference toxicant tests must be conducted at 34 ± 2‰.

12.10.3 START OF THE TEST

12.10.3.1 Prior to Beginning the Test

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

12.10.3.1.2 Just prior to test initiation (approximately 1 h), the temperature of a sufficient quantity of the sample to make the test solutions should be adjusted to the test temperature (13 or 15 ± 1EC) and maintained at that temperature during the addition of dilution water.

12.10.3.1.3 Increase the temperature of the water bath, room, or incubator to the required test temperature (13 or 15 ± 1EC).

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

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

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

12.10.3.2 Randomized Assignment of Mysids to Test Chambers

12.10.3.2.1 The juvenile mysids must be randomized before placing them into the test chambers. Pool all of the test juveniles into a 1-liter beaker. Using a 10-mL wide-bore pipet or fire-polished glass tube (approximately 2-3 mm inside diameter), place one or two juveniles into as many plastic cups as there are test chambers (including reference toxicant chambers). These cups should contain enough clean dilution seawater to maintain water quality and temperature during the transfer process (approximately 50 mL per cup). When each of the cups contains one or two juveniles, repeat the process, adding mysids until each cup contains 5 animals.

12.10.3.2.2 Carefully pour or pipet off excess water in the cups, leaving less than 5 mL with the test mysids. This 5 mL volume can be estimated visually after initial measurements. Carefully pour or pipet the juveniles into the test chambers immediately after reducing the water volume. Gently rocking the water back and forth before pouring may help prevent juveniles from clinging to the walls of the randomization cups. Juveniles can become trapped in drops; have a squirt bottle ready to gently rinse down any trapped mysids. If more than 5 mLs of water are added to the test solution with the juveniles, report the amount on the data sheet. Be sure that all water used in culture, transfer, and test solutions is within 1EC of the test temperature. Because of the small volumes involved in the transfer process, temperature control is best accomplished in a constant-temperature room.

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

12.10.4 LIGHT, PHOTOPERIOD, SALINITY AND TEMPERATURE

12.10.4.1 The light quality and intensity should be at ambient laboratory conditions are generally adequate. Light intensity should be 10-20 µE/m^2/s, or 50 to 100 foot candles (ft-c), with a 16 h light and 8 h dark cycle. A 30 minute phase-in/out period is recommended.

12.10.4.2 The water temperature in the test chambers should be maintained at 13 or 15 ± 1EC. It is critical that the test water temperature be maintained at 13 ± 1EC (for mysids collected north of Pt. Conception, California) or 15 ± 1EC (for mysids collected south of Pt. Conception, California). If a water bath is used to maintain the test temperature, the water depth surrounding the test cups should be as deep as possible without floating the chambers.

12.10.4.3 The test salinity should be in the range of 34 ± 2‰. The salinity should vary by no more than ±2‰ among the chambers on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.

12.10.4.4 Rooms or incubators with high volume ventilation should be used with caution because the volatilization of the test solutions and evaporation of dilution water may cause wide fluctuations in salinity. Covering the test chambers with clean polyethylene plastic may help prevent volatilization and evaporation of the test solutions.

12.10.5 DISSOLVED OXYGEN (DO) CONCENTRATION

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

12.10.6 FEEDING

12.10.6.1 ***Artemia*** nauplii are prepared as described above.

12.10.6.2 The feeding rates in the test beakers should be closely controlled to avoid overfeeding and fouling of test

solutions. Add 40 newly hatched ***Artemia*** nauplii per mysid per day. ***Artemia*** nauplii should be well rinsed with clean seawater and concentrated so that no more than one mL of seawater is added during feeding. (Use a 100-µm-mesh screen tube for rinsing and concentrating the nauplii; see Section 12.6.24.3). Test performance may be enhanced by feeding half the ration twice daily. If mysids die during the course of the experiment, the ration should be reduced proportionally. The mysids should not be fed on day 7.

12.10.7 DAILY CLEANING OF TEST CHAMBERS

12.10.7.1 Before the renewal of test solutions, uneaten and dead ***Artemia***, dead mysids and other debris are removed from the bottom of the test chambers with a pipette. As much of the uneaten ***Artemia*** as possible should be removed from each chamber to ensure that the mysids eat primarily newly hatched nauplii. By placing the test chambers on a light box, inadvertent removal of live mysids can be greatly reduced because they can be more easily seen. If a mysid is lost during siphoning, note the test chamber from it came, and reduce the initial count from five to four for that chamber when calculating survival at the end of the test.

12.10.8 OBSERVATIONS DURING THE TEST

12.10.8.1 Routine Chemical and Physical Observations

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

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

12.10.8.1.3 Record all the measurements on the data sheet.

12.10.8.2 Routine Biological Observations

12.10.8.2.1 The number of live mysids are counted and recorded each day. Dead animals and excess food should be removed with a pipette before test solutions are renewed. This is necessary to avoid cannibalism and to prevent fouling of test solutions.

12.10.8.2.2 Protect the mysids from unnecessary disturbance during the test by carrying out the daily test observations, solution renewals, and removal of the dead mysids, carefully. Make sure the mysids remain immersed during the performance of the above operations.

12.10.9 TEST SOLUTION RENEWAL

12.10.9.1 The test duration is 7 days. Because effluent toxicity may change over short time periods in test chambers, the test solutions must be renewed after 48 h and 96 h. Prepare renewal test solutions in the same way as initial test solutions. Remove three quarters of the original test solution from each chamber, taking care to avoid losing or damaging mysids. This can be done by siphoning with a small-bore (2 to 3 mm) fire-polished glass tube or pipet. Attach the glass tube to clear plastic tubing fitted with a pinch clamp so that the siphon flow can be stopped quickly if necessary to release entrained mysids. It is convenient to siphon old solutions into a small (500 mL) chamber in order to check to make sure that no mysids have been inadvertently removed during solution renewals. If a mysid is siphoned, return it to the test chamber and note it on the data sheet. Follow the chamber randomization sheet to siphon first from the controls, then work sequentially to the highest test concentration to avoid cross-contamination.

12.10.9.2 To minimize disturbance to the juvenile mysids, refill the chambers to the 200-mL mark by carefully siphoning new test solution into the test chambers using small diameter plastic tubing attached to a bent clean glass rod that directs incoming solution upward or to the side to slow the current and minimize turbulence.

12.10.9.3 The effluent or receiving water used in the test is stored in an incubator or refrigerator at 4EC. Plastic chambers such as 8-20 L cubitainers have proven suitable for effluent collection and storage. For on-site toxicity studies no more than 24 h should elapse between collection of the effluent and

use in a toxicity test (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

12.10.9.4 Approximately 1 h before test initiation, a sufficient quantity of effluent or receiving water sample is warmed to 13 ± 1EC or 15 ± 1EC to prepare the test solutions. A sufficient quantity of effluent should be warmed to make the test solutions.

12.10.10 TERMINATION OF THE TEST

12.10.10.1 Ending the Test

12.10.10.1.1 Record the time the test is terminated.

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

12.10.10.1.3 On the last day of the test, examine each test chamber, and remove and record any dead mysids. Sum the cumulative total of all mortalities observed in each test chamber over the 7 days of the test, subtract this from the initial number of mysids (5), and verify the number of survivors. Immobile mysids that do not respond to a stimulus are considered dead. The stimulus should be two or three gentle prods with a disposable pipet. Mysids that exhibit any response clearly visible to the naked eye are considered living. The most commonly observed movement in moribund mysids is a quick contraction of the abdomen. This or any other obvious movement qualifies a mysid as alive.

12.10.10.2 Weighing

12.10.10.2.1 To prepare mysids for weighing at the end of the exposure period, remove any remaining dead mysids, then carefully pour the contents of the test chamber through a small mesh screen (<300µm). Count the mysids before screening, and take care to keep track of them on the screen. Make sure mortality counts have already been recorded. Briefly dip the screen containing the mysids in deionized water to rinse away the salt. Using fine point forceps, carefully transfer the mysids from the screen to a preweighed and labelled micro-weigh boat. Carefully fold the

foil weigh boats over the mysids to avoid loss while drying test organisms.

12.10.10.2.2 To prepare weigh boats prior to testing, write the test chamber number on each with a fine felt-tipped marker, dry the ink and weigh boat in a drying oven, allow the dry weigh boats to cool in a desiccator, weigh the weigh boats to the nearest 1 microgram (µg) on a microbalance, and record the weight and chamber number on the data sheet. Place the weighed weigh boats in a clean ziplock bag until ready to use for weighing mysids. The juvenile mysids are very small, and light (60 µg) relative to the weigh boats (4 mg). Take all precautions to make sure weigh boats remain clean and dry during weighing and subsequent storage, so that mysid weights may be accurately determined by subtraction.

12.10.10.2.3 When all mysids are loaded onto weigh boats, arrange them all in a dish, small tray or other small open chamber, and place them in a clean drying oven. Dry for at least 24 hours at 60EC or for at least 6 hours at 105EC. Remove the weigh boats with mysids from the drying oven and place them in a desiccator to cool for one hour. When cool, carefully weigh each weigh boat on a microbalance (accurate to 1 µg). Record the chamber number, mysid weight, weigh boat weight (recorded previously), and number of mysids per weigh boat (replicate) on the data sheet.

12.10.10.3 Endpoint

12.10.10.3.1 Growth is measured as dry weight of surviving mysids. All surviving mysids from a single replicate test chamber are pooled together and weighed, then this total weight is divided by the number of original mysids to obtain the mean dry weight per individual for each replicate, which is used for statistical analysis.

12.10.10.3.2 The percentage of surviving mysids in each chamber at the end of the test will be used for subsequent statistical analysis.

12.11 **SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA**

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

12.12 **ACCEPTABILITY OF TEST RESULTS**

12.12.1 Test results are acceptable only if all the following requirements are met:

(1) Control survival must be at least 75%.

(2) The average weight of control mysids must be at least 40 µg per mysid.

(3) Between replicate variability in the mortality data must be low enough that the minimum significant difference (%MSD) is less than 40% in the reference toxicant test.

(4) Between replicate variability in the weight data must be low enough that the %MSD is less than 50 µg in the reference toxicant test.

(5) Both the mortality NOEC and LC50 must be less than 100 µg/L zinc in the reference toxicant test.

12.13 **DATA ANALYSIS**

12.13.1 GENERAL

12.13.1.1 Tabulate and summarize the data. Table 4 presents a sample set of survival and growth data.

12.13.1.2 The endpoints of the mysid 7-day chronic test are based on the adverse effects on survival and growth. The LC50 and the IC25 are calculated using point estimation techniques (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). LOEC and NOEC values for survival and growth are obtained using a hypothesis testing approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981) (see Section 9). Separate analyses are

performed for the estimation of the LOEC and NOEC endpoints and for the estimation of the LC50 and IC25. Concentrations at which there is no survival in any of the test chambers are excluded from the statistical analysis of the NOEC and LOEC for survival and growth, but included in the estimation of the LC50 and IC25. See the Appendices for examples of the manual computations, and examples of data input and program output.

12.13.1.3 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

12.13.2 EXAMPLE OF ANALYSIS OF MYSID, ***HOLMESIMYSIS COSTATA***, SURVIVAL DATA

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

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

12.13.2.3 If equal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR THE MYSID, ***HOLMESIMYSIS COSTATA***, GROWTH AND SURVIVAL TEST WITH EFFLUENTS AND RECEIVING WATERS

1. Test type:	Static-renewal
2. Salinity:	34 ± 2‰
3. Temperature:	13 ± 1EC (mysids collected north of Pt. Conception) 15 ± 1EC (mysids collected south of Pt. Conception)
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 µE/m^2/s (Ambient laboratory illumination)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber:	1000 mL
8. Test solution volume:	200 mL
9. Renewal of test solutions:	75% renewal at 48 and 96 hours
10. Age of test organisms:	3 to 4 days post-hatch juveniles
11. No. organisms per test chamber:	5
12. No. replicate chambers per concentration:	5
13. No. mysids per concentration:	25
14. Source of food:	Newly hatched ***Artemia*** nauplii (less than 24 h old)
15. Feeding regime:	Feed 40 nauplii per larvae daily (dividing into morning and evening feedings)

16. Cleaning:	Siphon during test solution renewal
17. Aeration:	None unless DO falls below 4.0 mg/L, then gently aerate in all cups
18. Dilution water:	Uncontaminated 1-µm-filtered natural seawater or hypersaline brine prepared from natural seawater
19. Test concentrations:	Effluents: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
20. Dilution factor:	Effluents: **$**0.5 series Receiving waters: None, or **$**0.5
21. Test duration:	7 days
22. Endpoints:	Survival and growth
23. Test acceptability criteria:	**$**75% survival, average dry weight **$** 0.40 µg in the controls; survival MSD <40%; growth MSD <50 µg; and both survival and growth NOECs must be less than 100 µg/L with zinc
24. Sampling requirements:	For on-site tests, samples must be used within 24 h of the time they are removed from the sampling device (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests)
25. Sample volume required:	2 L per renewal

TABLE 4. DATA FOR HOLMESIMYSIS COSTATA 7-DAY SURVIVAL AND GROWTH TEST[1]

Treatment	Replicate Chamber	Total Mysids	No. Alive	Prop. Alive	Mean Weight
Control, Brine	1	5	5	1.00	0.051
	2	5	5	1.00	0.050
	3	5	5	1.00	0.040
	4	5	5	1.00	0.064
	5	5	5	1.00	0.039
Control, Dilution	1	5	5	1.00	0.048
	2	5	5	1.00	0.058
	3	5	5	1.00	0.047
	4	5	5	1.00	0.058
	5	5	5	1.00	0.051
1.80%		1	5	5	1.00
					0.055
	2	5	5	1.00	0.048
	3	5	5	1.00	0.042
	4	5	4	0.80	0.041
	5	5	5	1.00	0.052
3.20%		1	5	5	1.00
					0.057
	2	5	4	0.80	0.050
	3	5	5	1.00	0.046

t test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative.

12.13.2.4 Probit Analysis (Finney, 1971; see Appendix G) is used to estimate the concentration that causes a specified percent decrease in survival from the control. In this analysis, the total mortality data from all test replicates at a given concentration are combined. If the data do not fit the Probit model, the Spearman-Karber method, the Trimmed Spearman-Karber

method, or the Graphical method may be used to estimate the LC50 (see Appendices H-K).

12.13.2.5 The proportion of survival in each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix B. The raw and transformed data, means and variances of the transformed observations at each concentration including the control are listed in Table 5. A plot of the survival data is provided in Figure 3.

12.13.2.6 Test for Normality

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

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

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

Where: $\&_i$ = the ith centered observation

$\&$ = the overall mean of the centered observations

n = the total number of centered observations.

12.13.2.6.3 For this set of data, n = 25

$$\& = \frac{1}{25}(0.001) = 0.00$$

D = 0.227

12.13.2.6.4 Order the centered observations from smallest to largest:

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

Where $X^{(i)}$ is the ith ordered observation. These ordered observations are listed in Table 7.

TABLE 5. MYSID, HOLMESIMYSIS COSTATA, SURVIVAL DATA

	Replicate	Control	Concentration (%) 1.80	3.20	5.60	10.00
	1	1.00	1.00	1.00	0.80	0.20
	2	1.00	1.00	0.80	1.00	0.00
RAW	3	1.00	1.00	1.00	1.00	0.00
	4	1.00	0.80	1.00	0.80	0.00
	5	1.00	1.00	0.80	0.80	0.00
ARC SINE	1	1.345	1.345	1.345	1.107	0.464
SQUARE	2	1.345	1.345	1.107	1.345	0.225
ROOT	3	1.345	1.345	1.345	1.345	0.225
TRANS-	4	1.345	1.107	1.345	1.107	0.225
FORMED	5	1.345	1.345	1.107	1.107	0.225
Mean($\bar{x}_i$)		1.345	1.297	1.250	1.202	0.273
S_i^2		0.000	0.011	0.017	0.017	0.011
i		1	2	3	4	5

12.13.2.6.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2,...., a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 25 and k = 12. The a_i values are listed in Table 8.

12.13.2.6.6 Compute the test statistic, W, as follows:

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

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 8.

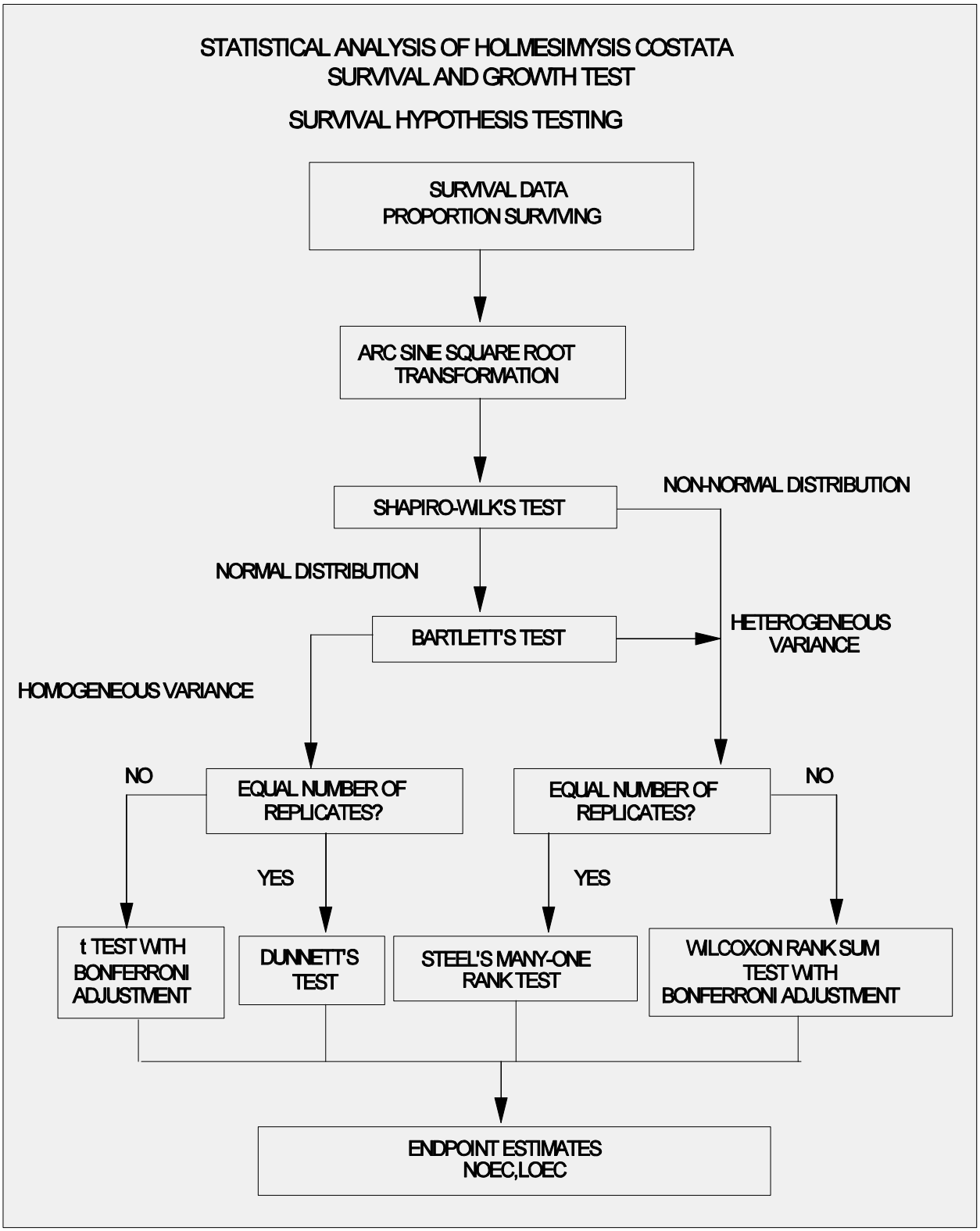

Figure 1. Flowchart for statistical analysis of mysid, ***Holmesimysis costata***, survival data by hypothesis testing.

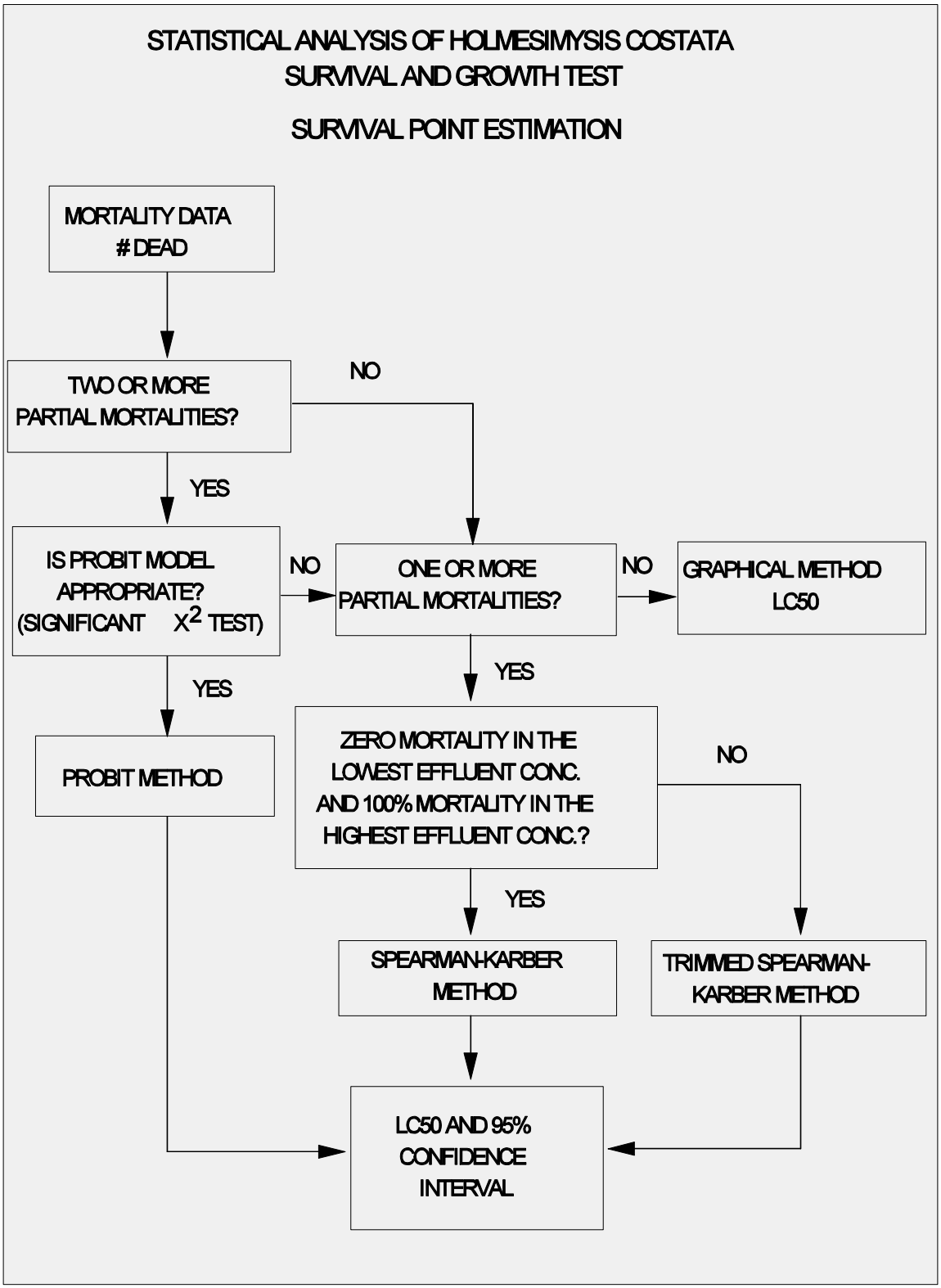

Figure 2.Flowchart for statistical analysis of mysid, *Holmesimysis costata*, survival data by point estimation.

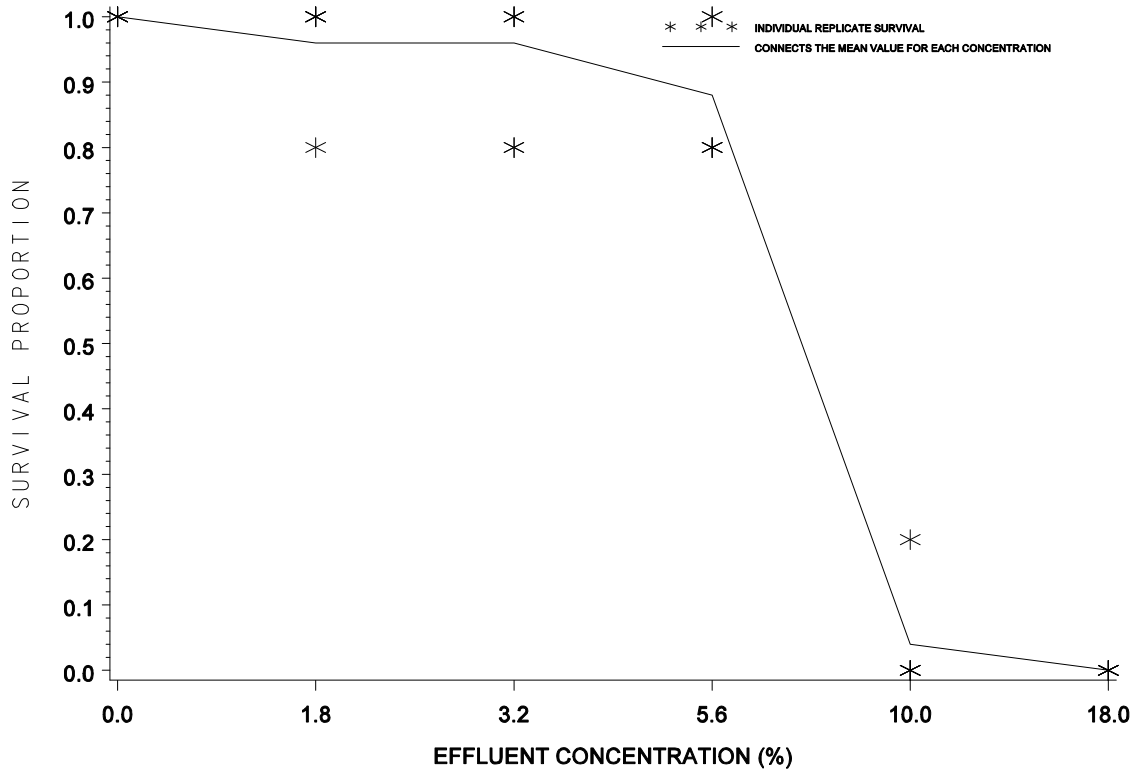

Figure 3. Plot of survival of mysids, ***Holmesimysis costata***, at each treatment

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

Replicate	Control (Dilution)	Concentration 1.80	3.20	5.60	10.00
1	0.000	0.048	0.095	-0.095	0.191
2	0.000	0.048	-0.143	0.143	-0.048
3	0.000	0.048	0.095	0.143	-0.048
4	0.000	-0.190	0.095	-0.095	-0.048
5	0.000	0.048	-0.143	-0.095	-0.048

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

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.190	14	0.000
2	-0.143	15	0.000
3	-0.143	16	0.048
4	-0.095	17	0.048
5	-0.095	18	0.048
6	-0.095	19	0.048
7	-0.048	20	0.095
8	-0.048	21	0.095
9	-0.048	22	0.095
10	-0.048	23	0.143
11	0.000	24	0.143
12	0.000	25	0.191
13	0.000		

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

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4450	0.381	$X^{(25)} - X^{(1)}$
2	0.3069	0.286	$X^{(24)} - X^{(2)}$
3	0.2543	0.286	$X^{(23)} - X^{(3)}$
4	0.2148	0.190	$X^{(22)} - X^{(4)}$
5	0.1822	0.190	$X^{(21)} - X^{(5)}$
6	0.1539	0.190	$X^{(20)} - X^{(6)}$
7	0.1283	0.096	$X^{(19)} - X^{(7)}$
8	0.1046	0.096	$X^{(18)} - X^{(8)}$
9	0.0823	0.096	$X^{(17)} - X^{(9)}$
10	0.0610	0.096	$X^{(16)} - X^{(10)}$
11	0.0403	0.000	$X^{(15)} - X^{(11)}$
12	0.0200	0.000	$X^{(14)} - X^{(12)}$

For this data in this example:

$$W = \frac{1}{0.227} (0.4708)^2 = 0.976$$

12.13.2.6.7 The decision rule for this test is to compare W as calculated in Subsection 6.6 with the critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For this set of data, the critical value at a significance level of 0.01 and n = 25 observations is 0.888. Since W = 0.976 is greater than the critical value, conclude that the data are normally distributed.

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

12.13.2.6.9 Since the data do not meet the assumption of homogeneity of variance, Steel's Many-one Rank Test will be used to analyze the survival data.

12.13.2.7 Steel's Many-one Rank Test

12.13.2.7.1 For each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign the ranks (1, 2, ... , 10) to the ordered observations with a rank of 1 assigned to the smallest observation, rank of 2 assigned to the next larger observation, etc. If ties occur when ranking, assign the average rank to each tied observation.

12.13.2.7.2 An example of assigning ranks to the combined data for the control and 1.80% concentration is given in Table 9. This ranking procedure is repeated for each control/concentration combination. The complete set of rankings is summarized in Table 10. The ranks are then summed for each concentration level, as shown in Table 11.

12.13.2.7.3 For this example, determine if the survival in any of the concentrations is significantly lower than the survival in the control. If this occurs, the rank sum at that concentration would be significantly lower than the rank sum of the control. Thus compare the rank sums for the survival at each of the various concentration levels with some "minimum" or critical rank sum, at or below which the survival would be considered significantly lower than the control. At a significance level of 0.05, the minimum rank sum in a test with four concentrations (excluding the control) is 17(See Table 5, Appendix E).

12.13.2.8.1 The data used to calculate the LC50 is summarized in Table 12. For this example, although there are two concentrations with partial mortalities, the chi-square test for heterogeneity was significant, indicating that Probit Analysis is inappropriate for this set of data. Inspection of the data reveals that the smoothed, adjusted proportion mortality for the lowest concentration will not be zero, indicating that the Trimmed Spearman-Karber Method is recommended to calculated the LC50 for this dataset.

12.13.2.8.2 For the Trimmed Spearman-Karber analysis, run the USEPA Trimmed Spearman-Karber program, TSK. An example of the program output is provided in Figure 4.

TABLE 9. ASSIGNING RANKS TO THE CONTROL AND 1.80% CONCENTRATION LEVEL FOR STEEL'S MANY-ONE RANK TEST

Rank	Transformed Proportion of Total Mortality	Concentration
1	1.107	1.80%
6	1.345	Control
6	1.345	Control
6	1.345	Control
6	1.345	Control
6	1.345	Control
6	1.345	1.80%
6	1.345	1.80%
6	1.345	1.80%
6	1.345	1.80%

TABLE 10. TABLE OF RANKS[1]

		Concentration (%)			
Replicate	Control	1.80	3.20	5.60	10.0
1	1.345(6,6.5,7,8)	1.345(6)	1.345(6.5)	1.107(2)	0.464(5)
2	1.345(6,6.5,7,8)	1.345(6)	1.107(1.5)	1.345(7)	0.225(2.5)
3	1.345(6,6.5,7,8)	1.345(6)	1.345(6.5)	1.345(7)	0.225(2.5)
4	1.345(6,6.5,7,8)	1.107(1)	1.345(6.5)	1.107(2)	0.225(2.5)
5	1.345(6,6.5,7,8)	1.345(6)	1.107(1.5)	1.107(2)	0.225(2.5)

[1]*Control ranks are given in the order of the concentration with which they were ranked.*

TABLE 11. RANK SUMS

Concentration	Rank Sum
1.80	25.0
3.20	22.5
5.60	20.0
10.00	15.0

TABLE 12. DATA FOR TRIMMED SPEARMAN-KARBER ANALYSIS

		Concentration (%)				
	Control	*1.80*	*3.20*	*5.60*	*10.0*	*18.0*
No Dead	*0*	*1*	*2*	*3*	*24*	*25*
No Exposed	*25*	*25*	*25*	*25*	*25*	*25*

12.13.3 EXAMPLE OF ANALYSIS OF MYSID, ***HOLMESIMYSIS COSTATA*** ***G***ROWTH DATA

12.13.3.1 Formal statistical analysis of the growth data is outlined in Figure 5. The response used in the statistical analysis is mean weight per surviving organism per replicate. The IC25 can be calculated for the growth data via a point

TRIMMED SPEARMAN-KARBER METHOD. VERSION 1.5

DATE: TEST NUMBER: 1 DURATION: 7 days

TOXICANT : Effluent
SPECIES: Holmesimysis costata

RAW DATA:	*Concentration (%)*	*Number Exposed*	*Mortalities*
---	*----*		
	.00	*25*	*0*
	1.80	*25*	*1*
	3.20	*25*	*2*
	5.60	*25*	*3*
	10.00	*25*	*24*
	18.00	*25*	*25*

SPEARMAN-KARBER TRIM: 4.00%

SPEARMAN-KARBER ESTIMATES: LC50: 6.95
95% LOWER CONFIDENCE: 6.22
95% UPPER CONFIDENCE: 7.76

Figure 4. Output for USEPA Trimmed Spearman-Karber Program, version 1.5.

estimation technique (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). Hypothesis testing can be used to obtain an NOEC and LOEC for growth. Concentrations above the NOEC for survival are excluded from the hypothesis test for growth effects.

12.13.3.2 The statistical analysis using hypothesis tests consists of a parametric test, Dunnett's Procedure, and a nonparametric test, Steel's Many-one Rank Test. The underlying

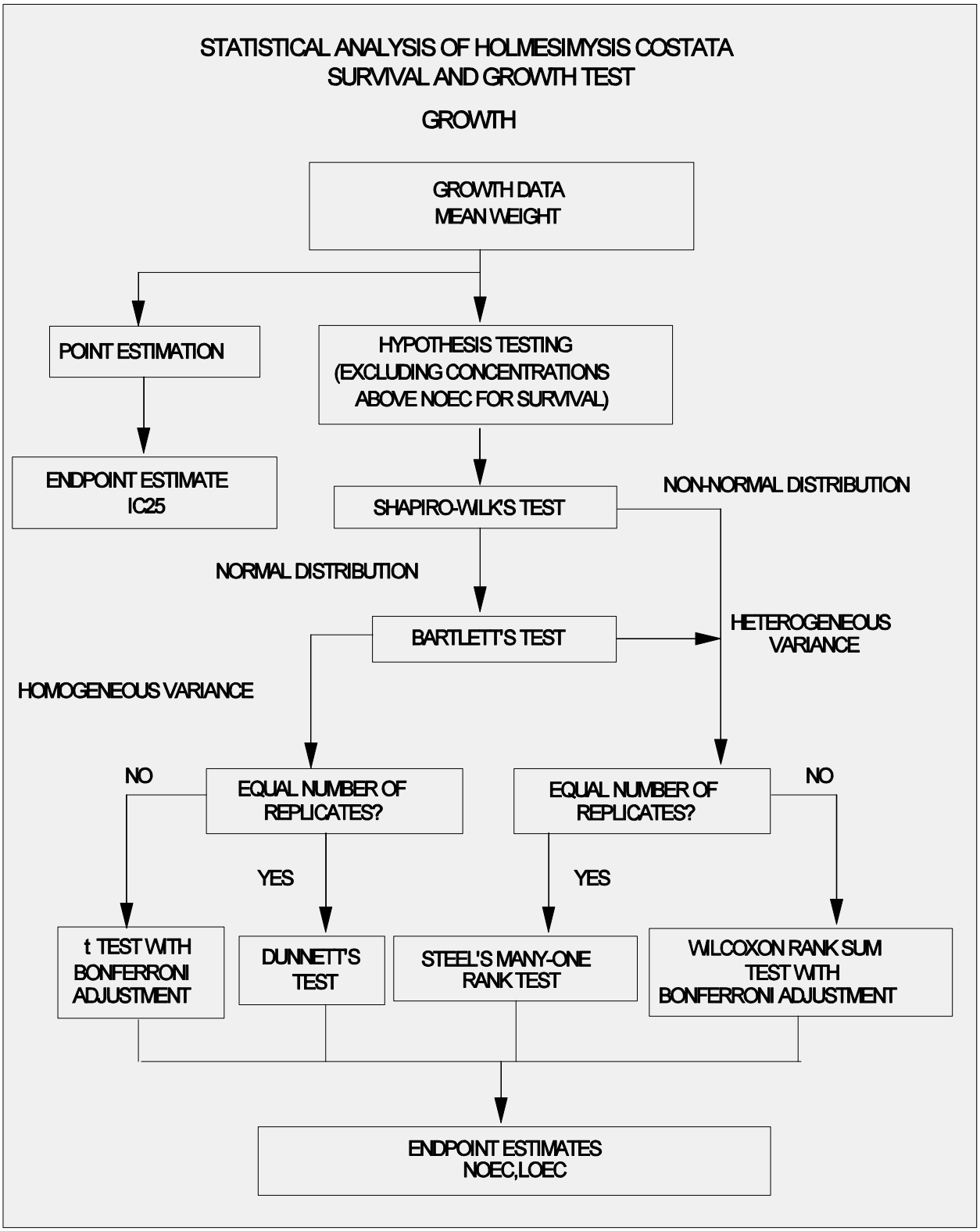

Figure 5. Flowchart for statistical analysis of mysid, *Holmesimysis costata*, growth data.

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

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

12.13.3.4 The data, mean and variance of the observations at each concentration including the control for this example are listed in Table 13. A plot of the data is provided in Figure 6. Since there is significant mortality in the 10.0% concentration, its effect on growth is not considered.

12.13.3.5 Test for Normality

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

12.13.3.5.2 Calculate the denominator, D, of the statistic:

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

TABLE 13. MYSID, HOLMESIMYSIS COSTATA, GROWTH DATA

Replicate	Control	Concentration (%) 1.80	3.20	5.60	10.0
1	0.048	0.055	0.057	0.041	0.033
2	0.058	0.048	0.050	0.040	0.000
3	0.047	0.042	0.046	0.041	0.000
4	0.058	0.041	0.043	0.043	0.000
5	0.051	0.052	0.045	0.040	0.000
$Mean(\bar{Y}_i)$	0.052	0.048	0.048	0.041	0.007
S_i^2	0.0000283	0.0000373	0.0000307	0.0000015	0.000218
i	1	2	3	4	5

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

Replicate	Control	Concentration (%) 1.80	3.20	5.60
1	-0.004	0.007	0.009	0.000
2	0.006	0.000	0.002	-0.001
3	-0.005	-0.006	-0.002	0.000
4	0.006	-0.007	-0.005	0.002
5	-0.001	0.004	-0.003	-0.001

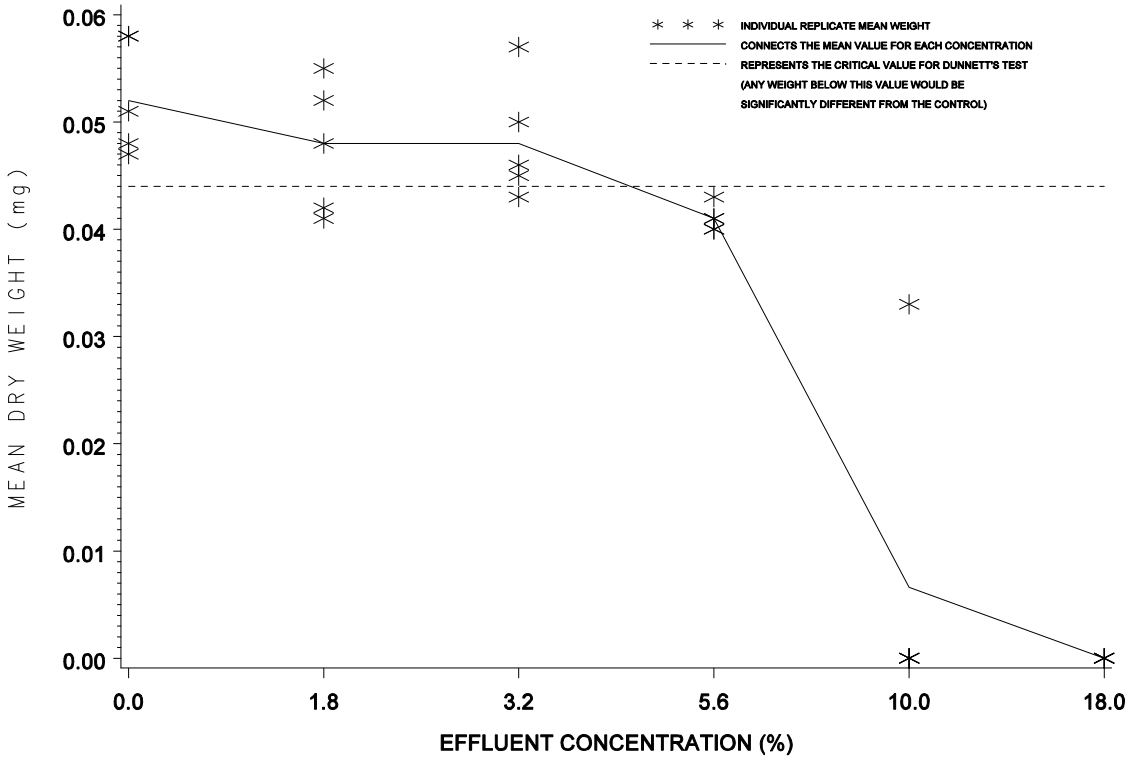

Figure 6. Plot of growth data for mysid, ***Holmesimysis costata***, test.

Where: X_i = the ith centered observation

& = the overall mean of the centered observations

n = the total number of centered observations

12.13.3.5.3 For this set of data, n = 20

$$\text{\&} = \frac{1}{20}(0.001) = 0.000$$

D = 0.000393

12.13.3.5.4 Order the centered observations from smallest to largest

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

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 15.

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

i	$X^{(i)}$	*i*	$X^{(i)}$
1	*-0.007*	*11*	*0.000*
2	*-0.006*	*12*	*0.000*
3	*-0.005*	*13*	*0.000*
4	*-0.005*	*14*	*0.002*
5	*-0.004*	*15*	*0.002*
6	*-0.003*	*16*	*0.004*
7	*-0.002*	*17*	*0.006*
8	*-0.001*	*18*	*0.006*
9	*-0.001*	*19*	*0.007*
10	*-0.001*	*20*	*0.009*

12.13.3.5.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2, ... a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 20 and k = 10. The a_i values are listed in Table 16.

12.13.3.5.6 Compute the test statistic, W, as follows:

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

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

$$W = \frac{1}{0.000393} (0.0194)^2 = 0.958$$

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

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	*0.4734*	*0.016*	$X^{(20)} - X^{(1)}$
2	*0.3211*	*0.013*	$X^{(19)} - X^{(2)}$
3	*0.2565*	*0.011*	$X^{(18)} - X^{(3)}$
4	*0.2085*	*0.011*	$X^{(17)} - X^{(4)}$
5	*0.1686*	*0.008*	$X^{(16)} - X^{(5)}$
6	*0.1334*	*0.005*	$X^{(15)} - X^{(6)}$
7	*0.1013*	*0.004*	$X^{(14)} - X^{(7)}$
8	*0.0711*	*0.001*	$X^{(13)} - X^{(8)}$
9	*0.0422*	*0.001*	$X^{(12)} - X^{(9)}$
10	*0.0140*	*0.001*	$X^{(11)} - X^{(10)}$

12.13.3.5.7 The decision rule for this test is to compare W as calculated in Subsection 12.13.3.5.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For this set of data, the critical value at a significance level of

0.01 and n = 20 observations is 0.868. Since W = 0.958 is greater than the critical value, conclude that the data are normally distributed.

12.13.3.6 Test for Homogeneity of Variance

12.13.3.6.1 The test used to examine whether the variation in mean weight of the mysids is the same across all concentration levels including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{[(\sum_{i=1}^{p} V_i) \ln \overline{S}^2 - \sum_{i=1}^{p} V_i \ln S_i^2]}{C}$$

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

p = number of concentration levels including the control

ln = $\log_e$

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

n_i = the number of replicates for concentration i.

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

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

12.13.3.6.2 For the data in this example (See Table 13), all concentrations including the control have the same number of replicates (n_i = 5 for all i). Thus, V_i = 4 for all i.

12.13.3.6.3 Bartlett's statistic is therefore:

$$B = [(16)\ln(0.0000245) - 4\sum_{i=1}^{p}\ln(S_i^2)]/1.104$$

$$= [16(-10.617) - 4(-44.470)]/1.104$$

$$= [-169.872 - (-177.880)]/1.104$$

$$= 7.254$$

12.13.3.6.4 B is approximately distributed as chi-square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with three degrees of freedom, is 9.210. Since B = 7.254 is less than the critical value of 9.210, conclude that the variances are not different.

12.13.3.7 Dunnett's Procedure

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

TABLE 17. ANOVA TABLE

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

Where: p = number of concentration levels including the control

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

n_i = number of observations in concentration i

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

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

$$SSW = SST - SSB$$ Within Sum of Squares

G = the grand total of all sample observations,

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

T_i = the total of the replicate measurements for concentration i

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

12.13.3.7.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = 5$

$N = 20$

$T_1 = Y_{11} + Y_{12} + \ldots + Y_{15} = 0.262$
$T_2 = Y_{21} + Y_{22} + \ldots + Y_{25} = 0.238$
$T_3 = Y_{31} + Y_{32} + \ldots + Y_{35} = 0.241$
$T_4 = Y_{41} + Y_{42} + \ldots + Y_{45} = 0.205$
$G = T_1 + T_2 + T_3 + T_4 = 0.946$

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

$$= \frac{1}{5}(0.225) - \frac{(0.946)^2}{20} = 0.000254$$

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

$$= 0.0455 - \frac{(0.946)^2}{} = 0.000754$$

$SSW = SST - SSB$

$= 0.000754 - 0.000254 = 0.000500$

$S_B^2 = SSB/(p-1) = 0.000254/(4-1) = 0.0000847$

$S_W^2 = SSW/(N-p) = 0.000500/(20-4) = 0.0000313$

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

TABLE 18. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	*df*	*Sum of Squares (SS)*	*Mean Square (MS) (SS/df)*
Between	*3*	*0.000254*	*0.0000847*
Within	*16*	*0.000500*	*0.0000313*
Total	*19*	*0.000754*	

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

$$t_i = \frac{(\overline{Y_1} - \overline{Y_i})}{S_w\sqrt{(1/n_1) + (1/n_i)}}$$

Where: $\overline{Y}_i$ = mean weight for concentration i

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

S_W = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i

12.13.3.7.5 Table 19 includes the calculated t values for each concentration and control combination. In this example, comparing the 1.80% concentration with the control the calculation is as follows:

$$t_2 = \frac{(0.052 - 0.048)}{[0.00559\sqrt{(1/5)+(1/5)}]}$$

$$= 1.131$$

TABLE 19. CALCULATED t VALUES

Concentration (ppb)	*i*	t_i
1.80	*2*	*1.131*
3.20	*3*	*1.131*
5.60	*4*	*3.111*

12.13.3.7.6 Since the purpose of this test is to detect a significant reduction in mean weight, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix C. For an overall alpha level of 0.05, 16 degrees of freedom for error and three concentrations (excluding the control) the approximate critical value is 2.23. The mean weight for concentration "i" is considered significantly less than the mean weight for the control if t_i is greater than the critical value. Therefore, the 5.60% concentration has significantly lower mean weight than the control. Hence the NOEC and the LOEC for growth are 3.20% and 5.60%, respectively.

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

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

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

S_W = the square root of the within mean square

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

n_1 = the number of replicates in the control.

12.13.3.7.8 In this example:

$$MSD = 2.23(0.00559)\sqrt{(1/5)+(1/5)}$$

$$= 2.23\ (0.00559)(0.632)$$

$$= 0.00788$$

12.13.3.7.9 Therefore, for this set of data, the minimum difference that can be detected as statistically significant is 0.00788 mg.

12.13.3.7.10 This represents a 15.2% reduction in mean weight from the control.

12.13.3.8 Calculation of the ICp

12.13.3.8.1 The growth data from Table 13 are utilized in this example. As seen in the table, the observed means are monotonically non-increasing with respect to concentration. Therefore, the smoothed means will be simply the corresponding observed mean. The observed means are represented by $\bar{Y}_i$ and the smoothed means by M_i. Table 20 contains the smoothed means and Figure 7 gives a plot of the smoothed response curve.

12.13.3.8.2 An IC25 can be estimated using the Linear Interpolation Method. A 25% reduction in weight, compared to the controls, would result in a mean weight of 0.039 mg, where $M_1(1-p/100) = 0.052(1-25/100)$. Examining the smoothed means and their associated concentrations (Table 20), the response, 0.039 mg, is bracketed by $C_4 = 5.60\%$ and $C_5 = 10.0\%$.

12.13.3.8.5 Using the equation in Section 4.2 from Appendix L, the estimate of the IC25 is calculated as follows:

$$ICp = C_j\%[M_1(1\&p/100)\&M_j]\frac{(C_{(j\%1)}\&C_j)}{(M_{(j\%1)}\&M_j)}$$

$$IC25 = 5.60 + [0.052(1 - 25/100) - 0.041] \frac{(10.0 - 5.60)}{(0.0066 - 0.041)}$$

$$= 5.86\%.$$

12.13.3.8.7 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 5.86%. The empirical 95.0% confidence interval for the true mean was 4.9440% to 6.2553%. The computer program output for the IC25 for this data set is shown in Figure 8.

TABLE 20. MYSID, HOLMESIMYSIS COSTATA, MEAN GROWTH RESPONSE AFTER SMOOTHING

Toxicant Conc. (%)	i	Response Means Y_i (mg)	Smoothed Means M_i (mg)
Control	1	0.052	0.052
1.80	2	0.048	0.048
3.20	3	0.048	0.048
5.60	4	0.041	0.041
10.00	5	0.0066	0.0066
18.00	6	0.000	0.000

12.14 **PRECISION AND ACCURACY**

12.12.1 PRECISION

12.12.1.1 Single-Laboratory Precision

12.12.1.1.1 Data on the single laboratory precision of the ***Holmesimysis costata*** growth and survival test with zinc sulfate

are shown in Table 21. NOECs for mysid survival were either 32 or 56 µg/L Zn. There was also good agreement among LC50s, with a coefficient of variation of 14%. Mysids did not exhibit a growth response at zinc concentrations below those causing significant mortality; NOEC values for growth were always greater than or equal to the highest zinc concentration. IC50 values for growth could not be calculated.

12.12.1.2 Multi-laboratory Precision

12.12.1.2.1 The multi-laboratory data indicate a similar level of test precision (Table 22). The four multi-laboratory tests were conducted over a two year period, and each used split effluent samples tested at two laboratories. Survival NOEC values were the same for both laboratories in three of the four tests, with the NOECs varying by one concentration in the fourth test. The mean coefficient of variation between LC50 values from different laboratories was 21%. The two available comparisons of growth NOEC values indicate similar responses at both laboratories. Growth was the more sensitive indicator of toxicity in three of the four effluent tests.

12.14.2 ACCURACY

12.14.2.1 The accuracy of toxicity tests cannot be determined.

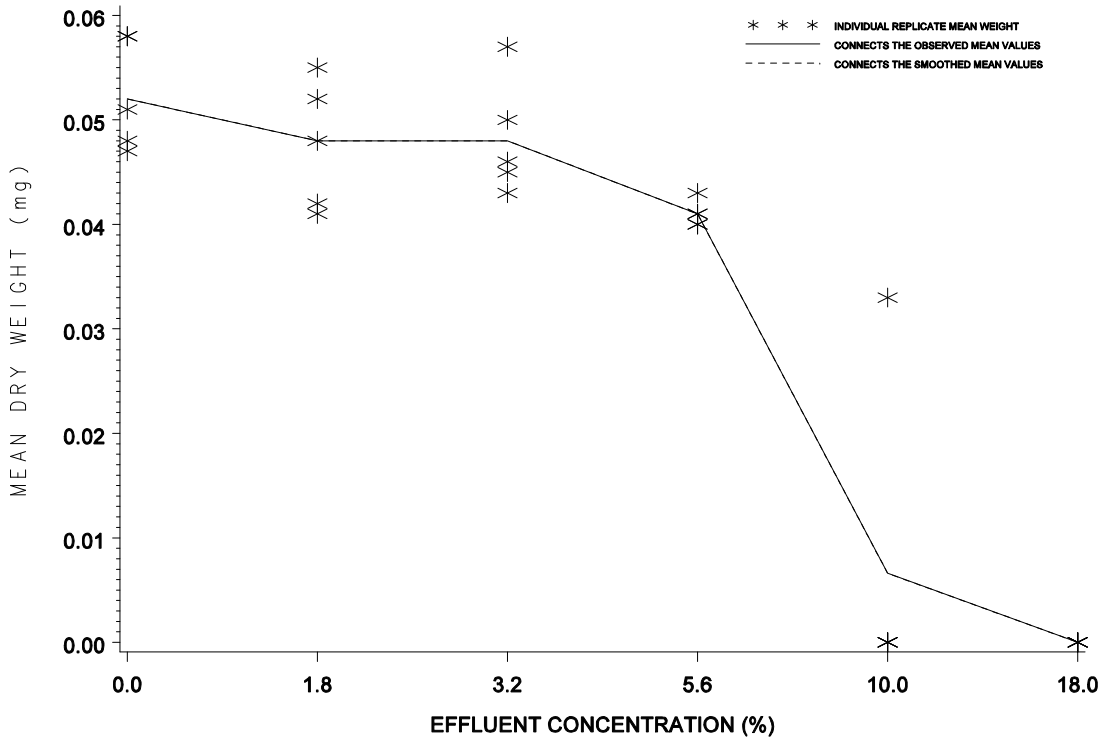

Figure 7. Plot of raw data, observed means, and smoothed means for the mysid, ***Holmesimysis costata***, Growth data from tables 13 and 20

```
Conc. ID            1         2         3         4         5         6
-----------------------------------------------------------------------
Conc. Tested        0      1.80      3.20      5.60      10.0      18.0
-----------------------------------------------------------------------
Response  1      .048      .055      .057      .041      .033         0
Response  2      .058      .048      .050      .040         0         0
Response  3      .047      .042      .046      .041         0         0
Response  4      .058      .041      .043      .043         0         0
Response  5      .051      .052      .045      .040         0         0
-----------------------------------------------------------------------
*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Effluent
Test Start Date:    Test Ending Date:
Test Species: mysid, Holmesimysis costata
Test Duration:          7 days
DATA FILE: mysid.icp
OUTPUT FILE:  mysid.i25
-----------------------------------------------------------------------
Conc.     Number     Concentration     Response      Std.       Pooled
 ID     Replicates               %       Means       Dev.   Response Means
-----------------------------------------------------------------------
  1          5             0.000          0.052      0.005      0.052
  2          5             1.800          0.048      0.006      0.048
  3          5             3.200          0.048      0.006      0.048
  4          5             5.600          0.041      0.001      0.041
  5          5            10.000          0.007      0.015      0.007
  6          5            18.000          0.000      0.000      0.000
-----------------------------------------------------------------------
The Linear Interpolation Estimate:     5.8174   Entered P Value: 25
-----------------------------------------------------------------------
Number of Resamplings:   80
The Bootstrap Estimates Mean:   5.8205 Standard Deviation:     0.2673
Original Confidence Limits:   Lower:     4.9440 Upper:     6.2553
Expanded Confidence Limits:   Lower:     4.5073 Upper:     6.4743
Resampling time in Seconds:     0.22  Random_Seed: 526805435
```

Figure 8. Output for USEPA Linear Interpolation Program for the IC25.

TABLE 21. SINGLE LABORATORY PRECISION DATA FOR THE MYSID, ***HOLMESIMYSIS COSTATA*** GROWTH AND SURVIVAL TEST WITH ZINC (ZN µG/L) SULFATE AS THE REFERENCE TOXICANT

Test	Survival		Growth
	NOEC	LC50	NOEC
1	32	47	>32
2	32	59	>32
3	56	62	>56
4	56	65	>56
N	4	4	4
Mean	44	58	>44
SD		7.9	
CV (%)		14	

No growth effect was observed in zinc concentrations below those causing significant mortality (10, 18, 32, 56 and 100 µg/L).

All tests were conducted at MPSL.

TABLE 22. MULTI-LABORATORY PRECISION DATA FOR THE MYSID, ***HOLMESIMYSIS COSTATA*** GROWTH AND SURVIVAL TEST WITH SPLIT EFFLUENT (%) ON THE SAME DATE.

Test	Effluent Type	Lab	Survival NOEC	Survival LC50	Growth NOEC
1	BKME	OSU	1.0	1.8	0.5[L]
1	BKME	MPSL	1.0	1.3	0.5[L]
			CV=26%		
2	POTW	ATL	3.2	4.1	>3.2[L]
2	POTW	MPSL	3.2	5.1	>3.2[L]
			CV=14%		
3	POTW	SRH	10.0	12.8	na
3	POTW	MPSL	10.0	11.7	3.2[W]
			CV=6%		
4	POTW	SRH	10.0	15.8	5.6[W]
4	POTW	MPSL	5.6	9.1	3.2[W]
			CV=38%		

Mean Interlaboratory CV= 21%

[L] Length was measured as the growth endpoint in tests 1 and 2,
[W] Weight was measured in test 3 and 4.
na Data was not available.

OSU is the Oregon State University Laboratory at the Hatfield Marine Science Center in Newport Oregon.

ATL is Aquatic Testing Laboratory in Ventura, California.

SRH is S.R. Hansen and Associates in Concord, California.

MPSL is the Marine Pollution Studies Laboratory near Monterey, California.

APPENDIX I. MYSID TEST: STEP-BY-STEP SUMMARY

PREPARATION OF TEST SOLUTIONS

A. Determine test concentrations and appropriate dilution water based on NPDES permit conditions and guidance from the appropriate regulatory agency.

B. Prepare effluent test solutions by diluting well mixed unfiltered effluent using volumetric flasks and pipettes. Use hypersaline brine where necessary to maintain all test solutions at 34 ± 2‰. Include brine controls in tests that use brine.

C. Prepare a zinc reference toxicant stock solution (10,000 µg/L) by adding 0.0440 g of zinc sulfate ($ZnSO_4 \cdot 7H_2O$) to 1 liter of reagent water.

D. Prepare zinc reference toxicant solution of 0 (control) 10, 18, 32, 56 and 100 µg/L by adding 0, 1.0 1.8, 3.2, 5.6 and 10.0 mL of stock solution, respectively, to a 1-L volumetric flask and filling to 1-L with dilution water.

E. Sample effluent and reference toxicant solutions for physical/chemical analysis. Measure salinity, pH and dissolved oxygen from each test concentration.

F. Randomize numbers for test chambers and record the chamber numbers with their respective test concentrations on a randomization data sheet. Store the data sheet safely until after the test samples have been analyzed.

G. Place test chambers in a water bath or environmental chamber set to 13 or 15EC and allow temperature to equilibrate.

H. Measure the temperature daily in one random replicate (or separate chamber) of each test concentration. Monitor the temperature of the water bath or environmental chamber continuously.

I. At the end of the test, measure salinity, pH, and dissolved oxygen concentration from each test concentration.

PREPARATION AND ANALYSIS OF TEST ORGANISMS

A. Four to five days prior to the beginning of the toxicity test, isolate approximately 150 gravid female mysids in a screened (2-mm-mesh) compartment within an aerated 80-liter aquarium (15EC). Add a surplus of ***Artemia*** nauplii (200 per mysid, static; 500 per mysid, flow-through) to stimulate overnight release of juveniles. Add blades of kelp as habitat.

B. Isolate the newly released juveniles by slowly siphoning into a screen-tube (150-µm-mesh, 25 cm diam.) immersed in a bucket of clean seawater. Transfer juveniles into additional screen-tubes or static 4-liter beakers at a density of approximately 50 juveniles per liter. Juveniles should be fed five to ten newly released ***Artemia*** nauplii per juvenile per day and a pinch (10 to 20 mg) of ground Tetramin® flake food per 100 juveniles per day. Maintain the juveniles for three days at 13 to 15EC, changing the water at least once in static chambers.

C. After three days, begin randomized introduction of juveniles into the test chambers. Place one or two mysids at a time into as many plastic cups as there are test chambers. Repeat the process until each cup has exactly five juvenile mysids.

D. Eliminate excess water from the cups (no more than 5 mL should remain) and pipet the mysids into the test chambers using a wide bore glass tube or pipet (approximately 3 mm ID). Make sure no mysids are left in the randomization cups. Count the number of juveniles in each test chamber to verify that each has five.

E. Remove all dead mysids daily, and add 40 newly hatched ***Artemia*** nauplii/mysid/day, adjusting feeding to account for mysid mortality.

F. At 48 and 96 hours, renew 75% of the test solution in each chamber.

G. After 7 days, count and record the number of live and dead mysids in each chamber. After counting, use the randomization sheet to assign the correct test concentration to each chamber. Remove all dead mysids.

H. Carefully pour the contents of each test chamber through a small mesh screen (<300µm). Count the mysids and record before screening. Briefly dip the screen containing the mysids in fresh water to rinse away the salt. Carefully transfer the mysids from the screen to a prenumbered, preweighed micro-weigh boat using fine-tipped forceps. Dry for 24 hours at 60EC. Weigh each weigh boat on a microbalance (accurate to 1 µg). Record the chamber number, mysid weight, weigh boat weight (recorded previously), and number of mysids per weigh boat (replicate) on the data sheet.

I. Analyze the data.

J. Include standard reference toxicant point estimate values in the standard quality control charts.

Data Sheet for Juvenile Holmesimysis Toxicity Test

Test Start Date: Start Time: Mysid Source
Test End Date: End Time: Collection/Arrival Date:
Reference Toxicant: Mysid Age at Start:
Sample Source:

Test Cont. #	Toxic Conc.	Number Alive							Total Number Alive	Total Number at Start	Notes and Initials
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7			
1											
2											
3											
4											
5											
6											
7											
8											
9											
10											
11											
12											
13											
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33											
34											
35											

Computer Data Storage
Disk:
File:

Note: See juvenile growth data on separate sheet.

Data Sheet for Weighing Juvenile Mysids

Test Start Date:

Start Time:

Mysid Source :

Test End Date:

End Time:

Collection/Arrival Date:

Reference Toxicant:

Mysid Age at Start:

Sample Source:

Sample Type:

Test Container Number	Site Code or Concentration	Foil Number	Foil Weight (µg)	Total Weight (µg)	Mysid Wt (Total - Foil) (mg)	Number of Mysids	Weight per Mysid (µg)
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
14							
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33							
34							
35							

Computer Data Storage

Disk:

File:

Note: See mysid mortality data on separate sheet.

SECTION 13

PACIFIC OYSTER, *Crassostrea gigas* AND MUSSEL, *Mytilus sp.* EMBRYO-LARVAL DEVELOPMENT TEST METHOD

Adapted from a method developed by
Gary A. Chapman, U.S. EPA, ORD Newport, OR
and Debra L. Denton, U.S. EPA, Region IX

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13.8 Calibration and Standardization
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13.10 Test Procedures
13.11 Summary of Test Conditions and Test Acceptability Criteria
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Appendix I Step-by Step Summary

SECTION 13

PACIFIC OYSTER, *CRASSOSTREA GIGAS*, AND MUSSEL, *MYTILUS SPP.* EMBRYO-LARVAL DEVELOPMENT TEST

13.1 SCOPE AND APPLICATION

13.1.1 This method estimates the chronic toxicity of effluents and receiving waters to the embryos and larvae of several bivalve molluscs, the Pacific oyster (*Crassostrea gigas*) and the mussels (*Mytilus edulis*, *M. californianus*, *M. galloprovincialis*, or *M. trossulus*) in a 48-h static non-renewal exposure. The effects include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms.

13.1.2 Detection limits of the toxicity of an effluent or chemical substance are organism dependent.

13.1.3 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling, and because the test chambers are not sealed, highly volatile and highly degradable toxicants in the source may not be detected in the test.

13.1.4 This test is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

13.1.5 This method should be restricted to use by, or under the supervision of, professionals experienced in aquatic toxicity testing. Specific experience with any toxicity test is usually needed before acceptable results become routine.

13.2 SUMMARY OF METHOD

13.2.1 The method provides step-by-step instructions for performing a 48-h static non-renewal toxicity test using embryos and larvae of the test species to determine the toxicity of

substances in marine and estuarine waters. The test endpoint is normal shell development and should include mortality as a measure of adverse effect.

13.3 **INTERFERENCES**

13.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities and Equipment, and Supplies).

13.3.2 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests).

13.4 **SAFETY**

13.4.1 See Section 3, Health and Safety

13.5 **APPARATUS AND EQUIPMENT**

13.5.1 Tanks, trays, or aquaria -- for holding and acclimating adult pacific oysters and mussels, e.g., standard salt water aquarium or Instant Ocean Aquarium (capable of maintaining seawater at 10-20EC), with appropriate filtration and aeration system.

13.5.2 Air pump, air lines, and air stones -- for aerating water containing broodstock or for supplying air to test solutions with low dissolved oxygen.

13.5.3 Constant temperature chambers or water baths -- for maintaining test solution temperature and keeping dilution water supply, gametes, and embryo stock suspensions at test temperature prior to the test.

13.5.4 Water purification system -- Millipore Super-Q, Deionized water (DI) or equivalent.

13.5.5 Refractometer -- for determining salinity.

13.5.6 Hydrometer(s) -- for calibrating refractometer.

13.5.7 Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

13.5.8 Thermometer, National Bureau of Standards Certified (see USEPA METHOD l70.l, USEPA, l979) -- to calibrate laboratory thermometers.

13.5.9 pH and DO meters -- for routine physical and chemical measurements.

13.5.10 Standard or micro-Winkler apparatus -- for determining DO (optional) and calibrating the DO meter.

13.5.11 Winkler bottles -- for dissolved oxygen determinations.

13.5.12 Balance -- Analytical, capable of accurately weighing to 0.0001 g.

13.5.13 Fume hood -- to protect the analyst from effluent or formaldehyde fumes.

13.5.14 Glass stirring rods -- for mixing test solutions.

13.5.15 Graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 50-l000 mL for making test solutions. (Note: not to be used interchangeably for gametes or embryos and test solutions).

13.5.16 Volumetric flasks -- Class A, borosilicate glass or non-toxic plastic labware, 100-1000 mL for making test solutions.

13.5.17 Pipets, automatic -- adjustable, to cover a range of delivery volumes from 0.010 to 1.000 mL.

13.5.18 Pipet bulbs and fillers -- PROPIPET® or equivalent.

13.5.19 Wash bottles -- for reagent water, for topping off graduated cylinders, for rinsing small glassware and instrument electrodes and probes.

13.5.20 Wash bottles -- for dilution water.

13.5.21 20-liter cubitainers or polycarbonate water cooler jugs -- for making hypersaline brine.

13.5.22 Cubitainers, beakers, or similar chambers of non-toxic composition for holding, mixing, and dispensing dilution water and other general non-effluent, non-toxicant contact uses. These should be clearly labeled and not used for other purposes.

13.5.23 Beakers, 50 mL -- for pooling surrogate water samples for chemistry measurements at the end of the test.

13.5.24 Beakers, 250 mL borosilicate glass -- for preparation of test solutions.

13.5.25 Beakers, 1,000 mL borosilicate glass -- for mixing gametes for fertilization of eggs.

13.5.26 Inverted or compound microscope -- for inspecting gametes and making counts of embryos and larvae. The use of an inverted scope is not required, but recommended. Its use reduces the exposure of workers to hazardous fumes (formalin or glutaraldehyde) during the counting of larvae and reduces sample examination time. Alternatively, a Sedgewick-Rafter cell may be used on a regular compound scope.

13.5.27 Counter, two unit, 0-999 -- for recording counts of embryos and larvae.

13.5.28 A perforated plunger -- for maintaining a homogeneous suspension of embryos.

13.5.29 Nytex screens, ca. 75 µm and ca. 37 µm -- for rinsing gametes to separate individual gametes from larger material; for retaining eggs, embryos, or larvae.

13.5.30 60 µm NITEX® filter -- for filtering receiving water.

13.6 **REAGENTS AND SUPPLIES**

13.6.1 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests).

13.6.2 Data sheets (one set per test) -- for data recording (see Figure 1).

13.6.3 Tape, colored -- for labelling test chambers and containers.

13.6.4 Markers, water-proof -- for marking containers, etc.

13.6.5 Parafilm -- to cover graduated cylinders and vessels containing gametes, embryos.

13.6.6 Gloves, disposable -- for personal protection from contamination.

13.6.7 Pipets, serological -- 1-10 mL, graduated.

13.6.8 Pipet tips -- for automatic pipets.

13.6.9 Coverslips -- for microscope slides.

13.6.10 Lens paper -- for cleaning microscope optics.

13.6.11 Laboratory tissue wipes -- for cleaning and drying electrodes, microscope slides, etc.

13.6.12 Disposable countertop covering -- for protection of work surfaces and minimizing spills and contamination.

13.6.13 pH buffers 4, 7, and 10 (or as per instructions of instrument manufacturer) -- for standards and calibration check (see USEPA Method 150.1, USEPA, 1979).

13.6.14 Membranes and filling solutions -- for dissolved oxygen probe (see USEPA Method 360.1, USEPA, 1979), or reagents for modified Winkler analysis.

13.6.15 Laboratory quality assurance samples and standards -- for the above methods.

13.6.16 Test chambers -- 30 mL, four chambers per concentration. The chambers should be borosilicate glass or nontoxic disposable

plastic labware. The test may be performed in other sized chambers as long as the density of embryos is the same.

13.6.17 Formaldehyde, 37% (Concentrated Formalin) -- for preserving larvae. Note: formaldehyde has been identified as a carcinogen and is irritating to skin and mucous membranes. It should not be used at a concentration higher than necessary to achieve morphological preservation of larvae for counting and only under conditions of maximal ventilation and minimal opportunity for volatilization into room air.

13.6.19 Reference toxicant solutions (see Section 13.10.2.4 and Section 4, Quality Assurance).

13.6.20 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms (see Section 5, Facilities, Equipment, and Supplies and Section 7, Dilution Water).

13.6.21 Effluent and receiving water -- see Section 8, Effluent and Surface Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests.

13.6.22 Dilution water and hypersaline brine -- see Section 7, Dilution Water and Section 13.6.24, Hypersaline Brines. The dilution water should be uncontaminated 1-µm-filtered natural seawater. Hypersaline brine should be prepared from dilution water.

13.6.23 HYPERSALINE BRINES

13.6.23.1 Most industrial and sewage treatment effluents entering marine and estuarine systems have little measurable salinity. Exposure of larvae to these effluents will usually require increasing the salinity of the test solutions. It is important to maintain an essentially constant salinity across all treatments. In some applications it may be desirable to match the test salinity with that of the receiving water (See Section 7.1). Two salt sources are available to adjust salinities -- artificial sea salts and hypersaline brine (HSB) derived from natural seawater. Use of artificial sea salts is necessary only when high effluent concentrations preclude salinity adjustment by HSB alone.

13.6.23.2 Hypersaline brine (HSB) can be made by concentrating natural seawater by freezing or evaporation. HSB should be made from high quality, filtered seawater, and can be added to the effluent or to reagent water to increase salinity. HSB has several desirable characteristics for use in effluent toxicity testing. Brine derived from natural seawater contains the necessary trace metals, biogenic colloids, and some of the microbial components necessary for adequate growth, survival, and/or reproduction of marine and estuarine organisms, and it can be stored for prolonged periods without any apparent degradation. However, even if the maximum salinity HSB (100‰) is used as a diluent, the maximum concentration of effluent (0‰) that can be tested is 66% effluent at 34‰ salinity (see Table 1).

13.6.23.3 High quality (and preferably high salinity) seawater should be filtered to at least 10 µm before placing into the freezer or the brine generator. Water should be collected on an incoming tide to minimize the possibility of contamination.

13.6.23.4 Freeze Preparation of Brine

13.6.23.4.1 A convenient container for making HSB by freezing is one that has a bottom drain. One liter of brine can be made from four liters of seawater. Brine may be collected by partially freezing seawater at -10 to -20EC until the remaining liquid has reached the target salinity. Freeze for approximately six hours, then separate the ice (composed mainly of fresh water) from the remaining liquid (which has now become hypersaline).

13.6.23.4.2 It is preferable to monitor the water until the target salinity is achieved rather than allowing total freezing followed by partial thawing. Brine salinity should never exceed 100‰. It is advisable not to exceed about 70‰ brine salinity unless it is necessary to test effluent concentrations greater than 50%.

13.6.23.4.3 After the required salinity is attained, the HSB should be filtered through a 1 µm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at

TABLE 1. MAXIMUM EFFLUENT CONCENTRATION (%) THAT CAN BE TESTED BY ADDING DILUTION WATER ONLY OR BRINE ONLY (WITHOUT ADDITION OF DRY SEA SALTS), GIVEN VARIOUS EFFLUENT SALINITIES, DILUTION WATER SALINITIES, AND BRINE SALINITIES, AND MAINTAINING 30‰ TEST SALINITY.

Effl.	Dilution Water Salinity ‰					Brine Salinity ‰				
‰	31	32	33	34	35	60	70	80	90	100
0	3.23	6.25	9.09	11.76	14.29	50.00	57.14	62.50	66.67	70.00
1	3.33	6.45	9.38	12.12	14.71	50.85	57.97	63.29	67.42	70.71
2	3.45	6.67	9.68	12.50	15.15	51.72	58.82	64.10	68.18	71.43
3	3.57	6.90	10.00	12.90	15.63	52.63	59.70	64.94	68.97	72.16
4	3.70	7.14	10.34	13.33	16.13	53.57	60.61	65.79	69.77	72.92
5	3.85	7.41	10.71	13.79	16.67	54.55	61.54	66.67	70.59	73.68
10	4.76	9.09	13.04	16.67	20.00	60.00	66.67	71.43	75.00	77.78
15	6.25	11.76	16.67	21.05	25.00	66.67	72.73	76.92	80.00	82.35
20	9.09	16.67	23.08	28.57	33.33	75.00	80.00	83.33	85.71	87.50
25	16.67	28.57	37.50	44.44	50.00	85.71	88.89	90.91	92.31	93.33

4EC (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

13.6.23.5 Heat Preparation of Brine

13.6.23.5.1 The ideal container for making brine using heat-assisted evaporation of natural seawater is one that (1) has a high surface to volume ratio, (2) is made of a non-corrosive material, and (3) is easily cleaned (fiberglass containers are ideal). Special care should be used to prevent any toxic materials from coming in contact with the seawater being used to generate the brine. If a heater is immersed directly into the seawater, ensure that the heater materials do not corrode or leach any substances that would contaminate the brine. One successful method is to use a thermostatically controlled heat exchanger made from fiberglass. If aeration is needed, use only oil-free air compressors to prevent contamination.

13.6.23.5.2 Before adding seawater to the brine generator, thoroughly clean the generator, aeration supply tube, heater, and any other materials that will be in direct contact with the brine. A good quality biodegradable detergent should be used, followed by several (at least three) thorough reagent water rinses.

13.6.23.5.3 Seawater should be filtered to at least 10 µm before being put into the brine generator. The temperature of the seawater is increased slowly to 40EC. The water should be aerated to prevent temperature stratification and to increase water evaporation. The brine should be checked daily (depending on the volume being generated) to ensure that the salinity does not exceed 100‰ an that the temperature does not exceed 40EC. Additional seawater may be added to the brine to obtain the volume of brine required.

13.6.23.5.4 After the required salinity is attained, the HSB should be filtered through a 1 µm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4EC (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

13.6.23.6 Artificial Sea Salts

13.6.23.6.1 No data from mussel or oyster tests using sea salts or artificial seawater (e.g., GP2) are available for evaluation at this time, and their use must be considered provisional.

13.6.23.7 Dilution Water Preparation from Brine

13.6.23.7.1 Although salinity adjustment with brine is the preferred method, the use of high salinity brines and/or reagent water has sometimes been associated with discernible adverse effects on test organisms. For this reason, it is recommended that only the minimum necessary volume of brine and reagent water be used to offset the low salinity of the effluent, and that brine controls be included in the test. The remaining dilution water should be natural seawater. Salinity may be adjusted in one of two ways. First, the salinity of the highest effluent test concentration may be adjusted to an acceptable salinity, and then serially diluted. Alternatively, each effluent concentration can be prepared individually with appropriate volumes of effluent and brine.

13.6.23.7.2 When HSB and reagent water are used, thoroughly mix together the reagent water and HSB before mixing in the effluent. Divide the salinity of the HSB by the expected test salinity to determine the proportion of reagent water to brine. For example, if the salinity of the brine is 100‰ and the test is to be conducted at 30‰, 100‰ divided by 30‰ = 3.33. The proportion of brine is 1 part in 3.33 (one part brine to 2.33 parts reagent water). To make 1 L of dilution water at 30‰ salinity from a HSB of 100‰, 300 mL of brine and 700 mL of reagent water are required. Verify the salinity of the resulting mixture using a refractometer.

13.6.23.8 Test Solution Salinity Adjustment

13.6.23.8.1 Table 2 illustrates the preparation of test solutions (up to 50% effluent) at 34‰ by combining effluent, HSB, and dilution water. Note: if the highest effluent concentration does not exceed 50% effluent, it is convenient to prepare brine so that the sum of the effluent salinity and brine salinity equals 68‰; the required brine volume is then always

equal to the effluent volume needed for each effluent concentration as in the example in Table 2.

13.6.23.8.2 Check the pH of all test solutions and adjust to within 0.2 units of dilution water pH by adding, dropwise, dilute hydrochloric acid or sodium hydroxide (see Section 8.8.9, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

13.6.23.8.3 To calculate the amount of brine to add to each effluent dilution, determine the following quantities: salinity of the brine (SB, in ‰), the salinity of the effluent (SE, in ‰), and volume of the effluent to be added (VE, in mL). Then use the following formula to calculate the volume of brine (VB, in mL) to be added:

$$VB = VE \times (30 - SE)/(SB - 30)$$

13.6.23.8.4 This calculation assumes that dilution water salinity is 30 ± 2‰.

13.6.23.9 Preparing Test Solution**s**

13.6.23.9.1 Ten mL of test solution are needed for each test container. To prepare test solutions at low effluent concentrations (<6%), effluents may be added directly to dilution water. For example, to prepare 1% effluent, add 1.0 mL of effluent to a 100-mL volumetric flask using a volumetric pipet or calibrated automatic pipet. Fill the volumetric flask to the 100-mL mark with dilution water, stopper it, and shake to mix. Pour into a (150-250 mL) beaker and stir. Distribute equal volumes into the replicate test chambers. The remaining test solution can be used for chemistry.

13.6.23.9.2 To prepare a test solution at higher effluent concentrations, hypersaline brine must usually be used. For example, to prepare 40% effluent, add 400 mL of effluent to a 1-liter volumetric flask. Then, assuming an effluent salinity of 2‰ and a brine salinity of 66‰, add 400 mL of brine (see equation above and Table 2) and top off the flask with dilution water. Stopper the flask and shake well. Pour into a (100-250

TABLE 2. EXAMPLES OF EFFLUENT DILUTION SHOWING VOLUMES OF EFFLUENT (AT X‰), BRINE, AND DILUTION WATER NEEDED FOR ONE LITER OF EACH TEST SOLUTION.

FIRST STEP: Combine brine with reagent water or natural seawater to achieve a brine of 68-x‰ and, unless natural seawater is used for dilution water, also a brine-based dilution water of 34‰.

SERIAL DILUTION:
Step 1. Prepare the highest effluent concentration to be tested by adding equal volumes of effluent and brine to the appropriate volume of dilution water. An example using 40% is shown.

Effluent Conc. (%)	Effluent x‰	Brine (68-x)‰	Dilution Water* 34‰
40	800 mL	800 mL	400 mL

Step 2. Make serial dilutions from the highest test concentration.

Effluent Conc. (%)	Effluent Source	Dilution Water* (34‰)
20	1000 mL of 40%	1000 mL
10	1000 mL of 20%	1000 mL
5	1000 mL of 10%	1000 mL
2.5	1000 mL of 5%	1000 mL
Control	none	1000 mL

INDIVIDUAL PREPARATION:

Effluent Conc. (%)	Effluent x‰	Brine (68-x)‰	Dilution Water* 34‰
40	400 mL	400 mL	200 mL
20	200 mL	200 mL	600 mL
10	100 mL	100 mL	800 mL
5	50 mL	50 mL	900 mL
2.5	25 mL	25 mL	950 mL
Control	none	none	1000 mL

*May be natural seawater or brine-reagent water equivalent.

mL) beaker and stir. Distribute equal volumes into the replicate test chambers. The remaining test solution can be used for chemistry.

13.6.23.10 Brine Controls

13.6.23.10.1 Use brine controls in all tests where brine is used. Brine controls contain the same volume of brine as does the highest effluent concentration using brine, plus the volume of reagent water needed to reproduce the hyposalinity of the effluent in the highest concentration, plus dilution water. Calculate the amount of reagent water to add to brine controls by rearranging the above equation, (See, 13.6.23.8.3) setting SE = 0, and solving for VE.

$$VE = VB \times (SB - 30)/(30 - SE)$$

If effluent salinity is essentially 0‰, the reagent water volume needed in the brine control will equal the effluent volume at the highest test concentration. However, as effluent salinity and effluent concentration increase, less reagent water volume is needed.

13.6.24 TEST ORGANISMS, OYSTERS AND MUSSELS

13.6.24.1 The test organisms for this test are the Pacific, oyster, ***Crassostrea gigas,*** or mussels, ***Mytilus spp.*** (at least twelve per test). Pacific oysters are native to Japan, but have been cultured commercially on the west coast of the United States for over a century.

13.6.24.2 Species Identification

13.6.24.2.1 The three species of mussels included in this method are presumably native to the west coast. The California mussel (***Mytilus californianus***) is distributed along the exposed rocky coast from Alaska to Baja California and is found from intertidal areas to 150 feet depth. The other two mussels included in this method (***M. trossulus*** and ***M. galloprovinciallis***) are common in sheltered waters such as bays and estuaries and were previously considered to be west coast populations of ***Mytilus edulis***. The two species are both present in central California, with ***M.***

galloprovincialis reported from San Francisco Bay to Baja California, and ***M. trossulus*** reported from Monterey to Alaska.

13.6.24.2.2 Test organisms should be identified to species using morphological features in recognized keys. Separation of the "***M. edulis***" complex, (***M. trossulus***, and ***M. galloprovinciallis***) may not be possible without electrophoretic characterization. The geographic source of the ***Mytilus spp***. broodstock must be reported.

13.6.24.3 Obtaining Broodstock

13.6.24.3.1 Adult oysters (***Crassostrea gigas***) and mussels (***Mytilus spp***.) can be obtained from commercial suppliers and the mussels can also be collected from the field. Organisms are best shipped in damp towels or seaweed and kept cool (4-12EC). Note: if practical, check the sex ratio of brood stock or request such information from a commercial supplier. A highly skewed sex ratio could result in poor embryo yield.

13.6.24.4 Broodstock Culture and Handling

13.6.24.4.1 The adult bivalves are maintained in glass aquaria or fiberglass troughs or tanks. These are supplied continuously (approximately 5 L/min) with natural seawater, or salt water prepared from commercial sea salts is recirculated. The animals are checked daily and any obviously unhealthy animals are discarded. Prior to spawning, the animals should be brushed or gently scraped to remove barnacles and other encrusting organisms; this alleviates problems of egg and sperm contamination, especially through potential barnacle spawning.

13.6.24.4.2 Although ambient temperature seawater is usually acceptable for holding, recommended temperatures are 14-15EC for oyster and 8EC for mussels; conditioning bivalves to spawning condition usually requires holding for from 1-8 weeks at a higher temperature (20EC for oysters, 15-18EC for mussels).

13.6.24.4.3 Natural seawater ($30‰) is used to maintain the adult animals and as a control water in the tests.

13.6.24.4.4 Adult animals used in field studies are transported in insulated boxes or coolers packed with wet kelp or paper

toweling. Upon arrival at the field site, aquaria are filled with control water, loosely covered with a styrofoam sheet and allowed to equilibrate to the holding temperature before animals are added.

13.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

13.7.1 See Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

13.8 CALIBRATION AND STANDARDIZATION

13.8.1 See Section 4, Quality Assurance.

13.9 QUALITY CONTROL

13.9.1 See Section 4, Quality Assurance.

13.10 TEST PROCEDURES

13.10.1 TEST DESIGN

13.10.1.1 The test consists of at least four replicates of five effluent concentrations plus a dilution water control. Tests that use brine to adjust salinity must also contain four replicates of a brine control. In addition, at least six extra count controls are prepared in dilution water and the number of embryos in each are counted at the time of test initiation. These counts provide an average initial embryo density that is used in the calculation of test results (see 13.13.1.3). Extra replicates are recommended for water chemistry during the tests (see Section 13.8 and Table 3).

13.10.1.2 Effluent concentrations are expressed as percent effluent.

13.10.2 TEST SOLUTIONS

13.10.2.1 Receiving waters

13.10.2.1.1 The sampling point is determined by the objectives of the test. At estuarine and marine sites, samples are usually

collected at mid-depth. Receiving water toxicity is determined with samples used directly as collected or with samples passed through a 60 µm NITEX® filter and compared without dilution, against a control. Using four replicate chambers per test, each containing 10 mL, and 400 mL for chemical analysis, would require approximately 440 mL of sample per test.

13.10.2.2 Effluents

14.10.2.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of at least 0.5 is commonly used. A dilution factor of 0.5 provides hypothesis test discrimination of ± 100%, and testing of a 16 fold range of concentrations. Hypothesis test discrimination shows little improvement as dilution factors are increased beyond 0.5 and declines rapidly if smaller dilution factors are used. USEPA recommends that one of the five effluent treatments must be a concentration of effluent mixed with dilution water which corresponds to the permittee's instream waste concentration (IWC). At least two of the effluent treatments must be of lesser effluent concentration than the IWC, with one being at least one-half the concentration of the IWC. If 100‰ HSB is used as a diluent, the maximum concentration of effluent that can be tested will be 70% at 30‰ salinity.

13.10.2.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12% and 1.56%).

13.10.2.2.3 The volume in each test chamber is 10 mL.

13.10.2.2.4 Effluent dilutions should be prepared for all replicates in each treatment in one container to minimize variability among the replicates. Dispense into the appropriate effluent test chambers.

13.10.2.3 Dilution Water

13.10.2.3.1 Dilution water should be uncontaminated 1-µm-filtered natural seawater or hypersaline brine (prepared from uncontaminated natural seawater) plus reagent water (see Section

7, Dilution Water). Natural seawater may be uncontaminated receiving water. This water is used in all dilution steps and as the control water.

13.10.2.4 Reference Toxicant Test

13.10.2.4.1 Reference toxicant tests should be conducted as described in Quality Assurance (see Section 4.7).

13.10.2.4.2 The preferred reference toxicant for oysters and mussels is copper chloride ($CuCl_2 \cdot H_2O$). Reference toxicant tests provide an indication of the sensitivity of the test organisms and the suitability of the testing laboratory (see Section 4 Quality Assurance). Another toxicant may be specified by the appropriate regulatory agency. Prepare a copper reference toxicant stock solution (2,000 mg/L) by adding 5.366 g of copper chloride ($CuCl_2 \cdot 2H_2O$) to 1 liter of reagent water. For each reference toxicant test prepare a copper sub-stock of 3 mg/L by diluting 1.5 mL of stock to one liter with reagent water. Alternatively, certified standard solutions can be ordered from commercial companies.

13.10.2.4.3 Prepare a control (0 µg/L) plus four replicates each of at least five consecutive copper reference toxicant solutions (e.g., from the series 3.0, 4.4, 6.5, 9.5, 13.9, 20.4, and 30.0 µg/L, by adding 0.10, 0.15, 0.22, 0.32, 0.46, 0.68, and 1.00 mL of sub-stock solution, respectively, to 100-mL volumetric flasks and filling to 100-mL with dilution water). Start with control solutions and progress to the highest concentration to minimize contamination.

13.10.2.4.4 If the effluent and reference toxicant tests are to be run concurrently, then the tests must use embryos from the same spawn. The tests must be handled in the same way and test solutions delivered to the test chambers at the same time. Reference toxicant tests must be conducted at 30 ± 2‰.

13.10.3 COLLECTION OF GAMETES FOR THE TEST

13.10.3.1 Spawning Induction

13.10.3.1.1 Select at least a dozen bivalves and place them into a container filled with seawater (ca. 20°C for oysters, 15°C for

mussels) and allow time for them to resume pumping (ca. 30 minutes). Mussels will often start pumping following immersion if they have been kept out of water and refrigerated overnight prior to spawning.

TABLE 3. EXAMPLE OF TYPICAL TEST ARRAY SHOWING NUMBER AND TYPES OF TREATMENT CHAMBERS REQUIRED.

TREATMENT	Test Vials	Chemistry Vials
Count Control	6	0
Brine Control	4	1-3
Dilution Water Control	4	1-3
Effluent conc. 1	4	1-3
Effluent conc. 2	4	1-3
Effluent conc. 3	4	1-3
Effluent conc. 4	4	1-3
Effluent conc. 5	4	1-3
TOTAL Chambers = 41-55	34	7-21

13.10.3.1.2 Over a 15-20 minute period, increase the temperature (do not exceed 32EC for oysters, or 20EC for mussels), checking for spawning.

13.10.3.1.3 If no spawning occurs after 30 minutes, replace the water with some at the original temperature and after 15 minutes again increase the temperature as in 13.10.3.2. Although ASTM (1993) cautions against it, the addition of algae into the water can often stimulate spawning of bivalves; if this method is used, the organisms should be moved to clean water once spawning begins. Mussels can also be induced to spawn by injection of 0.5 M KCl into the posterior adductor muscle. Oysters can be induced to spawn by the addition of heat-killed sperm about one hour after initial temperature increase.

13.10.3.2 Pooling Gametes

13.10.3.2.1 When individuals are observed to be shedding gametes, remove each spawner from the tank and place each in a separate container (20EC water for oysters, 15EC for mussels). Alternatively, bivalves can be placed into individual chambers initially (at temperatures per 13.10.5.2) and these placed into a water bath that provides the desired maximum temperature.

13.10.3.2.2 Early in the spawning process, examine a small sample of the gametes from each spawner to confirm sex and to see if the gametes are of adequate quality.

13.10.3.2.3 Place a small amount of sperm from each male onto a microscope slide (well slides work nicely). Examine the sperm for motility; use sperm from those males with the better sperm motility.

13.10.3.2.4 A small sample of the eggs from each female should be examined for the presence of significant quantities of poor eggs (vacuolated, small, or abnormally shaped). If good quality eggs are available from one or more females, questionable batches of eggs should not be used for the test. It is more important to use high quality eggs than it is to use a pooled population of eggs.

13.10.3.2.5 Sperm and egg suspensions that are to be used for preparing the embryo stock should be passed through Nytex screen (ca. 75 Fm) to separate out clumps of gametes or extraneous material.

13.10.3.2.6 The pooled eggs are placed into a 1 L beaker and sufficient dilution water added to achieve an egg density of about 5,000-8,000 eggs/mL (objects are just discernible when viewed through the egg suspension) in about 800-900 mL water volume.

13.10.3.3 Fertilization

13.10.3.3.1 Sperm density may vary from one spawning to the next. It is important to use enough sperm to achieve a high percent egg fertilization, but too many sperm can cause polyspermy with resultant abnormal development. To achieve an acceptable level of sperm, several egg suspensions of equal density should be fertilized using a range of sperm volumes,

e.g., 100 mL of egg suspension plus 1, 3, and 10 mL of sperm suspension. This test fertilization should be accomplished within 1 hour of spawning. Use the eggs with the lowest amount of sperm giving normal embryo development after 1.5-2.5 hours after fertilization, as determined by microscopic examination. Usually >90% of the eggs should be fertilized; oysters should have changed from the tear-drop shaped egg to a round single cell zygote; mussels should show a single polar body; or embryos of either species should have advanced to the two-cell stage.

13.10.4 START OF THE TEST

13.10.4.1 Prior to Beginning the Test

13.10.4.1.1 The test should begin as soon as possible, preferably within 24 h of sample collection. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity tests unless permission is granted by the permitting authority. In no case should the sample be used in a test more than 72 h after sample collection (see Section 8 Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Test).

13.10.4.1.2 Just prior to test initiation (approximately 1 h), the temperature of a sufficient quantity of the sample to make the test solutions should be adjusted to the test temperature (18 or 20 ± 1EC) and maintained at that temperature during the addition of dilution water.

13.10.4.1.3 Increase the temperature of the water bath, room, or incubator to the required test temperature (18 or 20 ± 1EC).

13.10.4.1.4 Randomize the placement of test chambers in the temperature-controlled water bath, room, or incubator at the beginning of the test, using a position chart. Assign numbers for the position of each test chamber using a random numbers or similar process (see Appendix A, for an example of randomization). Maintain the chambers in this configuration throughout the test, using a position chart. Record these numbers on a separate data sheet together with the concentration and replicate numbers to which they correspond. Identify this sheet with the date, test organism, test number, laboratory, and

investigator's name, and safely store it away until after the oysters or mussels have been examined at the end of the test.

13.10.4.1.5 Note: Loss of the randomization sheet would invalidate the test by making it impossible to analyze the data afterwards. Make a copy of the randomization sheet and store separately. Take care to follow the numbering system exactly while filling chambers with the test solutions.

13.10.4.1.6 Arrange the test chambers randomly in the water bath or controlled temperature room. Once chambers have been labeled randomly, they can be arranged in numerical order for convenience, since this will also ensure random placement of treatments.

13.10.4.2 Estimation of Embryo Density

13.10.4.2.1 Adjust the embryo suspension to a density of 1,500-3,000/mL. Confirm by counting chamber counts on 1 mL subsamples from a stirred suspension of embryos. Final larval density of 15/mL will provide reasonable precision (150 larvae) and be easier to count than 300 larvae. Add 0.1 mL of the embryo suspension to 10 mL of test solution into each of the randomized test vials. It is extremely important (for a consistent embryo density among test chambers) to maintain a homogeneous distribution of embryos in the stock suspension by regular, slow oscillation of a perforated plunger during embryo distribution.

13.10.4.3 Initial Density Counts

13.10.4.3.1 If tests are conducted on small volumes, using an inverted microscope, the total number of embryos injected into the count controls should be determined as soon as the test has been started. If larger test volumes are used, with counts based upon subsamples, the embros should be resuspended in the water using a perforated plunger. Then subsamples are taken (e.g., 5-10 mL) and the total number of embryos counted in the subsample. Two methods for these counts are to use a counting chamber of the same volume as the subsample, or to screen the embryos using a 37 Fm screen and backwash with a smaller volume for small counting chambers. In either procedure, appropriate multiple rinsing is needed to achieve quantitative transfer of embryos.

13.10.4.3.2 Initial counts are required to determine survival in the controls and other treatments. High coefficients of variability in initial counts make survival estimates inexact and may actually decrease the sensitivity of the test.

13.10.4.4 Incubation

13.10.4.4.1 Cover and incubate the chambers in an environmental chamber or by partial immersion in a temperature-controlled water bath for 48 hours.

13.10.4.4.2 At the end of the 48-hour incubation period, examine a count control test chamber (or control test vial if the count controls were transferred to a counting chamber to make the initial counts) under a microscope to check for complete development of control organisms. If development is complete, the test should be ended. If development does not appear to be complete, the test should be continued until complete development occurs (but not beyond 54 hours total test duration).

13.10.5 LIGHT, PHOTOPERIOD, SALINITY AND TEMPERATURE

13.10.5.1 The light quality and intensity should be at ambient laboratory conditions. Light intensity should be 10-20 µE/m2/s, or 50 to 100 foot candles (ft-c), with a 16 h light and 8 h dark cycle.

13.10.5.2 The water temperature in the test chambers should be maintained at 18 or 20 ± 1EC. If a water bath is used to maintain the test temperature, the water depth surrounding the test cups should be as deep as possible without floating the chambers.

13.10.5.3 The test salinity should be in the range of 30 ± 2‰. The salinity should vary by no more than ±2‰ among the chambers on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.

13.10.5.4 Rooms or incubators with high volume ventilation should be used with caution because the volatilization of the test solutions and evaporation of dilution water may cause wide fluctuations in salinity. Covering the test chambers with clean

polyethylene plastic may help prevent volatilization and evaporation of the test solutions.

13.10.6 DISSOLVED OXYGEN (DO) CONCENTRATION

13.10.6.1 Aeration may affect the toxicity of effluent and should be used only as a last resort to maintain a satisfactory DO. The DO concentration should be measured on new solutions at the start of the test (Day 0). The DO should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). If it is necessary to aerate, all treatments and the control should be aerated. The aeration rate should not exceed that necessary to maintain a minimum acceptable DO and under no circumstances should it exceed 100 bubbles/minute, using a pipet with a 1-2 mm orifice, such as a 1 mL KIMAX® serological pipet No. 37033, or equivalent.

13.10.7 OBSERVATIONS DURING THE TEST

13.10.7.1 Routine Chemical and Physical Observations

13.10.7.1.1 DO is measured at the beginning of the exposure period in each test concentration and in the control.

13.10.7.1.2 Temperature, pH, and salinity are measured at the beginning of the exposure period in each test concentration and in the control. Temperature should also be monitored continuously or observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be measured in a sufficient number of test chambers at the end of the test to determine temperature variation in the environmental chamber.

13.10.7.1.3 Record all the measurements on the data sheet.

13.10.8 TERMINATION OF THE TEST

13.10.8.1 Ending the Test

13.10.8.1.1 Record the time the test is terminated.

13.10.8.1.2 The pH, dissolved oxygen, and salinity are measured

at the end of the exposure period in one test chamber at each concentration and in the control. If small electrodes are used, these measurements can be performed in a single extra replicate vial set up specifically for this measurement. Measurements should not be made in vials that are to be counted, as larvae may adhere to electrodes, possibly biasing larval counts.

13.10.8.2 Sample Preservation

13.10.8.2.1 To terminate the test, add 0.25 mL of concentrated formalin (37% formaldehyde). It is advisable not to shake the contents at any time following test termination because the larvae may stick to the edge of the chambers. Simply allow the preservative to mix passively and the larvae to settle out. The use of glutaraldehyde instead of formalin is likely to be acceptable, but as no record of its use with this test is known, care should be taken to confirm that glutaraldehyde kills, preserves, and produces no artifacts that would confound the test results.

13.10.8.2.2 Note: Formaldehyde has been identified as a carcinogen and both glutaraldehyde and formaldehyde are irritating to skin and mucus membranes. Neither should be used at higher concentrations than needed to achieve morphological preservation and only under conditions of maximal ventilation and minimal opportunity for volatilization into room air.

13.10.8.3 Counting

13.10.8.3.1 After addition of preservative, observe all the larvae in each test vial. This can be done by examining the contents of each test vial with an inverted microscope at about 40X-50X magnification or by quantitative transfer of all larvae onto a counting chamber and counting using a compound microscope at about 100X. Using the mechanical stage, carefully count and score all larvae as either normal or abnormal. If substantial numbers of completely developed shells without meat are observed (i.e., > 5 percent of normal larvae), then these shells should be enumerated separately (as dead larvae). "Larvae possessing misshapened or otherwise malformed shells are considered normal, provided development has been completed" (ASTM, 1994). Record the final counts on the data sheet.

13.10.8.3.2 If the number of larvae observed appears to be low in relation to the number inoculated at the beginning of the test, this signifies either mortality and dissolution, or possible adherence to the walls of the vials or incomplete transfer to the counting chamber. Inspect the vials for evidence of the latter two occurrences.

13.10.8.4 Endpoint

13.10.8.4.1 The percentage of embryos that did not survive and develop to live larvae with completely developed shells (i.e., abnormal or dead organisms) is calculated for each treatment replicate (See 13.13.1.3). All larvae are considered live unless they are merely empty shells "without meat" (ASTM, 1994); embryos and larvae that are not yet in the D-hinge stage are counted as abnormal, even if they may have died during the test. Embryos and larvae that die and disintegrate during the test are estimated from initial embryo counts (See N' in 13.13.1.3).

13.10.8.4.2 Unless used as the dilution water, natural seawater controls are only used to check the relative performance of the dilution water controls (e.g., brine controls) required for salinity adjustment. Statistical analysis should use the appropriate dilution water control data.

13.11 **SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA**

13.11.1 A summary of test conditions and test acceptability criteria is listed in Table 4.

TABLE 4. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR, ***CRASSOSTREA GIGAS*** and ***MYTILUS SPP.***, EMBRYO-LARVAL DEVELOPMENT TEST WITH EFFLUENTS AND RECEIVING WATERS

1. Test type:	Static non-renewal
2. Salinity:	30 ± 2‰
3. Temperature:	20 ± 1EC (oysters) 15 or 18 ± 1EC (mussels)*
4. Light quality:	Ambient laboratory light

5. Light intensity:	l0-20 uE/m2/s (Ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	30 mL
8. Test solution volume:	10 mL
9. No. larvae per chamber:	150-300
10. No. replicate chambers per concentration:	4 (plus 3 chemistry vials)
11. Dilution water:	Uncontaminated 1-µm-filtered natural seawater or hypersaline brine prepared from natural seawater
12. Test concentrations:	Effluents: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
13. Dilution factor:	Effluents: **$**0.5 Receiving waters: None or **$**0.5
14. Test duration:	48 hours (or until complete development up to 54 hours)
15. Endpoint:	Survival and normal shell development
16. Test acceptability criteria:	Control survival must be **$**70% for oyster embryos or **$**50% for mussel embryos in control vials; **$**90% normal shell development in surviving controls; and must achieve a %MSD of <25%

17. Sampling requirements:	One sample collected at test initiation, and preferably used within 24 h of the time it is removed from the sampling device (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests)
18. Sample volume required:	1 L per test

*Mussel embryo-larval tests were commonly conducted at 15EC (ASTM, 1994). Experience has shown that many laboratories in northern Californa, Oregon, and Washington often fail to achieve adequate control development at 15EC in 48 hours. It is acceptable to conduct the test at 15EC with the permission of the regulatory authority. Developmental rates may be dependent upon species, local population characteristics, or other factors.

13.12 **ACCEPTABILITY OF TEST RESULTS**

13.12.1 For tests to be considered acceptable, the following requirements must be met:

(1) The mean survival must be at least 70% for oysters or at least 50% for mussels in the controls.

(2) The percent normal must be at least 90% in the surviving controls.

(3) The minimum significant difference (%MSD) is <25% relative to the control.

13.13 **DATA ANALYSIS**

13.13.1 **GENERAL**

13.13.1.1 Tabulate and summarize the data. Calculate the proportion of normally developed larvae for each replicate. A sample set of test data is listed in Table 5.

13.13.1.2 Final calculations are based upon counts of normal larvae and total larvae at test termination, and mean initial embryo count.

13.13.1.3 The percentage of embryos that did not survive or develop to live larvae with completely developed shells (i.e., abnormal or dead organisms) is calculated for each treatment replicate (including controls) using the formula:

$$A = \frac{100\ (N' - B')}{N'}$$

where:

A = percent abnormal and dead organisms
B' = the adjusted number of normal larvae at the end of the test
N' = the initial number of embryos in the test chambers expressed as the mean of the initial counts; and: if N > N', where

N = the actual number of larvae at the end of the test then: $B' = B\ (N' / N)$

where: B = the actual number of normal larvae at the end of the test but, when N # N', then: B' = B

The means of "A" are obtained for each treatment concentration, and the latter are corrected for control response using Abbott's formula, as follows:

$$E = \frac{100\ (A - M)}{100 - M}$$

where:

E = the mean percent abnormal/dead corrected for controls
A = the mean percent abnormal/dead
M = the value of A for the controls.

13.13.1.4 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

TABLE 5. DATA FROM BIVALVE DEVELOPMENT TEST

Copper Concentration (µg/L)	Replicate	Initial Density	Number Surviving	Number Normal	Proportion Normal
Control	A	25	22	22	1.00
	B	25	25	24	0.96
	C	25	25	25	1.00
	D	30	30	29	0.97
0.13	A	25	23	22	0.96
	B	30	30	29	0.97
	C	25	25	25	1.00
	D	25	24	23	0.96
0.25	A	25	25	23	0.92
	B	25	19	18	0.95
	C	25	21	19	0.90
	D	25	23	22	0.96
0.50	A	25	11	10	0.91
	B	25	14	13	0.93
	C	25	17	15	0.88
	D	25	15	14	0.93
1.00	A	25	8	7	0.88
	B	25	6	5	0.83
	C	25	8	7	0.88
	D	25	11	9	0.82
2.00	A	25	2	2	1.00
	B	25	3	2	0.67
	C	25	4	3	0.75
	D	25	5	2	0.40

13.13.1.5 The endpoints of toxicity tests using bivalves are based on the reduction in proportion of normally developed larvae. The IC25 is calculated using the Linear Interpolation Method (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). LOEC and NOEC values for larval development are obtained using a hypothesis testing approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981) (see Section 9). Separate analyses are performed for the estimation of the LOEC and NOEC endpoints and for the estimation of the IC25. See the Appendices for examples of the manual computations, and examples of data input and program output.

13.13.2 EXAMPLE OF ANALYSIS OF BIVALVE EMBRYO-LARVAL DEVELOPMENT DATA

13.13.2.1 Formal statistical analysis of the embryo-larval development is outlined in Figure 1. The response used in the analysis is the proportion of normally developed surviving larvae in each test or control chamber. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the IC25 endpoint. Concentrations at which there is no normal development in any of the test chambers are excluded from statistical analysis of the NOEC and LOEC, but included in the estimation of the IC25.

13.13.2.2 For the case of equal numbers of replicates across all concentrations and the control, the evaluation of the NOEC and LOEC endpoints is made via a parametric test, Dunnett's Procedure, or a nonparametric test, Steel's Many-one Rank Test, on the arc sine square root transformed data. Underlying assumptions of Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test, and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are estimated by the parametric procedure.

13.13.2.3 If unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a *t* test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative.

13.13.2.4 Example of Analysis of Embryo-Larval Development Data

13.13.2.4.1 Since the response of interest is the proportion of normally developed surviving larvae, each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix B. Because there are varying numbers of survivors in the replicates, the adjustment for response proportions of zero or one will not be made. The raw and transformed data, means and variances of the transformed observations at each effluent concentration and control are listed in Table 5. The data are plotted in Figure 2.

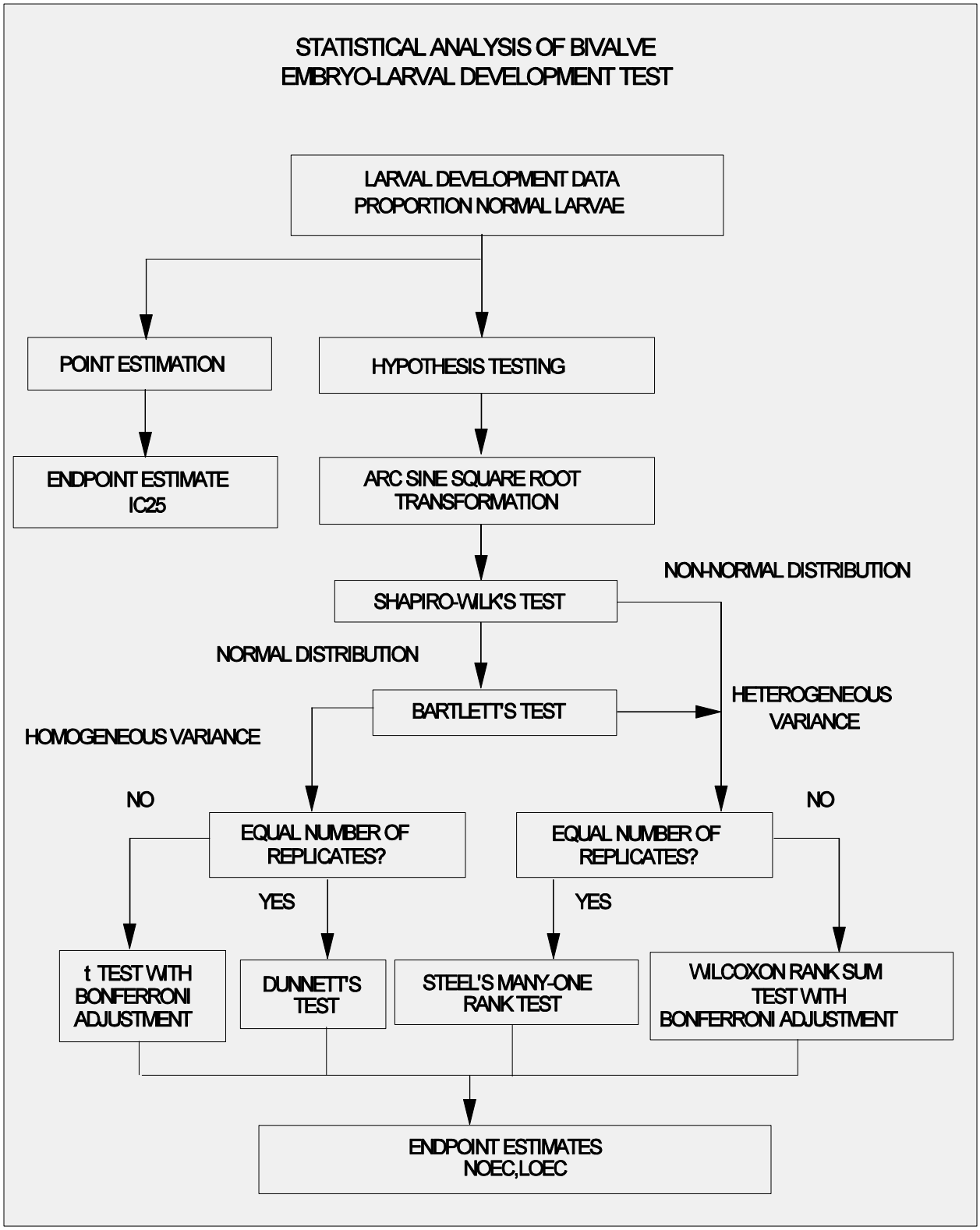

Figure 1. Flowchart for statistical analysis of the pacific oyster, *Crassostrea gigas*, and mussel, *Mytilus spp.*, development data.

13.13.2.5 Test for Normality

13.13.2.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 6.

TABLE 6. BIVALVE EMBRYO-LARVAL DEVELOPMENT DATA

		Copper Concentration (µg/L)					
	Replicate	Control	0.13	0.25	0.50	1.00	2.00
	A	1.00	0.96	0.92	0.91	0.88	1.00
RAW	B	0.96	0.97	0.95	0.93	0.83	0.67
	C	1.00	1.00	0.90	0.88	0.88	0.75
	D	0.97	0.96	0.96	0.93	0.82	0.40
ARC SINE	A	1.571	1.369	1.284	1.266	1.217	1.571
SQUARE ROOT	B	1.369	1.397	1.345	1.303	1.146	0.959
TRANSFORMED	C	1.571	1.571	1.249	1.217	1.217	1.047
	D	1.397	1.369	1.369	1.303	1.133	0.685
Mean ($\bar{X}_i$)		1.477	1.427	1.312	1.272	1.178	1.066
S_i^2		0.01191	0.00945	0.00303	0.00166	0.00203	0.13733
i		1	2	3	4	5	6

13.13.2.5.2 Calculate the denominator, D, of the statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation
$\bar{X}$ = the overall mean of the centered observations
n = the total number of centered observations

13.13.2.5.3 For this set of data, n = 24

$$\bar{X} = \frac{1}{24}(-0.002) = 0.000$$

D = 0.4963

13.13.2.5.4 Order the centered observations from smallest to largest

$$X^{(1)} \# X^{(2)} \# \ldots \# X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 7

TABLE 7. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.381	13	-0.019
2	-0.108	14	-0.006
3	-0.107	15	0.031
4	-0.080	16	0.031
5	-0.063	17	0.033
6	-0.058	18	0.039
7	-0.058	19	0.039
8	-0.055	20	0.057
9	-0.045	21	0.094
10	-0.032	22	0.094
11	-0.030	23	0.144
12	-0.028	24	0.505

13.13.2.5.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2, ... a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 24 and k = 12. The a_i values are listed in Table 8.

13.13.2.5.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i (X^{(n-i+1)} - X^{(i)})\right]^2$$

The differences, $X^{(n-i+1)} - X^{(i)}$, are listed in Table 8. For the data in this example:

$$W = \frac{1}{0.4963} (0.6322)^2 = 0.805$$

TABLE 8. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4493	0.886	$X^{(24)} - X^{(1)}$
2	0.3098	0.252	$X^{(23)} - X^{(2)}$
3	0.2554	0.201	$X^{(22)} - X^{(3)}$
4	0.2154	0.174	$X^{(21)} - X^{(4)}$
5	0.1807	0.120	$X^{(20)} - X^{(5)}$
6	0.1512	0.097	$X^{(19)} - X^{(6)}$
7	0.1245	0.097	$X^{(18)} - X^{(7)}$
8	0.0997	0.088	$X^{(17)} - X^{(8)}$
9	0.0764	0.076	$X^{(16)} - X^{(9)}$
10	0.0539	0.063	$X^{(15)} - X^{(10)}$
11	0.0321	0.024	$X^{(14)} - X^{(11)}$
12	0.0107	0.009	$X^{(13)} - X^{(12)}$

13.13.2.5.7 The decision rule for this test is to compare W as calculated in Subsection 5.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For the data in this example, the critical value at a significance level of 0.01 and n = 24 observations is 0.884. Since W = 0.805 is less than the critical value, conclude that the data are not normally distributed.

13.13.2.5.8 Since the data do not meet the assumption of normality, Steel's Many-one Rank Test will be used to analyze the embryo-larval development data.

13.13.2.6 Steel's Many-one Rank Test

13.13.2.6.1 For each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign the ranks (1, 2, ... , 8) to the ordered observations with a rank of 1 assigned to the smallest observation, rank of 2 assigned to the next larger observation, etc. If ties occur when ranking, assign the average rank to each tied observation.

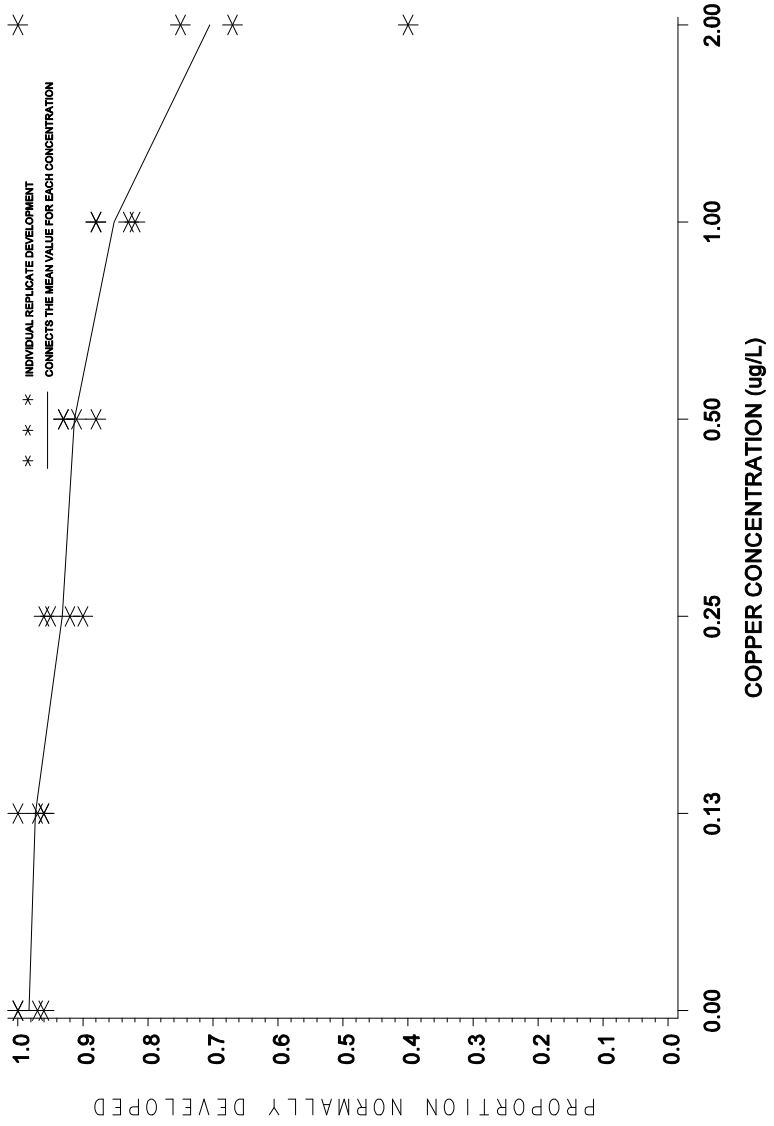

Figure 2. Plot of mean proportion of normaly developed bivalve larvae.

13.13.2.6.2 An example of assigning ranks to the combined data for the control and 0.13 µg/L concentration is given in Table 9. This ranking procedure is repeated for each control/concentration combination. The complete set of rankings is summarized in Table 10. The ranks are then summed for each concentration level, as shown in Table 11.

TABLE 9. ASSIGNING RANKS TO THE CONTROL AND 0.13 µg/L CONCENTRATION LEVEL FOR STEEL'S MANY-ONE RANK TEST

Rank	Transformed Proportion Normal	Concentration
2	1.369	0.13 µg/L
2	1.369	0.13 µg/L
2	1.369	Control
4.5	1.397	0.13 µg/L
4.5	1.397	Control
7	1.571	0.13 µg/L
7	1.571	Control
7	1.571	Control

13.13.2.6.3 For this example, determine if the survival in any of the concentrations is significantly lower than the survival in the control. If this occurs, the rank sum at that concentration would be significantly lower than the rank sum of the control. Thus compare the rank sums for the survival at each of the various concentration levels with some "minimum" or critical rank sum, at or below which the survival would be considered significantly lower than the control. At a significance level of 0.05, the minimum rank sum in a test with five concentrations (excluding the control) and four replicates is 10 (See Table 5, Appendix E).

13.13.2.6.4 Since the rank sums for the 0.50 µg/L and 1.00 µg/L concentration levels are equal to the critical value, the proportions of normal development in those concentrations are considered significantly less than that in the control. Since no other rank sum is less than or equal to the critical value, no

TABLE 10. TABLE OF RANKS[1]

Replicate	Control	Copper Concentration (µg/L) 0.13	0.25
1	1.571(7,7.5,7.5,7.5,7)	1.369(2)	1.284(2)
2	1.369(2,4.5,5,5,4)	1.397(4.5)	1.345(3)
3	1.571(7,7.5,7.5,7.5,7)	1.571(7)	1.249(1)
4	1.397(4.5,6,6,6,5)	1.369(2)	1.369(4.5)

Replicate	Copper Concentration (µg/L) (Continued) 0.50	1.00	2.00
1	1.266(2)	1.217(3.5)	1.571(7)
2	1.303(3.5)	1.146(2)	0.959(2)
3	1.217(1)	1.217(3.5)	1.047(3)
4	1.303(3.5)	1.133(1)	0.685(1)

[1]Control ranks are given in the order of the concentration with which they were ranked.

TABLE 11. RANK SUMS

Concentration µg/L Copper)	Rank Sum
0.13	15.5
0.25	10.5
0.50	10.0
1.00	10.0

other concentration has a significantly lower proportion normal than the control. Because the 0.50 µg/L concentration shows significantly lower normal development than the control while the

higher 2.00 µg/L concentration does not, these test results are

considered to have an anomalous dose-response relationship and it is recommended that the test be repeated. If an NOEC and LOEC must be determined for this test, the lowest concentration with significant growth impairment versus the control is considered to the LOEC for growth. Thus, for this test, the NOEC and LOEC would be 0.25 µg/L and 0.50 µg/L, respectively.

13.13.2.7 Calculation of the ICp

13.13.2.7.1 The embryo-larval development data in Table 4 are utilized in this example. As can be seen from Table 4 and Figure 2, the observed means are monotonically non-increasing with respect to concentration (mean response for each higher concentration is not less than or equal to the mean response for the previous concentration and the responses between concentrations do not follow a linear trends). Therefore, it is not necessary to smooth the means prior to calculating the IC. The observed means, represented by $\&_i$ become the corresponding smoothed means, M_i. Table 12 contains the response means and smoothed means and Figure 3 gives a plot of the smoothed response curve.

TABLE 12. BIVALVE MEAN LARVAL DEVELOPMENT RESPONSE AFTER SMOOTHING

Copper Conc. (µg/L)	i	Response Means, $\&_i$ (proportion)	Smoothed Means, M_i (proportion)
Control	1	0.983	0.983
0.13	2	0.973	0.973
0.25	3	0.932	0.932
0.50	4	0.913	0.913
1.00	5	0.852	0.852
2.00	6	0.705	0.705

13.13.2.7.2 An IC25 can be estimated using the Linear Interpolation Method. A 25% reduction in mean proportion of normally developed larvae, compared to the controls, would result

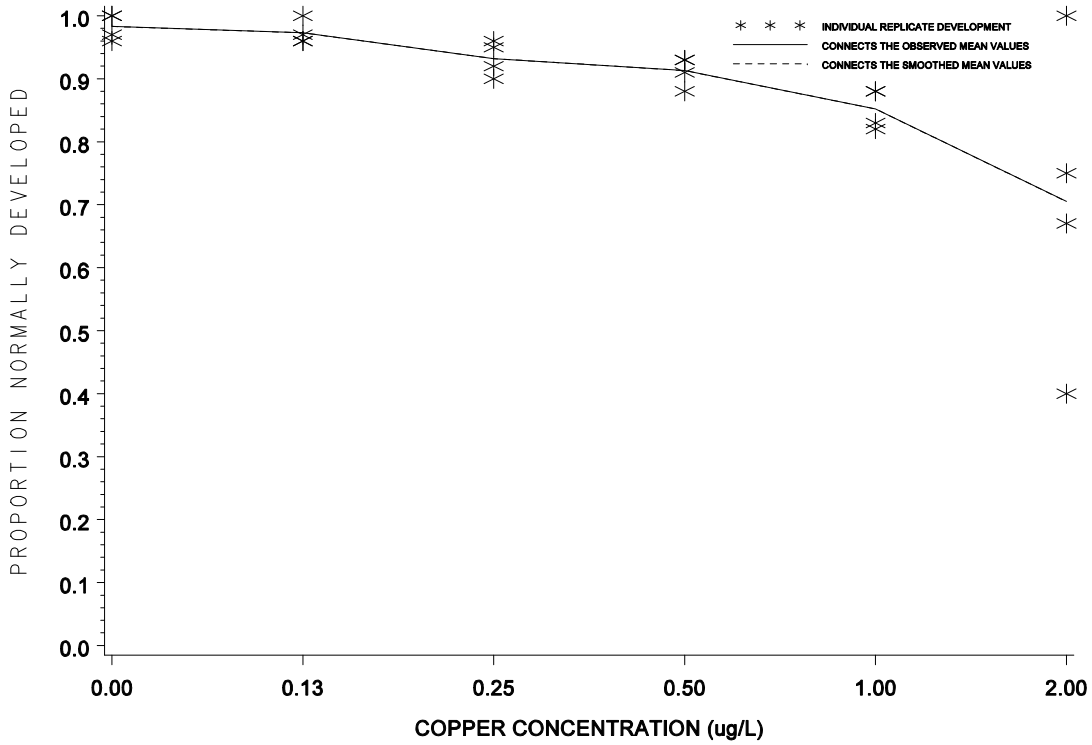

Figure 3. Plot of raw data, observed means, and smoothed means for the bivalve development data from Tables 4 and 12.

in a mean proportion of 0.737, where $M_1(1-p/100) = 0.983(1-25/100)$. Examining the means and their associated concentrations (Table 12), the response, 0.737, is bracketed by C_4 = 1.00 µg/L copper and C_5 = 2.00 µg/L copper.

13.13.2.7.3 Using the equation from Section 4.2 in Appendix L, the estimate of the IC25 is calculated as follows:

$$ICp \text{ ' } C_j\%[M_1(1\&p/100)\&M_j]\frac{(C_{(j\%1)}\&C_j)}{(M_{(j\%1)}\&M_j)}$$

IC25 = 1.00 + [0.983(1 - 25/100) - 0.852] (2.00 - 1.00)
S))))))))))))Q
(0.705 - 0.852)
= 1.78 µg/L.

13.13.2.7.4 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 1.7839 µg/L. The empirical 95.0% confidence interval for the true mean was not available because the number of resamples which generated an IC25 estimate was not an even multiple of 40. The computer program output for the IC25 for this data set is shown in Figure 4.

13.14 PRECISION AND ACCURACY

13.14.1 PRECISION

13.14.1.1 Single-Laboratory Precision

13.14.1.1.1 Single-laboratory precision data for the ***Mytilus spp.*** with the reference toxicant cadmium and lyophilized pulp mill effluent with natural seawater are provided in Tables 4-5. The coefficient of variation, based on EC25, is 32.8% to 45.0% for cadmium and 14.2% to 30.6% for lyophilized pulp mill effluent. Single-laboratory precision data for the ***Crassostrea gigas*** with the reference toxicant cadmium and lyophilized pulp mill effluent with natural seawater are provided in Tables 6-7. The coefficient of variation, based on EC25, is 18.5% to 80.4% for cadmium and 20.8% to 43.3% for lyophilized pulp mill effluent.

13.14.1.2 Multi-laboratory Precision

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	0.13	0.25	0.50	1.00	2.00
Response 1	1.00	0.96	0.92	0.91	0.88	1.00
Response 2	0.96	0.97	0.95	0.93	0.83	0.67
Response 3	1.00	1.00	0.90	0.88	0.88	0.75
Response 4	0.97	0.96	0.96	0.93	0.82	0.40

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Copper
Test Start Date: Test Ending Date:
Test Species: bivalve
Test Duration: 48 hours
DATA FILE: bivalve.icp
OUTPUT FILE: bivalve.i25

Conc. ID	Number Replicates	Concentration ug/L	Response Means	Std. Dev.	Pooled Response Means
1	4	0.000	0.983	0.021	0.983
2	4	0.130	0.973	0.019	0.973
3	4	0.250	0.932	0.028	0.932
4	4	0.500	0.913	0.024	0.913
5	4	1.000	0.852	0.032	0.852
6	4	2.000	0.705	0.247	0.705

The Linear Interpolation Estimate: 1.7839 Entered P Value: 25

Number of Resamplings: 80Those resamples not used had estimates above the highest concentration/ %Effluent.

The Bootstrap Estimates Mean: 1.6188 Standard Deviation: 0.1758

No Confidence Limits can be produced since the number of resamples generated is not a multiple of 40.
Resampling time in Seconds: 0.17 Random_Seed: -232404862

Figure 4. ICPIN program output for the IC25.

13.14.1.2.1 Multi-laboratory precision data for ***Mytilus spp***. with the reference toxicant, cadmium and lyophilized pulp mill effluent are provided in Tables 12-13. The coefficient of variation for cadmium EC25 is 23.6%, and for effluent EC25 is 14.4% based on five laboratories. Multi-laboratory precision data for ***Crassostrea gigas*** with the reference toxicant, cadmium and lyophilized pulp mill effluent are provided in Tables 14-15. The coefficient of variation is 21.3% for cadmium EC25 and 14.2% for lyophilized pulp mill effluent EC25, based on results from five laboratories.

13.14.2 ACCURACY

13.14.2.1 The accuracy of toxicity tests cannot be determined.

TABLE 12. SINGLE AND MULTI-LABORATORY PRECISION OF THE MUSSEL, ***MYTILUS SPP.***, DEVELOPMENT TEST PERFORMED WITH CADMIUM CHLORIDE (CD MG/L) AS A REFERENCE TOXICANT

Month	Lab A	Lab B	Lab C	Lab D	Lab E
Oct-92	2.35	1.06	2.42	4.20	4.77
Nov-92	0.86	3.49	3.89	2.21	2.39
Dec-92	1.79	2.51	no data	2.27	3.73
Jan-93	3.69	2.25	6.77	no data	1.57
Feb-93	2.81	2.91	5.85	3.75	3.05
Mar-93	3.71	2.64	2.62	4.89	no data

Mean	2.54	2.48	4.31	3.46	3.10
SD	1.11	0.81	1.94	1.19	1.23
CV (%)	43.9	32.8	45.0	34.3	40.0

# of Labs	Statistic	EC25
5	Mean (N=5) SD CV(%)	3.18 0.75 23.6

These data are from: Pastorok, et al. (1994), West Coast Marine Species Chronic Protocol Variability Study, PTI Environmental Services, Prepared for Washington Department of Ecology, February, 1994.

TABLE 13. SINGLE AND MULTI-LABORATORY PRECISION OF THE MUSSEL, ***MYTILUS SPP.***, DEVELOPMENT TEST PERFORMED WITH LYOPHILIZED PULP MILL EFFLUENT (%) AS THE TOXICANT

Month	Lab A	Lab B	Lab C	Lab D	Lab E
Oct-92	1.78	1.40	2.02	1.83	1.85
Nov-92	1.57	1.94	2.70	1.98	no data
Dec-92	1.74	1.88	3.08	no data	1.87
Jan-93	3.17	2.03	2.46	1.07	no data
Feb-93	1.66	no data	no data	no data	no data
Mar-93	1.85	1.66	1.72	1.82	no data

Mean	1.96	1.78	2.40	1.68	1.86
SD	0.60	0.25	0.54	0.41	0.28
CV (%)	30.6	14.2	22.5	24.5	1.4

# of Labs	Statistic	EC25
5	Mean (n=5) SD CV(%)	1.93 0.28 14.4

These data are from: Pastorok, et al. (1994) West Coast Marine Species Chronic Protocol Variability Study, PTI Environmental Services, Prepared for Washington Department of Ecology, February, 1994.

TABLE 14. SINGLE AND MULTI-LABORATORY PRECISION OF THE OYSTER, ***CRASSOSTREA GIGAS***, DEVELOPMENT TEST PERFORMED WITH CADMIUM CHLORIDE (CD MG/L) AS A REFERENCE TOXICANT

Month	Lab A	Lab B	Lab C	Lab D	Lab E
July-92	1.04	1.54	0.50	0.41	0.56
Aug-92	0.31	1.38	0.30	0.35	no data
Sept-92	0.68	0.20	0.49	no data	no data
Apr-93	no data	0.45	0.51	no data	0.95
May-93	0.46	0.30	1.05	0.52	0.83
June-93	0.26	1.55	0.93	no data	0.83
July-93	0.28	0.82	0.66	1.56	0.90

Mean	0.51	0.89	0.63	0.71	0.81
SD	0.31	0.59	0.27	0.57	0.15
CV (%)	60.6	66.7	42.1	80.4	18.5

# of Labs	Statistic	EC25
5	Mean (n=5) SD CV(%)	0.71 0.15 21.3

These data are from: Pastorok, et al. (1994), West Coast Marine Species Chronic Protocol Variability Study, PTI Environmental Services, Prepared for Washington Department of Ecology, February, 1994.

TABLE 15. SINGLE AND MULTI-LABORATORY PRECISION OF THE OYSTER, ***CRASSOSTREA GIGAS***, DEVELOPMENT TEST PERFORMED WITH LYOPHILIZED PULP MILL EFFLUENT (%) AS THE TOXICANT

Month	Lab A	Lab B	Lab C	Lab D	Lab E
July-92	no data	0.91	1.28	no data	1.43
Aug-92	1.21	1.09	0.98	0.61	no data
Sept-92	0.76	1.66	0.83	no data	no data
Apr-93	0.80	1.10	1.61	1.66	no data
May-93	1.21	0.65	1.90	0.93	0.93
June-93	1.09	1.32	1.72	0.83	0.98
July-93	0.82	0.80	1.56	1.67	1.04

Mean	0.98	1.08	1.41	1.14	1.10
SD.	0.21	0.34	0.40	0.49	0.23
CV (%)	21.6	31.4	28.0	43.3	20.8

# of Labs	Statistic	EC25
5	Mean (n=5) SD CV(%)	1.14 0.16 14.2

These data are from: Pastorok, et al., (1994), West Coast Marine Species Chronic Protocol Variability Study, PTI Environmental Services, Prepared for Washington Department of Ecology, February, 1994.

APPENDIX I. BIVALVE TEST: STEP-BY-STEP SUMMARY

PREPARATION OF TEST SOLUTIONS

A. Determine test concentrations and appropriate dilution water based on NPDES permit conditions and guidance from the appropriate regulatory agency.

B. Prepare effluent test solutions by diluting well mixed unfiltered effluent using volumetric flasks and pipettes. Use hypersaline brine where necessary to maintain all test solutions at 30 ± 2‰. Include brine controls in tests that use brine.

C. Prepare a copper reference toxicant stock solution.

D. Prepare a series copper reference toxicant concentrations.

E. Sample effluent and reference toxicant solutions for physical/chemical analysis. Measure salinity, pH and dissolved oxygen from each test concentration.

F. Randomize numbers for test chambers and record the chamber numbers with their respective test concentrations on a randomization data sheet. Store the data sheet safely until after the test samples have been analyzed.

G. Place test chambers in a water bath or environmental chamber set to 18 or 20EC and allow temperature to equilibrate.

H. Measure the temperature daily in one random replicate (or separate chamber) of each test concentration. Monitor the temperature of the water bath or environmental chamber continuously.

I. At the end of the test, measure salinity, pH, and dissolved oxygen concentration from each test concentration.

PREPARATION AND ANALYSIS OF TEST ORGANISMS

A. Determine test concentrations and appropriate dilution water based on NPDES permit requirements and guidance from the appropriate regulatory agency.

B. Prepare test solutions by diluting well mixed unfiltered effluent using volumetric pipettes. Use hypersaline brine where necessary to maintain all test solutions at 30 ± 2‰. Include brine controls in tests that use brine.

C. Sample effluent and reference toxicant solutions for physical/chemical analysis. Measure salinity, pH and dissolved oxygen from each test concentration.

D. Randomize numbers for test chambers and record the chamber numbers with their respective test concentrations on a randomization data sheet. Store the data sheet safely until after the test samples have been analyzed.

E. Place test chambers in a water bath or environmental chamber set to 18 or 20EC as appropriate for the test species and allow temperature to equilibrate.

F. Measure the test solution temperature daily in a randomly located blank test chamber. Monitor the temperature of the water bath or environmental chamber continuously.

PREPARATION AND ANALYSIS OF TEST ORGANISMS

A. Obtain test organisms and hold or condition as necessary for spawning.

B. On day of test, spawn organisms, examine gametes, pool good eggs, pool good sperm.

C. Fertilize subsets of eggs with a range of sperm concentrations to obtain >90% embryogenesis without polyspermy.

D. Adjust embryo stock suspension density to 1500-3000/mL.

E. Introduce organisms to test chambers (150-300 embryos in 0.1 mL of stock).

F. Count all embryos in each of six extra controls set up for determining mean embryo density and variation. Return these to the test for later examination for developmental rate in controls.

G. Near the end of the 48-hour incubation period examine several of the extra controls to determine if development has reached the prodisoconch stage. If yes, terminate the test at 48 hours; if no, continue the test for up to 54 hours as required for complete development.

H. Terminate the test by addition of formalin.

I. Count larvae and record the number of normal prodisoconch larvae and other larvae in each test vial.

J. Analyze the data.

K. Include standard reference toxicant point estimate values in the standard quality control charts.

Sample data sheet for embryo microscopic examination.

BIVALVE DEVELOPMENT TEST: RESULTS

Bioassay No._____ Date_____

Counter_____

Number	Sample	Abnormal	Normal	%Normal	Notes

SECTION 14

RED ABALONE, *Haliotis rufescens* LARVAL DEVELOPMENT TEST METHOD

Adapted from a method developed by
John W. Hunt and Brian S. Anderson
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Marine Pollution Studies Laboratory
34500 Coast Route 1, Monterey, CA 93940

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Appendix I Step-by Step Summary

SECTION 14

RED ABALONE, *HALIOTUS RUFESCENS*
LARVAL DEVELOPMENT TEST METHOD

14.1 SCOPE AND APPLICATION

14.1.1 This method estimates the chronic toxicity of effluents and receiving waters to the larvae of red abalone, ***Haliotis rufescens*** during a 48-h static non-renewal exposure. The effects include the synergistic, antagonistic, and additive effects of all chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms.

14.1.2 Detection limits of the toxicity of an effluent or chemical substance are organism dependent.

14.1.3 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling and because the test chambers are not sealed, highly volatile and highly degradable toxicants in the source may not be detected in the test.

14.1.4 This method is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

14.1.5 This method should be restricted to use by, or under the supervision of, professionals experienced in aquatic toxicity testing. Specific experience with any toxicity test is usually needed before acceptable results become routine.

14.2 SUMMARY OF METHOD

14.2.1 This method provides the step-by-step instructions for performing a 48-h static non-renewal test using early development of abalone larvae to determine the toxicity of substances in marine and estuarine waters. The test endpoint is normal shell development.

14.3 **INTERFERENCES**

14.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities and Equipment, and Supplies).

14.3.2 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests).

14.4 **SAFETY**

14.4.1 See Section 3, Health and Safety.

14.5 **APPARATUS AND EQUIPMENT**

14.5.1 Tanks, trays, or aquaria -- for holding and acclimating adult red abalone, e.g., standard salt water aquarium or Instant Ocean Aquarium (capable of maintaining seawater at 10-20EC), with appropriate filtration and aeration system.

14.5.2 Air pump, air lines, and air stones -- for aerating water containing broodstock or for supplying air to test solutions with low dissolved oxygen.

14.5.3 Constant temperature chambers or water baths -- for maintaining test solution temperature and keeping dilution water supply, gametes, and embryo stock suspensions at test temperature (15EC) prior to the test.

14.5.4 Water purification system -- Millipore Super-Q, Deionized water (DI) or equivalent.

14.5.5 Refractometer -- for determining salinity.

14.5.6 Hydrometer(s) -- for calibrating refractometer.

14.5.7 Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

14.5.8 Thermometer, National Bureau of Standards Certified (see USEPA METHOD l70.l, USEPA, l979) -- to calibrate laboratory thermometers.

14.5.9 pH and DO meters -- for routine physical and chemical measurements.

14.5.10 Standard or micro-Winkler apparatus -- for determining DO (optional) and calibrating the DO meter.

14.5.11 Winkler bottles -- for dissolved oxygen determinations.

14.5.12 Balance -- Analytical, capable of accurately weighing to 0.0001 g.

14.5.13 Fume hood -- to protect the analyst from effluent or formaldehyde fumes.

14.5.14 Glass stirring rods -- for mixing test solutions.

14.5.15 Graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 50-l000 mL for making test solutions. (Note: not to be used interchangeably for gametes or embryos and test solutions).

14.5.16 Volumetric flasks -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making test solutions.

14.5.17 Pipets, automatic -- adjustable, to cover a range of delivery volumes from 0.010 to 1.000 mL.

14.5.18 Pipet bulbs and fillers -- PROPIPET® or equivalent.

14.5.19 Wash bottles -- for reagent water, for topping off graduated cylinders, for rinsing small glassware and instrument electrodes and probes.

14.5.20 Wash bottles -- for dilution water.

14.5.21 20-liter cubitainers or polycarbonate water cooler jugs -- for making hypersaline brine.

14.5.22 Cubitainers, beakers, or similar chambers of non-toxic composition for holding, mixing, and dispensing dilution water and other general non-effluent, non-toxicant contact uses. These should be clearly labeled and not used for other purposes.

14.5.23 Beakers, 1,000 mL borosilicate glass -- for mixing gametes for fertilization of eggs.

14.5.24 Beakers, 250 mL borosilicate glass -- for preparation of test solutions.

14.5.25 Counter, two unit, 0-999 -- for recording counts of larvae.

14.5.26 Inverted or compound microscope -- for inspecting gametes and making counts of larvae.

14.5.27 Perforated plunger -- for stirring egg solutions.

14.5.28 Supply of ***Macrocystis*** or other macroalgae (if holding broodstock for longer than 5 days) -- for feeding abalone.

14.5.29 Stainless steel butter knife, rounded smooth-edged blade (for handling adult abalone). Abalone irons and plastic putty knives have also been used successfully.

14.5.30 Sieve or screened tube, approximately 37 µm-mesh -- for retaining larvae at the end of the test.

14.5.31 60 µm NITEX® filter -- for filtering receiving water.

14.6 **REAGENTS AND SUPPLIES**

14.6.1 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests).

14.6.2 Data sheets (one set per test) -- for data recording (See Appendix I).

14.6.3 Tape, colored -- for labelling test chambers and containers.

14.6.4 Markers, water-proof -- for marking containers, etc.

14.6.5 Parafilm -- to cover graduated cylinders and vessels containing gametes, embryos.

14.6.6 Gloves, disposable -- for personal protection from contamination.

14.6.7 Pipets, serological -- 1-10 mL, graduated.

14.6.8 Pipet tips -- for automatic pipets.

14.6.9 Coverslips -- for microscope slides.

14.6.10 Lens paper -- for cleaning microscope optics.

14.6.11 Laboratory tissue wipes -- for cleaning and drying electrodes, microscope slides, etc.

14.6.12 Disposable countertop covering -- for protection of work surfaces and minimizing spills and contamination.

14.6.13 pH buffers 4, 7, and 10 (or as per instructions of instrument manufacturer) -- for standards and calibration check (see USEPA Method 150.1, USEPA, 1979).

14.6.14 Membranes and filling solutions -- for dissolved oxygen probe (see USEPA Method 360.1, USEPA, 1979), or reagents for modified Winkler analysis.

14.6.15 Laboratory quality assurance samples and standards -- for the above methods.

14.6.16 Test chambers -- 600 mL, five chambers per concentration. The chambers should be borosilicate glass (for effluents) or nontoxic disposable plastic labware (for reference toxicants). To avoid contamination from the air and excessive evaporation of test solutions during the test, the chambers should be covered during the test with safety glass plates or a plastic sheet (6 mm thick).

14.6.17 Formaldehyde, 37% (Concentrated Formalin) -- for preserving larvae. Note: formaldehyde has been identified as a

carcinogen and is irritating to skin and mucous membranes. It should not be used at a concentration higher than necessary to achieve morphological preservation of larvae for counting and only under conditions of maximal ventilation and minimal opportunity for volatilization into room air.

14.6.18 Tris (hydroxymethyl) aminomethane and hydrogen peroxide (for H_2O_2 spawning method) -- for spawning abalone.

14.6.19 Reference toxicant solutions (see Subsection 14.10.2.4 and see Section 4, Quality Assurance).

14.6.20 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms (see Section 5, Facilities, Equipment, and Supplies and Section 7, Dilution Water).

14.6.21 Effluent and receiving water -- see Section 8, Effluent and Surface Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests.

14.6.22 Dilution water and hypersaline brine -- see Section 7, Dilution Water and Section 14.6.24, Hypersaline Brines. The dilution water should be uncontaminated 1-µm-filtered natural seawater. Hypersaline brine should be prepared from dilution water.

14.6.23 HYPERSALINE BRINES

14.6.23.1 Most industrial and sewage treatment effluents entering marine and estuarine systems have little measurable salinity. Exposure of larvae to these effluents will usually require increasing the salinity of the test solutions. It is important to maintain an essentially constant salinity across all treatments. In some applications it may be desirable to match the test salinity with that of the receiving water (See Section 7.1). Two salt sources are available to adjust salinities -- artificial sea salts and hypersaline brine (HSB) derived from natural seawater. Use of artificial sea salts is necessary only when high effluent concentrations preclude salinity adjustment by HSB alone.

14.6.23.2 Hypersaline brine (HSB) can be made by concentrating natural seawater by freezing or evaporation. HSB should be made from high quality, filtered seawater, and can be added to the effluent or to reagent water to increase salinity. HSB has several desirable characteristics for use in effluent toxicity testing. Brine derived from natural seawater contains the necessary trace metals, biogenic colloids, and some of the microbial components necessary for adequate growth, survival, and/or reproduction of marine and estuarine organisms, and it can be stored for prolonged periods without any apparent degradation. However, even if the maximum salinity HSB (100‰) is used as a diluent, the maximum concentration of effluent (0‰) that can be tested is 66% effluent at 34‰ salinity (see Table 1).

14.6.23.3 High quality (and preferably high salinity) seawater should be filtered to at least 10 µm before placing into the freezer or the brine generator. Water should be collected on an incoming tide to minimize the possibility of contamination.

14.6.23.4 Freeze Preparation of Brine

14.6.23.4.1 A convenient container for making HSB by freezing is one that has a bottom drain. One liter of brine can be made from four liters of seawater. Brine may be collected by partially freezing seawater at -10 to -20EC until the remaining liquid has reached the target salinity. Freeze for approximately six hours, then separate the ice (composed mainly of fresh water) from the remaining liquid (which has now become hypersaline).

14.6.23.4.2 It is preferable to monitor the water until the target salinity is achieved rather than allowing total freezing followed by partial thawing. Brine salinity should never exceed 100‰. It is advisable not to exceed about 70‰ brine salinity unless it is necessary to test effluent concentrations greater than 50%.

14.6.23.4.3 After the required salinity is attained, the HSB should be filtered through a 1 µm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at

4EC (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

14.6.23.5 Heat Preparation of Brine

TABLE 1. MAXIMUM EFFLUENT CONCENTRATION (%) THAT CAN BE TESTED AT 34‰ WITHOUT THE ADDITION OF DRY SALTS GIVEN THE INDICATED EFFLUENT AND BRINE SALINITIES.

Effluent Salinity ‰	Brine 60 ‰	Brine 70 ‰	Brine 80 ‰	Brine 90 ‰	Brine 100 ‰
0	43.33	51.43	57.50	62.22	66.00
1	44.07	52.17	58.23	62.92	66.67
2	44.83	52.94	58.97	63.64	67.35
3	45.61	53.73	59.74	64.37	68.04
4	46.43	54.55	60.53	65.12	68.75
5	47.27	55.38	61.33	65.88	69.47
10	52.00	60.00	65.71	70.00	73.33
15	57.78	65.45	70.77	74.67	77.65
20	65.00	72.00	76.67	80.00	82.50
25	74.29	80.00	83.64	86.15	88.00

14.6.23.5.1 The ideal container for making brine using heat-assisted evaporation of natural seawater is one that (1) has a high surface to volume ratio, (2) is made of a non-corrosive material, and (3) is easily cleaned (fiberglass containers are ideal). Special care should be used to prevent any toxic materials from coming in contact with the seawater being used to generate the brine. If a heater is immersed directly into the seawater, ensure that the heater materials do not corrode or leach any substances that would contaminate the brine. One successful method is to use a thermostatically controlled heat

exchanger made from fiberglass. If aeration is needed, use only oil-free air compressors to prevent contamination.

14.6.23.5.2 Before adding seawater to the brine generator, thoroughly clean the generator, aeration supply tube, heater, and any other materials that will be in direct contact with the brine. A good quality biodegradable detergent should be used, followed by several (at least three) thorough reagent water rinses.

14.6.23.5.3 Seawater should be filtered to at least 10 µm before being put into the brine generator. The temperature of the seawater is increased slowly to 40EC. The water should be aerated to prevent temperature stratification and to increase water evaporation. The brine should be checked daily (depending on the volume being generated) to ensure that the salinity does not exceed 100‰ and that the temperature does not exceed 40EC. Additional seawater may be added to the brine to obtain the volume of brine required.

14.6.23.5.4 After the required salinity is attained, the HSB should be filtered through a 1 µm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4EC (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

14.6.23.6 Artificial Sea Salts

14.6.23.6.1 No data from red abalone tests using sea salts or artificial seawater (e.g., GP2) are available for evaluation at this time, and their use must be considered provisional.

14.6.23.7 Dilution Water Preparation from Brine

14.6.23.7.1 Although salinity adjustment with brine is the preferred method, the use of high salinity brines and/or reagent water has sometimes been associated with discernible adverse effects on test organisms. For this reason, it is recommended that only the minimum necessary volume of brine and reagent water be used to offset the low salinity of the effluent, and that

brine controls be included in the test. The remaining dilution water should be natural seawater. Salinity may be adjusted in one of two ways. First, the salinity of the highest effluent test concentration may be adjusted to an acceptable salinity, and then serially diluted. Alternatively, each effluent concentration can be prepared individually with appropriate volumes of effluent and brine.

14.6.23.7.2 When HSB and reagent water are used, thoroughly mix together the reagent water and HSB before mixing in the effluent. Divide the salinity of the HSB by the expected test salinity to determine the proportion of reagent water to brine. For example, if the salinity of the brine is 100‰ and the test is to be conducted at 34‰, 100‰ divided by 34‰ = 2.94. The proportion of brine is 1 part plus 1.94 parts reagent water. To make 1 L of dilution water at 34‰ salinity from a HSB of 100‰, 340 mL of brine and 660 mL of reagent water are required. Verify the salinity of the resulting mixture using a refractometer.

14.6.23.8 Test Solution Salinity Adjustment

14.6.23.8.1 Table 2 illustrates the preparation of test solutions (up to 50% effluent) at 34‰ by combining effluent, HSB, and dilution water. Note: if the highest effluent concentration does not exceed 50% effluent, it is convenient to prepare brine so that the sum of the effluent salinity and brine salinity equals 68‰; the required brine volume is then always equal to the effluent volume needed for each effluent concentration as in the example in Table 2.

14.6.23.8.2 Check the pH of all test solutions and adjust to within 0.2 units of dilution water pH by adding, dropwise, dilute hydrochloric acid or sodium hydroxide (see Section 8.8.9, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

14.6.23.8.3 To calculate the amount of brine to add to each effluent dilution, determine the following quantities: salinity of the brine (SB, in ‰), the salinity of the effluent (SE, in ‰), and volume of the effluent to be added (VE, in mL). Then use the following formula to calculate the volume of brine (VB, in mL) to be added:

$$VB = VE \times (34 - SE)/(SB - 34)$$

14.6.23.8.4 This calculation assumes that dilution water salinity is 34 ± 2‰.

14.6.23.9 Preparing Test Solutions

14.6.23.9.1 Two hundred mL of test solution are needed for each test chamber. To prepare test solutions at low effluent concentrations (<6%), effluents may be added directly to dilution water. For example, to prepare 1% effluent, add 10 mL of effluent to a 1-liter volumetric flask using a volumetric pipet or calibrated automatic pipet. Fill the volumetric flask to the 1-L mark with dilution water, stopper it, and shake to mix. Distribute equal volumes into the replicate test chambers.

14.6.23.9.2 To prepare a test solution at higher effluent concentrations, hypersaline brine must usually be used. For example, to prepare 40% effluent, add 400 mL of effluent to a 1-liter volumetric flask. Then, assuming an effluent salinity of 2‰ and a brine salinity of 66‰, add 400 mL of brine (see equation above and Table 2) and top off the flask with dilution water. Stopper the flask and shake well. Distribute equal volumes into the replicate test chambers.

14.6.23.10 Brine Controls

14.6.23.10.1 Use brine controls in all tests where brine is used. Brine controls contain the same volume of brine as does the highest effluent concentration using brine, plus the volume of reagent water needed to reproduce the hyposalinity of the effluent in the highest concentration, plus dilution water. Calculate the amount of reagent water to add to brine controls by rearranging the above equation, (See, 16.6.23.8.3) setting SE = 0, and solving for VE.

$$VE = VB \times (SB - 34)/(34 - SE)$$

If effluent salinity is essentially 0‰, the reagent water volume needed in the brine control will equal the effluent volume at the highest test concentration. However, as effluent salinity and effluent concentration increase, less reagent water volume is needed.

14.6.24 TEST ORGANISMS

14.6.24.1 The test organisms used for this test are red abalone, ***Haliotis rufescens***. This large gastropod mollusc is harvested commercially in southern California and supports a popular recreational fishery throughout the state. It consumes a variety of seaweeds and small incidental organisms, and is an important food source for sea otters, lobsters, and octopods (Hines and Pearse 1892). Abalone are "broadcast" spawners that reproduce by equivalent.ejecting large numbers of gametes into the water column, where fertilization takes place externally. Free-swimming larvae hatch as trochophores, then undergo torsion while passing through a veliger stage. Abalone larvae do not feed during their one to three weeks in the plankton, but exist on energy stored in the yolk sack, supplemented perhaps by the uptake of dissolved amino acids. Once larvae come into contact with suitable substrate, they metamorphose and begin to consume benthic algae using a rasp-like tongue (the radula). Red abalone become reproductive after about two years at a length of about 7 cm, and can live for at least 25 years, growing to 30 cm in length. Refer to Hahn (1989) for a review of abalone life history and culture to Martin et al. (1977), Morse et al (1979) and Hunt and Anderson (1989 and 1993) for previous toxicity studies.

14.6.24.2 Species Identification

14.6.24.2.1 Broodstock should be positively identified to species. Epipodal characteristics provide the best means of identification. All California haliotids have a lacey epipodial fringe, except for the red and black abalone, which have smooth, lobed epipodia. The red abalone can be distinguished from the black by shell coloration and by the number of respiratory pores in the shell (reds have 3 to 4, blacks have 5 to 8). For further information on abalone taxonomy consult Owen et al. (1971), and Morris et al. (1980).

14.6.24.3 Obtaining Broodstock

14.6.24.3.1 Mature red abalone broodstock can be collected from rocky substrates from the intertidal to depths exceeding 30 meters. They are found most commonly in crevices in areas where there is an abundance of macroalgae. State collection permits are usually required for collecting abalone. Collection of

TABLE 2. EXAMPLES OF EFFLUENT DILUTION SHOWING VOLUMES OF EFFLUENT (x‰), BRINE, AND DILUTION WATER NEEDED FOR ONE LITER OF EACH TEST SOLUTION.

FIRST STEP: Combine brine with reagent water or natural seawater to achieve a brine of 68-x‰ and, unless natural seawater is used for dilution water, also a brine-based dilution water of 34‰.

SERIAL DILUTION:

Step 1. Prepare the highest effluent concentration to be tested by adding equal volumes of effluent and brine to the appropriate volume of dilution water. An example using 40% is shown.

Effluent Conc. (%)	Effluent x‰	Brine (68-x)‰	Dilution Water* 34‰
40	800 mL	800 mL	400 mL

Step 2. Use either serially prepared dilutions of the highest test concentration or individual dilutions of 100% effluent.

Effluent Conc. (%)	Effluent Source	Dilution Water* (34‰)
20	1000 mL of 40%	1000 mL
10	1000 mL of 20%	1000 mL
5	1000 mL of 10%	1000 mL
2.5	1000 mL of 5%	1000 mL
Control	none	1000 mL

INDIVIDUAL PREPARATION

Effluent Conc. (%)	Effluent x‰	Brine (68-x)‰	Dilution Water* 34‰
40	400 mL	400 mL	200 mL
20	200 mL	200 mL	600 mL
10	100 mL	100 mL	800 mL
5	50 mL	50 mL	900 mL
2.5	25 mL	25 mL	950 mL
Control	none	none	1000 mL

*May be natural seawater or brine-reagent water.

abalone is regulated by California law. Collectors must obtain a scientific collectors permit from the California Department of Fish and Game and observe any regulations regarding collection, transfer, and maintenance of abalone broodstock.

14.6.24.3.2 While abalone captured in the wild can be induced to spawn, those grown or conditioned in the laboratory have been more dependable. Commercial mariculture facilities in California produce large numbers of abalone, and distribution systems exist to supply live spawners to a number of market areas. In any case, broodstock should be obtained from sources free of contamination by toxic substances to avoid genetic or physiological preadaptation to pollutants.

14.6.24.3.3 Abalone broodstock can be transported for short time periods from the field or supply facility in clean covered plastic buckets filled with seawater. Use compressed air, or battery powered pumps to supply aeration. Compressed oxygen is not recommended because bubbled oxygen may induce unintended spawning (Morse et al., 1977). Maintain water temperatures within 3EC of the temperature at the collecting site. Four abalone in a 15-liter bucket should remain healthy for up to four hours under these conditions.

14.6.24.3.4 Abalone can be transported for up to 30 hours in sealed, oxygen-filled plastic bags containing moist (seawater) polyfoam sponges (Hahn, 1989). Cut the polyfoam into sections (about 20 X 40 cm) and allow them to soak in clean seawater for a few minutes. New sponges should be leached in seawater for at least 24 hours. Rinse the sponges in fresh seawater and wring them out well. Place the polyfoam inside double plastic trash bags, then place the abalone on the moist foam. It is important that there is no standing water in the bags. Put the abalone bags into an ice chest (10 to 15 liter), fill the bags with pure oxygen, squeeze the bags to purge out all the air, then refill with oxygen (approximately three liters of oxygen gas will support eight abalone). Seal the bags (air-tight) with a tie or rubber band. Wrap two small (one-liter) blue ice blocks in sections of newspaper (about 15 pages thick) for insulation, and place the wrapped blue ice in a sealed plastic bag in the chest on top of the abalone bags. Fill any remaining space with packing and seal the box for shipping. Avoid transporting the ice chest in temperatures below freezing or above 30EC.

14.6.24.4 Broodstock Culture and Handling

14.6.24.4.1 At the testing facility, place the abalone in aerated tanks with flowing seawater (1 to 2 liter/min). With high water quality, water flow, and aeration, abalone 8 to 10 cm long can be kept at a density of one per liter of tank space or one per 100 cm^2 of tank surface area, whichever provides the lower density. Density should be cut to a maximum of 0.5 per liter in recirculating systems and to a maximum of 0.25 per liter in static tanks. Tanks should be covered for shade and to prevent escape. Drain and rinse culture tanks twice weekly to prevent build-up of detritus. Remove any dead abalone immediately, and drain and scrub its tank.

14.6.24.4.2 Ideal maintenance temperature is 15 ± 1EC, the toxicity test temperature (see also Leighton, 1974). If broodstock are to be held for longer than 5 days at the testing facility, feed broodstock with blades of the giant kelp, ***Macrocystis***. Feed to slight excess; large amounts of uneaten algae will foul culture water. If ***Macrocystis*** is unavailable, other brown algae (***Nereocystis***, ***Egregia***, ***Eisenia***) or any fleshy red algae can be substituted (Hahn, 1989).

14.6.24.4.3 Recirculating tanks should be equipped with biological or activated carbon filtration systems and oyster shell beds to maintain water quality. Measure the ammonia content of static or recirculating seawater daily to monitor the effectiveness of the filtration system. Un-ionized ammonia concentrations should not exceed 20 µg/liter and total ammonia concentrations should not exceed 1.0 mg/liter. Supply constant aeration and temperature control. Add only a few blades of algal food at each cleaning to prevent its accumulation and decay.

14.6.24.4.4 When handling abalone, use a rounded, dull-bladed stainless-steel butter knife, abalone iron, or plastic putty knife to release the animal's grip on the substrate. Gently slide the flat dull blade under the foot at the posterior end near the beginning of the shell whorl, and slide it under about two-thirds of the foot. Apply constant pressure to keep the front edge of the blade against the substrate and not up into the foot. Quickly and gently lift the foot off the substrate. A smooth deliberate motion is more effective and less damaging than repeated prying.

14.6.24.4.5 Assess the reproductive condition of the broodstock by examining the gonads, located under the right posterior edge of the shell. An abalone placed upside down on a flat surface will soon relax and begin moving the foot trying to right itself. Take advantage of this movement and use the dull blade to bend the foot away from the gonad area for inspection. The female ovary is jade green, the male testes are cream-colored. When the gonad fully envelopes the dark blue-gray conical digestive gland and is bulky along its entire length, the abalone is ready for spawning (Hahn, 1989). Ripe (recrudescent) spawners have a distinct color difference between the gray digestive gland and the green or cream-colored gonad. Less developed gonads appear gray (in females) or brown (in males).

14.6.24.4.6 Abalone 7 to 10 cm in shell length are recommended in broodstock. They are easier to handle than larger ones, and can be spawned more often (approximately every four months under suitable culture conditions; Ault, 1985). Though spawning fewer eggs than larger abalone, 10 cm abalone will produce over 100,000 eggs at a time (Ault, 1985). Twenty to thirty-five thousand eggs are needed for a single toxicant test, depending on test design. For further information of red abalone culture, see Ebert and Houk (1984) or Hahn (1989).

14.6.24.5 Culture Materials

14.6.24.5.1 See Section 4, Quality Assurance Section for a discussion of suitable materials to be used in laboratory culture of abalone. Be sure all new materials are properly leached in seawater before use. After use, all culture materials should be washed in soap and water, then rinsed with seawater before reuse.

14.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

14.7.1 See Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

14.8 CALIBRATION AND STANDARDIZATION

14.8.1 See Section 4, Quality Assurance.

14.9 QUALITY CONTROL

14.9.1 See Section 4, Quality Assurance.

14.10 **TEST PROCEDURES**

14.10.1 TEST DESIGN

14.10.1.1 The test consists of at least five effluent concentrations plus a dilution water control. Tests that use brine to adjust salinity must also contain five replicates of a brine control.

14.10.1.2 Effluent concentrations are expressed as percent effluent.

14.10.2 TEST SOLUTIONS

14.10.2.1 Receiving waters

14.10.2.1.1 The sampling point is determined by the objectives of the test. At estuarine and marine sites, samples are usually collected at mid-depth. Receiving water toxicity is determined with samples used directly as collected or with samples passed through a 60 µm NITEX® filter and compared without dilution, against a control. Using five replicate chambers per test, each containing 200 mL would require approximately 1 L of sample per test.

14.10.2.2 Effluents

14.10.2.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of at least 0.5 is commonly used. A dilution factor of 0.5 provides hypothesis test discrimination of ± 100%, and testing of a 16 fold range of concentrations. Hypothesis test discrimination shows little improvement as dilution factors are increased beyond 0.5 and declines rapidly if smaller dilution factors are used. **USEPA recommends that one of the five effluent treatments must be a concentration of effluent mixed with dilution water which corresponds to the permittee's instream waste concentration (IWC).** At least two of the effluent treatments must be of lesser effluent concentration than the IWC,

with one being at least one-half the concentration of the IWC. If 100‰ HSB is used as a diluent, the maximum concentration of effluent that can be tested will be 66% at 34‰ salinity.

14.10.2.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12% and 1.56%).

14.10.2.2.3 The volume in each test chamber is 200 mL.

14.10.2.2.4 Effluent dilutions should be prepared for all replicates in each treatment in one container to minimize variability among the replicates. Dispense into the appropriate effluent test chambers.

14.10.2.3 Dilution Water

14.10.2.3.1 Dilution water should be uncontaminated 1-µm-filtered natural seawater or hypersaline brine prepared from uncontaminated natural seawater plus reagent water (see Section 7, Dilution Water). Natural seawater may be uncontaminated receiving water. This water is used in all dilution steps and as the control water.

14.10.2.4 Reference Toxicant Test

14.10.2.4.1 Reference toxicant tests should be conducted as described in Quality Assurance (see Section 4.7).

14.10.2.4.2 The preferred reference toxicant for red abalone is zinc sulfate ($ZnSO_4 \cdot H_2O$). Reference toxicant tests provide an indication of the sensitivity of the test organisms and the suitability of the testing laboratory (see Section 4 Quality Assurance). Another toxicant may be specified by the appropriate regulatory agency. Prepare a 10,000 µg/L zinc stock solution by adding 0.0440 g of zinc sulfate ($ZnSO_4 \cdot H_2O$) to one liter of reagent water in a polyethylene volumetric flask. Alternatively, certified standard solutions can be ordered from commercial companies.

14.10.2.4.3 Reference toxicant solutions should be five replicates each of 0 (control), 10, 18, 32, and 56, and 100 µg/L total zinc. Prepare one liter of each concentration by adding 0,

1.0, 1.8, 3.2, 5.6, and 10.0 mL of stock solution, respectively, to one-liter volumetric flasks and fill with dilution water. Start with control solutions and progress to the highest concentration to minimize contamination.

14.10.2.4.4 If the effluent and reference toxicant tests are to be run concurrently, then the tests must use embryos from the same spawn. The tests must be handled in the same way and test solutions delivered to the test chambers at the same time. Reference toxicant tests must be conducted at 34 ± 2‰.

14.10.3 COLLECTION OF GAMETES FOR THE TEST

14.10.3.1 Spawning Induction

14.10.3.1.1 Note: Before beginning the spawning induction process, be sure that test solutions will be mixed, sampled, and temperature equilibrated in time to receive the newly fertilized eggs. Spawning induction generally takes about three hours, but if embryos are ready before test solutions are at the proper temperature, the delay may allow embryos to develop past the one-cell stage before transfer to the toxicant. Transfer can then damage the embryos, leading to unacceptable test results.

14.10.3.1.2 Culture work (spawning, etc.) and toxicant work should be done in separate laboratory rooms, and care should be taken to avoid contaminating organisms prior to testing.

14.10.3.1.3 Ripe abalone can be induced to spawn by stimulating the synthesis of prostoglandin-endoperoxide in the reproductive tissues (Morse et al., 1977). This can be done in two ways: addition of hydrogen peroxide to seawater buffered with Tris (Morse et al., 1977), or irradiation of seawater with ultraviolet light (Kikuchi and Uki, 1974). The first method is preferable for small laboratories because it avoids the cost and maintenance requirements of a UV system. If a UV system is available, this method may be preferable because it is simple, does not use chemicals that could accidentally harm larvae, and is considered to be less likely to force gametes from unripe adults.

14.10.3.1.4 If brood stock are shipped to the laboratory by a supplier, it is important to allow two days or more for laboratory acclimation before spawning induction; this should

increase the probability of achieving a successful spawn of viable gametes. Always bring brood stock up to acclimation temperature slowly to avoid premature spawning.

14.10.3.2 Hydrogen Peroxide Method

14.10.3.2.1 Select four ripe male abalone and four ripe females. Clean their shells of any loose debris. Place the males in one clean polyethylene bucket and the females in another. Cover the buckets with a tight fitting perforated lid, supply the chambers with flowing or recirculating (1 liter/minute) 20-µm-filtered seawater (15EC), and leave the animals without food for 24 to 48 hours to acclimate and eliminate wastes. If flowing seawater is unavailable, keep the spawners in larger (>30 liter) aquaria with aeration at 15 ± 1EC for 24 hours without food to eliminate wastes. Three hours prior to the desired spawning time, drain the buckets, wipe and rinse out mucus and debris, and refill with 6 liters of 1 µm-filtered seawater. If abalone have been kept in larger aquaria, put them in the buckets at this time. Check the abalone from time to time to make sure they remain underwater. Add air stones to the buckets and keep them aerated until spawning begins.

14.10.3.2.1 Dissolve 12.1 g of Tris into 50 mL of reagent water. When the Tris has dissolved completely, mix the hydrogen peroxide (H_2O_2) solution in a separate flask by pouring 10 mL of fresh* refrigerated H_2O_2 (30%) into 40 mL of refrigerated reagent water (1:5 dilution). Pour 25 mL of Tris solution and 25 mL of H_2O_2 solution into each of the spawning buckets (male and female). Stir well to mix; the final concentration in the spawning buckets will be approximately 6 mM Tris (pH = 9.1) and 5 m H_2O_2. Allow the abalone to remain in contact with the chemicals for 2.5 hours at 15 ± 1EC. The chemical reaction is temperature dependent (three hours of contact with H_2O_2 would be necessary at 11EC). Temperatures higher than 15EC are not recommended for spawning. Maintain constant aeration. Since females often begin spawning after the males, it may be useful to induce male spawning 15-30 minutes later, however egg quality should not be compromised if females spawn first (See 14.10.3.3.2 below).

*Note: Hydrogen peroxide loses potency over time. Purchase reagent or certified grade H_2O_2 in small containers (100

mL). Store unopened containers for no more than one year, and discard open containers after one month. Mark the purchase date and opening date on all containers, and keep all containers refrigerated.

14.10.3.2.3 After 2.5 hours, empty the spawning buckets, rinse them well, and refill them to the top with fresh dilution water seawater at the same temperature (15 ± 1EC). Keep the containers clean by siphoning away mucus and debris. Maintain constant aeration until spawning begins, then remove the air stones. The abalone begin spawning about three hours after the introduction of the chemicals (at 15 ± 1EC). Eggs are dark green and are visible individually to the naked eye, sperm appear as white clouds emanating from the respiratory pores.

14.10.3.2.4 If spawning begins before the chemicals have been removed, drain the buckets immediately, discarding any gametes. Rinse the buckets thoroughly and refill with clean, dilution water seawater (15 ± 1EC). Use only the gametes subsequently spawned in clean water for testing.

14.10.3.3 UV Irradiation Method

14.10.3.3.1 Select four ripe male abalone and four ripe females. Clean their shells of any debris. Place the males in one clean polyethylene bucket and the females in another. Cover the buckets with a tight fitting perforated lid, supply the containers with flowing or recirculating (1 liter/minute) 20-µm-filtered seawater (15 ± 1EC), and leave the animals without food for 24 to 48 hours to acclimate and eliminate wastes. If flowing seawater is unavailable, keep the spawners in larger (>30 liter) aquaria with aeration at 15 ± 1EC for 24 hours. Three hours prior to the desired spawning time, drain the buckets, wipe and rinse out mucus and debris, and refill with just enough water to cover the abalone (which should all be placed in the bottom of the bucket). Begin slowly filling the buckets with dilution water seawater (15 ± 1EC) that has passed through the UV sterilization unit. Flow rates to each of the buckets should be 150 mL/min. A low total flow rate (300 mL/minute) in the UV unit is necessary to permit sufficient seawater irradiation. (The sterilization unit should be cleaned and the UV bulb replaced at least once annually). Place the buckets in a water bath at 15 ± 1EC to counter the temperature increase caused by the slow

passage of the water past the UV lamp. Check the containers periodically, and keep them clean by siphoning out any debris. After three hours (± about 1/2 hour), abalone should begin spawning by ejecting clouds of gametes into the water. Eggs are dark green and are visible individually to the naked eye, sperm appear as white clouds emanating from the respiratory pores.

14.10.3.3.2 Note: If past experience or other factors indicate difficulties in achieving synchronous spawning, it may be helpful to induce a second group of females about an hour after the first. This will increase the chances of providing fresh eggs (less than one hour old) for fertilization if males spawn late (see below). Senescence of sperm is seldom a problem because males continue spawning over a longer period of time.

14.10.3.4 Pooling Gametes

14.10.3.4.1 Although it is not necessary, it is preferable to have more than one abalone of each sex spawn. To increase the probability of multiple spawners without risking senescence of the gametes, allow one-half hour after the first individual of the second sex begins to spawn before initiating fertilization. For example, if males spawn first, wait one-half hour after the first female spawns before fertilizing eggs. In most cases this will provide time for more than one of each sex to spawn. More important than multiple spawning, however, is avoiding delay of fertilization. Eggs should be fertilized within one hour of release (Uki and Kikuchi 1974). All sperm should be pooled, and all eggs should be pooled prior to fertilization. This can be accomplished by gentle swirling within the spawning buckets. Note: Take care to avoid contaminating eggs with sperm prior to the intended fertilization time. It is important that development is synchronous among all test embryos.

14.10.3.5 Fertilization

14.10.3.5.1 As the females spawn, allow the eggs to settle to the bottom. If necessary, gently stir to evenly distribute the eggs. Siphon out and discard any eggs that appear clumped together. Eggs are ready to transfer to a third (fertilization) bucket when either: (1) one-half hour has passed since the first individual of the second sex has spawned (2) multiple individuals of each sex have spawned, or 3) there are too many eggs on the

bottom of the bucket to allow evenly distributed eggs to avoid each other. Slowly siphon eggs into a third clean polyethylene bucket containing one or two liters of dilution water seawater (15 ± 1EC). Siphon carefully to avoid damaging the eggs and to avoid collecting any debris from the spawning container. Siphon about 100,000 eggs, enough to make a single even layer on the container bottom. Each egg should be individually distinguishable, and not touching other eggs. If excess eggs are available, siphon them into a second fertilization bucket to be used as a reserve. Keep all containers at 15 ± 1EC. Make sure that water temperatures differ by no more than 1EC when transferring eggs or sperm from one container to another.

14.10.3.5.2 As the males spawn, siphon sperm from directly above the respiratory pore and collect this in a 500 mL flask with filtered seawater. Keep the flask at 15 ± 1EC, and use it as a back-up in case the males stop spawning. If spawning continues renew this reserve every 15 minutes. Usually the males will continue spawning, turning the water in the bucket milky white. As long as the males continue spawning, partially drain and refill the bucket every 15 minutes, replacing old sperm-laden water with fresh seawater (15 ± 1EC). Use the freshest sperm possible for fertilization.

14.10.3.5.3 Make sure eggs are fertilized within one hour of release (Uki and Kikuchi, 1974, see note after Section 14.8.5.2). To fertilize the eggs, collect about 200 mL of sperm-laden water in a small beaker. The sperm concentration in the beaker does not have to be exact, just enough to give a slightly cloudy appearance (approximately 1 to 10 X 10^6 cells/mL in the fertilization bucket). See Hahn (1989) for further information on sperm concentrations and the method for fertilization. Pour the sperm solution into the fertilization bucket containing the clean isolated eggs. Using a hose fitted with a clean glass tube, add dilution water seawater to the fertilization bucket at a low flow rate (<1 liter/min; 15 ± 1EC). Use the water flow to gently roil the eggs to allow them to mix with the sperm and fertilize. When the bucket is about half-full and eggs are evenly mixed, stop the water flow and allow the eggs to settle to the bottom of the bucket (about 15 minutes). Fertilization is then complete.

14.10.3.5.4 Note: Once fertilized eggs have settled to the bottom of the bucket (15 minutes after addition of sperm), the following steps (rinsing, concentrating, and counting the embryos) must proceed without delay to assure that embryos are transferred into the test solutions within about one hour. Embryos must be delivered to the test chambers before the first cell division takes place. (Multicellular embryos are more susceptible to damage in handling, and test endpoint analysis assumes that the first cell division takes place in the toxicant solution).

14.10.3.5.5 After embryos have settled, carefully pour or siphon off the water from above the settled embryos to remove as much of the sperm laden water as possible without losing substantial numbers of embryos. Slowly refill the bucket with dilution water seawater (15 ± 1EC). Allow the embryos to settle, and siphon them into a tall 1000 mL beaker for counting. Siphon at a slow flow rate, and move the siphon along the bottom of the bucket quickly to pick up a large number of embryos in the short amount of time it takes to fill the beaker. Examine a sample of the embryos at 100X magnification. One to one hundred sperm should be visible around the circumference of each embryo, 15 sperm per egg is optimal. If sperm are so dense that the embryos appear fuzzy (>>100 sperm/egg), the abalone may develop abnormally and should not be used.

14.10.4 START OF THE TEST

14.10.4.1 Prior to Beginning the Test

14.10.4.1.1 The test should begin as soon as possible, preferably within 24 h of sample collection. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity tests unless permission is granted by the permitting authority. In no case should the sample be used in a test more than 72 h after sample collection (see Section 8 Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Test).

14.10.4.1.2 Just prior to test initiation (approximately 1 h), the temperature of a sufficient quantity of the sample to make

the test solutions should be adjusted to the test temperature (15 ± 1EC) and maintained at that temperature during the addition of dilution water.

14.10.4.1.3 Increase the temperature of the water bath, room, or incubator to the required test temperature (15 ± 1EC).

14.10.4.1.4 Randomize the placement of test chambers in the temperature-controlled water bath, room, or incubator at the beginning of the test, using a position chart. Assign numbers for the position of each test chamber using a random numbers or similar process (see Appendix A, for an example of randomization). Maintain the chambers in this configuration throughout the test, using a position chart. Record these numbers on a separate data sheet together with the concentration and replicate numbers to which they correspond. Identify this sheet with the date, test organism, test number, laboratory, and investigator's name, and safely store it away until after the abalone have been examined at the end of the test.

14.10.4.1.5 Note: Loss of the randomization sheet would invalidate the test by making it impossible to analyze the data afterwards. Make a copy of the randomization sheet and store separately. Take care to follow the numbering system exactly while filling chambers with the test solutions.

14.10.4.1.6 Arrange the test chambers randomly in the water bath or controlled temperature room. Once chambers have been labeled randomly and filled with test solutions, they can be arranged in numerical order for convenience, since this will also ensure random placement of treatments.

14.10.4.2 Estimation of Embryo Density

14.10.4.2.1 Evenly mix the embryos in the 1000 mL beaker by gentle vertical stirring with a clean perforated plunger. Never allow embryos to settle densely in the bottom of the beaker, and take care not to crush embryos while stirring. Take a sample of the evenly suspended embryos using a 1 mL wide bore graduated pipet. Hold the pipet up to the light and count the individual embryos using a hand counter. Alternatively, empty the contents of the pipet onto a Sedgewick-Rafter slide and count embryos under low magnification on a compound scope. Discard the sampled

embryos after counting. Density of embryos in the beaker should be between 200 and 300 embryos/mL. Dilute if the concentration is too high, let embryos settle and pour off excess water if concentration is too low. Take the mean of five samples from this solution to estimate the number of embryos per milliliter.

14.10.4.3 Delivery of Fertilized Embryos

14.10.4.3.1 Using the estimated embryo density in the 1000 mL beaker, calculate the volume of water that contains 1000 embryos. Remove 1000 embryos (or less for smaller volumes, see Section 14.10.1.3) by drawing the appropriate volume of water from the well-mixed beaker using a 10 mL wide bore pipet. Deliver the embryos into the test chambers directly from the pipet making sure not to touch the pipet to the test solution. Stir the embryo beaker with the plunger before taking aliquots. The temperature of the embryo suspension must be within 1EC of the temperature of the test solution. (As above, all solutions are kept at 15 ± 1EC). Record the volume of water delivered into the test chambers with the embryos. Embryos must be delivered into the test solutions within one hour of fertilization. Immediately after the embryos have been delivered, take a sample from the embryo beaker and examine it under 100X magnification. All embryos should still be in the one-cell stage; record any observations to the contrary on the data sheet.

14.10.4.4 Incubation

14.10.4.4.1 Incubate test organisms for 48 hours in the test chambers at 15 ± 1EC under low lighting (approximately 10 $\mu E/m^2/s$) with 16L:8D photoperiod. Fertilized embryos become trochophore larvae, hatch, and develop into veliger larvae in the test solutions during the exposure period.

14.10.5 LIGHT, PHOTOPERIOD, SALINITY AND TEMPERATURE

14.10.5.1 The light quality and intensity should be at ambient laboratory conditions. Light intensity should be 10-20 $\mu E/m^2/s$, or 50 to 100 foot candles (ft-c), with a 16 h light and 8 h dark cycle.

14.10.5.2 The water temperature in the test chambers should be maintained at 15 ± 1EC. If a water bath is used to maintain the

test temperature, the water depth surrounding the test cups should be as deep as possible without floating the chambers.

14.10.5.3 The test salinity should be in the range of 34 ± 2‰. The salinity should vary by no more than ±2‰ among the chambers on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.

14.10.5.4 Rooms or incubators with high volume ventilation should be used with caution because the volatilization of the test solutions and evaporation of dilution water may cause wide fluctuations in salinity. Covering the test chambers with clean polyethylene plastic may help prevent volatilization and evaporation of the test solutions.

14.10.6 DISSOLVED OXYGEN (DO) CONCENTRATION

14.10.6.1 Aeration may affect the toxicity of effluent and should be used only as a last resort to maintain a satisfactory DO. The DO concentration should be measured on new solutions at the start of the test (Day 0). The DO should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). If it is necessary to aerate, all treatments and the control should be aerated. The aeration rate should not exceed that necessary to maintain a minimum acceptable DO and under no circumstances should it exceed 100 bubbles/minute, using a pipet with a 1-2 mm orifice, such as a 1 mL KIMAX® serological pipet No. 37033, or equivalent.

14.10.7 OBSERVATIONS DURING THE TEST

14.10.7.1 Routine Chemical and Physical Observations

14.10.7.1.1 DO is measured at the beginning of the exposure period in one test chamber at each test concentration and in the control.

14.10.7.1.2 Temperature, pH, and salinity are measured at the beginning of the exposure period in one test chamber at each concentration and in the control. Temperature should also be monitored continuously or observed and recorded daily for at

least two locations in the environmental control system or the samples. Temperature should be measured in a sufficient number of test chambers at the end of the test to determine temperature variation in the environmental chamber.

14.10.7.1.3 Record all the measurements on the data sheet.

14.10.8 TERMINATION OF THE TEST

14.10.8.1 Ending the Test

14.10.8.1.1 Record the time the test is terminated.

14.10.8.1.2 Temperature, pH, dissolved oxygen, and salinity are measured at the end of the exposure period in one test chamber at each concentration and in the control.

14.10.8.2 Sample Preservation

14.10.8.2.1 After 48 hours exposure, the abalone larvae are fixed in formalin or glutaraldehyde. The two methods for sample preservation are described. Be sure that samples for physicochemical measurements have been taken before further processing of test solutions.

14.10.8.2.2 At the end of the 48-hour incubation period, remove each test chamber, swirl the solution to suspend all the larvae, and pour the entire contents through a 37 µm-mesh screen. The test solution is discarded and the larvae are retained on the screen. Using streams of filtered seawater from a squeeze bottle, rinse the larvae from the screen through a funnel into 25 mL screw cap vials. Be careful not to hit the larvae directly with the streams of water; rough handling during transfer may cause fragmentation of the larvae, making counting more difficult and less accurate. Add enough buffered formalin to preserve larvae in a 5% solution (some laboratories have successfully preserved larvae with lower formalin concentrations. Under-preserved larvae disintegrate quickly, however, and whole tests may have to be rejected if larvae have not been adequately fixed). Addition of formalin is more accurate if the vials are premarked with lines showing the volume of sample and the volume

of formalin to be added. Alternatively, a 0.05% final glutaraldehyde solution may be substituted. Larvae should be counted within two weeks.

14.10.8.2.3 Note: Formaldehyde has been identified as a carcinogen and both glutaraldehyde and formaldehyde are irritating to skin and mucus membranes. Neither should be used at higher concentrations than needed to achieve morphological preservation and only under conditions of maximal ventilation and minimal opportunity for volatilization into room air.

14.10.8.3 Counting

14.10.8.3.1 To count the larvae using a standard compound microscope, pipet all the larvae from the bottom of the preservation vial onto a Sedgewick-Rafter counting cell. Examine 100 larvae from each vial under 100X magnification. To best characterize the sample and to avoid bias, select groups of larvae one field of vision at a time, moving to the next field without looking through the lens. Be careful to work across the slide in one direction to avoid recounting the same areas. Count the number of normal and abnormal larvae using hand counters. The percent normal larvae is calculated as the number normal divided by the total number counted. After counting, use a funnel to return the larvae to the vial for future reference.

14.10.8.4 Endpoint

14.10.8.4.1 Examine the shape of the larval shell to distinguish normal from abnormal larvae. Count veliger larvae as normal if they have smoothly curved larval shells that are striated with calcareous deposits and are somewhat opaque. It is common for normal larvae to have a slight curved indentation near the leading edge of the shell. A single indentation is this area is counted as normal.

14.10.8.4.2 Larvae with both multiple indentations and an obvious lack of calcification (i.e. clear appearance in at least part of the shell) are counted as abnormal. The combination of these two features indicates inhibition of a biological process (lack of calcification) and actual damage to the organism (indentations) allowed by the thin shell. Refer to the accompanying photographs (Figure 1) for classification of

marginally deformed larvae. The following types of larvae are also counted as abnormal: (1) larvae that have arrested development (from one cell through trochophore stage), (2) larvae with obvious severe deformations, (3) larvae with broken shells, (4) larval shells separated from the rest of the animal, and (5) larvae found remaining in the egg membrane (however, take care to distinguish these from larvae that may have come in contact with loose egg cases). Record all counts and the test chamber number on the data sheet.

14.11 **SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA**

14.14.1 A summary of test conditions and test acceptability criteria is listed in Table 3.

14.12 **ACCEPTABILITY OF TEST RESULTS**

14.12.1 Test results are acceptable only if all the following requirements are met:

(1) the mean larval normality must be at least 80% in the controls.

(2) the response from 56 µg/L zinc treatment must be significantly different from the control response.

(3) the minimum significant difference (%MSD) is <20% relative to the control for the reference toxicant.

14.13 **DATA ANALYSIS**

14.13.1 GENERAL

14.13.1.1 Tabulate and summarize the data. Calculate the proportion of larvae with normally developed shells for each replicate. A sample set of test data is listed in Table 4.

14.13.1.2 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

FIGURE 1. 48-HOUR-OLD ABALONE VELIGER LARVAE

Figures 1A -D Provided by John Hunt, Institute of Marine Sciences. Photocopied from:

"Marine Bioassay Project Procedures Manual of October, 1990." California State Water Resources Control Board.

The following three pages show 12 photographs of 48-hour-old abalone veliger larvae from effluent toxicity tests. All larvae were taken from intermediate effluent concentrations and were chosen to represent "borderline" cases (i.e. larvae that were slightly affected and are therefore, difficult to categorize as normal or abnormal). In most cases, larvae from lower and higher effluent concentrations are more easily categorized than those shown here; in the lower concentrations they are obviously without shell abnormalities and in the higher concentrations they are severely deformed. These photographs are presented as a visual reference to help standardize test analysis and eliminate bias in the interpretation of marginally deformed larvae. All larvae on the left-hand side of these pages were counted as normal, all larvae on the right-hand side were counted as abnormal.

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR, ***HALIOTIS RUFESCENS***, LARVAL DEVELOPMENT TEST WITH EFFLUENTS AND RECEIVING WATERS

1. Test type:	Static non-renewal
2. Salinity:	34 ± 2‰
3. Temperature:	15 ± 1EC
4. Light quality:	Ambient laboratory light
5. Light intensity:	10 µE/m^2/s (Ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	600 mL
8. Test solution volume:	200 mL/replicate
9. Larvae density per chamber:	5-10 per mL
10. No. replicate chambers per concentration:	5
11. Dilution water:	Uncontaminated 1-µm-filtered natural seawater or hypersaline brine plus reagent water
12. Test concentrations:	Effluents: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
13. Dilution factor:	Effluents: $0.5 Receiving waters: None or $0.5
14. Test duration:	48 h
15. Endpoint:	Normal shell development

16. Test acceptability criteria:	$80% normal shell development in the controls; must have statistical significant effect at 56 µg/L zinc; must achieve a %MSD of <20%
17. Sampling requirements:	One sample collected at test initiation, and preferably used within 24 h of the time it is removed from the sampling device (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests)
18. Sample volume required:	2 L per test

14.13.1.3 The endpoints of toxicity tests using the red abalone are based on the reduction in proportion of normal shell development. The IC25 is calculated using the Linear Interpolation Method (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). LOEC and NOEC values for larval development are obtained using a hypothesis testing approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981) (see Section 9). Separate analyses are performed for the estimation of the LOEC and NOEC endpoints and for the estimation of the IC25. See the Appendices for examples of the manual computations, and examples of data input and program output.

14.13.2 EXAMPLE OF ANALYSIS OF RED ABALONE, ***HALIOTUS RUFESCENS***, LARVAL DEVELOPMENT DATA

14.13.2.1 Formal statistical analysis of the larval development is outlined in Figure 2. The response used in the analysis is the proportion of larvae with normally developed shells in each test or control chamber. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the IC25 endpoint. Concentrations at which there is no normal shell development in any of the test chambers are excluded from statistical analysis of the NOEC and LOEC, but included in the estimation of the IC25.

TABLE 4. DATA FROM RED ABALONE, ***HALIOTUS RUFESCENS***, DEVELOPMENT TEST

Effluent Concentration (%)	Replicate	No. Larvae Counted	Number Normal	Proportion Normal
Brine	A	100	100	1.00
Control	B	100	98	0.98
	C	100	100	1.00
	D	100	99	0.99
	E	100	99	0.99
Dilution	A	100	99	0.99
Control	B	100	99	0.99
	C	100	99	0.99
	D	100	100	1.00
0.56	A	100	99	0.99
	B	100	99	0.99
	C	100	98	0.98
	D	100	100	1.00
	E	100	100	1.00
1.00	A	100	99	0.99
	B	100	100	1.00
	C	100	99	0.99
	D	100	99	0.99
	E	100	100	1.00
1.80	A	100	99	0.99
	B	100	99	0.99
	C	100	99	0.99
	D	100	98	0.98
	E	100	97	0.97
3.20	A	100	39	0.39
	B	100	57	0.57
	C	100	61	0.61
	D	100	65	0.65
	E	100	80	0.80
5.60	A	100	0	0.00
	B	100	0	0.00
	C	100	0	0.00
	D	100	0	0.00
	E	100	0	0.00
10.00	A	100	0	0.00
	B	100	0	0.00
	C	100	0	0.00
	D	100	0	0.00
	E	100	0	0.00

14.13.2.2 For the case of equal numbers of replicates across all concentrations and the control, the evaluation of the NOEC and LOEC endpoints is made via a parametric test, Dunnett's Procedure, or a nonparametric test, Steel's Many-one Rank Test, on the arc sine square root transformed data. Underlying assumptions of Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test, and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are estimated by the parametric procedure.

14.13.2.3 If unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a ***t*** test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative.

14.13.2.4 Comparison of Brine and Dilution Controls

14.13.2.4.1 This example uses toxicity data from a red abalone, ***Haliotus rufescens***, larval development test performed with effluent. The response of interest is the proportion of larvae with normally developed shells, thus each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix B. Because the example test was run using both brine and dilution controls, the two controls must first be tested for significant differences in the normal shell development proportions. The raw and transformed data, means and variances of the transformed observations for the two controls are listed in Table 5.

14.13.2.4.2 Tests for Normality

14.13.2.4.2.1 In the two sample situation, the distributional assumption is that each sample comes from a normally distributed population. Thus in comparing the brine and dilution controls, the data for each concentration must be separately checked for normality. When the two response groups are tested separately, it is not necessary to center the data.

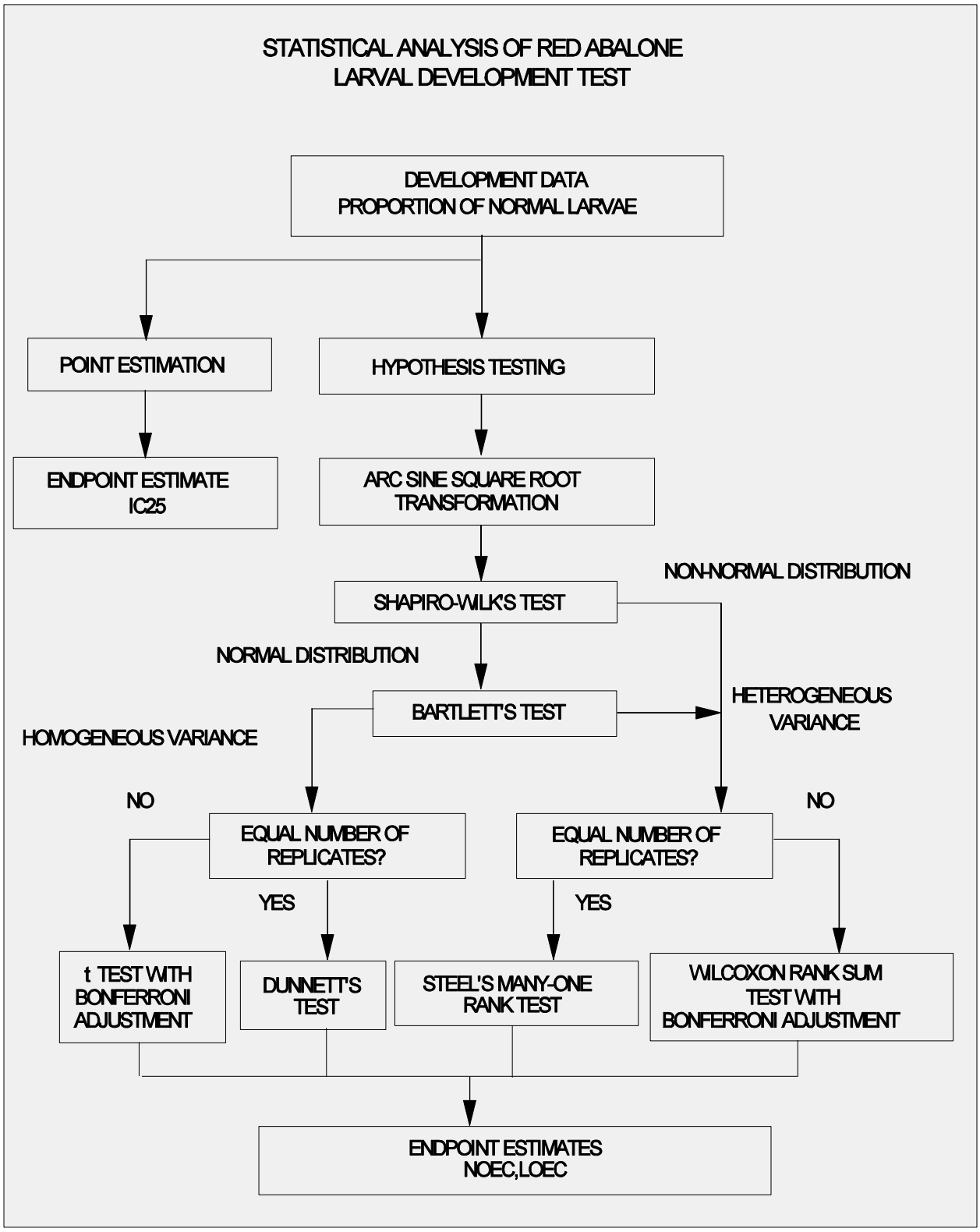

Figure 2. Flowchart for statistical analysis of red abalone, *Haliotus rufescens*, development data.

TABLE 5. RED ABALONE, ***HALIOTUS RUFESCENS***, LARVAL DEVELOPMENT DATA FROM BRINE AND DILUTION CONTROLS

	Replicate	Brine Control	Dilution Control
RAW	A	1.00	0.99
	B	0.98	0.99
	C	1.00	0.99
	D	0.99	1.00
	E	0.99	
ARC SINE SQUARE ROOT TRANSFORMED	A	1.521	1.471
	B	1.429	1.471
	C	1.521	1.471
	D	1.471	1.521
	E	1.471	
Mean ($\bar{X}_i$)		1.483	1.484
S_i^2		0.00152	0.000625
i		1	2

14.13.2.4.2.2 Calculate the denominator, D, of the statistic for each control group:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

$\bar{X}$ = the overall mean of the centered observations

n = the total number of centered observations

14.13.2.4.2.3 For the brine control data,

n = 5

$$\& = \frac{1}{5}(7.413) = 1.483$$

$$D = 0.00609$$

For the dilution control data,

$$n = 4$$

$$\& = \frac{1}{4}(5.934) = 1.484$$

$$D = 0.00191$$

14.13.2.4.2.4 Order the observations for each control group from smallest to largest

$$X^{(1)} \# X^{(2)} \# \ldots \# X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for the two groups in this example are listed in Table 6.

TABLE 6. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

Brine Control		Dilution Control	
i	$X^{(i)}$	i	$X^{(i)}$
1	1.429	1	1.471
2	1.471	2	1.471
3	1.471	3	1.471
4	1.521	4	1.521
5	1.521		

14.13.2.4.2.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2, ... a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the datasets in this example, n = 5 and k = 2 for the brine control group, and n = 4 and k = 2 for the dilution control group. The a_i values are listed in Table 7.

TABLE 7. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
Brine Control Group			
1	0.6646	0.092	$X^{(5)} - X^{(1)}$
2	0.2413	0.050	$X^{(4)} - X^{(3)}$
Dilution Control Group			
1	0.6872	0.050	$X^{(4)} - X^{(1)}$
2	0.1667	0.000	$X^{(3)} - X^{(2)}$

14.13.2.4.2.6 Compute the test statistic, W, for each group as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i\left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

The differences, $X^{(n-i+1)} - X^{(i)}$, are listed in Table 7. For the data in the brine example:

$$W = \frac{1}{0.00609}\ (0.07321)^2 = 0.880$$

For the data in the dilution example:

$$W = \frac{1}{0.00191}\ (0.03436)^2 = 0.618$$

14.13.2.4.2.7 The decision rule for this test is to compare W as

calculated in Subsection 2.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For the data in the brine control, the critical value at a significance level of 0.01 and n = 5 observations is 0.686. Since W = 0.880 is greater than the critical value, conclude that the brine control data are normally distributed. For the data in the dilution control, the critical value at a significance level of 0.01 and n = 4 observations is 0.687. Since W = 0.618 is less than the critical value, conclude that the dilution control data are not normally distributed.

14.13.2.4.2.8 Since the dilution control data does not meet the normality assumption, the Wilcoxon Rank Sum Test will be used to compare the responses in the two control groups.

14.13.2.4.3 Wilcoxon Rank Sum Test

14.13.2.4.3.1 To perform the Wilcoxon Rank Sum test, combine the data from the two groups and arrange in order from smallest to largest. Assign the rands (1, 2, ..., 9) to the ordered observations with a rank of 1 assigned to the smallest observation, rank of 2 assigned to the next larger observation, etc. If ties occur when ranking, assign the average rank to each tied observation. A table of the ranks is given in Table 8.

TABLE 8. ASSIGNING RANKS TO THE BRINE AND DILUTION CONTROLS FOR THE WILCOXON RANK SUM TEST

Rank	Transformed Proportion Normal	Control Group
1	1.429	Brine
4	1.471	Brine
4	1.471	Brine
4	1.471	Dilution
4	1.471	Dilution
4	1.471	Dilution
8	1.521	Brine
8	1.521	Dilution
8	1.521	Dilution

14.13.2.4.3.2 The ranks are then summed for both of the control groups. For this data, the sum of the ranks in the brine control group is 25 and the sum of the ranks in the dilution control group is 20.

14.13.2.4.3.3 For this situation, we wish to determine if the proportions of normally developed larvae in the two control groups are significantly different. To do this, compare the rank sum of the group with the smaller sample size with some "minimum" or critical rank sum, at or below which the devlopment in the controls would be considered significantly different. At a significance level of 0.05, the minimum rank sum in a test with five replicates in one group and and four replicates in the other is 11 (See Snedecor and Cochran, 1980).

14.13.2.4.3.4 The dilution control sample size is smaller than the sample size of the brine control group so its rank sum is compared to the critical value. Since its rank sum of 20 is greater than the critical value of 11, conclude that the development proportions for the two control groups are not significantly different.

14.13.2.5 Example of Analysis of Larval Development Data

14.13.2.5.1 Since the responses in the two control groups are not significantly different, only the dilution control group will be used in the analysis of the shell development responses for the effluent concentrations. As above, each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix B. The raw and transformed data, means and variances of the transformed observations at each effluent concentration and dilution control are listed in Table 9. The data are plotted in Figure 3. Since there is 100% abnormality in all replicates for the 5.6% and 10.0% concentrations, they are not included in the statistical analysis and are considered qualitative abnormality effects.

14.13.2.6 Test for Normality

14.13.2.6.1 The first step of the test for normality is to center the observations by subtracting the mean of all

observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 10.

14.13.2.6.2 Calculate the denominator, D, of the statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

$\bar{X}$ = the overall mean of the centered observations

n = the total number of centered observations

14.13.2.6.3 For this set of data, n = 24

$$\bar{X} = \frac{1}{24}(-0.004) = 0.000$$

D = 0.1127

14.13.2.6.4 Order the centered observations from smallest to largest

$$X^{(1)} \le X^{(2)} \le \ldots \le X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 11.

14.13.2.6.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2, ... a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 24 and k = 12. The a_i values are listed in Table 12.

14.13.2.5.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i (X^{(n-i+1)} - X^{(i)})\right]^2$$

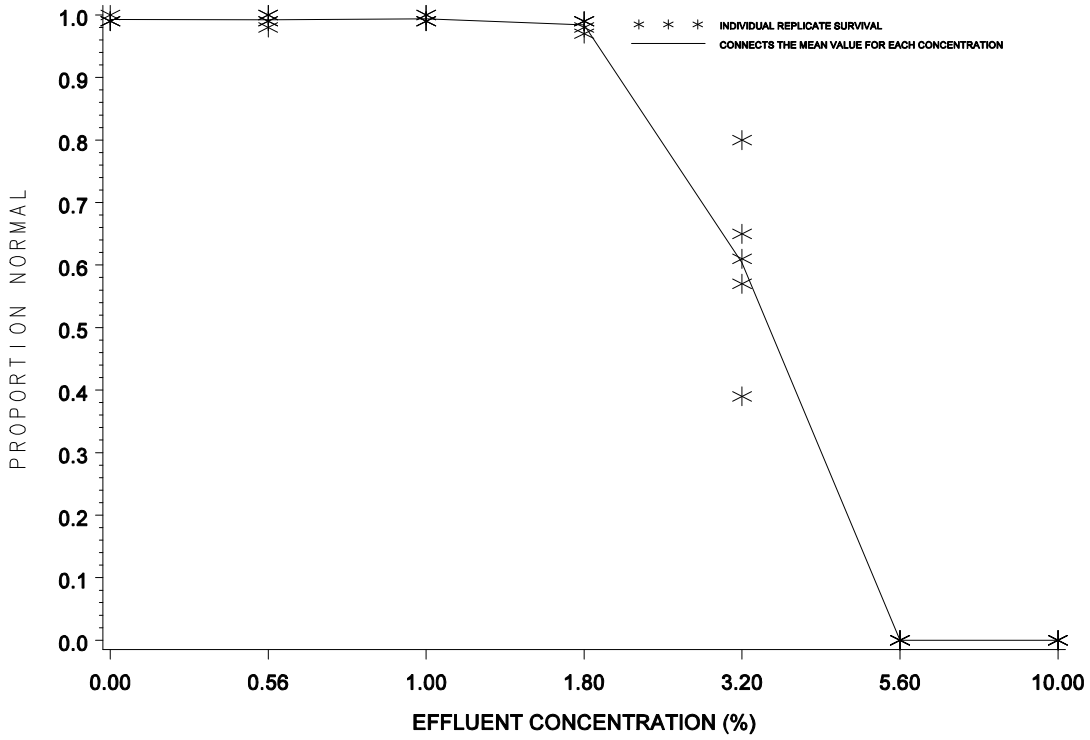

Figure 3. Plot of mean proportion of normally developed red abalone, *Haliotus rufescens* larvae.

TABLE 9. RED ABALONE, ***HALIOTUS RUFESCENS***, SHELL DEVELOPMENT DATA

		Effluent Concentration (%)						
	Dilution							
	Replicate	Control	0.56	1.00	1.80	3.20	5.6	10.0
	A	0.99	0.99	0.99	0.99	0.39	0	0
RAW	B	0.99	0.99	1.00	0.99	0.57	0	0
	C	0.99	0.98	0.99	0.99	0.61	0	0
	D	1.00	1.00	0.99	0.98	0.65	0	0
	E		1.00	1.00	0.97	0.80	0	0
ARC SINE	A	1.471	1.471	1.471	1.471	0.674	-	-
SQUARE ROOT	B	1.471	1.471	1.521	1.471	0.856	-	-
TRANSFORMED	C	1.471	1.429	1.471	1.471	0.896	-	-
	D	1.521	1.521	1.471	1.429	0.938	-	-
	E		1.521	1.521	1.397	1.107	-	-
Mean ($\bar{x}_i$)		1.484	1.483	1.491	1.448	0.894	-	-
S_i^2		0.000625	0.001523	0.000750	0.001137	0.024288	-	-
i		1	2	3	4	5	6	7

TABLE 10. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

	Effluent Concentration (%)				
Replicate	Control	0.56	1.00	1.80	3.20
A	-0.013	-0.012	-0.020	0.023	-0.220
B	-0.013	-0.012	0.030	0.023	-0.038
C	-0.013	-0.054	-0.020	0.023	0.002
D	0.037	0.038	-0.020	-0.019	0.044
E		0.038	0.030	-0.051	0.213

TABLE 11. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.220	13	-0.012
2	-0.054	14	0.002
3	-0.051	15	0.023
4	-0.038	16	0.023
5	-0.020	17	0.023
6	-0.020	18	0.030
7	-0.020	19	0.030
8	-0.019	20	0.037
9	-0.013	21	0.038
10	-0.013	22	0.038

TABLE 12. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4493	0.433	$X^{(24)} - X^{(1)}$
2	0.3098	0.098	$X^{(23)} - X^{(2)}$
3	0.2554	0.089	$X^{(22)} - X^{(3)}$
4	0.2145	0.076	$X^{(21)} - X^{(4)}$
5	0.1807	0.057	$X^{(20)} - X^{(5)}$
6	0.1512	0.050	$X^{(19)} - X^{(6)}$
7	0.1245	0.050	$X^{(18)} - X^{(7)}$
8	0.0997	0.042	$X^{(17)} - X^{(8)}$
9	0.0764	0.036	$X^{(16)} - X^{(9)}$
10	0.0539	0.036	$X^{(15)} - X^{(10)}$

The differences, $X^{(n-i+1)} - X^{(i)}$, are listed in Table 12. For the data in this example:

$$W = \frac{1}{0.1127} (0.2974)^2 = 0.7848$$

14.13.2.5.7 The decision rule for this test is to compare W as calculated in 14.13.2.5.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For the data in this example, the critical value at a significance level of 0.01 and n = 24 observations is 0.884. Since W = 0.7848 is less than the critical value, conclude that the data are not normally distributed.

14.13.2.5.8 Since the data do not meet the assumption of normality, the Wilcoxon Rank Sum Test with the Bonferroni Adjustment will be used to analyze the shell development data.

14.13.2.6 Wilcoxon Rank Sum Test with the Bonferroni Adjustment

14.13.2.6.1 For each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign the ranks (1, 2, ... , 9) to the ordered observations with a rank of 1 assigned to the smallest observation, rank of 2 assigned to the next larger observation, etc. If ties occur when ranking, assign the average rank to each tied observation.

14.13.2.6.2 An example of assigning ranks to the combined data for the control and 0.56% concentration is given in Table 13. This ranking procedure is repeated for each control/concentration combination. The complete set of rankings is summarized in Table 14. The ranks are then summed for each concentration level, as shown in Table 15.

14.13.2.6.3 For this example, determine if the survival in any of the concentrations is significantly lower than the survival in the control. If this occurs, the rank sum at that concentration would be significantly lower than the rank sum of the control. Thus compare the rank sums for the survival at each of the various concentration levels with some "minimum" or critical rank sum, at or below which the survival would be considered significantly lower than the control. At a significance level of 0.05, the minimum rank sum in a test with four concentrations (excluding the control), four control replicates and five concentration replicates is 15 (See Table 5, Appendix F).

TABLE 13. ASSIGNING RANKS TO THE CONTROL AND 0.56% CONCENTRATION LEVEL FOR THE WILCOXON RANK SUM TEST WITH THE BONFERRONI ADJUSTMENT

Rank	Transformed Proportion Normal	Concentration
1	1.429	0.56 %
4	1.471	0.56 %
4	1.471	0.56 %
4	1.471	Control
4	1.471	Control
4	1.471	Control
8	1.521	0.56 %
8	1.521	0.56 %
8	1.521	Control

TABLE 14. TABLE OF RANKS[1]

Replicate	Control	Effluent Concentration (%) 0.56	1.00	1.80	3.20
1	1.471(4,3.5,5.5,7)	1.471(4)	1.471(3.5)	1.471(5.5)	0.674(1)
2	1.471(4,3.5,5.5,7)	1.471(4)	1.521(8)	1.471(5.5)	0.856(2)
3	1.471(4,3.5,5.5,7)	1.429(1)	1.471(3.5)	1.471(5.5)	0.896(3)
4	1.521(8,8,9,9)	1.521(8)	1.471(3.5)	1.429(2)	0.938(4)
5		1.521(8)	1.521(8)	1.397(1)	1.107(5)

[1]Control ranks are given in the order of the concentration with which they were ranked.

TABLE 15. RANK SUMS

Concentration (% Effluent)	Rank Sum
0.56	25.0
1.00	26.5
1.80	19.5
3.20	15.0

14.13.2.6.4 Since the rank sum for the 3.20% concentration level is equal to the critical value, the proportion normal in that concentration is considered significantly less than that in the control. Since no other rank sum is less than or equal to the critical value, no other concentration has a significantly lower proportion normal than the control. Hence, the NOEC and the LOEC are 1.80% and 3.20%, respectively.

14.13.2.7 Calculation of the ICp

14.13.2.7.1 The shell development data in Table 4 are utilized in this example. As can be seen from Table 4 and Figure 4, the observed means are not monotonically non-increasing with respect to concentration (mean response for each higher concentration is not less than or equal to the mean response for the previous concentration and the responses between concentrations do not follow a linear trends). Therefore, the means are smoothed prior to calculating the IC. In the following discussion, the observed means are represented by $\bar{x}_i$ and the smoothed means by M_i.

14.13.2.7.2 Starting with the control mean, $\bar{x}_1 = 0.993$ and $\bar{x}_2 = 0.992$, we see that $\bar{x}_1 > \bar{x}_2$. Set $M_1 = \bar{x}_1$. Comparing $\bar{x}_2$ to $\bar{x}_3$, $\bar{x}_2 < \bar{x}_3$.

14.13.2.7.3 Calculate the smoothed means:

$$M_2 = M_3 = (\bar{x}_2 + \bar{x}_3)/2 = 0.993$$

14.13.2.7.4 Since $\bar{x}_7 = 0 < \bar{x}_6 = 0 < \bar{x}_5 = 0.604 < \bar{x}_4 = 0.984 < \bar{x}_3 = 0.993$, set $M_3 = 0.993$, $M_4 = 0.984$, $M_5 = 0.604$, $M_6 = 0$, and set $M_7 = 0$.

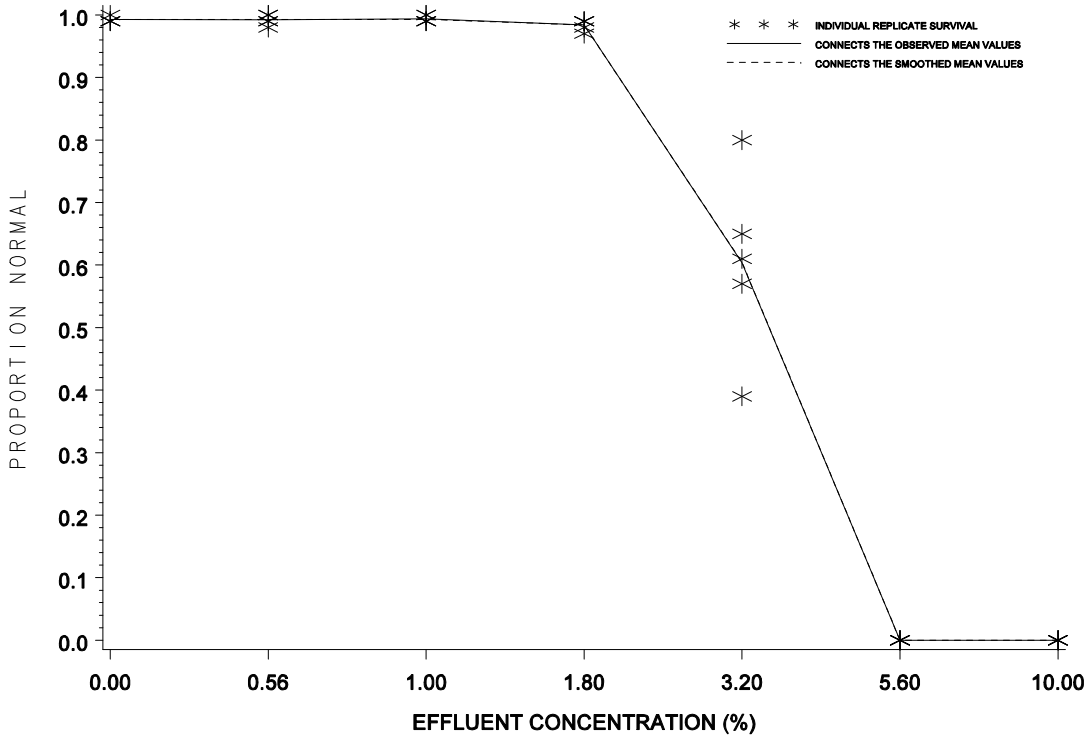

Figure 4. Plot of raw data, observed means, and smoothed means for red abalone, *Haliotus rufescens*, development data from Tables 4 and 16.

TABLE 16. RED ABALONE, ***HALIOTUS RUFESCENS***, MEAN SHELL DEVELOPMENT RESPONSE AFTER SMOOTHING

Effluent Conc. (%)	i	Response Means, $\&_i$ (proportion)	Smoothed Means, M_i (proportion)
Control	1	0.993	0.993
0.56	2	0.992	0.993
1.00	3	0.994	0.993
1.80	4	0.984	0.984
3.20	5	0.604	0.604
5.60	6	0.000	0.000
10.00	7	0.000	0.000

14.13.2.7.5 Table 16 contains the response means and smoothed means and Figure 4 gives a plot of the smoothed response curve.

14.13.2.7.6 An IC25 can be estimated using the Linear Interpolation Method. A 25% reduction in mean proportion of fertilized eggs, compared to the controls, would result in a mean proportion of 0.745, where $M_1(1-p/100) = 0.993(1-25/100)$. Examining the means and their associated concentrations (Table 16), the response, 0.745, is bracketed by $C_4 = 1.80\%$ effluent and $C_5 = 3.20\%$ effluent.

14.13.2.7.7 Using the equation from Section 4.2 in Appendix L, the estimate of the IC25 is calculated as follows:

$$ICp = C_j\%[M_1(1\&p/100)\&M_j]\frac{(C_{(j\%1)}\&C_j)}{(M_{(j\%1)}\&M_j)}$$

$$IC25 = 1.8 + [0.993(1 - 25/100) - 0.984] \quad (3.2 - 1.8)$$
$$S)))))))))))Q$$
$$(0.604 - 0.984)$$
$$= 2.68\%.$$

14.13.2.7.8 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 2.6818%. The empirical 95.0% confidence interval for the true

mean was 2.5000% to 3.1262 %. The computer program output for the IC25 for this data set is shown in Figure 5.

14.14 **PRECISION AND ACCURACY**

14.14.1 PRECISION

14.14.1.1 Single-Laboratory Precision

14.14.1.1.1 Data on the single laboratory precision of the ***Haliotis rufescens*** larval development method using zinc sulfate are shown in Table 17. Zinc concentrations were 18, 32, 56, and 100 µg/L. All tests were conducted at the Marine Pollution Studies Laboratory. There was good agreement among test EC50s, with a coefficient of variation of 8%.

14.14.1.2 Multi-laboratory Precision

14.14.1.2.1 The multi-laboratory data indicate a similar level of test precision Table 18. Data are presented for four interlaboratory trials in which either two or three laboratories tested both split effluent samples and reference toxicants. The mean coefficient of variation between EC50 values from different laboratories was 15%.

14.14.2 ACCURACY

14.14.2.1 The accuracy of toxicity tests cannot be determined.

```
Conc. ID        1    2    3    4    5    6    7
-----------------------------------------------
Conc. Tested    0  .56    1  1.8  3.2  5.6   10
-----------------------------------------------
Response  1   .99  .99  .99  .99  .39    0    0
Response  2   .99  .99 1.00  .99  .57    0    0
Response  3   .99  .98  .99  .99  .61    0    0
Response  4  1.00 1.00  .99  .98  .65    0    0
Response  5       1.00 1.00  .97  .80    0    0
-----------------------------------------------
*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Effluent
Test Start Date:    Test Ending Date:
Test Species: Red Abalone
Test Duration:        48 hours
DATA FILE: abalone.icp
OUTPUT FILE: abalone.i25
-------------------------------------------------------------------
Conc.     Number     Concentration     Response      Std.       Pooled
 ID     Replicates                %      Means       Dev.   Response Means
-------------------------------------------------------------------------
  1          4             0.000          0.993      0.005      0.993
  2          5             0.560          0.992      0.008      0.993
  3          5             1.000          0.994      0.005      0.993
  4          5             1.800          0.984      0.009      0.984
  5          5             3.200          0.604      0.148      0.604
  6          5             5.600          0.000      0.000      0.000
  7          5            10.000          0.000      0.000      0.000
-------------------------------------------------------------------------
The Linear Interpolation Estimate:     2.6818   Entered P Value: 25
-------------------------------------------------------------------------
Number of Resamplings:   80
The Bootstrap Estimates Mean:   2.7085 Standard Deviation:     0.1510
Original Confidence Limits:   Lower:     2.5000 Upper:     3.1262
Expanded Confidence Limits:   Lower:     2.4091 Upper:     3.3484
Resampling time in Seconds:     0.27  Random_Seed: -770872716
```

Figure 5. ICPIN program output for the IC25.

TABLE 17. SINGLE LABORATORY PRECISION DATA FOR THE RED ABALONE, ***HALIOTIS RUFESCENS*** LARVAL DEVELOPMENT TEST WITH ZINC (ZN FG/L) SULFATE AS A REFERENCE TOXICANT

Test Date	NOEC (µg/L)	EC50 (µg/L)
March 1990	32	42[1]
May 1990	32	39[1]
January 1991	18	34[1]
February 1991	18	40[2]
Mean		38.4
SD		3.0
CV (%)		7.8

1 Source: Hunt et al., 1991
2 Source: Anderson et al., 1994

TABLE 18. MULTI-LABORATORY PRECISION OF THE RED ABALONE, ***HALIOTIS RUFESCENS*** LARVAL DEVELOPMENT TEST PERFORMED WITH ZINC (ZN FG/L) SULFATE AND EFFLUENT (%) AS THE TOXICANTS

Test Date	Toxicant	Lab	NOEC	EC50	CV
March 1990	Effluent	A	>3.2%	nc	
March 1990	Effluent	B	>3.2%	nc	
March 1990	Effluent	C	0.32%	1.83	nc
March 1990	Zinc	A	32	41	
March 1990	Zinc	B	18	28	
March 1990	Zinc	C	18	31	20%
May 1990	Effluent	A	3.2%	4.7	
May 1990	Effluent	D	1.8%	3.5	
May 1990	Effluent	C	3.2%	3.8	16%
May 1990	Zinc	A	32	39	
May 1990	Zinc	D	32	46	
May 1990	Zinc	C	32	37	12%
January 1991	Effluent	A	<0.56%	1.5	
January 1991	Effluent	C	1.25%	1.8	13%
January 1991	Zinc	A	18	34	
January 1991	Zinc	C	32	48	24%
January 1991	Effluent	A	1.0%	2.7	
January 1991	Effluent	C	1.8%	2.8	3.0%

Mean Interlaboratory CV = 15% Interlaboratory CV based on 6 tests for which CV values could be calculated. Source: Hunt et al., 1991.

nc = indicates that the CV could not be calculated because only one laboratory observed a 50% effect and calculated an EC50.

APPENDIX I. RED ABALONE TEST: STEP-BY-STEP SUMMARY

PREPARATION OF TEST SOLUTIONS

A. Determine test concentrations and appropriate dilution water based on NPDES permit conditions and guidance from the appropriate regulatory agency.

B. Prepare effluent test solutions by diluting well mixed unfiltered effluent using volumetric flasks and pipettes. Use hypersaline brine where necessary to maintain all test solutions at 34 ± 2‰. Include brine controls in tests that use brine.

C. Prepare a zinc reference toxicant stock solution (10,000 µg/L) by adding 0.0440 g of zinc sulfate ($ZnSO_4 \cdot 7H_2O$) to 1 liter of reagent water.

D. Prepare zinc reference toxicant solution of 0 (control) 10, 18, 32, 56 and 100 µg/L by adding 0, 1.0 1.8, 3.2, 5.6 and 10.0 mL of stock solution, respectively, to a 1-L volumetric flask and filling to 1-L with dilution water.

E. Sample effluent and reference toxicant solutions for physical/chemical analysis. Measure salinity, pH and dissolved oxygen from each test concentration.

F. Randomize numbers for test chambers and record the chamber numbers with their respective test concentrations on a randomization data sheet. Store the data sheet safely until after the test samples have been analyzed.

G. Place test chambers in a water bath or environmental chamber set to 15EC and allow temperature to equilibrate.

H. Measure the temperature daily in one random replicate (or separate chamber) of each test concentration. Monitor the temperature of the water bath or environmental chamber continuously.

I. At the end of the test, measure salinity, pH, and dissolved oxygen concentration from each test concentration.

PREPARATION AND ANALYSIS OF TEST ORGANISMS

A. Obtain test organisms and hold or condition as necessary for spawning.

B. Induce four male and four female abalone to spawn using either H_2O_2 and Tris or UV irradiated seawater (300 mL/min flow rate through the UV unit). All solutions should be maintained at 15 ± 1EC.

C. Siphon eggs into a fertilization bucket. Add 200 mL of sperm-laden water to fertilize the eggs. Wash the fertilized eggs at least twice by slowly decanting and refilling the chamber with fresh filtered seawater. Temperatures should vary by no more than 1EC between waters used in mixing and refilling.

D. Suspend the embryos evenly in a 1000 mL beaker and count five samples in a 1 mL pipet to estimate embryo density.

E. Pipet 1000 fertilized embryos into each 200 mL test chamber. Be sure temperatures in the embryo beaker and the solutions are at 15 ± 1EC. Incubate for 48 h. For smaller-sized chambers, use proportionately fewer embryos.

F. At the end of the 48 h period, pour the entire test solution with larvae through a 37 µm-meshed screen. Wash larvae from the screen into 25 mL vials. Add buffered formalin to preserve the larvae in a 5% solution or glutaraldehyde for a 0.05% solution. Cap the flask and invert gently to mix.

G. Pipet a sample from each vial onto a Sedgwick-Rafter counting slide and count 100 larvae. Return the larvae to the vials for future reference.

H. Count the number of normal larvae for each replicate and divide by the total counted.

I. Analyze the data.

J. Include standard reference toxicant point estimate values in the standard quality control charts.

Salinity Adjustment Worksheet for Abalone

Date Sampled: Batch:
Date Adjusted: Region:

$\frac{VS \quad (TS-SS)}{(SB-TS)}$ SS = Salinity of Sample VB = Volume of Brine
VS = Volume of Sample SB = Salinity of Brine
TS = Target Salinity (34±2%)

VDW = VBL - VBS VDW = Volume of Dilution Water (Adjusted to 34±2%)
VBL = Largest Volume of Brine added to adjust salinity
VBS = Volume of Brine added to each Sample

Total Volume = VB added + VDW added
(Total volume should be the same for all samples)

Site Code (ID Org #) or concentration	Initial Salinity	TS	Vol. of Brine	Vol.Dil. Water	Total Volume	Final Salinity	Precision and Accuracy for Refractometer
		34±2					
		34±2					
		34±2					
		34±2					
		34±2					
		34±2					
		34±2					
		34±2					
		34±2					
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		34±2					
		34±2					
		34±2					
		34±2					

Initials: Double Checked:

Data Sheet for Mollusc Larval Development Toxicity Test

Test Start Date: Start Time: Mollusc Species:

Test End Date: End Time: Collection/Arrival Date:

Reference Toxicant:Reference Toxicant: Broodstock Source:

Sample Source:

Sample Type: Effluent Ref Tox Solid Elutriate Pore Water WaterSample Type: Effluent Ref Tox Solid Elutriate Pore Water Water

Test Cont. #	Station Code or Concentration	Sample ID #	After 48 hours		Notes
			Normal Larvae	Abnormal Larvae	
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					
16					
17					
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32					
33					
34					
35					

Computer Data StorageComputer Data Storage Disk:
File:

Notes

SECTION 15

PURPLE URCHIN, *Strongylocentrotus purpuratus* AND SAND DOLLAR, *Dendraster excentricus* LARVAL DEVELOPMENT TEST METHOD

Adapted from a method developed by
Steven Bay and Darrin Greenstein
Southern California Coastal Water Research Project
Westminster, CA 92683

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Appendix I Step-by Step Summary

SECTION 15

SEA URCHIN, *Strongylocentrotus purpuratus* AND SAND DOLLAR, *Dendraster excentricus* LARVAL DEVELOPMENT TEST

15.1 SCOPE AND APPLICATION

15.1.1 This method estimates the chronic toxicity of effluents and receiving waters to the developing embryos of the purple sea urchin, *Strongylocentrotus purpuratus*, and the sand dollar, *Dendraster excentricus*, during a 72-h static non-renewal exposure. The effects include the synergistic, antangonistic, and additive effects of all chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms.

15.1.2 Detection limits of the toxicity of an effluent or chemical substance are organism dependent.

15.1.3 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling and because the test chambers are not sealed, highly volatile and highly degradable toxicants in the source may not be detected in the test.

15.1.4 This test is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

15.1.5 This method should be restricted to use by, or under the supervision of, professionals experienced in aquatic toxicity testing. Specific experience with any toxicity test is usually needed before acceptable results become routine.

15.2 SUMMARY OF METHOD

15.2.1 The method provides the step-by-step instructions for

performing a 72-h static non-renewal test using the early development of the purple sea urchin, ***Strongylocentrotus purpuratus***, and the sand dollar, ***Dendraster excentricus***, to determine the toxicity of substances in marine and estuarine

waters. The test endpoint is normal larval development and may include mortality if modified for total counts at test initiation and termination.

15.3 **INTERFERENCES**

15.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities, Equipment, and Supplies).

15.3.2 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling and Sample Handling, and Sample Preparation for Toxicity Tests).

15.4 **SAFETY**

15.4.1 See Section 3, Health and Safety

15.5 **APPARATUS AND EQUIPMENT**

15.5.1 Tanks, trays, or aquaria -- for holding and acclimating adult sea urchins and sand dollars, e.g., standard salt water aquarium or Instant Ocean Aquarium (capable of maintaining seawater at 10-20EC), with appropriate filtration and aeration system.

15.5.2 Air pump, air lines, and air stones -- for aerating water containing broodstock or for supplying air to test solutions with low dissolved oxygen.

15.5.3 Constant temperature chambers or water baths -- for maintaining test solution temperature and keeping dilution water supply, gametes, and embryo stock suspensions at test temperature (15EC) prior to the test.

15.5.4 Water purification system -- Millipore Super-Q, Deionized water (DI) or equivalent.

15.5.5 Refractometer -- for determining salinity.

15.5.6 Hydrometer(s) -- for calibrating refractometer.

15.5.7 Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

15.5.8 Thermometer, National Bureau of Standards Certified (see USEPA METHOD l70.l, USEPA, l979) -- to calibrate laboratory thermometers.

15.5.9 pH and DO meters -- for routine physical and chemical measurements.

15.5.10 Standard or micro-Winkler apparatus -- for determining DO (optional) and calibrating the DO meter.

15.5.11 Winkler bottles -- for dissolved oxygen determinations.

15.5.12 Balance -- Analytical, capable of accurately weighing to 0.0001 g.

15.5.13 Fume hood -- to protect the analyst from effluent or formaldehyde fumes.

15.5.14 Glass stirring rods -- for mixing test solutions.

15.5.15 Graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 50-l000 mL for making test solutions. (Note: not to be used interchangeably for gametes or embryos and test solutions).

15.5.16 Volumetric flasks -- Class A, borosilicate glass or non-toxic plastic labware, 100-1000 mL for making test solutions.

15.5.17 Pipets, automatic -- adjustable, to cover a range of delivery volumes from 0.010 to 1.000 mL.

15.5.18 Pipet bulbs and fillers -- PROPIPET® or equivalent.

15.5.19 Wash bottles -- for reagent water, for topping off graduated cylinders, for rinsing small glassware and instrument electrodes and probes.

15.5.20 Wash bottles -- for dilution water.

15.5.21 20-liter cubitainers or polycarbonate water cooler jugs -- for making hypersaline brine.

15.5.22 Cubitainers, beakers, or similar chambers of non-toxic composition for holding, mixing, and dispensing dilution water and other general non-effluent, non-toxicant contact uses. These should be clearly labeled and not used for other purposes.

15.5.23 Beakers, 5-10 mL borosilicate glass -- for collecting sperm from sand dollars.

15.5.24 Beakers, 250 mL borosilicate glass -- for preparation of test solutions.

15.5.25 Beakers, 100 mL borosilicate glass -- for spawning; to support sea urchins and to collect sea urchin and sand dollar eggs.

15.5.26 Beakers, 1,000 mL borosilicate glass -- for rinsing and settling sea urchin eggs.

16.5.27 Vortex mixer -- to mix sea urchin semen in tubes prior to sampling.

15.5.28 Compound microscope -- for examining gametes, counting sperm cells (200-400x), eggs and embryos and (100x), and examining larvae. Dissecting scopes are sometimes used to count eggs at a lower magnification. One piece of equipment worthy of a special mention is an inverted microscope. The use of an inverted scope is not required, but recommended. Its use reduces the exposure of workers to hazardous fumes (formalin or glutaraldehyde) during the counting of larvae and reduces sample examination time. Alternatively, a Sedgewick-Rafter cell may be used on a regular compound scope.

15.5.29 Counter, two unit, 0-999 -- for recording sperm, egg, embryo, and larval counts.

15.5.30 Sedgwick-Rafter counting chamber -- for counting egg and embryo stock and examining larval development at the end of the test.

15.5.31 Centrifuge tubes, test tubes, or vials -- for holding semen.

15.5.32 Hemacytometers, Neubauer -- for counting sperm.

15.5.33 Siphon hose (3 mm i.d.) -- for removing wash water from settled eggs.

15.5.34 Perforated plunger -- for maintaining homogeneous distribution of eggs and embryos during sampling and distribution to test chambers.

15.5.35 Enamel or plastic tray -- for optional spawning platform.

15.5.36 Nitex® screening (0.5mm mesh) -- cleaning egg solutions.

15.5.37 60 µm NITEX® filter -- for filtering receiving waters.

15.6 **REAGENTS AND SUPPLIES**

15.6.1 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests).

15.6.2 Data sheets (one set per test) -- for data recording (see Figures 1-4).

15.6.3 Tape, colored -- for labelling test chambers and containers.

15.6.4 Markers, water-proof -- for marking containers, etc.

15.6.5 Parafilm -- to cover graduated cylinders and vessels containing gametes and embryos.

15.6.6 Gloves, disposable -- for personal protection from contamination.

15.6.7 Pipets, serological -- 1-10 mL, graduated.

15.6.8 Pipet tips -- for automatic pipets. Note: pipet tips for handling semen should be cut off to produce an opening about 1 mm in diameter; pipet tips for handling eggs should be cut off to produce an opening about 2 mm in diameter. This is necessary to provide smooth flow of the viscous semen, accurate sampling of eggs, and to prevent injury to eggs passing through a restricted opening. A clean razor blade can be used to trim pipet tips.

15.6.9 Coverslips -- for microscope slides.

15.6.10 Lens paper -- for cleaning microscope optics.

15.6.11 Laboratory tissue wipes -- for cleaning and drying electrodes, microscope slides, etc.

15.6.12 Disposable countertop covering -- for protection of work surfaces and minimizing spills and contamination.

15.6.13 pH buffers 4, 7, and 10 (or as per instructions of instrument manufacturer) -- for standards and calibration check (see USEPA Method 150.1, USEPA, 1979).

15.6.14 Membranes and filling solutions -- for dissolved oxygen probe (see USEPA Method 360.1, USEPA, 1979), or reagents for modified Winkler analysis.

15.6.15 Laboratory quality assurance samples and standards -- for the above methods.

15.6.16 Test chambers -- 30-mL glass scintillation vials with polypropylene caps, four chambers per concentration.

15.6.17 Formaldehyde, 10%, in seawater -- for preserving larvae. Note: formaldehyde has been identified as a carcinogen and is irritating to skin and mucous membranes. It should not be used at a concentration higher than necessary to achieve morphological preservation of larvae for counting and only under conditions of maximal ventilation and minimal opportunity for volatilization into room air.

15.6.18 Glutaraldehyde, 1% in seawater -- for preserving larvae.

Figure 1. Sample experiment set-up sheet.

SEA URCHIN EMBRYO DEVELOPMENT TEST

EXPERIMENT DESCRIPTION

Bioassay No._____ Start Date_____ Start time_____

Investigator_____ End Date_____ End Time_____

Sample Numbers	Sample	Concentration	Other Information

Figure 2. Sample worksheet for urchin spawning information.

SEA URCHIN DEVELOPMENT TEST

SPAWNING WORKSHEET

Bioassay no._____ Date _____

Spawning

No.	Injection time	Sex	Accepted? (Comments)
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			

Sperm density

#sperm counted=_____

_____

_____ mean=_____

_____

(mean)_____ x (5 x 10^6)=_____ sperm/mL

Egg dilution

eggs counted=_____

_____ mean=_____

_____

(mean)_____ x 100=_____ eggs/mL in stock

eggs/mL in stock ÷ 1,000= _____ Egg dilution factor

Figure 3. Sample worksheet for sea urchin fertilization information.

SEA URCHIN DEVELOPMENT TEST
FERTILIZATION WORKSHEET

Bioassay No. _____
Date_____

_____mL eggs used _____mL dilution water used

Fertilization and initiation

_____mL in egg dilution x 1,000 eggs/mL
= _____eggs in dilution

_____eggs in dilution x 500 sperm/egg
= _____sperm needed

_____sperm needed ÷ _____sperm/mL in sperm dilution=_____mL sperm dilution needed

Percent fertilized after 10 min _____

Time of inoculation_____

Figure 4. Sample data sheet for embryo microscopic examination.

SEA URCHIN DEVELOPMENT TEST: RESULTS

Bioassay No._____ Date_____

Counter_____

Number	Sample	Abnormal	Normal	%Normal	Notes

15.6.19 Acetic acid, 10%, reagent grade, in filtered (10F) seawater -- for preparing killed sperm dilutions for sperm counts.

15.6.20 Haemo-Sol or equivalent cleaner -- for cleaning hemacytometer and cover slips.

15.6.21 0.5 M KCl solution -- for inducing spawning.

15.6.22 Syringe, disposable, 3 or 5 mL -- for injecting KCl into sea urchins and sand dollars to induce spawning.

15.6.23 Needles, 25 gauge -- for injecting KCl.

15.6.24 Pasteur pipets and bulbs -- for sampling eggs from spawning beakers.

15.6.25 Hematocrit capillary tubes -- for sampling sperm for examination and for loading hemacytometers.

15.6.26 Microscope well-slides -- for pre-test assessment of sperm activity and egg condition.

15.6.27 Reference toxicant solutions (see Section 15.10.2.4 and Section 4, Quality Assurance).

15.6.28 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms (see Section 5, Facilities, Equipment, and Supplies).

15.6.29 Effluent and receiving water -- see Section 8, Effluent and Surface Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests.

15.6.30 Dilution water and hypersaline brine -- see Section 7, Dilution Water and Section 15.6.24, Hypersaline Brines. The dilution water should be uncontaminated 1-µm-filtered natural seawater. Hypersaline brine should be prepared from dilution water.

15.6.31 HYPERSALINE BRINES

15.6.31.1 Most industrial and sewage treatment effluents

entering marine and estuarine systems have little measurable salinity. Exposure of larvae to these effluents will usually require increasing the salinity of the test solutions. It is important to maintain an essentially constant salinity across all treatments. In some applications it may be desirable to match the test salinity with that of the receiving water (See Section 7.1). Two salt sources are available to adjust salinities -- artificial sea salts and hypersaline brine (HSB) derived from natural seawater. Use of artificial sea salts is necessary only when high effluent concentrations preclude salinity adjustment by HSB alone.

15.6.31.2 Hypersaline brine (HSB) can be made by concentrating natural seawater by freezing or evaporation. HSB should be made from high quality, filtered seawater, and can be added to the effluent or to reagent water to increase salinity. HSB has several desirable characteristics for use in effluent toxicity testing. Brine derived from natural seawater contains the necessary trace metals, biogenic colloids, and some of the microbial components necessary for adequate growth, survival, and/or reproduction of marine and estuarine organisms, and it can be stored for prolonged periods without any apparent degradation. However, even if the maximum salinity HSB (100‰) is used as a diluent, the maximum concentration of effluent (0‰) that can be tested is 66% effluent at 34‰ salinity (see Table 1).

15.6.31.3 High quality (and preferably high salinity) seawater should be filtered to at least 10 µm before placing into the freezer or the brine generator. Water should be collected on an incoming tide to minimize the possibility of contamination.

15.6.31.4 Freeze Preparation of Brine

15.6.31.4.1 A convenient container for making HSB by freezing is one that has a bottom drain. One liter of brine can be made from four liters of seawater. Brine may be collected by partially freezing seawater at -10 to -20EC until the remaining liquid has reached the target salinity. Freeze for approximately six hours, then separate the ice (composed mainly of fresh water) from the remaining liquid (which has now become hypersaline).

TABLE 1. MAXIMUM EFFLUENT CONCENTRATION (%) THAT CAN BE TESTED AT 34‰ WITHOUT THE ADDITION OF DRY SALTS GIVEN THE INDICATED EFFLUENT AND BRINE SALINITIES.

Effluent Salinity ‰	Brine 60 ‰	Brine 70 ‰	Brine 80 ‰	Brine 90 ‰	Brine 100 ‰
0	43.33	51.43	57.50	62.22	66.00
1	44.07	52.17	58.23	62.92	66.67
2	44.83	52.94	58.97	63.64	67.35
3	45.61	53.73	59.74	64.37	68.04
4	46.43	54.55	60.53	65.12	68.75
5	47.27	55.38	61.33	65.88	69.47
10	52.00	60.00	65.71	70.00	73.33
15	57.78	65.45	70.77	74.67	77.65
20	65.00	72.00	76.67	80.00	82.50
25	74.29	80.00	83.64	86.15	88.00

15.6.31.4.2 It is preferable to monitor the water until the target salinity is achieved rather than allowing total freezing followed by partial thawing. Brine salinity should never exceed 100‰. It is advisable not to exceed about 70‰ brine salinity unless it is necessary to test effluent concentrations greater than 50%.

15.6.31.4.3 After the required salinity is attained, the HSB should be filtered through a 1 µm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4EC (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

15.6.31.5 Heat Preparation of Brine

15.6.31.5.1 The ideal container for making brine using heat-assisted evaporation of natural seawater is one that (1) has a high surface to volume ratio, (2) is made of a non-corrosive material, and (3) is easily cleaned (fiberglass containers are ideal). Special care should be used to prevent any toxic materials from coming in contact with the seawater being used to generate the brine. If a heater is immersed directly into the seawater, ensure that the heater materials do not corrode or leach any substances that would contaminate the brine. One successful method is to use a thermostatically controlled heat exchanger made from fiberglass. If aeration is needed, use only oil-free air compressors to prevent contamination.

15.6.31.5.2 Before adding seawater to the brine generator, thoroughly clean the generator, aeration supply tube, heater, and any other materials that will be in direct contact with the brine. A good quality biodegradable detergent should be used, followed by several (at least three) thorough reagent water rinses.

15.6.31.5.3 Seawater should be filtered to at least 10 µm before being put into the brine generator. The temperature of the seawater is increased slowly to 40EC. The water should be aerated to prevent temperature stratification and to increase water evaporation. The brine should be checked daily (depending on the volume being generated) to ensure that the salinity does not exceed 100‰ and that the temperature does not exceed 40EC. Additional seawater may be added to the brine to obtain the volume of brine required.

15.6.31.5.4 After the required salinity is attained, the HSB should be filtered through a 1 µm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4EC (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

15.6.31.6 Artificial Sea Salts

15.6.31.6.1 No data from sea urchin or sand dollar larval tests using sea salts or artificial seawater (e.g., GP2) are available

for evaluation at this time, and their use must be considered provisional.

15.6.31.7 Dilution Water Preparation from Brine

15.6.31.7.1 Although salinity adjustment with brine is the preferred method, the use of high salinity brines and/or reagent water has sometimes been associated with discernible adverse effects on test organisms. For this reason, it is recommended that only the minimum necessary volume of brine and reagent water be used to offset the low salinity of the effluent, and that brine controls be included in the test. The remaining dilution water should be natural seawater. Salinity may be adjusted in one of two ways. First, the salinity of the highest effluent test concentration may be adjusted to an acceptable salinity, and then serially diluted. Alternatively, each effluent concentration can be prepared individually with appropriate volumes of effluent and brine.

15.6.31.7.2 When HSB and reagent water are used, thoroughly mix together the reagent water and HSB before mixing in the effluent. Divide the salinity of the HSB by the expected test salinity to determine the proportion of reagent water to brine. For example, if the salinity of the brine is 100‰ and the test is to be conducted at 34‰, 100‰ divided by 34‰ = 2.94. The proportion is 1 part brine plus 1.94 parts reagent water. To make 1 L of dilution water at 34‰ salinity from a HSB of 100‰, 340 mL of brine and 660 mL of reagent water are required. Verify the salinity of the resulting mixture using a refractometer.

15.6.31.8 Test Solution Salinity Adjustment

15.6.31.8.1 Table 2 illustrates the preparation of test solutions (up to 50% effluent) at 34‰ by combining effluent, HSB, and dilution water. Note: if the highest effluent concentration does not exceed 50% effluent, it is convenient to prepare brine so that the sum of the effluent salinity and brine salinity equals 68‰; the required brine volume is then always equal to the effluent volume needed for each effluent concentration as in the example in Table 2.

15.6.31.8.2 Check the pH of all test solutions and adjust to within 0.2 units of dilution water pH by adding, dropwise, dilute

hydrochloric acid or sodium hydroxide (see Section 8.8.9, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

15.6.31.8.3 To calculate the amount of brine to add to each effluent dilution, determine the following quantities: salinity of the brine (SB, in ‰), the salinity of the effluent (SE, in ‰), and volume of the effluent to be added (VE, in mL). Then use the following formula to calculate the volume of brine (VB, in mL) to be added:

$$VB = VE \times (34 - SE)/(SB - 34)$$

15.6.31.8.4 This calculation assumes that dilution water salinity is 34 ± 2‰.

15.6.31.9 Preparing Test Solutions

15.6.31.9.1 Ten mL of test solution are needed for each test chamber. To prepare test solutions at low effluent concentrations (<6%), effluents may be added directly to dilution water. For example, to prepare 1% effluent, add 1.0 mL of effluent to a 100-mL volumetric flask using a volumetric pipet or calibrated automatic pipet. Fill the volumetric flask to the 100-mL mark with dilution water, stopper it, and shake to mix. Pour into a (150-250 mL) beaker and stir. Distribute equal volumes into the replicate test chambers. The remaining test solution can be used for chemistry.

15.6.31.9.2 To prepare a test solution at higher effluent concentrations, hypersaline brine must usually be used. For example, to prepare 40% effluent, add 400 mL of effluent to a 1-liter volumetric flask. Then, assuming an effluent salinity of 2‰ and a brine salinity of 66‰, add 400 mL of brine (see equation above and Table 2) and top off the flask with dilution water. Stopper the flask and shake well. Pour into a (100-250 mL) beaker and stir. Distribute equal volumes into the replicate test chambers. The remaining test solution can be used for chemistry.

TABLE 2. EXAMPLES OF EFFLUENT DILUTION SHOWING VOLUMES OF EFFLUENT (AT X‰), BRINE, AND DILUTION WATER NEEDED FOR ONE LITER OF EACH TEST SOLUTION.

FIRST STEP: Combine brine with reagent water or natural seawater to achieve a brine of 68-x‰ and, unless natural seawater is used for dilution water, also a brine-based dilution water of 34‰.

SERIAL DILUTION:

Step 1. Prepare the highest effluent concentration to be tested by adding equal volumes of effluent and brine to the appropriate volume of dilution water. An example using 40% is shown.

Effluent Conc. (%)	Effluent x‰	Brine (68-x)‰	Dilution Water* 34‰
40	800 mL	800 mL	400 mL

Step 2. Make serial dilutions from the highest test concentration.

Effluent Conc. (%)	Effluent Source	Dilution Water* (34‰)
20	1000 mL of 40%	1000 mL
10	1000 mL of 20%	1000 mL
5	1000 mL of 10%	1000 mL
2.5	1000 mL of 5%	1000 mL
Control	none	1000 mL

INDIVIDUAL PREPARATION:

Effluent Conc. (%)	Effluent x‰	Brine (68-x)‰	Dilution Water* 34‰
40	400 mL	400 mL	200 mL
20	200 mL	200 mL	600 mL
10	100 mL	100 mL	800 mL
5	50 mL	50 mL	900 mL
2.5	25 mL	25 mL	950 mL
Control	none	none	1000 mL

*May be natural seawater or brine-reagent water equivalent.

15.6.31.10 Brine Controls

15.6.31.10.1 Use brine controls in all tests where brine is used. Brine controls contain the same volume of brine as does the highest effluent concentration using brine, plus the volume of reagent water needed to reproduce the hyposalinity of the effluent in the highest concentration, plus dilution water. Calculate the amount of reagent water to add to brine controls by rearranging the above equation, (See, 15.6.31.8.3) setting SE = 0, and solving for VE.

$$VE = VB \times (SB - 34)/(34 - SE)$$

If effluent salinity is essentially 0‰, the reagent water volume needed in the brine control will equal the effluent volume at the highest test concentration. However, as effluent salinity and effluent concentration increase, less reagent water volume is needed.

15.6.32 TEST ORGANISMS, PURPLE URCHINS

15.6.32.1 Sea Urchins, ***Strongylocentrotus purpuratus*** (approximately 6 of each sex per test).

15.6.32.2 Species Identification

15.6.32.2.1 Although identification of purple sea urchins, ***Strogylocentrotus purpuratus***, is usually a simple matter of confirming general body color, size, and spine appearance, those unfamiliar with the species should seek confirmation from local experts.

15.6.32.3 Obtaining Broodstock

15.6.32.3.1 Adult sea urchins (***Strongylocentrotus purpuratus***) can be obtained from commercial suppliers or collected from uncontaminated intertidal areas. State collection permits are usually required for collection of sea urchins and collection is prohibited or restricted in some areas. The animals are best transported "dry," surrounded either by moist seaweed or paper towels dampened with seawater. Animals should be kept at approximately their collection or culture temperature to prevent thermal shock which can prematurely induce spawning.

15.6.32.4 Broodstock Culture and Handling

15.6.32.4.1 The adult sea urchins are maintained in glass aquaria or fiberglass tanks. The tanks are supplied continuously (approximately 5 L/min) with filtered natural seawater, or salt water prepared from commericial sea salts is recirculated. The animals are checked daily and any obviously unhealthy animals are discarded.

15.6.32.4.2 Although ambient temperature seawater is usually acceptable, maintaining sea urchins in spawning condition usually requires holding at a relatively constant temperature. The culture unit should be capable of maintaining a constant temperature between 10 and 14EC with a water temperature control device.

15.6.32.4.3 Food for sea urchins -- kelp, recommended, but not necessarily limited to, ***Laminaria sp.***, ***Hedophyllum sp.***, ***Nereocystis sp.***, ***Macrocystis sp.***, ***Egregia sp.***, ***Alaria sp.*** or romaine lettuce. The kelp should be gathered from known uncontaminated zones or obtained from commerical supply houses whose kelp comes from known uncontaminated areas, or romaine lettuce. Fresh food is introduced into the tanks at least several times a week. Sun dried (12-24 hours) or oven dried (60EC overnight) kelp, stores well at room temperature or frozen, rehydrates well and is adequate to maintain sea urchins for long periods. Decaying food and fecal pellets are removed as necessary to prevent fouling.

15.6.32.4.4 Natural seawater (>30‰) is used to maintain the adult animals and ($32‰) as a control water in the tests.

15.6.32.4.5 Adult male and female (if sexes known) animals used in field studies are transported in separate or partitioned insulated boxes or coolers packed with wet kelp or paper toweling. Upon arrival at the field site, aquaria (or a single partitioned aquarium) are filled with control water, loosely covered with a styrofoam sheet and allowed to equilibrate to the holding temperature before animals are added. Healthy animals will attach to the kelp or aquarium within hours.

15.6.32.4.6 To successfully maintain about 25 adult animals for seven days at a field site, 40-L glass aquaria using aerated,

recirculating, clean saline water (32‰) and a gravel bed filtration system, are housed within a water bath, such as an INSTANT OCEAN® Aquarium. The sexes should be held separately if possible.

15.6.33 TEST ORGANISMS, SAND DOLLARS

15.6.33.1 Sand Dollars, ***Dendraster excentricus***, (approximately 6 of each sex per test).

15.6.33.2 Species Identification

15.6.33.2.1 Although identification of sand dollars, ***Dendraster excentricus***, is usually a simple matter of confirming general body appearance, those unfamiliar with the species should seek confirmation from local experts.

15.6.33.3 Obtaining Broodstock

15.6.33.3.1 Adult sand dollars (***Dendraster excentricus***) can be obtained from commercial suppliers or collected from subtidal zones (most areas) or from intertidal zones of some sheltered waters (e.g., Puget Sound). State collection permits may be required for collection of sand dollars and collection prohibited or restricted in some areas. The animals are best transported "dry," surrounded either by moist seaweed or paper towels dampened with seawater. Animals should be kept at approximately their collection or culture temperature to prevent thermal shock which can prematurely induce spawning.

15.6.33.4 Broodstock Culture and Handling

15.6.33.4.1 The adult sand dollars are maintained in glass aquaria or fiberglass tanks. The tanks are supplied continuously (approximately 5 L/min) with filtered natural seawater, or saltwater prepared from commercial sea salts is recirculated. The animals are checked daily and any obviously unhealthy animals are discarded. For longer periods than a few days, several centimeters or more of a sand substrate may be desirable.

15.6.33.4.2 Although ambient temperature seawater is usually acceptable, maintaining sand dollars in spawning condition usually requires holding at a relatively constant temperature.

The culture unit should be capable of maintaining a constant temperature between 8 and 12EC with a water temperature control device.

15.6.33.4.3 Sand dollars will feed on suspended or benthic materials such as phytoplankton, benthic diatoms, etc. No reports of laboratory populations being maintained in spawning condition over several years are known. It is probably most convenient to obtain sand dollars, use them, and then discard them after they cease to produce good quality gametes.

15.6.33.4.4 Natural seawater (>30‰) is used to maintain the adult animals and ($32‰) as a control water in the tests.

15.6.33.4.5 Adult male and female (if sexes known) animals used in field studies are transported in separate or partitioned insulated boxes or coolers packed with wet kelp or paper toweling. Upon arrival at the field site, trays or aquaria (or a single partitioned aquarium) are filled with control water, loosely covered with a styrofoam sheet and allowed to equilibrate to the holding temperature before animals are added.

15.6.33.4.6 To successfully maintain about 25 adult animals for seven days at a field site, 40-L glass aquaria using aerated, recirculating, clean saline water (>30‰) are housed within a water bath, such as an INSTANT OCEAN® Aquarium. The sexes should be held separately if possible.

15.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION AND STORAGE

15.7.1 See Section 8, Effluent and Receiving Water Sampling and Sample Handling, and Sampling Preparation for Toxicity Tests.

15.8 CALIBRATION AND STANDARDIZATION

15.8.1 See Section 4, Quality Assurance.

15.9 QUALITY CONTROL

15.9.1 See Section 4, Quality Assurance.

15.10 TEST PROCEDURES

15.10.1 TEST DESIGN

15.10.1.1 The test consists of at least four replicates of five effluent concentrations plus a dilution water control. Tests that use brine to adjust salinity must also contain four replicates of a brine control.

15.10.1.2 Effluent concentrations are expressed as percent effluent.

15.10.2 TEST SOLUTIONS

15.10.2.1 Receiving waters

15.10.2.1.1 The sampling point is determined by the objectives of the test. At estuarine and marine sites, samples are usually collected at mid-depth. Receiving water toxicity is determined with samples used directly as collected or with samples passed through a 60 µm NITEX® filter and compared without dilution, against a control. Using four replicate chambers per test, each containing 10 mL, and 400 mL for chemical analysis, would require approximately 440 mL of sample per test.

15.10.2.2 Effluents

15.10.2.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of at least 0.5 is commonly used. A dilution factor of 0.5 provides hypothesis test discrimination of ± 100%, and testing of a 16 fold range of concentrations. Hypothesis test discrimination shows little improvement as dilution factors are increased beyond 0.5 and declines rapidly if smaller dilution factors are used. **USEPA recommends that one of the five effluent treatments must be a concentration of effluent mixed with dilution water which corresponds to the permittee's instream waste concentration (IWC).** At least two of the effluent treatments must be of lesser effluent concentration than the IWC, with one being at least one-half the concentration of the IWC. If 100% HSB is used as a diluent, the maximum concentration of effluent that can be tested will be 66% at 34‰ salinity.

15.10.2.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12% and 1.56%).

15.10.2.2.3 The volume in each test chamber is 10 mL.

15.10.2.2.4 Effluent dilutions should be prepared for all replicates in each treatment in one container to minimize variability among the replicates. Dispense into the appropriate effluent test chambers.

15.10.2.3 Dilution Water

15.10.2.3.1 Dilution water should be uncontaminated 1-µm-filtered natural seawater or hypersaline brine prepared from uncontaminated natural seawater plus reagent water (see Section 7, Dilution Water). Natural seawater may be uncontaminated receiving water. This water is used in all dilution steps and as the control water.

15.10.2.4 Reference Toxicant Test

15.10.2.4.1 Reference toxicant tests should be conducted as described in Quality Assurance (see Section 4.7).

15.10.2.4.2 The preferred reference toxicant for sea urchins and sand dollars is copper chloride ($CuCl_2 \cdot H_2O$). Reference toxicant tests provide an indication of the sensitivity of the test organisms and the suitability of the testing laboratory (see Section 4 Quality Assurance). Another toxicant may be specified by the appropriate regulatory agency. Prepare a copper reference toxicant stock solution (2,000 mg/L) by adding 5.366 g of copper chloride ($CuCl_2 \cdot 2H_2O$) to 1 liter of reagent water. For each reference toxicant test prepare a copper sub-stock of 3 mg/L by diluting 1.5 mL of stock to one liter with reagent water. Alternatively, certified standard solutions can be ordered from commercial companies.

15.10.2.4.3 Prepare a control (0 Fg/L) plus four replicates each of at least five consecutive copper reference toxicant solutions (e.g., from the series 3.0, 4.4, 6.5, 9.5, 13.9, 20.4, and 30.0 Fg/L, by adding 0.10, 0.15, 0.22, 0.32, 0.46, 0.68, and 1.00 mL of sub-stock solution, respectively, to 100-L volumetric flasks

and filling to 100-mL with dilution water). Alternatively, certified standard solutions can be ordered from commercial companies. Start with control solutions and progress to the highest concentration to minimize contamination.

15.10.2.4.4 If the effluent and reference toxicant tests are to be run concurrently, then the tests must use embryos from the same spawn. The tests must be handled in the same way and test solutions delivered to the test chambers at the same time. Reference toxicant tests must be conducted at 34 ± 2‰.

15.10.3 COLLECTION OF GAMETES FOR THE TEST

15.10.3.1 Spawning Induction

15.10.3.1.1 Pour seawater into 100 mL beakers and place in 15EC bath or room. Allow to come to temperature. Select a sufficient number of sea urchins or sand dollars (based upon recent or past spawning success) so that three of each sex are likely to provide gametes of acceptable quantity and quality for the test. During optimal spawning periods this may only require six animals, three of each sex, when the sexes are known from prior spawning. During other periods, especially if the sex is not known, many more animals may be required.

15.10.3.1.2 Care should be exercised when removing sea urchins from holding tanks so that damage to tube-feet is minimized. Following removal, sea urchins should be placed into a container lined with seawater-moistened paper towels to prevent reattachment.

15.10.3.1.3 Place each sand dollar, oral side up, on a 100 mL beaker filled with 15EC seawater or each sea urchin onto a clean tray covered with several layers of seawater moistened paper towels.

15.10.3.1.4 Handle sexes separately once known; this minimizes the chance of accidental egg fertilization. Throughout the test process, it is best if a different worker, different pipets, etc. are used for males (semen) and females (eggs). Frequent washing of hands is a good practice.

15.10.3.1.5 Fill a 3 or 5 mL syringe with 0.5 M KCl and inject

0.5 mL through the soft periostomal membrane of each sea urchin (See Figure 5) or into the oral opening each sand dollar. If sexes are known, use a separate needle for each sex. If sexes are not known, rinse the needle with hot tap water between each injection. This will avoid the accidental injection of sperm from males into females. Note the time of injection on the data sheet.

15.10.3.1.6 Spawning of sea urchins is sometimes induced by holding the injected sea urchin and gently shaking or swirling it for several seconds. This may provide an additional physical stimulus, or may aid in distributing the injected KCl.

15.10.3.1.7 Place the sea urchins onto the beakers or tray (oral side down). Place the sand dollars onto the beakers (oral side up). Females will release orange (sea urchins) or purple (sand dollars) eggs and males will release cream-colored semen.

15.10.3.1.8 As gametes begin to be shed, note the time on the data sheet and separate the sexes. Place male sand dollars with the oral side up atop a small (5-10 mL) glass beaker filled with 12EC seawater. Leave spawning sea urchin males on tray or beaker (oral side down) for semen collection. Female sand dollars and sea urchins are left to shed eggs into the 100-mL beakers.

15.10.3.1.9 If sufficient quantities of gametes are available, only collect gametes for the first 15 min after each animal starts releasing. This helps to insure good quality gametes. As a general guideline, do not collect gametes from any individual for more than 30 minutes after the first injection.

15.10.3.1.10 If no spawning occurs after 5 or 10 minutes, a second 0.5 mL injection may be tried. If animals do not produce sufficient gametes following injection of 1.0 mL of KCl, they should probably not be reinjected as this seldom results in acquisition of good quality gametes and may result in mortality of adult urchins.

15.10.3.1.11 Sections 15.10.4.2 and 15.10.6.4 describe collection and dilution of the sperm and eggs. While some of the gamete handling needs to be in a specific order, parts of the procedure can be done simultaneously while waiting for gametes to settle.

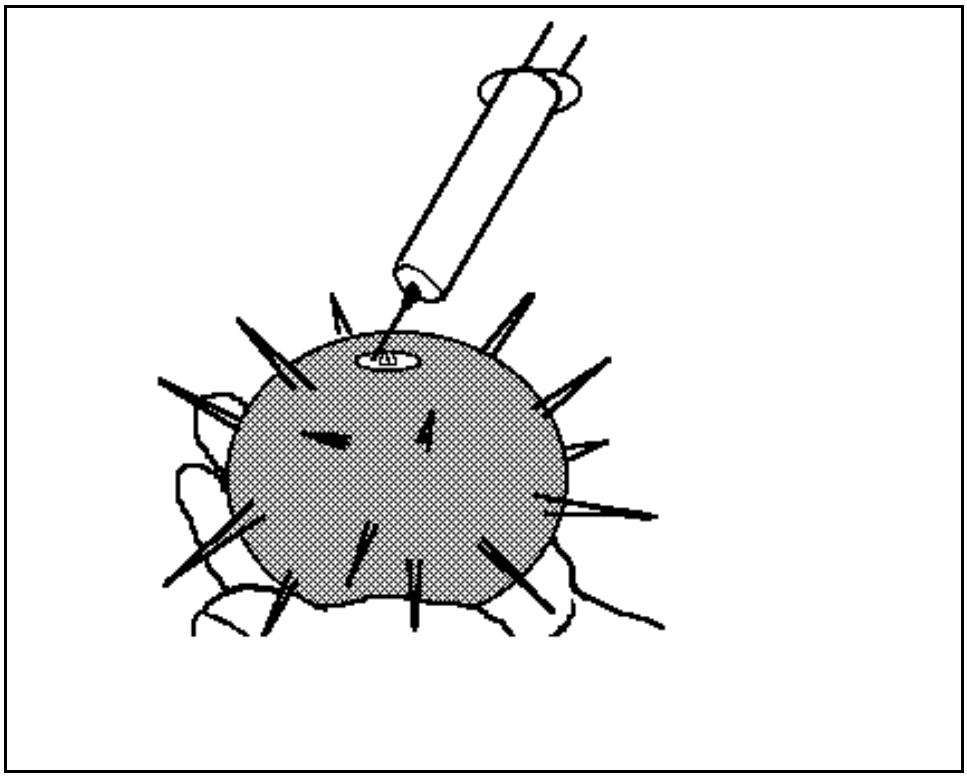


Figure 5. Showing the location and orientation used in the injection of KCl into sea urchins to stimulate spawning.

15.10.3.2 Collection of Sperm

15.10.3.2.1 Sea urchin semen should be collected dry (directly from the surface of the sea urchin), using either a Pasteur pipette or a 0.1 mL autopipette with the end of the tip cut off so that the opening is at least 2 mm. Pipette semen from each male into separate 1-15 mL conical test tubes, stored in an ice water bath.

15.10.3.3 Viability of Sperm

15.10.3.3.1 Early in the spawning process, place a very small amount of sperm from each male sea urchin or sand dollar into

dilution water on a microscope slide (well slides work nicely). Examine the sperm for motility; use sperm from males with high sperm motility.

15.10.3.4 Pooling of Sperm

15.10.3.4.1 Pool equal quantities of semen from each of the sea urchin males that has been deemed good. If possible, 0.025 mL should be pooled from each of those used and a total of at least 0.05 mL of pooled semen should be available. Sperm collected from good male sand dollars should be pooled after first decanting off the overlying water (the final sand dollar sperm density usually is between $2x10^9$ and $2x10^{10}$ sperm/mL).

15.10.3.5 Storage of Sperm

15.10.3.5.1 Cover each test tube or beaker with a cap or parafilm, as air exposure of semen may alter its pH through gas exchange and reduce the viability of the sperm. Keep sperm covered and on ice or refrigerated (<5EC). The sperm should be used within 4 h of collection.

15.10.4 PREPARATION OF SPERM DILUTION FOR USE IN THE TEST

15.10.4.1 Sperm Dilution

15.10.4.1.1 When ready to use sperm, mix by agitating the tube with a vortex mixer. Add about 0.025 mL of semen to a 100 mL beaker containing 50 mL of 15EC dilution water. Stir this solution thoroughly with a Pasteur pipette. A drop of egg solution from each female may be placed on a well slide and a small amount of sperm solution added to test fertilization. If no fertilization membrane forms on eggs from any female, then new gametes should be collected. Keep the sperm dilution covered and at 15EC until ready for use. This dilution should be used to fertilize the eggs within 1.5 hours of being made.

15.10.4.2 Sperm Density Determination

15.10.4.2.1 Take 0.5 mL subsample of the sperm solution and add it to 5 mL of 10% acetic acid in a 50 mL graduated cylinder, to kill the sperm. Bring the volume to 50 mL with dilution water. Mix by inversion and place one drop of the killed sperm solution

onto each side of a hemocytometer. Let sperm settle for about 15 minutes. Count the number of sperm in 80 small squares on each side of the hemocytometer. If the counts for each side are within 80% of one another, then take the mean of those two counts. If the counts are not that close, then refill the hemocytometer, recount and take the mean of the four counts. Use the following equations to determine sperm density and record the results on the spawning worksheet (Figure 2).

$$\#\text{sperm/mL} = \frac{(\text{dilution})(\text{count})(\text{hemacytometer conversion factor})(\text{mm3/mL})}{\text{number of squares counted}}$$

dilution=100
conversion factor=4000
mm^3/mL=1000
number of squares=80

Therefore:

$$\#\text{sperm/mL} = (\text{count})(5 \times 10^6) \qquad \text{(Equation 2A)}$$

15.10.5 PREPARATION OF EGG SUSPENSION FOR USE IN THE TEST

15.10.5.1 Acceptability of Eggs

15.10.5.1.1 Place a small sample of eggs from each female in the counting chamber and examine eggs with the microscope. Look for the presence of significant quantities of immature or abnormal appearing eggs (germinal vesicle present, unusually large or small or irregularly shaped). Do not use the eggs from females having more than 10% abnormal eggs or from females whose eggs did not fertilize during the test in Section 15.10.5.1.

15.10.5.2 Pooling of Eggs

15.10.5.2.1 Allow eggs to settle in the collection beakers. Decant some of the water from the collection beakers taking care not to pour off many eggs. Pour the remaining sea urchin eggs

through the Nitex® screen (to remove fecal material and other debris) into a 1 liter beaker. Repeat with each of the "good" females. Bring the volume up to about 600 mL with dilution water. Allow the eggs to settle to the bottom again. Siphon off about 400 mL of the overlying water and then bring back up to 600 mL with dilution water. Do not allow the temperature to rise above the 15EC test temperature; somewhat cooler temperatures for holding would be acceptable.

15.10.5.2.2 Pooled sand dollar eggs should be treated gently and no additional screening or rinsing step is recommended. Mix well once just before subsampling for egg stock calculations. This is best done in a large graduated cylinder appropriate for the number of eggs available. Cover with parafilm and invert gently several times.

15.10.5.3 Density of Eggs

15.10.5.3.1 Using a plunger, mix the sea urchin egg suspension well. While continuing to mix, remove a 10 mL sample and place in a 1 liter graduated cylinder. Bring the volume up to 1 liter with dilution water. Mix this dilution well and remove a 1 mL sample to a counting cell. Count all the eggs in the 1 mL sample. Repeat the process and take the mean of the two counts. Calculate the number of eggs per mL in the stock solution using Equation 3 and record the results.

# of eggs in count x 100= # eggs/mL in stock (Equation 3)

15.10.5.4 Dilution of Eggs

15.10.5.4.1 When using scintillation vials as the test chamber, the final concentration of eggs in the diluted stock must be 250 eggs/0.25 mL, which is equal to 1,000 eggs/mL. To calculate the dilution factor for the eggs, use Equation 4. (If larger test chambers are used, the total number of eggs used will be greater and the stock solution density may be adjusted, but the final concentration of eggs in the test solutions must remain 25 eggs/mL).

# of eggs/mL in stock ÷ 1,000= Dilution factor (Equation 4)

15.10.5.4.2 The dilution factor must be greater than one. If

not, concentrate the eggs and recount (starting at Section 15.4.5.3 The dilution factor minus 1 equals the number of parts of water that go with one part of eggs in the final dilution. For example: if the dilution factor were 5.3, then 4.3 parts of water would be used with 1 part eggs.

15.10.5.4.3 Make a dilution of the egg stock so that there is more than enough volume to perform the bioassay.

15.10.5.5 Fertilization of Eggs

15.10.5.5.1 The recommended initial sperm to egg ratio for fertilization of the eggs is 500:1. Calculate the volume of sperm dilution (Section 15.10.5.1) to add to the egg dilution, by using the following equations and record the results (Figure 3).

volume of egg dilution x 1,000 eggs/mL= total # of eggs in dilution (Equation 5A)

total # of eggs in dilution x 500 sperm/egg= # of sperm needed (Equation 5B)

# of sperm needed ÷ # sperm/mL in sperm dilution= mL of sperm solution (Equation 5C)

15.10.5.5.2 Add this volume of the sperm dilution to the egg dilution and mix gently with a plunger. Wait 10 min, then check for fertilization. If fertilization is not at least 90%, add a second volume of the sperm dilution. Wait 10 min and recheck. If fertilization is still not 90%, then the test must be restarted with different gametes.

15.10.5.5.3 The test should be initiated within 1 hour of fertilization being achieved.

15.10.6 START OF THE TEST

15.10.6.1 Prior to Beginning the Test

15.10.6.1.1 The test should begin as soon as possible, preferably within 24 h of sample collection. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity tests unless permission is granted by the permitting authority. In no case

should the sample be used in a test more than 72 h after sample collection (see Section 8 Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Test).

15.10.6.1.2 Just prior to test initiation (approximately 1 h), the temperature of a sufficient quantity of the sample to make the test solutions should be adjusted to the test temperature (15 ± 1EC) and maintained at that temperature during the addition of dilution water.

15.10.6.1.3 Increase the temperature of the water bath, room, or incubator to the required test temperature (15 ± 1EC).

15.10.6.1.4 Randomize the placement of test chambers in the temperature-controlled water bath, room, or incubator at the beginning of the test, using a position chart. Assign numbers for the position of each test chamber using a random numbers or similar process (see Appendix A, for an example of randomization). Maintain the chambers in this configuration throughout the test, using a position chart. Record these numbers on a separate data sheet together with the concentration and replicate numbers to which they correspond. Identify this sheet with the date, test organism, test number, laboratory, and investigator's name, and safely store it away until after the sea urchins or sand dollars have been examined at the end of the test.

15.10.6.1.5 Note: Loss of the randomization sheet would invalidate the test by making it impossible to analyze the data afterwards. Make a copy of the randomization sheet and store separately. Take care to follow the numbering system exactly while filling chambers with the test solutions.

15.10.6.1.6 Arrange the test chambers randomly in the water bath or controlled temperature room. Once chambers have been labeled randomly and filled with test solutions, they can be arranged in numerical order for convenience, since this will also ensure random placement of treatments.

15.10.6.1.6 If mortality is to be included as an endpoint, at least 5 extra control chambers should be set up and identified on the randomization sheet as initial count chambers.

15.10.6.2 Delivery of Fertilized Eggs

15.10.6.2.1 Gently mix the solution of fertilized eggs. Deliver 0.25 mL of egg solution to each vial, using an automatic pipette with the tip cut off to provide at least a 0.5 mm opening. Deliver the embryos into the test chambers directly from the pipette, taking care not to touch the pipette to the test solution. The egg solution temperature must be within 1EC of the test solutions. Keep the eggs well mixed during the delivery procedure.

15.10.6.3 Incubation

15.10.6.3.1 The embryos are incubated for 72 hours in the test chambers at 15 ± 1EC at ambient light level.

15.10.6.3.2 The optional extra control chambers for initial counts should be counted as soon as possible after test initiation. If they are sampled and counted in a non-destructive manner they may be returned to the test but used only as a check for larval developmental rate. They must not be used for for routine control counts at the end of the test.

15.10.7 LIGHT, PHOTOPERIOD, SALINITY AND TEMPERATURE

15.10.7.1 The light quality and intensity should be at ambient laboratory conditions. Light intensity should be 10-20 µE/m^2/s, or 50 to 100 foot candles (ft-c), with a 16 h light and 8 h dark cycle.

15.10.7.2 The water temperature in the test chambers should be maintained at 15 ± 1EC. If a water bath is used to maintain the test temperature, the water depth surrounding the test cups should be as deep as possible without floating the chambers.

15.10.7.3 The test salinity should be in the range of 34 ± 2‰. The salinity should vary by no more than ±2‰ among the chambers on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.

15.10.7.4 Rooms or incubators with high volume ventilation should be used with caution because the volatilization of the

test solutions and evaporation of dilution water may cause wide fluctuations in salinity. Covering the test chambers with clean polyethylene plastic may help prevent volatilization and evaporation of the test solutions.

15.10.8 DISSOLVED OXYGEN (DO) CONCENTRATION

15.10.8.1 Aeration may affect the toxicity of effluent and should be used only as a last resort to maintain a satisfactory DO. The DO concentration should be measured on new solutions at the start of the test (Day 0). The DO should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). If it is necessary to aerate, all treatments and the control should be aerated. The aeration rate should not exceed that necessary to maintain a minimum acceptable DO and under no circumstances should it exceed 100 bubbles/minute, using a pipet with a 1-2 mm orifice, such as a 1 mL KIMAX® serological pipet No. 37033, or equivalent.

15.10.9 OBSERVATIONS DURING THE TEST

15.10.9.1 Routine Chemical and Physical Observations

15.10.9.1.1 The DO should be measured in each test solution at the beginning of the exposure period.

15.10.9.1.2 The temperature, pH, and salinity should be measured in all each test solution at the beginning of the exposure period. Temperature should also be monitored continuously or observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be measured in a sufficient number of test chambers at the end of the test to determine temperature variation in the environmental chamber.

15.10.9.1.3 Record all the measurements on the data sheet.

15.10.9.2 Routine Biological Observations

15.10.9.2.1 Developing embryos do not need to be monitored during the test under normal circumstances.

15.10.10 TERMINATION OF THE TEST

15.10.10.1 Ending the Test

15.10.10.1.1 Record the time the test is terminated.

15.10.10.1.2 Temperature, pH, dissolved oxygen, and salinity are measured at the end of the exposure period in one test chamber at each concentration and in the control(s).

15.10.10.2 Sample Preservation

15.10.10.2.1 To terminate the test, add 1.0 mL of 37% (concentrated) buffered formalin to each sample to give a final formalin concentration of 4%. As an alternate fixative, 0.5 mL of 1.0% glutaraldehyde may be used, in each test chamber. Tightly cap and gently mix each chamber and store for later evaluation. (If the test is performed in larger chambers, a 10 mL subsample of well mixed test solution is to be taken from each chamber and preserved).

15.10.10.3 Counting

15.10.10.3.1 It is recommended that the embryos be examined within one week of preservation. Longer storage times may also be used, but run the risk of sample degradation due to improper preservation. Larvae can be counted directly in the scintillation vials using an inverted microscope. If an inverted scope is not available, then samples should be loaded into a Sedgewick-Rafter cell, as follows. The embryos should first be allowed to settle to the bottom of the sample chamber. All but about 1 mL of the overlying liquid should then be removed. All of the remaining liquid containing the embryos should then be transferred to the counting chamber. Whichever scope is used, the embryos should be examined at about 100x power. The first 100 embryos encountered are counted using a multi-unit handcounter to track normal versus abnormal larvae. Record the data by sample number on a data sheet (Figure 4).

15.10.10.3.2 Mortality can be determined only if: (1) all surviving larvae are counted (either in the test vials with an inverted microscope or by <u>total</u> transfer to a counting chamber); or (2) the test solution is stirred with a plunger and

quantitative subsampling is conducted followed by total larval counts on the subsample. The latter procedure requires homogeneous distribution of larvae in the test solution, quantitative transfer of larvae (without adherence to transfer hardware or test chambers), and accurate volume measurements. Mortality is most important to consider with point estimates (e.g., EC25) or when mortality occurs at the NOEC for normal development.

15.10.10.4 Endpoint

15.10.10.4.1 Normal Larvae

15.10.10.4.1.1 Normally developed pluteus larvae have several distinctive characteristics:

(1) The larvae should have a pyramid shape with a pair of skeletal rods that extend at least half the length of the long axis of the larvae (Figure 6D).

(2) The gut should be differentiated into three parts (Figure 6E). If the gut appears lobed and constricts distally in specimens with an obstructed view (e.g., Figure 6D), then normal gut development may be inferred.

(3) Development of post-oral arms has begun.

15.10.10.4.2 Abnormal Larvae

15.10.10.4.2.1 Larvae need only be scored as abnormal or normal to conduct the test, but the categories of abnormalities may be tracked as well. Abnormal larvae should fit into one of the following categories:

(1) Pathological prehatched: Embryos at the single or multi-cell stage with the fertilization membrane still visible.

(2) Pathological hatched: larvae that have no fertilization membrane and demonstrate an extensive

degree of malformation or necrosis. Most of these larvae appear as dark balls of cells or dissociated blobs of cells.

(3) Inhibited: larvae at the blastula or gastrula stage that have no gut differentiation or have no or underdeveloped skeleton. These larvae appear to be developing regularly, but are at a stage earlier than attained by control organisms (e.g., Figure 6A-C).

(4) Gut abnormalities: larvae whose overall appearance is normal, but have guts that are lacking, undifferentiated, abnormally shaped or project outside of the larvae (exogastrulated).

(5) Skeletal abnormalities: larvae whose overall appearance is normal, but have missing spicules, extraneous spicules or rods growing in abnormal directions. Note: Some larvae may exhibit a separation of the rods at the apex. This may be caused by preservation and should not be termed abnormal. Since the test is started with already fertilized eggs, any unfertilized eggs that are encountered should not be counted as either normal or abnormal, but should be ignored.

15.11 **SUMMARY OF TEST CONDITIONS**

15.11.1 A summary of test conditions and test acceptability criteria is listed in Table 3.

15.12 **ACCEPTABILITY OF TEST RESULTS**

15.12.1 Test results are acceptable only if all the following requirements are met:

(1) larval normality must be at least 80% in the controls.

(2) the minimum significant difference (%MSD) is #20% relative to the controls.

15.13 **DATA ANALYSIS**

15.13.1 GENERAL

15.13.1.1 Tabulate and summarize the data. Calculate the

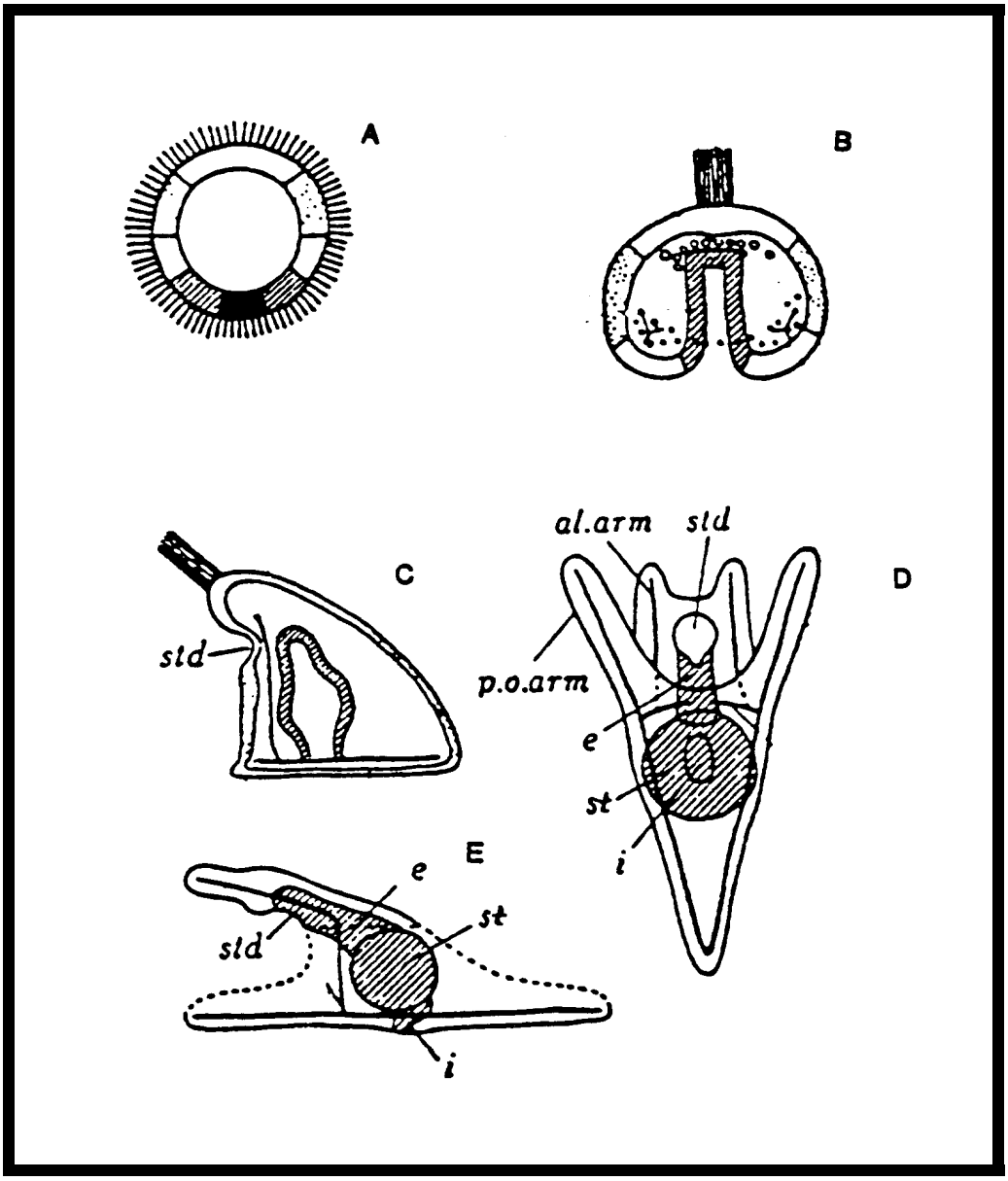


Figure 6. Stages of sea urchin embryo development (modified from Kume and Dan 1957). A. blastula; B. gastrula; C. prism; D. pluteus (frontal view); E. pluteus (lateral view). *al.arm*: anterior lateral arm, *e*: esophagus, *i*: intestine, *st*: stomach, *std*: stomodaeum.

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR THE PURPLE URCHIN, ***STRONGYLOCENTROTUS PURPURATUS***, AND SAND DOLLAR, ***DENDRASTER EXCENTRICUS*** EMBRYO DEVELOPMENT TEST WITH EFFLUENTS AND RECEIVING WATERS

1. Test type:	Static non-renewal
2. Salinity:	34 ± 2‰
3. Temperature:	15 ± 1EC
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 µE/m_2/s (Ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	30 mL
8. Test solution volume:	10 mL
9. No. replicate chambers per concentration:	4
10. Dilution water:	Uncontaminated 1-µm-filtered natural seawater or hypersaline brine prepared from natural seawater
11. Test concentrations:	Effluents: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
12. Dilution factor:	Effluents: $0.5 Receiving waters: 100% receiving water and a control
13. Test duration:	72 ± 2 hr
14. Endpoint:	Normal development; mortality can be included
15. Test acceptability criteria:	$80% normal shell development in the controls; must achieve a %MSD of <25%

16. Sampling requirements:	One sample collected at test initiation, and preferably used within 24 h of the time it is removed from the sampling device (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests)
17. Sample volume required:	1 L per test

proportion of normally developed larvae for each replicate. A sample set of test data is listed in Table 4.

15.13.1.2 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

15.13.1.3 The endpoints of toxicity tests using the purple sea urchin are based on the reduction in proportion of normally developed larvae. The IC25 is calculated using the Linear Interpolation Method (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). LOEC and NOEC values for development are obtained using a hypothesis testing approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981) (see Section 9). Separate analyses are performed for the estimation of the LOEC and NOEC endpoints and for the estimation of the IC25. See the Appendices for examples of the manual computations, and examples of data input and program output.

15.13.2 EXAMPLE OF ANALYSIS OF PURPLE SEA URCHIN, *STRONGYLOCENTROTUS PURPURATUS*, DEVELOPMENT DATA

15.13.2.1 Formal statistical analysis of the larval development data is outlined in Figure 7. The response used in the analysis is the proportion of normally developed larvae in each test or control chamber. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the IC25 endpoint. Concentrations at which there are no normally developed larvae in any of the test chambers are excluded from statistical analysis of the NOEC and LOEC, but included in the estimation of the IC25.

TABLE 4. DATA FROM PURPLE SEA URCHIN, ***STRONGYLOCENTROTUS PURPURATUS***, DEVELOPMENT TEST

Copper Concentration (µg/L)	Replicate	No. Larvae Exposed	No. Larvae Normally Developed	Proportion Normal
Control	A	100	87	0.87
	B	100	89	0.89
	C	100	81	0.81
	D	101	89	0.88
	E	74	62	0.84
3.2	A	110	98	0.89
	B	100	82	0.82
	C	100	91	0.91
	D	100	83	0.83
	E	100	89	0.89
5.6	A	102	86	0.84
	B	100	89	0.89
	C	100	85	0.85
	D	107	90	0.84
	E	100	85	0.85
10.0	A	100	70	0.70
	B	100	71	0.71
	C	100	77	0.77
	D	100	74	0.74
	E	100	87	0.87
18.0	A	100	7	0.07
	B	100	12	0.12
	C	100	14	0.14
	D	100	16	0.16
	E	100	10	0.10
32.0	A	101	0	0.00
	B	102	0	0.00
	C	100	0	0.00
	D	100	0	0.00
	E	100	0	0.00

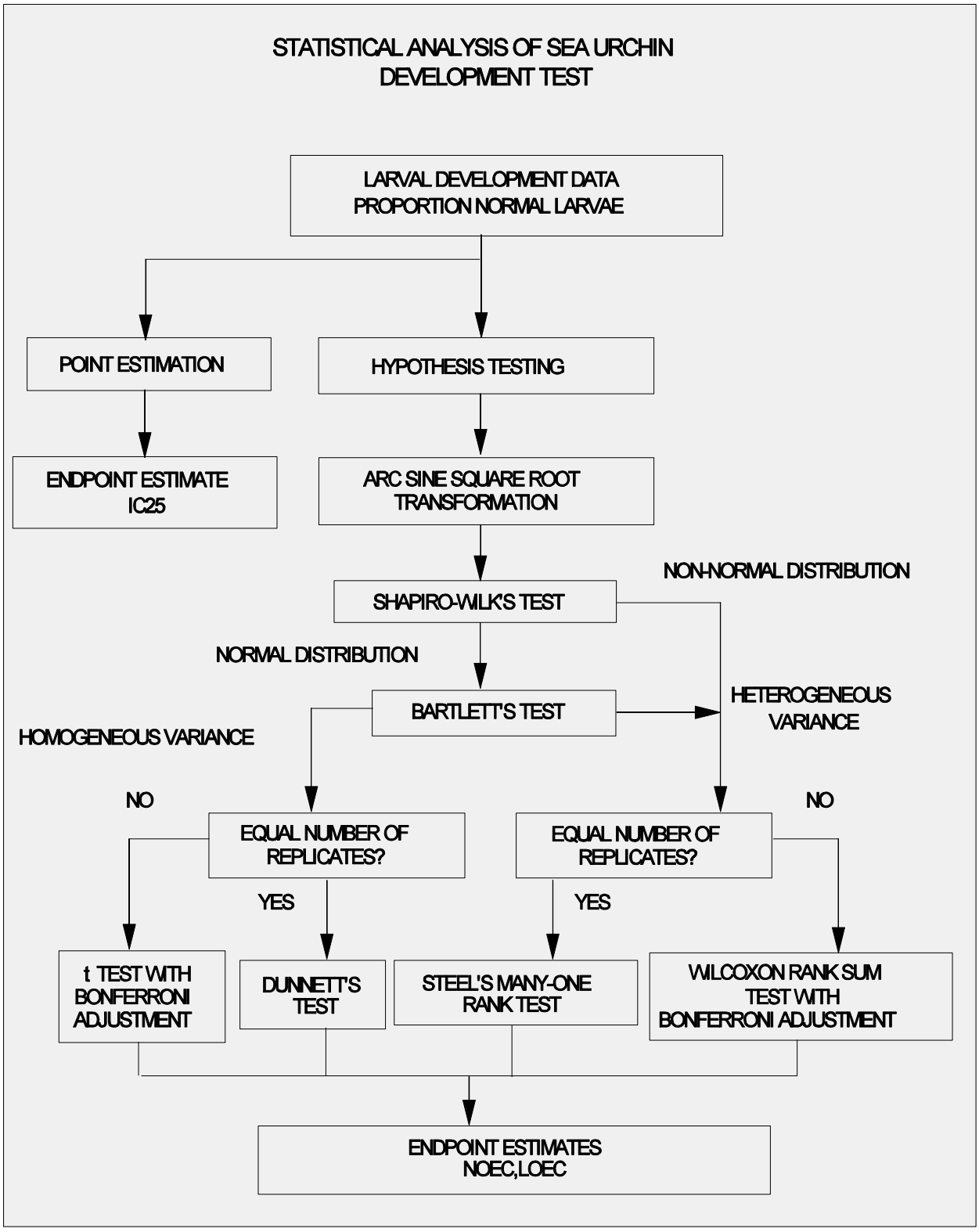


Figure 7. Flowchart for statistical analysis of sea urchin, *Strongylocentrotus purpuratus*, development test.

15.13.2.2 For the case of equal numbers of replicates across all concentrations and the control, the evaluation of the NOEC and LOEC endpoints is made via a parametric test, Dunnett's Procedure, or a nonparametric test, Steel's Many-one Rank Test, on the arc sine square root transformed data. Underlying assumptions of Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test, and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are estimated by the parametric procedure.

15.13.2.3 If unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a ***t*** test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative.

15.13.2.4 Example of Analysis of Development Data

15.13.2.4.1 This example uses toxicity data from a purple sea urchin, ***Strongylocentrotus purpuratus***, development test performed with copper. The response of interest is the proportion of normally developed larvae, thus each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix B. The raw and transformed data, means and variances of the transformed observations at each copper concentration and control are listed in Table 5. The data are plotted in Figure 8. Because there is zero normal development in all five replicates of the 32.0 µg/L copper concentration, it was not included in the statistical analysis and is considered a qualitative development effect.

15.13.2.5 Test for Normality

15.13.2.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 6.

15.13.2.5.2 Calculate the denominator, D, of the statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

& = the overall mean of the centered observations

n = the total number of centered observations

15.13.2.5.3 For this set of data, n = 25

$$\& = \frac{1}{25}(-0.001) = 0.000$$

D = 0.0680

TABLE 5. SEA URCHIN, ***STRONGYLOCENTROTUS PURPURATUS***, DEVELOPMENT DATA

		Copper Concentration (µg/L)				
	Replicate	Control	3.2	5.6	10.0	18.0
RAW	A	0.87	0.89	0.84	0.70	0.07
	B	0.89	0.82	0.89	0.71	0.12
	C	0.81	0.91	0.85	0.77	0.14
	D	0.88	0.83	0.84	0.74	0.16
	E	0.84	0.89	0.85	0.87	0.10
ARC SINE SQUARE ROOT TRANSFORMED	A	1.202	1.234	1.159	0.991	0.268
	B	1.234	1.133	1.234	1.002	0.354
	C	1.120	1.266	1.173	1.071	0.383
	D	1.217	1.146	1.159	1.036	0.412
	E	1.159	1.234	1.173	1.202	0.322
Mean ($\&_i$)		1.186	1.203	1.180	1.060	0.348
S_i^2		0.00215	0.00351	0.00097	0.00725	0.00311
i		1	2	3	4	5

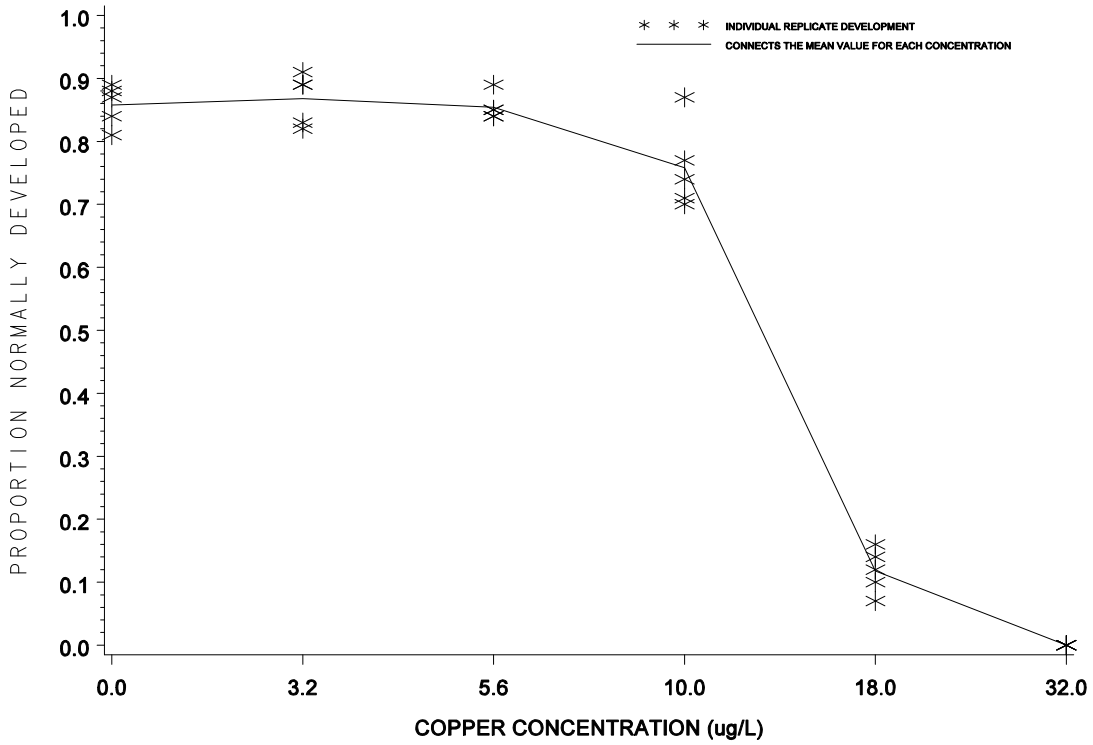


Figure 8. Plot of proportion of normally developed sea urchin, *Strongylocentrotus purpuratus*, larvae,

15.13.2.5.4 Order the centered observations from smallest to largest

$$X^{(1)} \# X^{(2)} \# \ldots \# X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 7.

TABLE 6. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

Replicate	Control	Copper Concentration (µg/L) 3.2	5.6	10.0	18.0
A	0.016	0.031	-0.021	-0.069	-0.080
B	0.048	-0.070	0.054	-0.058	0.006
C	-0.066	0.063	-0.007	0.011	0.035
D	0.031	-0.057	-0.021	-0.024	0.064
E	-0.027	0.031	-0.007	0.142	-0.026

15.13.2.5.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2, ... a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 25 and k = 12. The a_i values are listed in Table 8.

15.13.2.5.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i\left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

The differences, $X^{(n-i+1)} - X^{(i)}$, are listed in Table 8. For the data in this example:

$$W = \frac{1}{0.0680}\ (0.2545)^2 = 0.953$$

TABLE 7. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.080	14	0.006
2	-0.070	15	0.011
3	-0.069	16	0.016
4	-0.066	17	0.031
5	-0.058	18	0.031
6	-0.057	19	0.031
7	-0.027	20	0.035
8	-0.026	21	0.048
9	-0.024	22	0.054
10	-0.021	23	0.063
11	-0.021	24	0.064
12	-0.007	25	0.142
13	-0.007		

TABLE 8. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4450	0.222	$X^{(25)} - X^{(1)}$
2	0.3069	0.134	$X^{(24)} - X^{(2)}$
3	0.2543	0.132	$X^{(23)} - X^{(3)}$
4	0.2148	0.120	$X^{(22)} - X^{(4)}$
5	0.1822	0.106	$X^{(21)} - X^{(5)}$
6	0.1539	0.092	$X^{(20)} - X^{(6)}$
7	0.1283	0.058	$X^{(19)} - X^{(7)}$
8	0.1046	0.057	$X^{(18)} - X^{(8)}$
9	0.0823	0.055	$X^{(17)} - X^{(9)}$
10	0.0610	0.037	$X^{(16)} - X^{(10)}$
11	0.0403	0.032	$X^{(15)} - X^{(11)}$
12	0.0200	0.013	$X^{(14)} - X^{(12)}$

15.13.2.5.7 The decision rule for this test is to compare W as calculated in Subsection 5.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For the data in this example, the critical value at a significance level of 0.01 and n = 25 observations is 0.888. Since W = 0.953

is greater than the critical value, conclude that the data are normally distributed.

15.13.2.6 Test for Homogeneity of Variance

15.13.2.6.1 The test used to examine whether the variation in the proportion of normally developed larvae is the same across all copper concentrations including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{[(\sum_{i=1}^{P} V_i) \ln \bar{S}^2 - \sum_{i=1}^{P} V_i \ln S_i^2]}{C}$$

Where: V_i = degrees of freedom for each concentration and control,

$V_i = (n_i - 1)$

p = number of concentration levels including the control

n_i = the number of replicates for concentration i.

ln = $\log_e$

i = 1,2, ..., p where p is the number of concentrations including the control

$$\bar{S}^2 = \frac{(\sum_{i=1}^{P} V_i S_i^2)}{\sum_{i=1}^{P} V_i}$$

$$C = 1 + [3(p-1)]^{-1}[\sum_{i=1}^{P} 1/V_i - (\sum_{i=1}^{P} V_i)^{-1}]$$

15.13.2.6.2 For the data in this example (see Table 5), all concentrations including the control have the same number of replicates (n_i = 5 for all i). Thus, V_i = 4 for all i.

15.13.2.6.3 Bartlett's statistic is, therefore:

$$B = [(20)\ln(0.00340) - 4\sum_{i=1}^{P}\ln(S_i^2)] / 1.100$$

$$= [20(-5.6840) - 4(-29.4325)]/1.100$$

$$= 4.050/1.100$$

$$= 3.6818$$

15.13.2.6.4 B is approximately distributed as chi-square with p-1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with 4 degrees of freedom, is 13.28. Since B = 3.6818 is less than the critical value of 13.28, conclude that the variances are not different.

15.13.2.7 Dunnett's Procedure

15.13.2.7.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 9.

Where: p = number of concentration levels including the control

TABLE 9. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2 = SSW/(N-p)
Total	N - 1	SST	

N = total number of observations $n_1 + n_2 \ldots + n_p$

n_i = number of observations in concentration i

$$SSB = \sum_{i=1}^{P} T_i^2/n_i - G^2/N \quad \text{Between Sum of Squares}$$

$$SST = \sum_{i=1}^{P} \sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N \quad \text{Total Sum of Squares}$$

$$SSW = SST - SSB \quad \text{Within Sum of Squares}$$

G = the grand total of all sample observations, $G = \sum_{i=1}^{P} T_i$

T_i = the total of the replicate measurements for concentration i

Y_{ij} = the jth observation for concentration i (represents the proportion of normal larvae for concentration i in test chamber j)

15.13.2.7.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = 5$

$N = 25$

$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} + Y_{15} = 5.932$
$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} + Y_{25} = 6.013$
$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} + Y_{35} = 5.898$
$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} + Y_{45} = 5.302$
$T_5 = Y_{51} + Y_{52} + Y_{53} + Y_{54} + Y_{55} = 1.739$

$G = T_1 + T_2 + T_3 + T_4 + T_5 = 24.884$

$$SSB = \sum_{i=1}^{P} T_i^2/n_i - G^2/N$$
$$= (137.267)/5 - (24.884)^2/25 = 2.685$$

$$SST = \sum_{i=1}^{P} \sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$
$$= 27.521 - (24.884)^2/25 = 2.752$$

$SSW = SST - SSB = 2.752 - 2.685 = 0.067$

$S_B^2 = SSB/(p-1) = 2.685/(5-1) = 0.6713$

$S_W^2 = SSW/(N-p) = 0.067/(25-5) = 0.0034$

15.13.2.7.3 Summarize these calculations in the ANOVA table (Table 10).

TABLE 10. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	4	2.685	0.6713
Within	20	0.067	0.0034
Total	24	2.752	

15.13.2.7.4 To perform the individual comparisons, calculate the ***t*** statistic for each concentration, and control combination as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_w \sqrt{(1/n_1) + (1/n_i)}}$$

Where: $\bar{Y}_i$ = mean proportion normal larvae for concentration i

$\bar{Y}_1$ = mean proportion normal larvae for the control

S_W = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i.

Since we are looking for a decreased response from the control in the proportion of normally developed larvae, the concentration mean is subtracted from the control mean.

15.13.2.7.5 Table 11 includes the calculated t values for each concentration and control combination. In this example, comparing the 3.2 µg/L copper concentration with the control the calculation is as follows:

$$t_2 = \frac{(1.186 - 1.203)}{0.0583\sqrt{(1/5)+(1/5)}} = -0.461$$

TABLE 11. CALCULATED t VALUES

Copper Concentration (µg/L)	i	t_i
3.2	2	-0.461
5.6	3	0.163
10.0	4	3.417
18.0	5	22.727

15.13.2.7.6 Since the purpose of this test is to detect a significant decrease in the proportion of normally developed larvae, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix C. For an overall alpha level of 0.05, 20 degrees of freedom for error and four concentrations (excluding the control) the critical value is 2.30. The mean proportion of normally developed larvae for concentration i is considered significantly less than the mean proportion of normally developed larvae for the control if t_i is greater than the critical value. Therefore, the 10.0 µg/L and 18.0 µg/L concentrations have a significantly lower mean proportion of normally developed larvae than the control. Hence the NOEC is 5.6 µg/L copper and the LOEC is 10.0 µg/L copper.

15.13.2.7.7 To quantify the sensitivity of the test, the minimum significant difference (MSD) that can be statistically detected may be calculated:

$$MSD = d\,S_w\sqrt{(1/n_1)+(1/n)}$$

Where: d = the critical value for Dunnett's Procedure

S_W = the square root of the within mean square

n = the common number of replicates at each concentration (this assumes equal replication at each concentration)

n_1 = the number of replicates in the control.

15.13.2.7.8 In this example,

$$MSD \text{ ' } 2.30\,(0.0583)\sqrt{(1/5)\,\%\,(1/5)}$$

$$= 2.30\ (0.0583)(0.6325)$$

$$= 0.085$$

15.13.2.7.9 The MSD (0.085) is in transformed units. To determine the MSD in terms of proportion of normally developed larvae, carry out the following conversion.

1. Subtract the MSD from the transformed control mean.

$$1.186 - 0.085 = 1.101$$

2. Obtain the untransformed values for the control mean and the difference calculated in step 1 of 13.2.7.9.

$$[\ \text{Sine}\ (1.186)\ ]^2 = 0.859$$

$$[\ \text{Sine}\ (1.101)\ ]^2 = 0.795$$

3. The untransformed MSD (MSD_u) is determined by subtracting the untransformed values from step 2 in 14.2.7.9.

$$MSD_u = 0.859 - 0.795 = 0.064$$

15.13.2.7.10 Therefore, for this set of data, the minimum difference in mean proportion of normally developed larvae between the control and any copper concentration that can be detected as statistically significant is 0.064.

15.13.2.7.11 This represents a 7.5% decrease in the proportion of normally developed larvae from the control.

15.13.2.8 Calculation of the ICp

15.13.2.8.1 The development data in Table 4 are utilized in this example. As can be seen from Figure 9, the observed means are not monotonically non-increasing with respect to concentration.

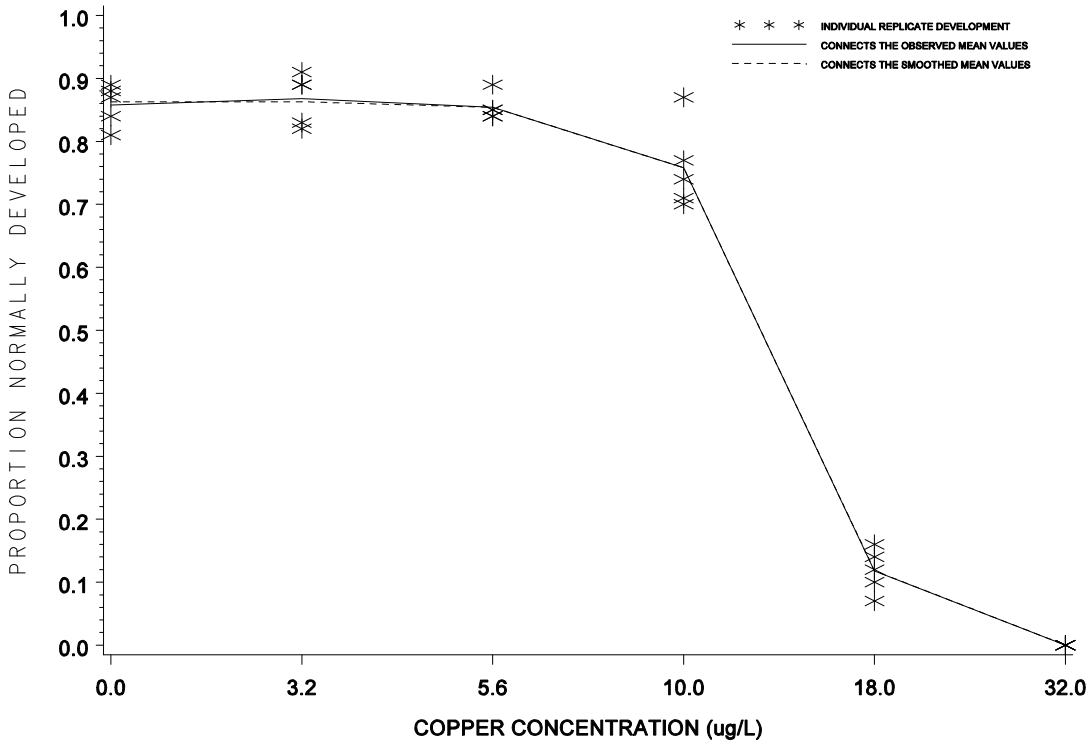


Figure 9. Plot of raw data, observed means, and smoothed means for the sea urchin, *Strogylocentrotus purpuratus*, larval development data from Tables 4 and 12.

Therefore, the means must be smoothed prior to calculating the IC.

15.13.2.8.2 Starting with the observed control mean, $\overline{Y}_1 = 0.858$, and the observed mean for the lowest copper concentration, $\overline{Y}_2 = 0.868$, we see that $\overline{Y}_1$ is less than $\overline{Y}_2$.

15.13.2.8.3 Calculate the smoothed means:

$$M_1 = M_2 = (\overline{Y}_1 + \overline{Y}_2)/2 = 0.863$$

15.13.2.8.4 Since $\overline{Y}_3 = 0.854 > \overline{Y}_4 = 0.758 > \overline{Y}_5 = 0.118 > \overline{Y}_6 = 0.0$, set $M_3 = 0.854$, $M_4 = 0.758$, $M_5 = 0.118$, and $M_6 = 0.0$. Table 12 contains the smoothed means and Figure 8 gives a plot of the smoothed means and the interpolated response curve.

15.13.2.8.5 An IC25 can be estimated using the Linear Interpolation Method. A 25% reduction in mean proportion of normally developed larvae, compared to the controls, would result in a mean proportion of 0.647, where $M_1(1-p/100) = 0.863(1-25/100)$. Examining the means and their associated concentrations (Table 12), the response, 0.647, is bracketed by $C_4 = 10.0$ µg/L copper and $C_5 = 18.0$ µg/L copper.

TABLE 12. SEA URCHIN, ***STRONYLOCENTROTUS PURPURATUS***, MEAN PROPORTION OF NORMALLY DEVELOPED LARVAE

Copper Conc. (µg/L)	i	Response Means, $\overline{Y}_i$ (proportion)	Smoothed Means, M_i (proportion)
Control	1	0.858	0.863
0.05	2	0.868	0.863
0.10	3	0.854	0.854
0.15	4	0.758	0.758
0.20	5	0.118	0.118
0.40	6	0.000	0.000

15.13.2.8.6 Using the equation from Section 4.2 in Appendix L, the estimate of the IC25 is calculated as follows:

$$ICp \text{ ' } C_j\%[M_1(1\&p/100)\&M_j]\frac{(C_{(j\%1)}\&C_j)}{(M_{(j\%1)}\&M_j)}$$

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	3.2	5.6	10	18	32
Response 1	.87	.89	.84	.70	.07	0
Response 2	.89	.82	.89	.71	.12	0
Response 3	.81	.91	.85	.77	.14	0
Response 4	.88	.83	.84	.74	.16	0
Response 5	.84	.89	.85	.87	.10	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Copper Chloride
Test Start Date: Test Ending Date:
Test Species: Purple Sea Urchin, Strongylocentrotus purpuratus
Test Duration: 72 hours
DATA FILE: urch_dev.icp
OUTPUT FILE: urch_dev.i25

Conc. ID	Number Replicates	Concentration ug/L	Response Means	Std. Dev.	Pooled Response Means
1	5	0.000	0.858	0.033	0.863
2	5	3.200	0.868	0.040	0.863
3	5	5.600	0.854	0.021	0.854
4	5	10.000	0.758	0.068	0.758
5	5	18.000	0.118	0.035	0.118
6	5	32.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 11.3844 Entered P Value: 25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 11.3702 Standard Deviation: 0.2898
Original Confidence Limits: Lower: 10.7785 Upper: 11.9375
Expanded Confidence Limits: Lower: 10.4756 Upper: 12.2141
Resampling time in Seconds: 0.16 Random_Seed: 83761380

Figure 10. ICPIN program output for the IC25.

$$IC25 = 10.0 + [0.863(1 - 25/100) - 0.758] (18.0 - 10.0)$$
$$S))))))))))))Q$$
$$(0.118 - 0.758)$$
$$= 11.38 \mu g/L.$$

15.13.2.8.7 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 11.3844 µg/L. The empirical 95.0% confidence interval for the true mean was 10.7785 µg/L to 11.9375 µg/L. The computer program output for the IC25 for this data set is shown in Figure 10.

15.14 **PRECISION AND ACCURACY**

15.14.1 PRECISION

15.14.1.1 Single Laboratory Precision

15.14.1.1.1 Data on the single-laboratory precision of the development test using copper as a reference toxicant is provided in Table 13. The NOEC varied by only one concentration interval indicating good precision. The coefficient of variation for the EC50 and EC25 were 22% and 21% indicating acceptable precision.

15.14.1.2 Multi-Laboratory Precision

15.14.1.2.1 Data on the multi-laboratory precision of the development test using copper as a reference toxicant is provided in Table 14. The NOEC for laboratory's A and B were identical. The difference in NOEC observed for lab C was probably due the wide range of concentrations used (See Footnote 4). The coefficient of variation for the EC50 was 39%, indicating acceptable interlaboratory precision.

15.14.2 ACCURACY

15.14.2.1 The accuracy of tox1icity tests cannot be determined.

Table 13. SINGLE-LABORATORY PRECISION OF THE PURPLE SEA URCHIN, ***STRONGYLOCENTROTUS PURPURATUS***, DEVELOPMENT TEST WITH COPPER (CU µG/L) SULFATE AS A REFERENCE TOXICANT[1].

Test Number	NOEC (µg/L)	EC50 (µg/L)	EC25 (µg/L)
1	10.0	19.4	15.1
2	10.0	18.3	15.4
3	5.6	10.8	9.0
4	5.6	14.3	11.0
5	5.6	16.8	12.9
Mean CV(%)		15.9 22.0	12.7 21.0

[1] Tests performed by Marine Pollution Studies Laboratory, Granite Canyon, Monterey California.

TABLE 14. MULTI-LABORATORY PRECISION OF THE PURPLE SEA URCHIN, ***STRONGYLOCENTROTUS PURPURATUS***, DEVELOPMENT TEST WITH COPPER (CU µG/L) SULFATE AS A REFERENCE TOXICANT.[1]

Lab	NOEC (µg/L)	EC50 (µg/L)
A[2]	10.0	22.5
B[3]	10.0	15.2
C[4]	1.8	10.1
Mean CV(%)		15.9 39.0

[1]Data from labs A and B are from an interlaboratory study using split reference toxicant samples and dilution water. Test performed in August, 1993. Test duration was 72 hr. Concentrations were 3.2, 5.6, 10, 18 and 32 Fg/L.

[2]Test performed by Southern California Coastal Water Research Project, Westminster, CA.

[3]Test performed by Marine Pollution Studies Laboratory, Granite Canyon, Monterey California.

[4] Test performed by MEC Analytical Systems, Inc., Tiburon, CA. Test performed in April, 1994. Test duration was 96 hr. Concentrations were 0.1, 0.32, 1.8, 18 and 56 Fg/L.

APPENDIX I. SEA URCHIN DEVELOPMENT: STEP-BY-STEP SUMMARY

PREPARATION OF TEST SOLUTIONS

A. Determine test concentrations and appropriate dilution water based on NPDES permit conditions and guidance from the appropriate regulatory agency.

B. Prepare effluent test solutions by diluting well mixed unfiltered effluent using volumetric flasks and pipettes. Use hypersaline brine where necessary to maintain all test solutions at 34 ± 2‰. Include brine controls in tests that use brine.

C. Prepare a copper reference toxicant stock solution.

D. Prepare a copper reference toxicant series. Add 10 mL of test solution each vial.

E. Sample effluent and reference toxicant solutions for physical/chemical analysis. Measure salinity, pH and dissolved oxygen of each test concentration.

F. Randomize numbers for test chambers and record the chamber numbers with their respective test concentrations on a randomization data sheet. Store the data sheet safely until after the test samples have been analyzed.

G. Place test chambers in a water bath or environmental chamber set to 15EC and allow temperature to equilibrate.

H. Measure the temperature daily in one random replicate (or separate chamber) of each test concentration. Monitor the temperature of the water bath or environmental chamber continuously.

I. At the end of the test, measure salinity, pH, and dissolved oxygen concentration from each test concentration.

PREPARTION AND ANALYSIS OF TEST ORGANISMS

A. Obtain test organisms and hold or condition as necessary for spawning.

B. Place six 100 mL beakers of dilution water in 15EC water bath or room. Select 6-8 sea urchins and place on tray covered with seawater moistened paper towels. Induce

spawning by injecting each sea urchin with 0.5 mL of 0.5 M KCl. Place animals back onto tray, oral side down.

C. When spawning begins, note time that each animal begins spawning. Leave males on tray for semen collection. Place spawning females oral side up on 100 mL beakers. Do not collect gametes more than 15 min after spawning begins.

D. Collect semen using either a Pasteur pipette or a 100 FL autopipette. Pipette semen from each male into a separate 5 mL conical test tube, stored in an ice water bath.

E. Check for the motility of sperm from each male.

F. Pool semen by pipetting equal amounts from each "good" male to another centrifuge tube. At least 0.025 mL should be taken from each male and a total of at least 0.05 mL should be available. Cover the tube and store in a refrigerator until ready for use.

G. Finish collecting eggs before diluting semen.

H. Mix pooled semen by agitating on a vortex mixer. Add about 0.025 mL of semen to a 100 mL beaker containing 50 mL of 15EC dilution water. Stir thoroughly with a Pasteur pipette. Test eggs from each female to determine if they can be fertilized.

I. Take 0.5 mL subsample of sperm dilution and add to 5 mL of 10% acetic acid in a 50 mL graduated cylinder. Bring to 50 mL with dilution water. Mix well by inversion and load a drop into each side of hemocytometer. Count the sperm in 80 small squares. Calculate the sperm density using Equation 2A.

J. Examine sample of eggs from each female. Do not use the eggs from any female whose eggs appear abnormal or that did not fertilize in Section G.

K. Decant water from eggs of each usable female and pour through Nitex® screen into a 1 liter beaker. Bring volume up to about 600 mL with dilution water. Allow to resettle, siphon about 400 mL of overlying water and bring back to 600 mL with dilution water.

L. Mix egg solution well and make an accurate 100x dilution using at least 10 mL of the egg solution. Mix the dilution well and count two different 1 mL subsamples in a counting

cell. Use the mean of the two counts in Equation 3 to determine the density of the egg stock.

M. Use Equation 4 to determine the egg dilution factor and make dilution of eggs with dilution water.

N. Use Equations 5 A-C to determine the volume of the sperm dilution that is necessary to fertilize the egg dilution. Add the appropriate volume of sperm and after 10 minutes, check fertilization success.

O. Gently mix the fertilized egg solution with a plunger and deliver 0.25 mL of egg solution to each vial. Make sure that the pipette tip is cut off to provide at least a 0.5 mm opening. Keep egg solution well mixed during addition period.

P. Incubate the embryos for 72 hours at 15 ± 1EC.

Q. Test termination and analysis

R. Perform water quality measurements as at the start.

S. After 72 hours, add 1.0 mL of 37% buffered formalin or 0.5 mL of 1.0% glutaraldehyde to each test chamber. Tightly cap and gently mix each vial.

T. Examine each sample with a microscope and determine the percentage of normally developed embryos.

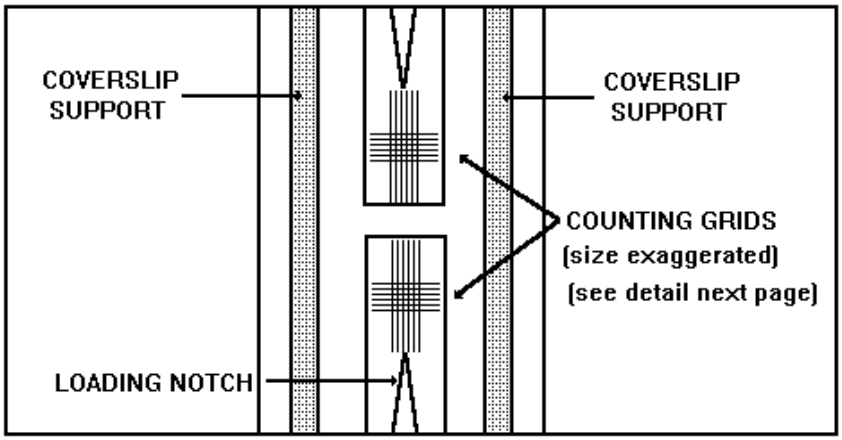
U. Analyze the data.

V. Include standard reference toxicant point estimate values in the standard quality control charts.

APPENDIX II. USING THE NEUBAUER HEMACYTOMETER TO ENUMERATE SEA URCHIN SPERM

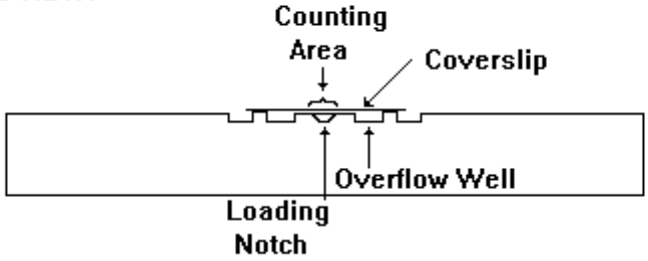
The Neubauer hemacytometer is a specialized microscope slide with two counting grids and a coverslip.

TOP VIEW:

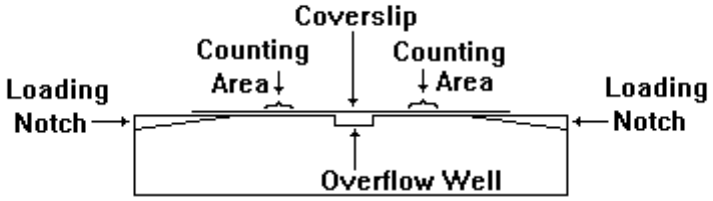


Together, the total area of each grid (1 mm^2) and the vertical distance between the grid and the coverslip (0.1 mm), provide space for a specific microvolume of aqueous sample (0.1 mm^3).

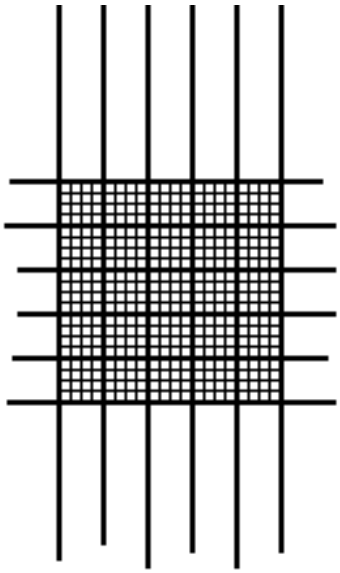
SIDE VIEW:



END VIEW THROUGH MID-CROSS SECTION:



This volume of liquid and the cells suspended therein (e.g., blood cells or sperm cells) represent 1/10,000th of the liquid volume and cell numbers of a full milliliter (cm^3) of the sampled material.



NEUBAUER
HEMACYTOMETER
GRID OF 400 SQUARES

If the full 400-squares of each grid are counted, this represents the number of sperm in 0.1 mm^3. Multiplying this value times 10 yields the sperm per mm^3 (and is the source of the hemacytometer factor of 4,000 squares/mm^3). If this product is multiplied by 1,000 mm^3/cm^3, the answer is the number of sperm in one milliliter of the sample. If the counted sample represents a dilution of a more concentrated original sample, the above answer is multiplied by the dilution factor to yield the cell density in the original sample. If the cells are sufficiently dense, it is not necessary to count the entire 400-square field, and the final calculation takes into account the number of squares actually counted:

$$\text{cells/mL} = \frac{\text{(dilution) (4,000 squares/mm}^3\text{) (1,000 mm}^3\text{/cm}^3\text{) (cell count)}}{\text{(number of squares counted)}}$$

Thus, with a dilution of 4000 (0.025 mL of semen in 100 mL of dilution water), 80 squares counted, and a count of 100, the calculation becomes:

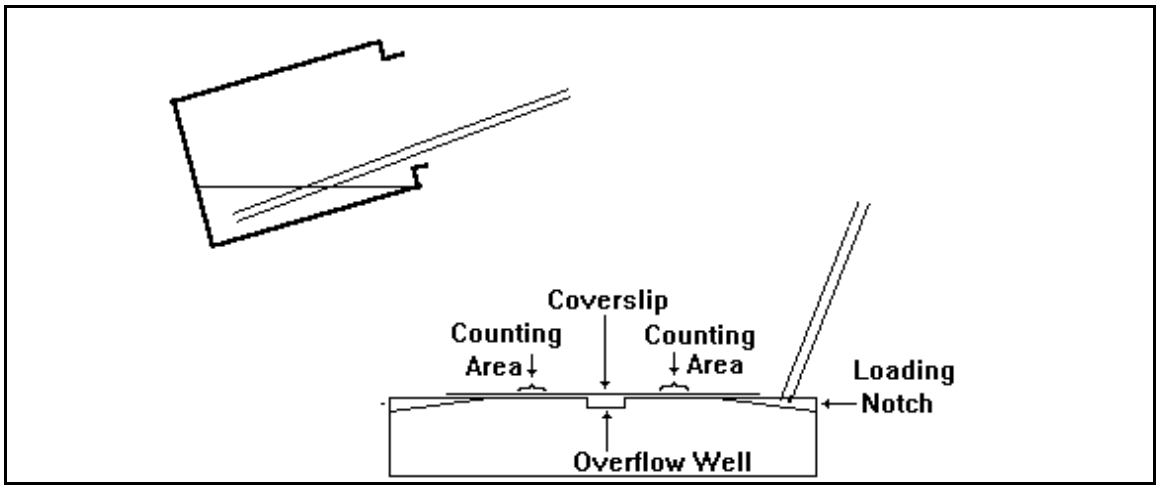
$$\text{cells/mL} = \frac{(4{,}000)\ (4{,}000)\ (1{,}000)\ (100)}{80}$$

$$= 20{,}000{,}000{,}000 \text{ cells/mL}$$

There are several procedures that are necessary for counts to be consistent within and between laboratories. These include mixing the sample, loading and emptying the hematocrit tube, cleaning the hemacytometer and cover slip, and actual counting procedures.

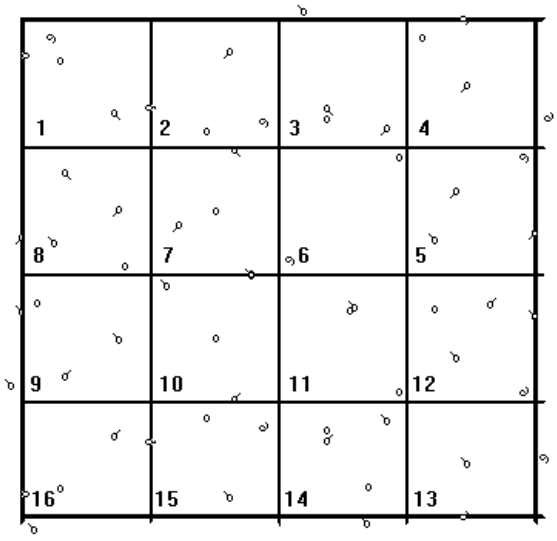
Obviously, if the sample is not homogeneous, subsamples can vary in sperm density. A few extra seconds in mixing can save a lot of wasted minutes in subsequent counting procedures. A full hematocrit tube empties more easily than one with just a little liquid, so withdraw a full sample. This can be expedited by tipping the sample vial.

Because the sperm are killed prior to sampling, they will slowly settle. For this reason, the sample in the hematocrit tube should be loaded onto the hemacytometer as rapidly as possible. Two replicate samples are withdrawn in fresh hematocrit tubes and loaded onto opposite sides of a hemacytometer.

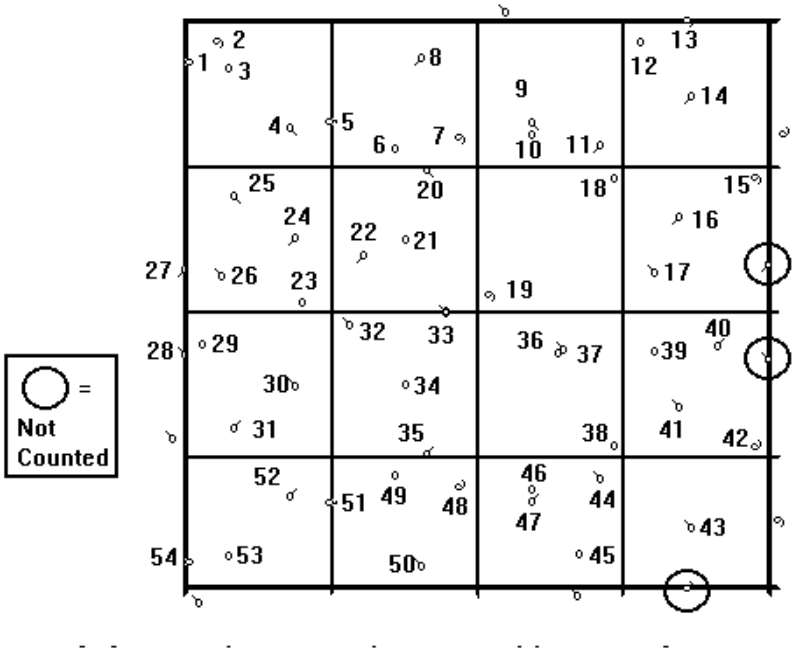


The loaded hemacytometer is left for 15 minutes to allow the sperm to settle onto the counting field. If the coverslip is moved after the samples are loaded, the hemacytometer should be rinsed and refilled with fresh sample. After 15 minutes, the hemacytometer is placed under a microscope and the counting grid located at 100x. Once the grid is properly positioned, the microscope is adjusted to 200x or 400x, and one of the corner squares is positioned for counting (any one of the four corners is appropriate). For consistency, use the same procedure each time (Many prefer to start in the upper left corner of the optical field, and this procedure will be used in the examples given below). Examine the first large square in the selected corner. If no sperm are visible, or if the sperm are so dense or clumped to preclude accurate counting, count a sample with a more appropriate dilution.

In making counts of sperm, it is necessary to adopt a consistent method of scanning the smaller squares and counting sperm that fall upon the lines separating the squares. Count the sperm in the small squares by beginning in the upper left hand corner (square 1) and proceding right to square 4, down to square 5, left to square 8, etc. until all 16 squares are counted.



Because sperm that appear <u>on</u> lines might be counted as being in either square, it is important to avoid double counting or non-counting. For this reason a convention is decided upon and used consistently: paraphrasing the instructions received with one (Hausser Scientific) counting chamber "to avoid counting (sperm) twice, the best practice is to count all touching the top and left, and none touching the lower and right, boundary lines." Whatever convention is chosen, it must be adhered to. The example below shows a sperm count based upon a selected convention of counting sperm that fall on the upper and left lines, but not on the lower or right lines:



In the above illustration, sperm falling on the lower and right lines are not counted. The count begins at the upper left as illustrated in the preceding figure. A typical count sequence is demonstrated by the numbers next to each sperm illustrated. Sperm identified as numbers 1, 5, 13, 20, 27, 28, 33, 51 and 54 touch lines and are counted as being in the square below them or to their right. The circled sperm are not counted as being in this field of 16 small squares (but they would be included in any counts of adjacent squares in which they would be on upper or left hand lines).

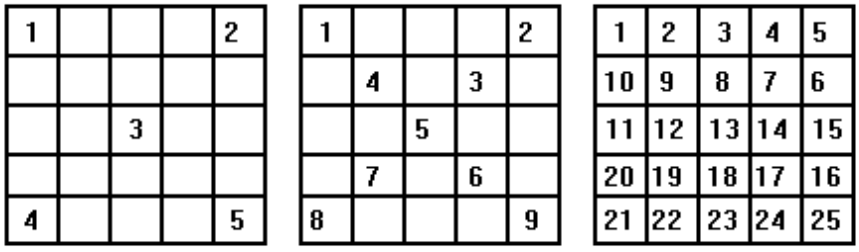
Once these counting conventions have been selected, it is advisable to follow another strict protocol outlining the number and sequence of large squares to be counted. Because the sperm may not be randomly distributed across the counting grid, it is recommended to count an array of squares covering the entire grid. The following procedure is recommended:

Count the number of sperm in the first large square.

1. If the number is less than 10, count all 25 squares using the same scanning pattern outlined above (left to right through squares 1 to 5, down to square 6, left through square 10, down to 11, etc.). See pattern no. 3.

2. If the number is between 10 and 19, count 9 large squares using pattern no. 2.

3. If the number is 20 or greater, count 5 large squares using pattern no. 1.



Pattern no. 1 Pattern no. 2 Pattern no. 3

The final consideration in achieving good replicate counts is keeping the hemacytometers and coverslips clean. They should be rinsed in distilled water soon after use. The coverslips should be stored in a good biocleanser such as hemasol. For an hour or so prior to use, the hemacytometer slides should also be soaked in the solution. Both slides and coverslips should then be rinsed off with reagent water, blotted dry with a lint-free tissue, and wiped with lens paper.

SECTION 16

PURPLE URCHIN, *Strongylocentrotus purpuratus* AND SAND DOLLAR, *Dendraster excentricus* FERTILIZATION TEST METHOD

Adapted from a method developed by
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SECTION 16

SEA URCHIN, *Strongylocentrotus purpuratus* AND SAND DOLLAR, *Dendraster excentricus* FERTILIZATION TEST

16.1 SCOPE AND APPLICATION

16.1.1 This method estimates the chronic toxicity of effluents and receiving waters to the gametes of sea urchins, (*Strongylocentrotus purpuratus*), or sand dollars (*Dendraster excentricus*) during a static non-renewal 20 minute sperm exposure and a subsequent 20 minute exposure period following the addition of eggs for measuring the fertilizing capacity of the sperm. The effects include the synergistic, antagonistic, and additive effects of all chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms.

16.1.2 The purpose of the test is to determine the concentrations of a test substance that reduce egg fertilization by exposed sperm relative to that attained by sperm in control solutions. Concentrations of materials adversely affecting egg fertilization under the conditions of this test are usually acutely and chronically toxic to one or more of several common marine test species and, by extension, are presumably acutely and chronically toxic to other of the many untested marine species.

16.1.3 Detection limits of the toxicity of an effluent or chemical substance are organism dependent.

16.1.4 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling and because the test chambers are not sealed, highly volatile and highly degradable toxicants in the source may not be detected in the test.

16.1.5 This test is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s),

consisting of one or more receiving water concentrations and a control.

16.1.6 This method should be restricted to use by, or under the supervision of, professionals experienced in aquatic toxicity testing. Specific experience with any toxicity test is usually needed before acceptable results become routine.

16.2 **SUMMARY OF METHOD**

16.2.1 The method provides the step-by-step instructions for exposing sperm suspensions (appropriate sperm density may first be determined in a trial fertilization test) to effluents or receiving waters for 20 minutes. Eggs are then added to the sperm suspensions and, twenty minutes after the eggs are added, the test is terminated by the addition of a preservative. The percent fertilization is determined by microscopic examination of 100 eggs in an aliquot of eggs from each treatment. The test endpoint is normal egg fertilization.

16.3 **INTERFERENCES**

16.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities, Equipment, and Supplies).

16.3.2 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling and Sample Handling, and Sample Preparation for Toxicity Tests).

16.4 **SAFETY**

16.4.1 See Section 3, Health and Safety

16.5 **APPARATUS AND EQUIPMENT**

16.5.1 Tanks, trays, or aquaria -- for holding and acclimating adult sea urchins and sand dollars, e.g., standard salt water aquarium or Instant Ocean Aquarium (capable of maintaining seawater at 10-20EC), with appropriate filtration and aeration system.

16.5.2 Air pump, air lines, and air stones -- for aerating water containing broodstock or for supplying air to test solutions with low dissolved oxygen.

16.5.3 Constant temperature chambers or water baths -- for maintaining test solution temperature and keeping dilution water supply, gametes, and embryo stock suspensions at test temperature (12EC) prior to the test. (Incubators are usually unsatisfactory because test tubes must be removed for addition of sperm and eggs and the small test volumes can rapidly change temperature at normal room temperatures.)

16.5.4 Water purification system -- Millipore Super-Q, Deionized water (DI) or equivalent.

16.5.5 Refractometer -- for determining salinity.

16.5.6 Hydrometer(s) -- for calibrating refractometer.

16.5.7 Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

16.5.8 Thermometer, National Bureau of Standards Certified (see USEPA METHOD l70.l, USEPA, l979) -- to calibrate laboratory thermometers.

16.5.9 pH and DO meters -- for routine physical and chemical measurements.

16.5.10 Standard or micro-Winkler apparatus -- for determining DO (optional) and calibrating the DO meter.

16.5.11 Winkler bottles -- for dissolved oxygen determinations.

16.5.12 Balance -- Analytical, capable of accurately weighing to 0.0001 g.

16.5.13 Fume hood -- to protect the analyst from effluent or formaldehyde fumes.

16.5.14 Glass stirring rods -- for mixing test solutions.

16.5.15 Graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 50-1000 mL for making test solutions. (Note: not to be used interchangeably for gametes or embryos and test solutions).

16.5.16 Volumetric flasks -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making test solutions.

16.5.17 Pipets, automatic -- adjustable, to cover a range of delivery volumes from 0.010 to 1.000 mL.

16.5.18 Pipet bulbs and fillers -- PROPIPET® or equivalent.

16.5.19 Wash bottles -- for reagent water, for topping off graduated cylinders, for rinsing small glassware and instrument electrodes and probes.

16.5.20 Wash bottles -- for dilution water.

16.5.21 20-liter cubitainers or polycarbonate water cooler jugs -- for making hypersaline brine.

16.5.22 Cubitainers, beakers, or similar chambers of non-toxic composition for holding, mixing, and dispensing dilution water and other general non-effluent, non-toxicant contact uses. These should be clearly labeled and not used for other purposes. Strong solutions of NaOH and formaldehyde should not be held for several month periods in Cubitainers: interaction or leaching into solutions of 0.1 N or 1 N NaOH used for pH adjustment of dilution water has caused poor egg fertilization; formaldehyde similarly stored has induced abberant partial membrane elevation in eggs.

16.5.23 Beakers, 5-10 mL borosilicate glass -- for collecting sperm from sand dollars.

16.5.24 Beakers, 100 mL borosilicate glass -- for spawning; to support sea urchins and to collect sea urchin and sand dollar eggs.

16.5.25 Beakers, 1,000 mL borosilicate glass -- for rinsing and settling sea urchin eggs.

16.5.26 Vortex mixer -- to mix sea urchin semen in tubes prior to sampling.

16.5.27 Compound microscope -- for examining gametes, counting sperm cells (200-400x) and eggs (100x), and examining fertilized eggs. Dissecting scopes are sometimes used to count eggs at a lower magnification.

16.5.28 Counter, two unit, 0-999 -- for recording sperm and egg counts.

16.5.29 Sedgwick-Rafter counting chamber -- for counting egg stock and examining eggs for fertilization at the end of the test.

16.5.30 Hemacytometers, Neubauer -- for counting sperm.

16.5.31 Siphon hose (3 mm i.d.) -- for removing wash water from settled eggs.

16.5.32 Centrifuge tubes, test tubes, or vials -- for holding semen.

16.5.33 Perforated plunger -- for maintaining homogeneous distribution of eggs during sampling and distribution to test tubes.

16.5.34 60 µm NITEX® filter -- for filtering receiving water.

16.6 **REAGENTS AND SUPPLIES**

16.6.1 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests).

16.6.2 Data sheets (one set per test) -- for data recording (see Figures 1 and 2).

16.6.3 Tape, colored -- for labelling test chambers and containers.

16.6.4 Markers, water-proof -- for marking containers, etc.

16.6.5 Parafilm -- to cover graduated cylinders and vessels containing gametes.

16.6.6 Gloves, disposable -- for personal protection from contamination.

16.6.7 Pipets, serological -- 1-10 mL, graduated.

16.6.8 Pipet tips -- for automatic pipets. Note: pipet tips for handling semen should be cut off to produce an opening about 1 mm in diameter; pipet tips for handling eggs should be cut off to produce an opening about 2 mm in diameter. This is necessary to provide smooth flow of the viscous semen, accurate sampling of eggs, and to prevent injury to eggs passing through a restricted opening. A clean razor blade can be used to trim pipet tips.

16.6.9 Coverslips -- for microscope slides.

16.6.10 Lens paper -- for cleaning microscope optics.

16.6.11 Laboratory tissue wipes -- for cleaning and drying electrodes, microscope slides, etc.

16.6.12 Disposable countertop covering -- for protection of work surfaces and minimizing spills and contamination.

16.6.13 pH buffers 4, 7, and 10 (or as per instructions of instrument manufacturer) -- for standards and calibration check (see USEPA Method 150.1, USEPA, 1979).

16.6.14 Membranes and filling solutions -- for dissolved oxygen probe (see USEPA Method 360.1, USEPA, 1979), or reagents for modified Winkler analysis.

16.6.15 Laboratory quality assurance samples and standards -- for the above methods.

16.6.16 Test chambers -- test tubes, borosilicate glass, 16 x 100 mm or 16 x 125 mm, with caps for conducting the test, four chambers per concentration.

Figure 1. Sample data sheet for spawning record.

Animal No.	Sex	Time Injected	Time Spawn	Comments
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				

Pooled eggs from female nos. _____.

Pooled (_____ mL) of sperm each from male nos. _____.

Figure 2. Sample data sheet for egg and sperm counts.

EGG COUNTS -

Sample	Dilution	Count	Eggs/mL

For 100 mL egg suspension at 2,240 eggs/mL use:

100 mL x 2,240 eggs/mL / (counted eggs/mL) = mL of egg stock

224,000 eggs / _____ eggs/mL = _____ mL

If required stock >100 mL, concentrate egg stock by settling the eggs and decanting off sufficient overlying water to retain:

(_____ eggs/mL / 2,240 eggs/mL) x 100 = _____ % volume

SPERM COUNTS -

Sample	Dilution	Count	Squares	Sperm/mL

$$\text{SPERM/mL} = \frac{(\text{DIL.FACT.})\ (\text{COUNT})\ (4000)\ (1000)}{(\text{NO. SQUARES COUNTED})}$$

16.6.17 Formaldehyde, 10%, in seawater -- for preserving eggs. Note: formaldehyde has been identified as a carcinogen and is irritating to skin and mucous membranes. It should not be used at a concentration higher than necessary to achieve morphological preservation of larvae for counting and only under conditions of maximal ventilation and minimal opportunity for volatilization into room air.

16.6.18 Glutaraldehyde, 1% in seawater -- for preserving eggs.

16.6.13 pH buffers 4, 7, and l0 (or as per instructions of
16.6.19 Acetic acid, 10%, reagent grade, in filtered (10F) seawater -- for preparing killed sperm dilutions for sperm counts.

16.6.20 Haemo-Sol or equivalent cleaner -- for cleaning hemacytometer and cover slips.

16.6.21 0.5 M KCl solution -- for inducing spawning.

16.6.22 Syringe, disposable, 3 or 5 mL -- for injecting KCl into sea urchins and sand dollars to induce spawning.

16.6.23 Needles, 25 gauge -- for injecting KCl.

16.6.24 Pasteur pipets and bulbs -- for sampling eggs from spawning beakers.

16.6.25 Hematocrit capillary tubes -- for sampling sperm for examination and for loading hemacytometers.

16.6.26 Microscope well-slides -- for pre-test assessment of sperm activity and egg condition.

16.6.27 Reference toxicant solutions (see 16.10.2.4 and Section 4, Quality Assurance).

16.6.28 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms (see Section 5, Facilities, Equipment, and Supplies and Section 7, Dilution Water).

16.6.29 Effluent and receiving water -- see Section 8, Effluent and Surface Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests.

16.6.30 Dilution water and hypersaline brine -- see Section 7, Dilution Water and Section 16.6.24, Hypersaline Brines. The dilution water should be uncontaminated 1-µm-filtered natural seawater. Hypersaline brine should be prepared from dilution water.

16.6.31 HYPERSALINE BRINES

16.6.31.1 Most industrial and sewage treatment effluents entering marine and estuarine systems have little measurable salinity. Exposure of larvae to these effluents will usually require increasing the salinity of the test solutions. It is important to maintain an essentially constant salinity across all treatments. In some applications it may be desirable to match the test salinity with that of the receiving water (See Section 7.1). Two salt sources are available to adjust salinities -- artificial sea salts and hypersaline brine (HSB) derived from natural seawater. Use of artificial sea salts is necessary only when high effluent concentrations preclude salinity adjustment by HSB alone.

16.6.31.2 Hypersaline brine (HSB) can be made by concentrating natural seawater by freezing or evaporation. HSB should be made from high quality, filtered seawater, and can be added to the effluent or to reagent water to increase salinity. HSB has several desirable characteristics for use in effluent toxicity testing. Brine derived from natural seawater contains the necessary trace metals, biogenic colloids, and some of the microbial components necessary for adequate growth, survival, and/or reproduction of marine and estuarine organisms, and it can be stored for prolonged periods without any apparent degradation. However, even if the maximum salinity HSB (100‰) is used as a diluent, the maximum concentration of effluent (0‰) that can be tested is 66% effluent at 34‰ salinity (see Table 1).

16.6.31.3 High quality (and preferably high salinity) seawater should be filtered to at least 10 µm before placing into the freezer or the brine generator. Water should be collected on an incoming tide to minimize the possibility of contamination.

16.6.31.4 Freeze Preparation of Brine

16.6.31.4.1 A convenient container for making HSB by freezing is one that has a bottom drain. One liter of brine can be made from four liters of seawater. Brine may be collected by partially freezing seawater at -10 to -20EC until the remaining liquid has reached the target salinity. Freeze for approximately six hours, then separate the ice (composed mainly of fresh water) from the remaining liquid (which has now become hypersaline).

16.6.31.4.2 It is preferable to monitor the water until the target salinity is achieved rather than allowing total freezing followed by partial thawing. Brine salinity should never exceed 100‰. It is advisable not to exceed about 70‰ brine salinity unless it is necessary to test effluent concentrations greater than 50%.

16.6.31.4.3 After the required salinity is attained, the HSB should be filtered through a 1 µm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4EC (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

16.6.31.5 Heat Preparation of Brine

16.6.31.5.1 The ideal container for making brine using heat-assisted evaporation of natural seawater is one that (1) has a high surface to volume ratio, (2) is made of a non-corrosive material, and (3) is easily cleaned (fiberglass containers are ideal). Special care should be used to prevent any toxic materials from coming in contact with the seawater being used to generate the brine. If a heater is immersed directly into the seawater, ensure that the heater materials do not corrode or leach any substances that would contaminate the brine. One successful method is to use a thermostatically controlled heat exchanger made from fiberglass. If aeration is applied, use only oil-free air compressors to prevent contamination.

16.6.31.5.2 Before adding seawater to the brine generator, thoroughly clean the generator, aeration supply tube, heater, and any other materials that will be in direct contact with the

brine. A good quality biodegradable detergent should be used, followed by several (at least three) thorough reagent water rinses.

16.6.31.5.3 Seawater should be filtered to at least 10 µm before being put into the brine generator. The temperature of the seawater is increased slowly to 40EC. The water should be aerated to prevent temperature stratification and to increase water evaporation. The brine should be checked daily (depending on the volume being generated) to ensure that the salinity does not exceed 100% and that the temperature does not exceed 40EC. Additional seawater may be added to the brine to obtain the volume of brine required.

16.6.31.5.4 After the required salinity is attained, the HSB should be filtered through a 1 µm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4EC (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

16.6.31.6 Artificial Sea Salts

16.6.31.6.1 No data from sea urchin or sand dollar fertilization tests using sea salts are available for evaluation at this time, and their use should be considered provisional. The use of GP2 artificial seawater (Table 2) has been found to provide control fertilization equal to that of natural seawater.

16.6.31.6.2 The GP2 reagent grade chemicals (Table 2) should be mixed with deionized (DI) water or its equivalent in a single batch, never by test concentration or replicate. The reagent water used for hydration should be between 21-26EC. The artificial seawater must be conditioned (aerated) for 24 h before use as the testing medium. If the solution is to be autoclaved, sodium bicarbonate is added after the solution has cooled. A stock solution of sodium bicarbonate is made up by dissolving 33.6 g $NaHCO_3$ in 500 mL of reagent water. Add 2.5 mL of this stock solution for each liter of the GP2 artificial seawater.

16.6.31.7 Dilution Water Preparation from Brine

16.6.31.7.1 Although salinity adjustment with brine is the preferred method, the use of high salinity brines and/or reagent water has sometimes been associated with discernible adverse effects on test organisms. For this reason, it is recommended that only the minimum necessary volume of brine and reagent water be used to offset the low salinity of the effluent, and that brine controls be included in the test. The remaining dilution water should be natural seawater. Salinity may be adjusted in one of two ways. First, the salinity of the highest effluent

test concentration may be adjusted to an acceptable salinity, and then serially diluted. Alternatively, each effluent concentration can be prepared individually with appropriate volumes of effluent and brine.

16.6.31.7.2 When HSB and reagent water are used, thoroughly mix together the reagent water and HSB before mixing in the effluent. Divide the salinity of the HSB by the expected test salinity to determine the proportion of reagent water to brine. For example, if the salinity of the brine is 100‰ and the test is to be conducted at 34‰, 100‰ divided by 34‰ = 2.94. Thus, the proportion is one part brine plus 1.94 parts reagent water). To make 1 L of dilution water at 34‰ salinity from a HSB of 100‰, 340 mL of brine and 660 mL of reagent water are required. Verify the salinity of the resulting mixture using a refractometer.

16.6.31.8 Test Solution Salinity Adjustment

16.6.31.8.1 Table 3 illustrates the preparation of test solutions (up to 50% effluent) at 34‰ by combining effluent, HSB, and dilution water. Note: if the highest effluent concentration does not exceed 50% effluent, it is convenient to prepare brine so that the sum of the effluent salinity and brine salinity equals 68‰; the required brine volume is then always equal to the effluent volume needed for each effluent concentration as in the example in Table 3.

16.6.31.8.2 Check the pH of all brine mixtures and adjust to within 0.2 units of dilution water pH by adding, dropwise, dilute hydrochloric acid or sodium hydroxide.

TABLE 1. MAXIMUM EFFLUENT CONCENTRATION (%) THAT CAN BE TESTED AT 34‰ WITHOUT THE ADDITION OF DRY SALTS GIVEN THE INDICATED EFFLUENT AND BRINE SALINITIES.

Effluent Salinity ‰	Brine 60 ‰	Brine 70 ‰	Brine 80 ‰	Brine 90 ‰	Brine 100 ‰
0	43.33	51.43	57.50	62.22	66.00
1	44.07	52.17	58.23	62.92	66.67
2	44.83	52.94	58.97	63.64	67.35
3	45.61	53.73	59.74	64.37	68.04
4	46.43	54.55	60.53	65.12	68.75
5	47.27	55.38	61.33	65.88	69.47
10	52.00	60.00	65.71	70.00	73.33
15	57.78	65.45	70.77	74.67	77.65
20	65.00	72.00	76.67	80.00	82.50
25	74.29	80.00	83.64	86.15	88.00

16.6.31.8.3 To calculate the amount of brine to add to each effluent dilution, determine the following quantities: salinity of the brine (SB, in ‰), the salinity of the effluent (SE, in ‰), and volume of the effluent to be added (VE, in mL). Then use the following formula to calculate the volume of brine (VB, in mL) to be added:

$$VB = VE \times (34 - SE)/(SB - 34)$$

16.6.31.8.4 This calculation assumes that dilution water salinity is 34 ± 2‰.

16.6.31.9 Preparing Test Solutions

TABLE 2. REAGENT GRADE CHEMICALS USED IN THE PREPARATION OF GP2 ARTIFICIAL SEAWATER FOR THE PURPLE URCHIN ***STRONGYLOCENTROTUS PURPURATUS***, AND SAND DOLLAR ***DENDRASTER EXCENTRICUS*** TOXICITY TEST[1,2]

Compound	Concentration (g/L)	Amount (g) Required for 20 L
NaCl	23.90	478.0
Na_2SO_4	4.00	80.0
KCl	0.698	13.96
KBr	0.100	2.00
$Na_2B_4O_7 \bullet 10\ H_2O$	0.039	0.78
$MgCl_2 \bullet 6\ H_2O$	10.80	216.0
$CaCl_2 \bullet 2\ H_2O$	1.50	30.0
$SrCl_2 \bullet 6\ H_2O$	0.025	0.490
$NaHCO_3$	0.193	3.86

[1]Modified GP2 from Spotte et al. (1984)
[2]The constituent salts and concentrations were taken from USEPA (1990b). The salinity is 34.0 g/L.

16.6.31.9.1 Five mL of test solution are needed for each test chamber. To prepare test solutions at low effluent concentrations (<6%), effluents may be added directly to dilution water. For example, to prepare 1% effluent, add 1.0 mL of effluent to a 100-mL volumetric flask using a volumetric pipet or calibrated automatic pipet. Fill the volumetric flask to the 100-mL mark with dilution water, stopper it, and shake to mix. Pour into a (150-250 mL) beaker and stir. Distribute equal volumes into the replicate test chambers. The remaining test solution can be used for chemistry.

16.6.31.9.2 To prepare a test solution at higher effluent concentrations, hypersaline brine must usually be used. For

example, to prepare 40% effluent, add 400 mL of effluent to a 1-liter volumetric flask. Then, assuming an effluent salinity of 2‰ and a brine salinity of 66‰, add 400 mL of brine (see equation above and Table 3) and top off the flask with dilution water. Stopper the flask and shake well. Pour into a (100-250 mL) beaker and stir. Distribute equal volumes into the replicate test chambers. The remaining test solution can be used for chemistry.

16.6.31.10 Brine Controls

16.6.31.10.1 Use brine controls in all tests where brine is used. Brine controls contain the same volume of brine as does the highest effluent concentration using brine, plus the volume of reagent water needed to reproduce the hyposalinity of the effluent in the highest concentration, plus dilution water. Calculate the amount of reagent water to add to brine controls by rearranging the above equation, (See, 16.6.33.8.3) setting SE = 0, and solving for VE.

$$VE = VB \times (SB - 34)/(34 - SE)$$

If effluent salinity is essentially 0‰, the reagent water volume needed in the brine control will equal the effluent volume at the highest test concentration. However, as effluent salinity and effluent concentration increase, less reagent water volume is needed.

16.6.32 TEST ORGANISMS, PURPLE URCHINS

16.6.32.1 Sea Urchins, ***Strongylocentrotus purpuratus*** (approximately 6 of each sex per test).

16.6.32.2 Adult sea urchins (***Strongylocentrotus purpuratus***) can be obtained from commercial suppliers or collected from uncontaminated intertidal or subtidal areas. State collection permits are usually required for collection of sea urchins and collection is prohibited or restricted in some areas. The animals are best transported "dry," surrounded either by moist seaweed or paper towels dampened with seawater. Animals should be kept at approximately their collection or culture temperature to prevent thermal shock which can prematurely induce spawning.

TABLE 3. EXAMPLES OF EFFLUENT DILUTION SHOWING VOLUMES OF EFFLUENT (AT X‰), BRINE, AND DILUTION WATER NEEDED FOR ONE LITER OF EACH TEST SOLUTION.

FIRST STEP: Combine brine with reagent water or natural seawater to achieve a brine of 68-x‰ and, unless natural seawater is used for dilution water, also a brine-based dilution water of 34‰.

SERIAL DILUTION:

Step 1. Prepare the highest effluent concentration to be tested by adding equal volumes of effluent and brine to the appropriate volume of dilution water. An example using 40% is shown.

Effluent Conc. (%)	Effluent x‰	Brine (68-x)‰	Dilution Water* 34‰
40	800 mL	800 mL	400 mL

Step 2. Use either serially prepared dilutions of the highest test concentration or individual dilutions of 100% effluent.

Effluent Conc. (%)	Effluent Source	Dilution Water* (34‰)
20	1000 mL of 40%	1000 mL
10	1000 mL of 20%	1000 mL
5	1000 mL of 10%	1000 mL
2.5	1000 mL of 5%	1000 mL
Control	none	1000 mL

INDIVIDUAL PREPARATION:

Effluent Conc. (%)	Effluent x‰	Brine (68-x)‰	Dilution Water* 34‰
40	400 mL	400 mL	200 mL
20	200 mL	200 mL	600 mL
10	100 mL	100 mL	800 mL
5	50 mL	50 mL	900 mL
2.5	25 mL	25 mL	950 mL
Control	none	none	1000 mL

*May be natural seawater or brine-reagent water equivalent.

16.6.32.3 The adult sea urchins are maintained in glass aquaria or fiberglass tanks. The tanks are supplied continuously (approximately 5 L/min) with filtered natural seawater, or salt water prepared from commericial sea salts is recirculated. The animals are checked daily and any obviously unhealthy animals are discarded.

16.6.32.4 Although ambient temperature seawater is usually acceptable, maintaining sea urchins in spawning condition usually requires holding at a relatively constant temperature. The culture unit should be capable of maintaining a constant temperature between 10 and 14EC with a water temperature control device.

16.6.32.5 Food for sea urchins -- kelp, recommended, but not necessarily limited to, ***Laminaria sp.***, ***Hedophyllum sp.***, ***Nereocystis sp.***, ***Macrocystis sp.***, ***Egregia sp.***, ***Alaria sp.*** or romaine lettuce. The kelp should be gathered from known uncontaminated zones or obtained from commerical supply houses whose kelp comes from known uncontaminated areas, or romaine lettuce. Fresh food is introduced into the tanks at least several times a week. Sun dried (12-24 hours) or oven dried (60EC overnight) kelp, stores well at room temperature or frozen, rehydrates well and is adequate to maintain sea urchins for long periods. Decaying food and fecal pellets are removed as necessary to prevent fouling.

16.6.32.6 Natural seawater (>30‰) is used to maintain the adult animals and ($32‰) as a control water in the tests.

16.6.32.7 Adult male and female (if sexes known) animals used in field studies are transported in separate or partitioned insulated boxes or coolers packed with wet kelp or paper toweling. Upon arrival at the field site, aquaria (or a single partitioned aquarium) are filled with control water, loosely covered with a styrofoam sheet and allowed to equilibrate to the holding temperature before animals are added. Healthy animals will attach to the kelp or aquarium within hours.

16.6.32.8 To successfully maintain about 25 adult animals for seven days at a field site, 40-L glass aquaria using aerated, recirculating, clean saline water (32‰) and a gravel bed filtration system, are housed within a water bath, such as an

INSTANT OCEAN® Aquarium. The sexes should be held separately if possible.

16.6.33 TEST ORGANISMS, SAND DOLLARS

16.6.33.1 Sand Dollars, ***Dendraster excentricus***, (approximately 6 of each sex per test).

16.6.33.2 Adult sand dollars (***Dendraster excentricus***) can be obtained from commercial suppliers or collected from subtidal zones (most areas) or from intertidal zones of some sheltered waters (e.g., Puget Sound). State collection permits may be required for collection of sand dollars and collection prohibited or restricted in some areas. The animals are best transported "dry," surrounded either by moist seaweed or paper towels dampened with seawater. Animals should be kept at approximately their collection or culture temperature to prevent thermal shock which can prematurely induce spawning.

16.6.33.3 The adult sand dollars are maintained in glass aquaria or fiberglass tanks. The tanks are supplied continuously (approximately 5 L/min) with filtered natural seawater, or saltwater prepared from commercial sea salts is recirculated. The animals are checked daily and any obviously unhealthy animals are discarded. For longer periods than a few days, several centimeters or more of a sand substrate may be desirable.

16.6.33.4 Although ambient temperature seawater is usually acceptable, maintaining sand dollars in spawning condition usually requires holding at a relatively constant temperature. The culture unit should be capable of maintaining a constant temperature between 8 and 12EC with a water temperature control device.

16.6.33.5 Sand dollars will feed on suspended or benthic materials such as phytoplankton, benthic diatoms, etc. No reports of laboratory populations being maintained in spawning condition over several years are known. It is probably most convenient to obtain sand dollars, use them, and then discard them after they cease to produce good quality gametes.

16.6.33.6 Natural seawater (>30‰) is used to maintain the adult animals and ($32‰) as a control water in the tests.

16.6.33.7 Adult male and female (if sexes known) animals used in field studies are transported in separate or partitioned insulated boxes or coolers packed with wet kelp or paper toweling. Upon arrival at the field site, trays or aquaria (or a single partitioned aquarium) are filled with control water, loosely covered with a styrofoam sheet and allowed to equilibrate to the holding temperature before animals are added.

16.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION AND STORAGE

16.7.1 Section 8, Effluent and Receiving Water Sampling and Sample Handling, and Sampling Preparation for Toxicity Tests.

16.8 CALIBRATION AND STANDARDIZATION

16.8.1 See Section 4, Quality Assurance.

16.9 QUALITY CONTROL

16.9.1 See Section 4, Quality Assurance.

16.10 TEST PROCEDURES

16.10.1 TEST DESIGN

16.10.1.1 The test consists of at least four effluent concentrations plus a dilution water control. Tests that use brine to adjust salinity must also contain four replicates of a brine control. In addition, four extra controls are prepared for egg controls.

16.10.1.2 Effluent concentrations are expressed as percent effluent.

16.10.2 TEST SOLUTIONS

16.10.2.1 Receiving waters

16.10.2.1.1 The sampling point is determined by the objectives of the test. At estuarine and marine sites, samples are usually collected at mid-depth. Receiving water toxicity is determined with samples used directly as collected or with samples passed

through a 60 µm NITEX® filter and compared without dilution, against a control. Using four replicate chambers per test, each containing 5 mL, and 400 mL for chemical analysis, would require approximately 420 mL or more of sample per test.

16.10.2.2 Effluents

16.10.2.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of at least 0.5 is commonly used. A dilution factor of 0.5 provides hypothesis test discrimination of ± 100%, and testing of a 16 fold range of concentrations. Hypothesis test discrimination shows little improvement as dilution factors are increased beyond 0.5 and declines rapidly if smaller dilution factors are used. **USEPA recommends that one of the five effluent treatments must be a concentration of effluent mixed with dilution water which corresponds to the permittee's instream waste concentration (IWC).** At least two of the effluent treatments must be of lesser effluent concentration than the IWC, with one being at least one-half the concentration of the IWC. If 100‰ HSB is used as a diluent, the maximum concentration of effluent that can be tested will be 66% at 34‰ salinity.

16.10.2.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12% and 1.56%).

16.10.2.2.3 The volume in each test chamber is 5 mL.

16.10.2.2.4 Effluent dilutions should be prepared for all replicates in each treatment in one container to minimize variability among the replicates. Dispense into the appropriate effluent test chambers.

16.10.2.3 Dilution Water

16.10.2.3.1 Dilution water should be uncontaminated 1-µm-filtered natural seawater, or hypersaline brine prepared from uncontaminated natural seawater plus reagent water; or sea salts (see Section 7, Dilution Water). Natural seawater may be uncontaminated receiving water. This water is used in all dilution steps and as the control water.

16.10.2.4 Reference Toxicant Test

16.10.2.4.1 Reference toxicant tests should be conducted as described in Quality Assurance (see Section 4.7).

16.10.2.4.2 The preferred reference toxicant for sea uchins and sand dollar is copper chloride ($CuCl_2$ºH_2O). Reference toxicant tests provide an indication of the sensitivity of the test organisms and the suitability of the testing laboratory (see Section 4 Quality Assurance). Another toxicant may be specified by the appropriate regulatory agency. Prepare a copper reference toxicant stock solution (2,000 mg/L) by adding 5.366 g of copper chloride ($CuCl_2$º2H_2O) to 1 liter of reagent water. For each reference toxicant test prepare a copper sub-stock of 3 mg/L by diluting 1.5 mL of stock to one liter with reagent water. Alternatively, certified standard solutions can be ordered from commercial companies.

16.10.2.4.3 Prepare a control (0 Fg/L) plus four replicates each of at least five consecutive copper reference toxicant solutions (e.g., from the series 3.0, 4.4, 6.5, 9.5, 13.9, 20.4, and 30.0 Fg/L, by adding 0.10, 0.15, 0.22, 0.32, 0.46, 0.68, and 1.00 mL of sub-stock solution, respectively, to 100-L volumetric flasks and filling to 100-mL with dilution water). Start with control solutions and progress to the highest concentration to minimize contamination.

16.10.2.4.4 If the effluent and reference toxicant tests are to be run concurrently, then the tests must use embryos from the same spawn. The tests must be handled in the same way and test solutions delivered to the test chambers at the same time. Reference toxicant tests must be conducted at 34 ± 2‰.

16.10.3 COLLECTION OF GAMETES FOR THE TEST

16.10.3.1 Spawning Induction

16.10.3.1.1 Pour seawater into 100 mL beakers and place in 12EC bath or room. Allow to come to temperature. Select a sufficient number of sea urchins or sand dollars (based upon recent or past spawning success) so that three of each sex are likely to provide gametes of acceptable quantity and quality for the test. During optimal spawning periods this may only require six animals, three

of each sex, when the sexes are known from prior spawning. During other periods, especially if the sex is not known, many more animals may be required.

16.10.3.1.2 Care should be exercised when removing sea urchins from holding tanks so that damage to tube-feet is minimized. Following removal, sea urchins should be placed into a container lined with seawater-moistened paper towels to prevent reattachment.

16.10.3.1.3 Place each sand dollar, oral side up, on a 100 mL beaker filled with 12EC seawater or each sea urchin onto a clean tray covered with several layers of seawater moistened paper towels.

16.10.3.1.4 Handle sexes separately once known; this minimizes the chance of accidental egg fertilization. Throughout the test process, it is best if a different worker, different pipets, etc. are used for males (semen) and females (eggs). Frequent washing of hands is a good practice.

16.10.3.1.5 Fill a 3 or 5 mL syringe with 0.5 M KCl and inject 0.5 mL through the soft periostomal membrane of each sea urchin (See Figure 3) or into the oral opening each sand dollar. If sexes are known, use a separate needle for each sex. If sexes are not known, rinse the needle with hot tap water between each injection. This will avoid the accidental injection of sperm from males into females. Note the time of injection (sample data sheet, Figure 1).

16.10.3.1.6 Spawning of sea urchins is sometimes induced by holding the injected sea urchin and gently shaking or swirling it for several seconds. This may provide an additional physical stimulus, or may aid in distributing the injected KCl.

16.10.3.1.7 Place the sea urchins onto the beakers or tray (oral side down). Place the sand dollars onto the beakers (oral side up). Females will release orange (sea urchins) or purple (sand dollars) eggs and males will release cream-colored semen.
16.10.3.1.8 As gametes begin to be shed, note the time on the data sheet and separate the sexes. Place male sand dollars with the oral side up atop a small (5-10 mL) glass beaker filled with 12EC seawater. Leave spawning sea urchin males on tray or beaker

(oral side down) for semen collection. Female sand dollars and sea urchins are left to shed eggs into the 100-mL beakers.

16.10.3.1.9 If sufficient quantities of gametes are available, only collect gametes for the first 15 min after each animal starts releasing. This helps to insure good quality gametes. As a general guideline, do not collect gametes from any individual for more than 30 minutes after the first injection.
16.10.3.1.10 If no spawning occurs after 5 or 10 minutes, a second 0.5 mL injection may be tried. If animals do not produce sufficient gametes following injection of 1.0 mL of KCl, they should probably not be reinjected as this seldom results in acquisition of good quality gametes and may result in mortality of adult urchins.

16.10.3.1.11 Collect the undiluted semen from each male sea urchin, using a 0.1 mL automatic pipet. Store the sperm from each male in a separate, labelled, conical, glass centrifuge tube, covered with a cap or parafilm, on ice. Air exposure of semen may alter its pH through gas exchange and reduce the viability of the sperm. Note: undiluted semen from ***Strongylocentrotus purpuratus*** typically contains about 4×10^{10} sperm/mL.

16.10.3.1.12 Sections 15.10.4.2 and 15.10.6.4 describe collection and dilution of the sperm and eggs. While some of the gamete handling needs to be in a specific order, parts of the procedure can be done simultaneously while waiting for gametes to settle.

16.10.3.2 Collection of Sperm

16.10.3.2.1 Sea urchin semen should be collected dry (directly from the surface of the sea urchin), using either a Pasteur pipette or a 0.1 mL autopipette with the end of the tip cut off so that the opening is at least 2 mm. Pipette semen from each male into separate 1-15 mL conical test tubes, stored in an ice water bath.

16.10.3.3 Viability of Sperm

16.10.3.3.1 Early in the spawning process, place a very small amount of sperm from each male sea urchin or sand dollar into

dilution water on a microscope slide (well slides work nicely). Examine the sperm for motility; use sperm from males with high sperm motility. It is more important to use high quality sperm than it is to use a pooled population of sperm.

16.10.3.4 Pooling of Sperm

16.10.3.4.1 Pool equal quantities of semen from each of the sea urchin males that has been deemed good. If possible, 0.025 mL should be pooled from each of those used and a total of at least 0.05 mL of pooled semen should be available. Sperm collected from good male sand dollars should be pooled after first decanting off the overlying water (the final sand dollar sperm density usually is between $2x10^9$ and $2x10^{10}$ sperm/mL).

16.10.3.5 Storage of Sperm

16.10.3.5.1 Cover each test tube or beaker with a cap or parafilm, as air exposure of semen may alter its pH through gas exchange and reduce the viability of the sperm. Keep sperm covered and on ice or refrigerated (<5EC). The sperm should be used in a toxicity test within 4 h of collection.

16.10.4 PREPARATION OF EGG SUSPENSION FOR USE IN THE TEST

16.10.4.1 Acceptability of Eggs

16.10.4.1.1 Prior to pooling, a small sample of the eggs from each female should be examined for the presence of significant quantities of poor eggs (vacuolated, small, or irregularly shaped) and mixed with good sperm to determine extent of fertilization. If good quality eggs are available from one or more females, questionable eggs should not be used for the test. It is more important to use high quality eggs than it is to use a pooled population of eggs.

16.10.4.2 Pooling of Eggs

16.10.4.2.1 Allow eggs to settle in the collection beakers. Decant some of the water from the collection beakers taking care not to pour off many eggs. The sea urchin eggs are pooled into a 1 L beaker, and the volume brought to 600 mL with 12EC dilution water. The eggs are suspended by swirling and the eggs allowed

to settle for 15 minutes at 12EC. About 500 mL of the overlying water are siphoned off, the volume brought back to 600 mL with more 12EC dilution water, and the eggs resuspended and allowed to settle for a second 15 minute period. After again siphoning off the overlying 500 mL, the rinsed eggs are gently transferred to either a 100 or a 250 mL graduated cylinder and brought to volume with 12EC dilution water. Eggs are stored at 12EC throughout the pre-test period.

16.10.4.2.2 Pooled sand dollar eggs should be treated gently and no additional rinsing step is recommended. Mix well once just before subsampling for egg stock calculations. This is best done in a large graduated cylinder appropriate for the number of eggs available. Cover with parafilm and invert gently several times.

16.10.4.3 Density of Eggs

16.10.4.3.1 Subsamples of the egg stock are then taken for determining egg density. Place 9 mL of dilution water in each of two 22 mL liquid scintillation vials. Label A and B. Place 1 mL of well-mixed egg stock into vial A. Mix well. (The remaining egg stock is covered with parafilm and stored at 12EC.) Transfer 1 mL of egg suspension from vial A to vial B. Mix contents of vial B and transfer 1 mL of egg suspension B into a Sedgewick-Rafter counting chamber. Count eggs under a compound microscope. If count is <30, count a 1 mL sample from vial A (see sample data sheet, Figure 2).

16.10.4.3.2 Prepare 100 mL of egg stock in dilution water at the final target concentration of 2,240 eggs/mL (224,000 eggs in 100 mL). If the egg stock is >2,240 eggs/mL (A >224 or B >30 eggs/mL), dilute the egg stock by transferring:

$$224{,}000 \text{ eggs} / \frac{D}{} \text{ eggs/mL} = \_\_\_\_\_ \text{mL}$$

of well-mixed egg stock to a 100 mL graduated cylinder and bring the total volume to 100 mL with dilution water where:

$$D = (\text{Count A}) \times 10 \text{ or } (\text{Count B}) \times 100.$$

If the egg stock is <2,240 eggs/mL (A <224 eggs/mL), concentrate the eggs by allowing them to settle and then decant enough water to retain the following percent of the original volume:

(D eggs/mL / 2,240) x 100 = % volume.

16.10.4.3.3 Check the egg stock density. Place 9 mL of dilution water into a 22 mL scintillation vial; add 1 mL of the final egg stock. Mix well and transfer 1 mL into a Sedgewick-Rafter counting chamber. The egg count should be between 200 and 245. Adjust egg stock volume and recheck counts if necessary to obtain counts within this range. Because some eggs (especially sand dollar eggs) may be sensitive to handling, it is advisable to separately prepare egg stocks for the fertilization trial and the definitive test (but use the same pooled batch of eggs).

16.10.5 PREPARATION OF SPERM DILUTION FOR USE IN THE (OPTIONAL) TRIAL FOR ESTIMATING APPROPRIATE SPERM DENSITY FOR TEST

16.10.5.1 A trial fertilization is recommended to reduce the likelihood of a failed test due to inadequate control fertilization or exceeding the maximum acceptable sperm density. However, two other alternative approaches are acceptable:

1) Conduct the test at a low enough sperm density that oversperming does not create test insensitivity. This can be met by using a confirmed sperm stock density of #5.6×10^6/mL (this is equivalent to a sperm:egg ratio of #500:1 at 200 eggs/mL); or

2) Conduct the test, but include two extra sets of controls, one set receiving only 0.050 mL of the sperm stock and the other receiving 0.2 mL of the sperm stock. The control fertilization in the 0.050 mL sperm stock controls must be at least 5% lower than that in the 0.2 mL sperm stock controls or the test is unacceptable. Confirm that the sperm stock density did not exceed the maximum acceptable density of 3.36×10^7 sperm/mL.

16.10.5.2 Fertilization trial is conducted to determine the sperm density that will provide about 80-100 percent control egg fertilization while avoiding significant "oversperming" that can reduce test sensitivity. Although usually expressed as a sperm:egg ratio (e.g., 1,000:1), because egg density is held constant at 200/mL, the sperm:egg ratio is also a measure of sperm density.

16.10.5.3 It is unacceptable to conduct a definitive toxicity test if the sperm:egg ratio exceeds 3,000:1. This is a cut-off based on gradual loss of test sensitivity at higher sperm densities, even in cases where control fertilization is considerably below 100 percent.

16.10.5.4 It is unnecessary to conduct trials for definitive toxicity tests at sperm:egg ratios below 500:1, because this ratio should never cause significant "oversperming."

16.10.5.5 Sperm density of sea urchin semen or sand dollar sperm suspension is checked by hemocytometer counts and a replicated series of nominal S:E ratios set up (3,000, 1288, 550, 234, and 100:1) based upon appropriate dilution calculations.

16.10.5.6 For sea urchins and sand dollars, prepare a killed sperm preparation for determining the dilution required to obtain a sperm stock (3.36×10^7 sperm/mL) for the maximum sperm density (6×10^5 sperm/200 eggs/mL--3,000:1) needed for the trial. A sperm density of about 1×10^7 is convenient to count. If the approximate sperm density is known, the dilution procedures outlined in Table 4 can be followed without initial sperm counts; the actual trial sperm density must still be determined by subsequent counts. For example (Table 4), if expected sperm density is ca. 5×10^8 dilute 0.2 mL of sperm to 10 mL, if ca. 5×10^9 dilute 0.2 mL of sperm to 100 mL (or 0.025 mL of sperm to 10 mL), if ca. 5×10^{10} dilute 0.040 mL to 200 mL. Table 4 is provided for guidance as a quick reference for dilution volumes if sperm density of pooled semen is can be reasonably estimated, and as a check for mathematical accuracy of formula calculations for sperm dilution.

16.10.5.7 Mix the pooled sea urchin semen (16.10.3.8) by agitating the centrifuge tube for about 5 seconds using a vortex mixer. Very slowly withdraw a subsample of semen using an automatic pipet, wipe off the outside of the pipet tip with tissue, and empty the pipet contents into an Erlenmeyer flask containing the appropriate volume (Table 4) of a sperm killing solution of 1% glacial acetic acid in dilution water (e.g., 10 mL of 10% glacial acetic acid plus 90 mL of dilution water).

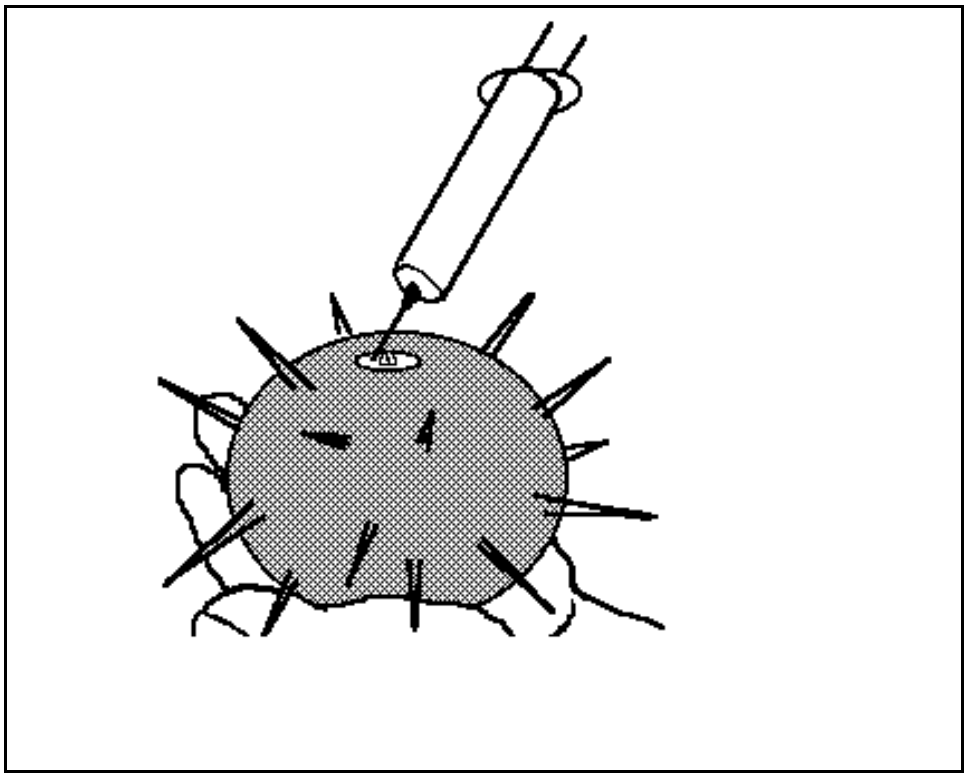


Figure 3. Showing the location and orientation used in the injection of KCl into sea urchins to stimulate spawning.

Repeatedly rinse the residual semen from the pipet tip by filling and emptying until no further cloudy solution is expelled from the pipet (this may require several dozen rinses). Cover the flask with parafilm and mix thoroughly by repeated inversion.

16.10.5.8 Mix the chilled suspension of pooled sand dollar sperm (16.10.5.6) using a stirring rod. Pipet the appropriate volume of sperm suspension (Table 4) into an Erlenmeyer flask containing the appropriate volume (Table 4) of a sperm killing solution of 1% glacial acetic acid in dilution water (e.g., 10 mL of 10% glacial acetic acid plus 90 mL of dilution water).

16.10.5.9 Transfer samples of well-mixed sperm suspension to both sides of two Neubauer hemacytometers. Let the sperm settle 15 min.

16.10.5.10 Count the sperm on one hemacytometer following procedures outlined in Appendix II. If the lower count is at

least 80% of the higher count use the mean count to estimate sperm density in semen and the required dilution volume for the test stock. If the two counts do not agree within 20%, count the two fields on the other hemacytometer. Calculate the sperm density in the semen or sperm suspension using the mean of all four counts unless one count can be eliminated as an obvious outlier.

16.10.5.11 Calculate the volume of dilution water necessary to dilute the sea urchin semen or the sand dollar sperm suspension to the sperm density (sperm/mL) required for the sperm stock for the trial. See Table 5 for recommended dilution procedures; it also provides a quick reference for dilution volumes once sperm density of pooled semen is known, or a check for mathematical accuracy of formula calculations for sperm dilution. Note: table values for sperm densities from $1x10^8$ to $9x10^9$ are for volume (mL) of sperm stock for total volume of 100 mL; table values for sperm densities $\geq 1x10^{10}$ are for dilution water volumes for 0.025 mL of semen. Table 5 is used as follows: given a sperm density in the semen stock (e.g., $4.7x10^9$) find the row containing the integer (characteristic) and the exponent ($4x10^9$) in the left hand column, then read across to the column coresponding to the mantissa (0.7). The value at the intersection of the row and column (0.71 mL) is the volume of semen per 100mL needed for sperm stock to achieve a 3000:1 sperm:egg ratio in the trial.

16.10.5.12 For the approximate sperm:egg ratios dilute the 3000:1 stock as follows:

1288:1	5 mL 3000:1 stock with 6.6 mL dilution water
550:1	2 mL 3000:1 stock with 9.9 mL dilution water
234:1	1 mL 3000:1 stock with 11.8 mL dilution water
100:1	0.5 mL 3000:1 stock with 16.5 mL dilution water

16.10.6 SPERM DENSITY TRIAL

16.10.6.1 The series of trial sperm:egg ratios should include 3,000:1 and several lower ratios. The ratios 100:1, 234:1, 550:1, 1288:1 and 3,000:1 are recommended because they evenly divide the log sperm:egg ratio. Fertilization appears to be a linear function of the log of sperm density (Figure 4). Recommended sperm dilution procedures are given in Table 5.

16.10.6.4 Quantitive evaluation of the sperm density trial should be obtained by counting 100 eggs from each tube until a suitable sperm density can be determined for the definitive test. Examples of sperm density selection are given in Table 6. Percent fertilization may be lower in the test than in the trial because the viability of the stored sperm may decrease during the period of the trial. If the sperm have very good viability (e.g., cases 1 and 2, Table 6), this loss of viability should be small. On the other hand, if viability is inherently poorer (cases 3, 4 and 5, Table 6), the loss of viability could be greater and probably should be taken into account in selecting the sperm density for the test. Case 6 (Table 6) represents a special case in which egg viability may affect the percent fertilization; in this case the asymptote of the fertilization curve is assumed to represent 100% fertilization for purposes of selection of sperm density for the test.

16.10.6.5 Prepare killed sperm preparations of the trial sperm stock suspensions to provide confirmation of the nominal sperm:egg ratios. It saves time if these can be prepared and loaded onto hemacytometers while the trial is being conducted. Alternatively, once the trial has been evaluated, the selected nominal sperm density can be confirmed by direct hemacytometer count.

16.10.6.6 Record all the counts made, select a target sperm:egg ratio for the test, and calculate the dilution of the stored sperm stock needed to provide the necessary sperm density for the definitive test.

16.10.6.7 Table 5 can be used for deriving the volumes needed for preparing the final sperm stock. For a pooled sperm suspension density of $4x10^9$ and a target sperm:egg ratio of 500:1, simply read the dilution for the 3000:1 sperm:egg ratio from Figure 5 (0.84 mL / 100 mL) and reduce the sperm volume by 3,000 / 500 = 6. In this case 0.84 / 6 = 0.14 mL; the dilution factor checks out (100 / 0.14 = 714).

TABLE 4. Dilution volume guide for initial count of sperm density to achieve recommended counting density of 1 x 10^7/mL.

Initial Sperm/mL	mL/10mL	mL/100mL	mL/200mL
1 x 10^8	1.000		
2 x 10^8	0.500		
3 x 10^8	0.333		
4 x 10^8	0.250		
5 x 10^8	0.200		
6 x 10^8	0.167		
7 x 10^8	0.143		
8 x 10^8	0.125		
9 x 10^8	0.111		
1 x 10^9	0.100	1.000	
2 x 10^9	0.050	0.500	1.000
3 x 10^9	0.033	0.333	0.667
4 x 10^9	0.025	0.250	0.500
5 x 10^9		0.200	0.400
6 x 10^9		0.167	0.333
7 x 10^9		0.143	0.286
8 x 10^9		0.125	0.250
9 x 10^9		0.111	0.222
1 x 10^{10}		0.100	0.200
2 x 10^{10}		0.050	0.100
3 x 10^{10}		0.033	0.067
4 x 10^{10}		0.025	0.050
5 x 10^{10}			0.040
6 x 10^{10}			0.033
7 x 10^{10}			0.029
8 x 10^{10}			0.025
9 x 10^{10}			0.022

Note: to obtain quantitatively repeatable samples of semen it is important that: (1) the pipet tip have an opening of at least 1 mm; (2) samples be withdrawn slowly to avoid cavitation and entrainment of air in the semen sample; (3) samples not include fragments of broken spines (which usually settle to the test tube bottom upon vortexing); and (4) wiping semen from the pipet tip with tissue be done with care to avoid wicking semen from within the pipet tip.

Bring the indicated volume of sperm stock to 100 mL

Density	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
1.00e+08	33.60	30.55	28.00	25.85	24.00	22.40	21.00	19.76	18.67	17.68
2.00e+08	16.80	16.00	15.27	14.61	14.00	13.44	12.92	12.44	12.00	11.59
3.00e+08	11.20	10.84	10.50	10.18	9.88	9.60	9.33	9.08	8.84	8.62
4.00e+08	8.40	8.20	8.00	7.81	7.64	7.47	7.30	7.15	7.00	6.86
5.00e+08	6.72	6.59	6.46	6.34	6.22	6.11	6.00	5.89	5.79	5.69
6.00e+08	5.60	5.51	5.42	5.33	5.25	5.17	5.09	5.01	4.94	4.87
7.00e+08	4.80	4.73	4.67	4.60	4.54	4.48	4.42	4.36	4.31	4.25
8.00e+08	4.20	4.15	4.10	4.05	4.00	3.95	3.91	3.86	3.82	3.78
9.00e+08	3.73	3.69	3.65	3.61	3.57	3.54	3.50	3.46	3.43	3.39
1.00e+09	3.36	3.05	2.80	2.58	2.40	2.24	2.10	1.98	1.87	1.77
2.00e+09	1.68	1.60	1.53	1.46	1.40	1.34	1.29	1.24	1.20	1.16
3.00e+09	1.12	1.08	1.05	1.02	0.99	0.96	0.93	0.91	0.88	0.86
4.00e+09	0.84	0.82	0.80	0.78	0.76	0.75	0.73	0.71	0.70	0.69
5.00e+09	0.67	0.66	0.65	0.63	0.62	0.61	0.60	0.59	0.58	0.57
6.00e+09	0.56	0.55	0.54	0.53	0.53	0.52	0.51	0.50	0.49	0.49
7.00e+09	0.48	0.47	0.47	0.46	0.45	0.45	0.44	0.44	0.43	0.43
8.00e+09	0.42	0.41	0.41	0.40	0.40	0.40	0.39	0.39	0.38	0.38
9.00e+09	0.37	0.37	0.37	0.36	0.36	0.35	0.35	0.35	0.34	0.34

To dilute dense semen: add 0.025 mL of semen into these volumes (mL) of dilution water

Density	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
1.00e+10	7.44	8.44	9.44	10.44	11.44	12.44	13.44	14.44	15.44	16.44
2.00e+10	14.88	15.88	16.88	17.88	18.88	19.88	20.88	21.88	22.88	23.88
3.00e+10	22.32	23.32	24.32	25.32	26.32	27.32	28.32	29.32	30.32	31.32
4.00e+10	29.76	30.76	31.76	32.76	33.76	34.76	35.76	36.76	37.76	38.76
5.00e+10	37.20	38.20	39.20	40.20	41.20	42.20	43.20	44.20	45.20	46.20
6.00e+10	44.64	45.64	46.64	47.64	48.64	49.64	50.64	51.64	52.64	53.64
7.00e+10	52.08	53.08	54.08	55.08	56.08	57.08	58.08	59.08	60.08	61.08
8.00e+10	59.52	60.52	61.52	62.52	63.52	64.52	65.52	66.52	67.52	68.52
9.00e+10	66.96	67.96	68.96	69.96	70.96	71.96	72.96	73.96	74.96	75.96

TABLE 5. DILUTION VOLUMES OF SPERM STOCK OF INDICATED DENSITY ($1.0X10^{8}$ TO $9.9X10^{10}$) TO ACHIEVE THE SPERM STOCK DENSITY ($3.36X10^{7}$) FOR A 3000:1 SPERM:EGG RATIO.

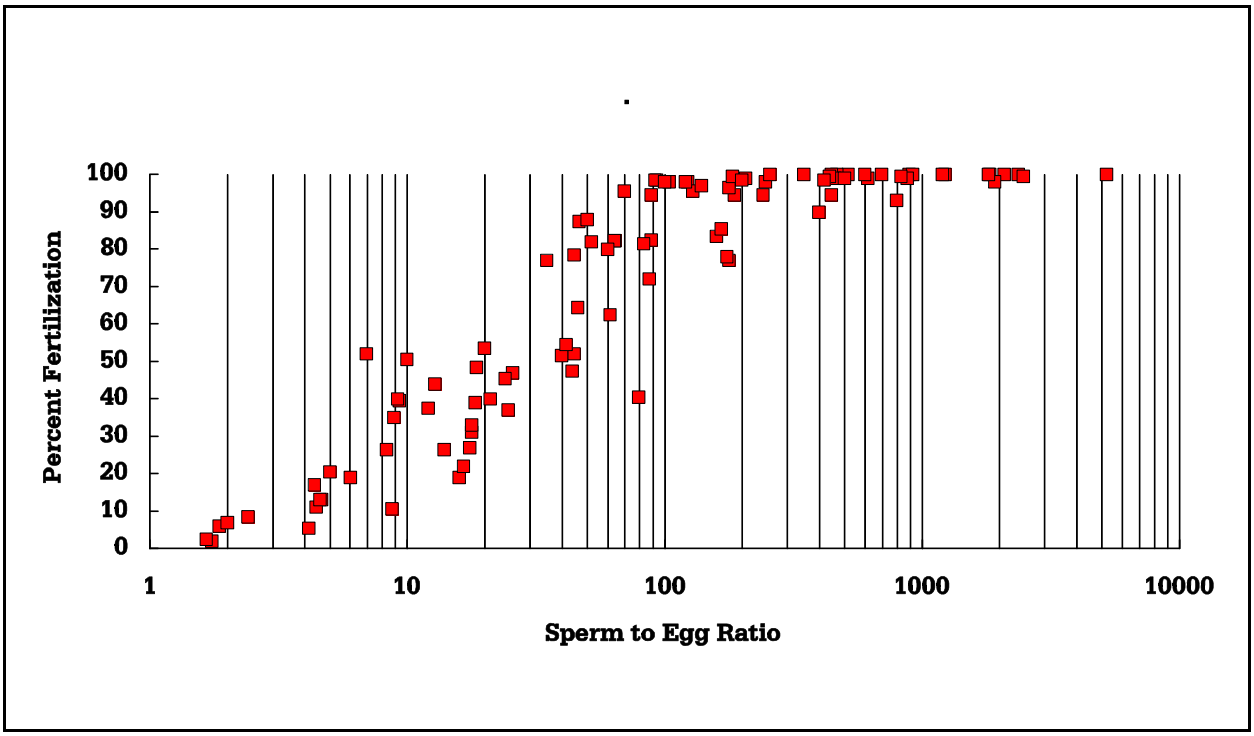


Figure 4. Relationship between sea urchin sperm:egg ration and percent control fertilization from 21 trials conducted by EPA (Feb-May 1991).

16.10.7 OBSERVATIONS DURING THE TEST

16.10.7.1 It is recommended that all observations be made on extra test solution remaining after the test tubes have been filled.

16.10.7.2 DO, pH, and salinity are measured at the beginning of the test. Due to the short duration of the test, no additional measurements of these parameters are required. Temperature is measured several times during the test as outlined in 16.10.7.

16.10.7.3 Record all measurements on the data sheet.

16.10.8 START OF THE DEFINITIVE TEST

16.10.8.1 Prior to Beginning the Test
produced good fertility, or if some produced good fertility.

TABLE 6. EXAMPLES OF RESULTS OF TRIAL FERTILIZATION TESTS WITH SPECIFIED SPERM DENSITIES AND TARGET SPERM DENSITY SELECTION (SPERM:EGG RATIO) FOR THE DEFINITIVE TEST.

sperm: egg	case 1	case 2	case 3	case 4	case 5	case 6
100:1	100*	95*	85	70	40	70
234:1	100	98	95*	80	64	85*
550:1	100	100	98	98*	82	89
1288:1	100	100	100	100	84	90
3000:1	100	100	100	100	88*	90

* recommended selection (interpolation to intermediate sperm:egg ratios may be used if found desirable)

1. If all trials exceed 90% fertilization, select 100:1 (case 1 and case 2).

2. If not all trials exceed 90% fertilization select the lowest sperm:egg ratio that does exceed 90% fertilization (case 3 and case 4).

3. If no trials exceed 90% fertilization, select the highest sperm:egg ratio (case 5) unless fertilization appears to become asymptotic below 100% (case 6).

4. If even the highest sperm:egg ratio fails to achieve 70% fertilization it is probable that an acceptable test cannot be conducted with these gametes.

   11,200 x target S:E ratio = target density; e.g., if target S:E = 500:1, target density = 11,200 x 500 = 5,600,000 sperm/mL. (11,200 = (1,120 eggs/tube)(0.1 mL of sperm stock/tube)).

   (stock sperm/mL)/(target sperm/mL) = dilution; e.g., if stock sperm has $4x10^9$ sperm/mL, then dilution = $4x10^9$ / $5.6x10^6$ = 714

16.10.8.1.1 The test should begin as soon as possible, preferably within 24 h of sample collection. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity tests unless

permission is granted by the permitting authority. In no case should the sample be used in a test more than 72 h after sample collection (see Section 8 Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Test).

16.10.8.1.2 Just prior to test initiation (approximately 1 h), the temperature of a sufficient quantity of the sample to make the test solutions should be adjusted to the test temperature (12 ± 1EC) and maintained at that temperature during the addition of dilution water.

16.10.8.1.3 Increase the temperature of the water bath, room, or incubator to the required test temperature (12 ± 1EC).

16.10.8.1.4 Randomize the placement of test chambers in the temperature-controlled water bath, room, or incubator at the beginning of the test, using a position chart. Assign numbers for the position of each test chamber using a random numbers or similar process (see Appendix A, for an example of randomization). Maintain the chambers in this configuration throughout the test, using a position chart. Record these numbers on a separate data sheet together with the concentration and replicate numbers to which they correspond. Identify this sheet with the date, test organism, test number, laboratory, and investigator's name, and safely store it away until after the sea urchins and sand dollars have been examined at the end of the test.

16.10.8.1.5 Note: Loss of the randomization sheet would invalidate the test by making it impossible to analyze the data afterwards. Make a copy of the randomization sheet and store separately. Take care to follow the numbering system exactly while filling chambers with the test solutions.

16.10.8.1.6 Arrange the test chambers randomly in the water bath or controlled temperature room. Once chambers have been labeled randomly, they can be arranged in numerical order for convenience, since this will also ensure random placement of treatments.

16.10.9.2 Sperm Exposure

16.10.9.2.1 Mix the iced sea urchin semen or sand dollar sperm suspension as described in 16.10.5.7 and 16.10.5.8 (do not kill the sperm). Combine the required volumes of sperm and dilution water and mix this sperm stock well by repeated inversion of the graduate cylinder or beaker. Begin test within 5 minutes. Table 5 (for 3000:1 sperm:egg ratio) can be used to aid in calculating appropriate volumes by reducing the sperm volume or increasing the dilution water volume by the factor:

f = 3000:1 / target sperm:egg ratio

16.10.9.2.2 The test tubes containing 5.0 mL of the various test solutions should have been equilibrated in a 12EC waterbath. Into each test tube, inject 0.100 mL of the sperm stock (except see 16.7.4 and 16.11.4) and note the time of first and last injection. It is important that the injection be performed with care that the entire volume goes directly into the test solution and not onto the side of the test tube. Similarly, the pipet tip should not touch the test solution or the side of the test tube, risking transfer of traces of test solution(s) into the sperm stock. Using repeated single 0.100 mL refill and injection, about 12 tubes per minute is a reasonable injection rate. More rapid rates of injection can be attained with repeating (single fill, multiple injection) pipets. Sperm injection rate (tubes/min) should not exceed that possible for egg injection.

16.10.9.2.3 Unless the test tubes are totally randomized, injection of sperm should be performed by replicate, i.e., the first set of replicates should receive sperm, then the second set, then the third set, etc. The sperm stock solution should be mixed frequently to maintain a homogeneous sperm stock.

16.10.9.2.4 Confirm the sperm density. Pipet 9 mL of sperm stock solution into a vial or test tube containing 1 mL of 10% acetic acid. Fill both sides of a hemacytometer with this dilution after mixing well. Let stand for 15 minutes. Count both sides of the hemacytometer using counting pattern no. 1 outlined in Appendix II and take the average count. For a sperm:egg ratio of 500:1 the stock sperm density will be 5,600,000 sperm/mL. (For counting pattern no. 1, this amounts to a total count of 102 sperm for the five large squares.) Calculate the sperm density in the sperm stock. If either: (1) the stock sperm density is greater than 33,600,000 sperm/mL (S:E

>3,000:1), or (2) the sperm density is more than 2x the target density, the test must be restarted with freshly diluted semen.

16.10.9.2.5 Check the temperature of the test solutions several times during the sperm exposure by including a temperature blank test tube containing 5 mL of dilution water with a thermometer.

16.10.9.3 Adding Eggs to the Test

16.10.9.3.1 Exactly 20 minutes after the sperm addition to the test was begun, begin to add the eggs, with every tube (including egg blanks - 11.7.4) receiving 0.5 mL of egg stock. Follow the same pattern of introduction for the eggs as used with the sperm so that each test tube has a sperm incubation period of 20 minutes. Note the time of start and finish of egg addition. This duration should be within one minute of that used for the sperm.

16.10.9.3.2 In order to maintain the same sperm:egg ratio in each test tube, the eggs must be maintained in a uniform distribution in the water column of the egg stock. Slow, gentle agitation of the egg stock in a beaker using a perforated plunger appears to be the best method of achieving a uniform distribution. Frequent inversion and mixing of egg stock in either a graduated cylinder or a multiple injection pipet may be acceptable.

16.10.9.3.3 The eggs should be injected using a pipet with an opening of at least 2 mm in order to avoid damaging the eggs and to provide sufficient flow to obtain a representative sample.

16.10.9.3.4 Two pair of egg blanks should be included in the test design, one at the beginning of the injection sequence (effluent blank) and one at the end of the injection sequence (egg blank). These tubes receive no sperm. The effluent blank contains the highest concentration of effluent and the egg blank contains dilution water. Examination of the effluent blank will indicate if the effluent induces a false fertilization membrane (a possible event, but probably rare) thus masking toxicity. Examination of the egg blank will indicate if accidentally fertilized eggs were used in the test (this is a minor factor unless a significant portion of the eggs were accidentally fertilized; it can indicate poor laboratory techniques). These

blanks are kept capped until the eggs are added in order to avoid contamination by sperm.

16.10.10 LIGHT, PHOTOPERIOD, SALINITY AND TEMPERATURE

16.10.10.1 The echinoderm fertilization test can be conducted in the dark or at ambient laboratory light levels. Due to its short duration, the fertilization test requires no photoperiod.

16.10.10.2 The water temperature in the test chambers should be maintained at 12 ± 1EC. If a water bath is used to maintain the test temperature, the water depth surrounding the test cups should be as deep as possible without floating the chambers. A sensor placed in a temperature blank vial with standard volume of test solution can provide a direct measure of test solution temperature, one which may be more stable than the temperature in the air or water in the medium surrounding the test vials. Do not measure temperatures directly in a test vial, but prepare and handle the temperature blank(s) exactly as the normal control vials. Record the temperature several times between the beginning and the end of the test.

16.10.10.3 The test salinity should be in the range of 34 ± 2‰. The salinity should vary by no more than ±2‰ among the chambers on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.

16.10.10.4 Rooms or incubators with high volume ventilation should be used with caution because the volatilization of the test solutions and evaporation of dilution water may cause wide fluctuations in salinity.

16.10.11 DISSOLVED OXYGEN (DO) CONCENTRATION

16.10.11.1 Aeration may affect the toxicity of effluent and should be used only as a last resort to maintain a satisfactory DO. The DO concentration should be measured on new solutions at the start of the test (Day 0). The DO should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). If it is necessary to aerate, all treatments and the control should be aerated. The aeration rate should not exceed that necessary

to maintain a minimum acceptable DO and under no circumstances should it exceed 100 bubbles/minute, using a pipet with a 1-2 mm orifice, such as a 1 mL KIMAX® serological pipet No. 37033, or equivalent.

16.10.12 OBSERVATIONS DURING THE TEST

16.10.12.1 Routine Chemical and Physical Observations

16.10.12.1.1 DO is measured at the beginning of the exposure period in one test chamber at each test concentration and in the control.

16.10.12.1.2 Temperature, pH, and salinity are measured at the beginning of the exposure period in one test chamber at each concentration and in the control. Temperature should also be monitored continuously or observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be measured in a sufficient number of test chambers at the end of the test to determine temperature variation in the environmental chamber.

16.10.12.1.3 Record all the measurements on the data sheet.

16.10.13 TERMINATION OF THE TEST

16.10.13.1 Ending the Test

16.10.13.1.1 Record the time the test is terminated.

16.10.13.1.2 Because of the short test duration water quality measurements are not necessary at the end.

16.10.13.2 Sample Preservation

16.10.13.2.1 Exactly 20 minutes after the egg addition, the test should be stopped by the addition of a fixative to kill the sperm and eggs (both unfertilized and fertilized [zygotes]) and to preserve the eggs for examination. Again, the time allotted to fixative addition should be about the same as that for sperm and egg addition and the sequence of addition the same as for the introduction of the gametes.

16.10.13.2.2 The choice of formaldehyde or glutaraldehyde is up to the individual laboratory. There are at least two acceptable procedures: (1) the EPA ***Arbacia*** method of adding 10% formaldehyde in dilution water at the rate of 2 mL to each test tube; or (2) the addition of 1% glutaraldehyde (vol/vol) in clean seawater at the rate of 0.5 mL to each test tube. Glutaraldehyde should be made up fresh each day. Because concentrated glutaraldehyde is commonly only 25% strength, 1% glutaraldehyde is obtained by diluting the concentrate by 25x (e.g., 4 mL + 96 mL seawater).

16.10.13.2.3 It must be noted that formaldehyde has been identified as a carcinogen and that both glutaraldehyde and formaldehyde are irritating to skin and mucous membranes. Neither should be used at higher concentrations than needed to achieve morphological preservation of eggs for counting and only under conditions of maximal ventilation and minimal opportunity for volatilization into room air. Before using either compound in this method, the user should consult the latest material safety data available.

16.10.13.3 Counting

16.10.13.3.1 Immediately after termination of the test, the tubes are capped (or otherwise covered) and the contents mixed by inversion. They can be stored at room temperature until the eggs are examined for fertilization. Counts should be completed within 48 hours and, if counts extend over two days, should be made by replicate, i.e., count all replicate 1 tubes, then replicate 2, etc.

16.10.13.3.2 At least 100 eggs from each test tube are examined and scored for the presence or absence of an elevated fertilization membrane. Newly fertilized eggs will almost always have a completely elevated membrane around the egg (See Figures 5 and 6). Often a double membrane appears in sea urchin eggs, but following storage, even of only several hours, the inner (hyaline) membrane may disappear. Fertilized eggs may touch the outer membrane, or the membrane(s) may partially collapse. Because these phenomena only occur after preservation, eggs with any elevation of the fertilization membrane are counted as fertilized.

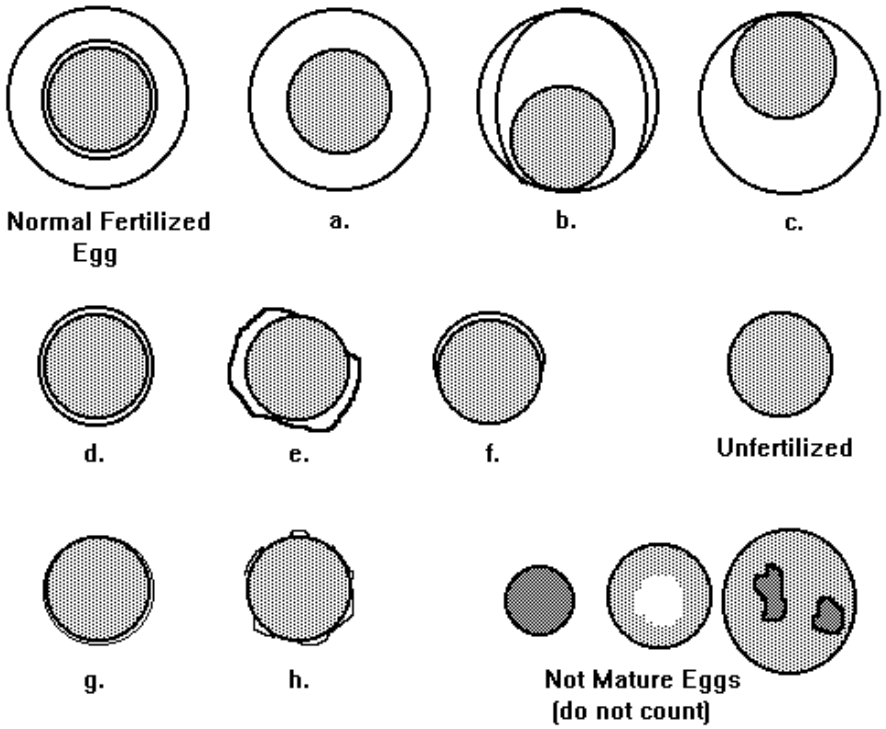


Figure 5. Examples of typical fertilized and unfertilized sea urchin eggs and a number of examples of atypical "fertilized" eggs (a through h). Normal fertilized eggs have an outer fertilization membrane and an inner hyaline membrane. After preservation, the hyaline membrane sometimes disappears (a); in other cases the egg is displaced from the center and contacts the perimeter either inside an enlarged hyaline envelope (b) or with no visible hyaline membrane (c). In some instances there appears to be only a slight elevation of the outer membrane or only the hyaline membrane appears, fully (d), partially (f), or only as a halo (g). In some batches of eggs the membrane(s) appear to be fragile and some collapse (e). In rare cases sperm appear to activate membrane elevation over only segments of the egg leading to a blistered appearance (h). When eggs appearing as those in examples f, g, and h are common in a test, the results should be examined closely to see if their occurrence appears to be dose-related (indicating an effect on fertilization), not dose-related (indicating a problem with egg quality or preservative), or is common in the effluent egg control (indicating an effluent- produced false fertilization). Eggs that are not mature are capable of being fertilized, but should never be counted. These include obviously smaller (often denser) eggs, normal sized eggs with a distinct, clear center, and very large eggs with often irregular color and density.

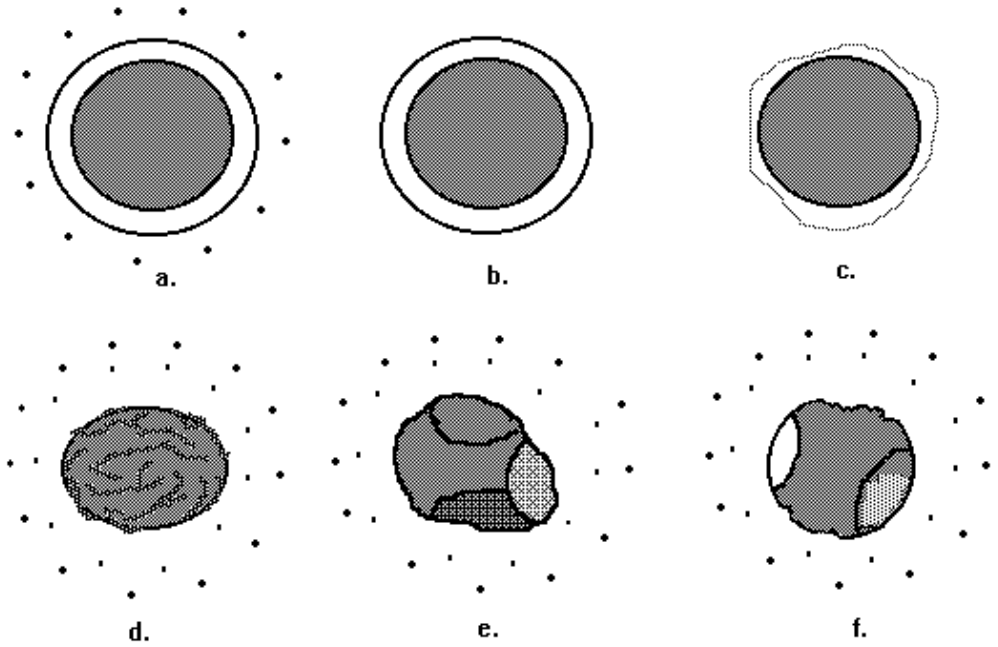


Figure 6. Examples of typical fertilized and unfertilized sand dollar eggs. Nearly all newly released eggs are characterized by a surrounding sphere of small purple chromatophores embedded within the transparent gelatinous coat surrounding the egg. The coat and the chromatophores may be lost or retained in the test and subsequent handling. Typical fertilized eggs are represented by (a) and (b). Some fertilized eggs (c) show only a wispy remnant of the fertilization membrane. Eggs when spawned usually appear as in (d) and (e) or somewhere in between. The more rounded "raisin" appearing egg in (d) is usually superior to the "asteroid" appearing egg in (e) although the latter can provide acceptable test results. However, the more irregularly shaped or vacuolated the eggs appear, the poorer the control fertilization is likely to be. The egg shown in (f), the "pitted olive," never shows a fertilization membrane and should not be counted.

16.10.13.3.3 It is convenient to concentrate the eggs prior to counting. If the eggs are allowed to completely settle (ca 30 minutes after termination and mixing), most of the overlying solution can be removed with a pipet, leaving the eggs concentrated in a much smaller volume. The eggs are then resuspended by filling and emptying a 1 mL pipet about 5 times from the remaining volume and finally transferring 1 mL of the egg suspension into a 1 mL Sedgewick-Rafter counting chamber (other volume counting chambers can be used).

16.10.13.3.4 Failure to completely resuspend the eggs can result in biasing the counts towards higher percent fertilization due to a tendency seen in rare batches of eggs in which unfertile eggs tend to be adhesive. This phenomenon may be further influenced by the choice of preservative, the strength of the preservative, and the period between preservation and counting. However, other sampling procedures may be used once demonstrated not to bias sampling and if no clumping of adhesive eggs is observed in a given test; for example, concentrated eggs may be picked up from the test tube and deposited in a small drop on a microscope slide, or eggs can be scored by examination with the test tubes laying on their sides and viewed at low power or with an inverted microscope.

16.10.13.4 Endpoint

16.10.13.4.1 In a count of at least 100 eggs, record the number of eggs with fertilization membranes and the number of eggs without fertilization membranes.

16.11 **SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA**

16.11.1 A summary of test conditions and test acceptability criteria is listed in Table 7.

TABLE 7. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR, ***STRONGYLOCENTROTUS PURPURATUS*** AND ***DENDRASTER EXCENTRICUS***, FERTILIZATION TEST WITH EFFLUENTS AND RECEIVING WATERS

1. Test type:	Static non-renewal
2. Salinity:	34 ± 2‰
3. Temperature:	12 ± 1EC
4. Light quality:	Ambient laboratory light during test preparation

5. Light intensity:	l0-20 uE/m^2/s (Ambient laboratory levels)
6. Test chamber size:	16 x 100 or 16 x 125 mm
7. Test solution volume:	5 mL
8. Number of spawners:	Pooled sperm from up to four males and pooled eggs from up to four females are used per test
9. No. egg and sperm cells per chamber:	About 1,120 eggs and not more than 3,360,000 sperm per test tube
10. No. replicate chambers per concentration:	4
11. Dilution water:	Uncontaminated 1-µm-filtered natural seawater or hypersaline brine prepared from natural seawater or artificial sea salts
12. Test concentrations:	Effluents: Minimum of 5 and a control Receiving waters: 100% a control
13. Dilution factor:	Effluents: **$**0.5 Receiving waters: None or **$**0.5
14. Test duration:	40 min (20 min plus 20 min)
15. Endpoint:	Fertilization of eggs
16. Test acceptability criteria:	**$**70% egg fertilization in controls; %MSD of <25%; and appropriate sperm counts
17. Sampling requirements:	One sample collected at test initiation, and preferably used within 24 h of the time it is removed from the sampling device (see Section 8, Effluent and Receiving Water Sampling, and Sample Preparation for Toxicity Tests)
18. Sample volume required:	1 L

16.12 **ACCEPTABILITY OF TEST RESULTS**

16.12.1 Test results are acceptable only if all the following requirements are met:

(1) Egg fertilization at the NOEC must be greater than 80% of that in the controls.

(2) The minimum significant difference (%MSD) is <25% relative to the control.

(3) The sperm count for the final sperm stock must not exceed 33,600,000/mL.

(4) If the sperm count for the final sperm stock is between 5,600,000 and 33,600,000/mL it must not exceed 2x of the target density from the trial, or if no target density was specified for the test (see 11.5.1), the high sperm density controls (0.2 mL sperm stock) must have at least 5% higher fertilization than the low sperm density controls (0.05 mL sperm stock).

(5) Dilution water egg blanks and effluent egg blanks should contain essentially no eggs with fertilization membranes.

16.13 **DATA ANALYSIS**

16.13.1 GENERAL

16.13.1.1 Tabulate and summarize the data. Calculate the proportion of fertilized eggs for each replicate. A sample set of test data is listed in Table 8.

16.13.1.2 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

16.13.1.3 The endpoints of toxicity tests using the sea urchin and the sand dollar are based on the reduction in proportion of eggs fertilized. The IC25 is calculated using the Linear Interpolation Method (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). LOEC and NOEC values for fecundity are obtained using a hypothesis testing approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981) (see Section 9). Separate analyses

are performed for the estimation of the LOEC and NOEC endpoints

and for the estimation of the IC25. See the Appendices for examples of the manual computations, and examples of data input and program output.

TABLE 8. DATA FROM SEA URCHIN, ***STRONGYLOCENTROTUS PURPURATUS***, FERTILIZATION TEST

Effluent Concentration (%)	Replicate	No. of Eggs Counted	No. of Eggs Fertilized	Proportion Fertilized
Control	A	100	97	0.97
	B	100	90	0.90
	C	100	100	1.00
0.05	A	100	100	1.00
	B	100	100	1.00
	C	100	98	0.98
0.10	A	100	100	1.00
	B	100	97	0.97
	C	100	99	0.99
0.15	A	100	98	0.98
	B	100	96	0.96
	C	100	97	0.97
0.20	A	100	94	0.94
	B	100	88	0.88
	C	100	97	0.97
0.40	A	100	43	0.43
	B	100	63	0.63
	C	100	46	0.46
0.60	A	100	2	0.02
	B	100	1	0.01
	C	100	9	0.09
0.80	A	100	0	0.00
	B	100	0	0.00
	C	100	0	0.00

16.13.2 EXAMPLE OF ANALYSIS OF SEA URCHIN, ***STRONGYLOCENTROTUS PURPURATUS***, AND SAND DOLLAR, ***DENDRASTER EXCENTRICUS***, FERTILIZATION DATA

16.13.2.1 Formal statistical analysis of the fertilization data is outlined in Figure 7.

The response used in the analysis is the proportion of fertilized eggs in each test or control chamber. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the IC25 endpoint. Concentrations at which there are no eggs fertilized in any of the test chambers are excluded from statistical analysis of the NOEC and LOEC, but included in the estimation of the IC25.

16.13.2.2 For the case of equal numbers of replicates across all concentrations and the control, the evaluation of the NOEC and LOEC endpoints is made via a parametric test, Dunnett's Procedure, or a nonparametric test, Steel's Many-one Rank Test, on the arc sine square root transformed data. Underlying assumptions of Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test, and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are estimated by the parametric procedure.

16.13.2.3 If unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a ***t*** test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative.

16.13.2.4 Example of Analysis of Fecundity Data

16.13.2.4.1 This example uses toxicity data from a sea urchin, ***Strongylocentrotus purpuratus***, fertilization test performed with effluent. The response of interest is the proportion of fertilized eggs, thus each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix B. The raw and transformed data, means and variances of the transformed observations at each effluent concentration and control are listed in Table 9. The data are plotted in Figure 8. Because there is zero fertilization in all three replicates for the 0.80% effluent concentration, it was not included in the statistical analysis and is considered a qualitative fecundity effect.

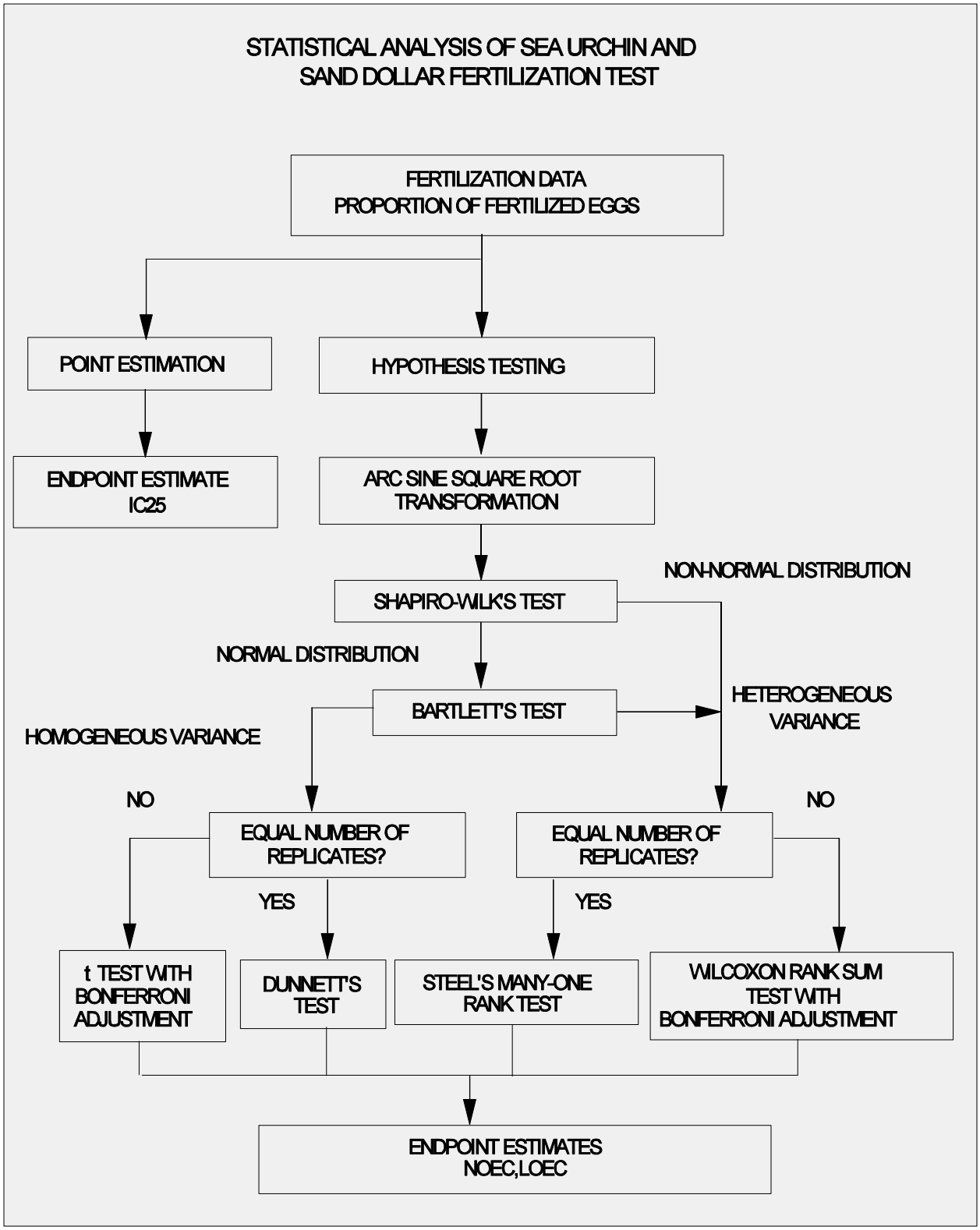


Figure 7. Flowchart for statistical analysis of sea urchin, *Strongylocentrotus purpuratus*, and sand dollar, *Dendraster excentricus*, test.

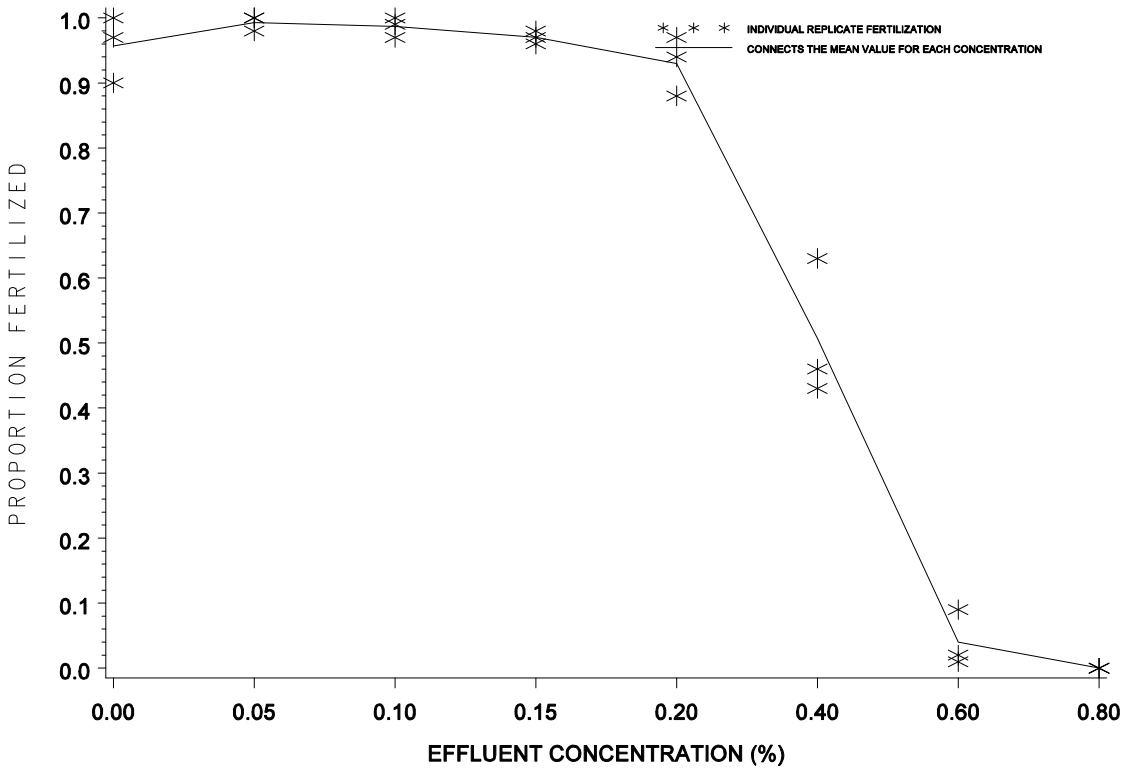


Figure 8. Plot of proportion of fertilized sea urchin, *Strongylocentrotus purpuratus*, eggs

16.13.2.5 Test for Normality

16.13.2.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 10.

TABLE 9. SEA URCHIN, ***STRONGYLOCENTROTUS PURPURATUS***, FERTILIZATION DATA

		Effluent Concentration (%)						
	Rep.	Control	0.05	0.10	0.15	0.20	0.40	0.60
	A	0.97	1.00	1.00	0.98	0.94	0.43	0.02
RAW	B	0.90	1.00	0.97	0.96	0.88	0.63	0.01
	C	1.00	0.98	0.99	0.97	0.97	0.46	0.09
ARC SINE	A	1.397	1.521	1.521	1.429	1.323	0.715	0.142
SQUARE ROOT	B	1.249	1.521	1.397	1.369	1.217	0.917	0.100
TRANSFORMED	C	1.521	1.429	1.471	1.397	1.397	0.745	0.305
Mean ($\bar{x}_i$)		1.389	1.490	1.463	1.398	1.312	0.792	0.182
S_i^2		0.01854	0.00282	0.00389	0.00090	0.00819	0.01188	0.01173
i		1	2	3	4	5	6	7

TABLE 10. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

	Effluent Concentration (%)						
Replicate	Control	0.05	0.10	0.15	0.20	0.40	0.60
A	0.008	0.031	0.058	0.031	0.011	-0.077	-0.040
B	-0.140	0.031	-0.066	-0.029	-0.095	0.125	-0.082
C	0.132	-0.061	0.008	-0.001	0.085	-0.047	0.123

16.13.2.5.2 Calculate the denominator, D, of the statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

$\bar{X}$ = the overall mean of the centered observations

n = the total number of centered observations

16.13.2.5.3 For this set of data, n = 21

$$\bar{X} = \frac{1}{21}(0.005) = 0.000$$

D = 0.1159

16.13.2.5.4 Order the centered observations from smallest to largest

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 11.

16.13.2.5.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2, ... a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 21 and k = 10. The a_i values are listed in Table 12.

16.13.2.5.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i (X^{(n-i+1)} - X^{(i)})\right]^2$$

The differences, $X^{(n-i+1)} - X^{(i)}$, are listed in Table 12. For the data in this example:

$$W = \frac{1}{0.1159}(0.3345)^2 = 0.9654$$

16.13.2.5.7 The decision rule for this test is to compare W as calculated in 2.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For the data in this

TABLE 11. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.140	12	0.008
2	-0.095	13	0.011
3	-0.082	14	0.031
4	-0.077	15	0.031
5	-0.066	16	0.031
6	-0.061	17	0.058
7	-0.047	18	0.085
8	-0.040	19	0.123
9	-0.029	20	0.125
10	-0.001	21	0.132
11	0.008		

TABLE 12. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4643	0.272	$X^{(21)} - X^{(1)}$
2	0.3185	0.220	$X^{(20)} - X^{(2)}$
3	0.2578	0.205	$X^{(19)} - X^{(3)}$
4	0.2119	0.162	$X^{(18)} - X^{(4)}$
5	0.1736	0.124	$X^{(17)} - X^{(5)}$
6	0.1399	0.092	$X^{(16)} - X^{(6)}$
7	0.1092	0.078	$X^{(15)} - X^{(7)}$
8	0.0804	0.071	$X^{(14)} - X^{(8)}$
9	0.0530	0.040	$X^{(13)} - X^{(9)}$
10	0.0263	0.009	$X^{(12)} - X^{(10)}$

example, the critical value at a significance level of 0.01 and n = 21 observations is 0.873. Since W = 0.9654 is greater than the critical value, conclude that the data are normally distributed.

16.13.2.6 Test for Homogeneity of Variance

16.13.2.6.1 The test used to examine whether the variation in the proportion of fertilized eggs is the same across all effluent

concentrations including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{[(\sum_{i=1}^{P} V_i) \ln \overline{S}^2 - \sum_{i=1}^{P} V_i \ln S_i^2]}{C}$$

Where: V_i = degrees of freedom for each concentration and control,

$V_i = (n_i - 1)$

p = number of concentration levels including the control

n_i = the number of replicates for concentration i.

ln = $\log_e$

i = 1,2, ..., p where p is the number of concentrations including the control

$$\overline{S}^2 = \frac{(\sum_{i=1}^{P} V_i S_i^2)}{\sum_{i=1}^{P} V_i} \qquad C = 1 + [3(p-1)]^{-1}[\sum_{i=1}^{P} 1/V_i - (\sum_{i=1}^{P} V_i)^{-1}]$$

16.13.2.6.2 For the data in this example (see Table 8), all effluent concentrations including the control have the same number of replicates (n_i = 3 for all i). Thus, V_i = 2 for all i.

16.13.2.6.3 Bartlett's statistic is, therefore:

$$B = [(14)\ln(0.008279) - 2\sum_{i=1}^{P} \ln(S_i^2)] / 1.1905$$

= [14(-4.7940) - 2(-36.1047)]/1.1905

= 5.0934/1.1905

= 4.2784

16.13.2.6.4 B is approximately distributed as chi-square with p-1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with 6 degrees of freedom, is 16.81. Since B = 4.2784 is less than the critical value of 16.81, conclude that the variances are not different.

16.13.2.7 Dunnett's Procedure

16.13.2.7.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 13.

TABLE 13. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2 = SSW/(N-p)
Total	N - 1	SST	

Where: p = number of concentration levels including the control

N = total number of observations $n_1 + n_2 \ldots + n_p$

n_i = number of observations in concentration i

$SSB = \sum_{i=1}^{P} T_i^2/n_i - G^2/N$ Between Sum of Squares

$SST = \sum_{i=1}^{P}\sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$ Total Sum of Squares

$SSW = SST - SSB$ Within Sum of Squares

G = the grand total of all sample observations, $G = \sum_{i=1} T_i$

T_i = the total of the replicate measurements for concentration i

Y_{ij} = the jth observation for concentration i (represents the proportion of fertilized eggs for concentration i in test chamber j)

16.13.2.7.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = n_6 = n_7 = 3$

$N = 21$

$T_1 = Y_{11} + Y_{12} + Y_{13} = 4.167$
$T_2 = Y_{21} + Y_{22} + Y_{23} = 4.471$
$T_3 = Y_{31} + Y_{32} + Y_{33} = 4.389$
$T_4 = Y_{41} + Y_{42} + Y_{43} = 4.194$
$T_5 = Y_{51} + Y_{52} + Y_{53} = 3.937$
$T_6 = Y_{61} + Y_{62} + Y_{63} = 2.377$
$T_7 = Y_{71} + Y_{72} + Y_{73} = 0.547$

$G = T_1 + T_2 + T_3 + T_4 + T_5 + T_6 + T_7 = 24.082$

$$SSB = \sum_{i=1}^{P} T_i^2/n_i - G^2/N$$
$$= (95.656)/3 - (24.082)^2/21 = 4.269$$

$$SST = \sum_{i=1}^{P}\sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$
$$= 32.001 - (24.082)^2/21 = 4.385$$

$SSW = SST - SSB = 4.385 - 4.269 = 0.116$

$S_B^2 = SSB/(p-1) = 4.269/(7-1) = 0.7115$

$S_W^2 = SSW/(N-p) = 0.116/(21-7) = 0.0083$

16.13.2.7.3 Summarize these calculations in the ANOVA table (Table 14).

16.13.2.7.4 To perform the individual comparisons, calculate the ***t*** statistic for each concentration, and control combination as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_w\sqrt{(1/n_1)+(1/n_i)}}$$

TABLE 14. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	6	4.269	0.7115
Within	14	0.116	0.0083
Total	20	4.385	

Where: $\bar{x}_i$ = mean proportion fertilized eggs for concentration i

$\bar{x}_1$ = mean proportion fertilized eggs for the control

S_W = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i.

Since we are looking for a decreased response from the control in the proportion of fertilized eggs, the concentration mean is subtracted from the control mean.

16.13.2.7.5 Table 15 includes the calculated ***t*** values for each concentration and control combination. In this example, comparing the 0.05% concentration with the control the calculation is as follows:

$$t_2 = \frac{(1.389 - 1.490)}{0.0911\sqrt{(1/3)+(1/3)}} = -1.358$$

16.13.2.7.6 Since the purpose of this test is to detect a significant decrease in the proportion of fertilized eggs, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix C. For an overall alpha level of 0.05, 14 degrees of freedom for error and six concentrations (excluding the control) the critical value is 2.53. The mean proportion of fertilized eggs for concentration i is considered significantly less than the mean proportion of

fertilized eggs for the control if t_i is greater than the critical value. Therefore, the 0.40% and 0.60% concentrations have a significantly lower mean proportion of fertilized eggs than the control. Hence the NOEC is 0.20% effluent and the LOEC is 0.40% effluent.

TABLE 15. CALCULATED t VALUES

Effluent Concentration (%)	i	t_i
0.05	2	-1.358
0.10	3	-0.995
0.15	4	-0.121
0.20	5	1.035
0.40	6	8.026
0.60	7	16.227

16.13.2.7.7 To quantify the sensitivity of the test, the minimum significant difference (MSD) that can be statistically detected may be calculated:

$$MSD = d\, S_w \sqrt{(1/n_1)\%(1/n)}$$

Where: d = the critical value for Dunnett's Procedure

S_W = the square root of the within mean square

n = the common number of replicates at each concentration (this assumes equal replication at each concentration)

n_1 = the number of replicates in the control.

16.13.2.7.8 In this example,

$$MSD = 2.53\,(0.0911)\sqrt{(1/3)\,\%\,(1/3)}$$

$$= 2.53\ (0.0911)(0.8165)$$

$$= 0.188$$

16.13.2.7.9 The MSD (0.188) is in transformed units. To determine the MSD in terms of proportion of fertilized eggs, carry out the following conversion.

1. Subtract the MSD from the transformed control mean.

$$1.389 - 0.188 = 1.201$$

2. Obtain the untransformed values for the control mean and the difference calculated in step 1 of 13.2.7.9.

$$[\ \text{Sine}\ (1.389)\ ]^2 = 0.967$$

$$[\ \text{Sine}\ (1.201)\ ]^2 = 0.869$$

3. The untransformed MSD (MSD_u) is determined by subtracting the untransformed values from step 2 in 14.2.7.9.

$$MSD_u = 0.967 - 0.869 = 0.098$$

16.13.2.7.10 Therefore, for this set of data, the minimum difference in mean proportion of fertilized eggs between the control and any effluent concentration that can be detected as statistically significant is 0.098.

16.13.2.7.11 This represents a 10.2% decrease in the proportion of fertilized eggs from the control.

16.13.2.8 Calculation of the ICp

16.13.2.8.1 The fertilization data in Table 7 are utilized in this example. As can be seen from Figure 8, the observed means are not monotonically non-increasing with respect to concentration. Therefore, the means must be smoothed prior to calculating the IC.

16.13.3.8.2 Starting with the observed control mean, $\overline{Y}_1 = 0.957$, and the observed mean for the lowest effluent concentration, $\overline{Y}_2 = 0.993$, we see that $\overline{Y}_1$ is less than $\overline{Y}_2$.

16.13.3.8.3 Calculate the smoothed means:

$$M_1 = M_2 = (\overline{Y}_1 + \overline{Y}_2)/2 = 0.975$$

16.13.3.8.4 Since $\overline{Y}_3 = 0.987$ is larger than M_2, average $\overline{Y}_3$ with the previous concentrations:

$$M_1 = M_2 = M_3 = (M_1 + M_2 + \overline{Y}_3)/3 = 0.979.$$

16.13.3.8.5 Since $M_3 > \overline{Y}_4 = 0.970 > \overline{Y}_5 = 0.930 > \overline{Y}_6 = 0.507 > \overline{Y}_7 = 0.040 > \overline{Y}_8 = 0.0$, set $M_4 = 0.970$, $M_5 = 0.930$, $M_6 = 0.507$, $M_7 = 0.040$, and $M_8 = 0.0$. Table 16 contains the smoothed means and Figure 10 gives a plot of the smoothed means and the interpolated response curve.

16.13.2.8.6 An IC25 can be estimated using the Linear Interpolation Method. A 25% reduction in mean proportion of fertilized eggs, compared to the controls, would result in a mean

proportion of 0.734, where $M_1(1-p/100)$ = 0.979(1-25/100). Examining the means and their associated concentrations (Table 16), the response, 0.734, is bracketed by C_5 = 0.20% effluent and C_6 = 0.40% effluent.

16.13.2.8.7 Using the equation from Section 4.2 in Appendix L, the estimate of the IC25 is calculated as follows:

$$ICp \text{ ' } C_j\%[M_1(1\&p/100)\&M_j]\frac{(C_{(j\%1)}\&C_j)}{(M_{(j\%1)}\&M_j)}$$

$$IC25 = 0.20 + [0.979(1 - 25/100) - 0.930] \frac{(0.40 - 0.20)}{(0.507 - 0.930)}$$

$$= 0.29\%.$$

TABLE 16. SEA URCHIN, ***STRONYLOCENTROTUS PURPURATUS***, MEAN PROPORTION OF FERTILIZED EGGS

Effluent Conc. (%)	i	Response Means, $\overline{Y}_i$ (proportion)	Smoothed Means, M_i (proportion)
Control	1	0.957	0.979
0.05	2	0.993	0.979
0.10	3	0.987	0.979
0.15	4	0.970	0.970
0.20	5	0.930	0.930
0.40	6	0.507	0.507
0.60	7	0.040	0.040
0.80	8	0.000	0.000

16.13.2.8.8 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 0.2925%. The empirical 95.0% confidence interval for the true mean was 0.2739% to 0.3241%. The computer program output for the IC25 for this data set is shown in Figure 10.

16.14 **PRECISION AND ACCURACY**

16.14.1 PRECISION

16.14.1.1 Single-Laboratory Precision

16.14.1.1.1 Single-laboratory precision data for ***Strongylocentrotus purpurtatus*** with the reference toxicant copper, tested in natural seawater, are provided in Table 17. The coefficient of variation based on the EC25 is 29%, and on EC50 is 24%, showing acceptable precision. Single-laboratory precision data for ***Dendraster excentricus*** with the reference toxicant copper, tested in natural seawater, are provided in Tables 18 and 19. The coefficient of variation based on the EC25, is 18% to 29% and EC50, is 21% to 33%, showing acceptable precision.

16.14.1.2 Multi-laboratory Precision

16.14.1.2.1 Multi-laboratory precision data for ***Strongylocentrotus purpuratus,*** with the reference toxicant copper, tested in natural seawater, are provided in Table 20. The coefficient of variation for the EC25 was 52%, based on data from five laboratories.

16.14.2 ACCURACY

16.14.2.1 The accuracy of toxicity tests cannot be determined.

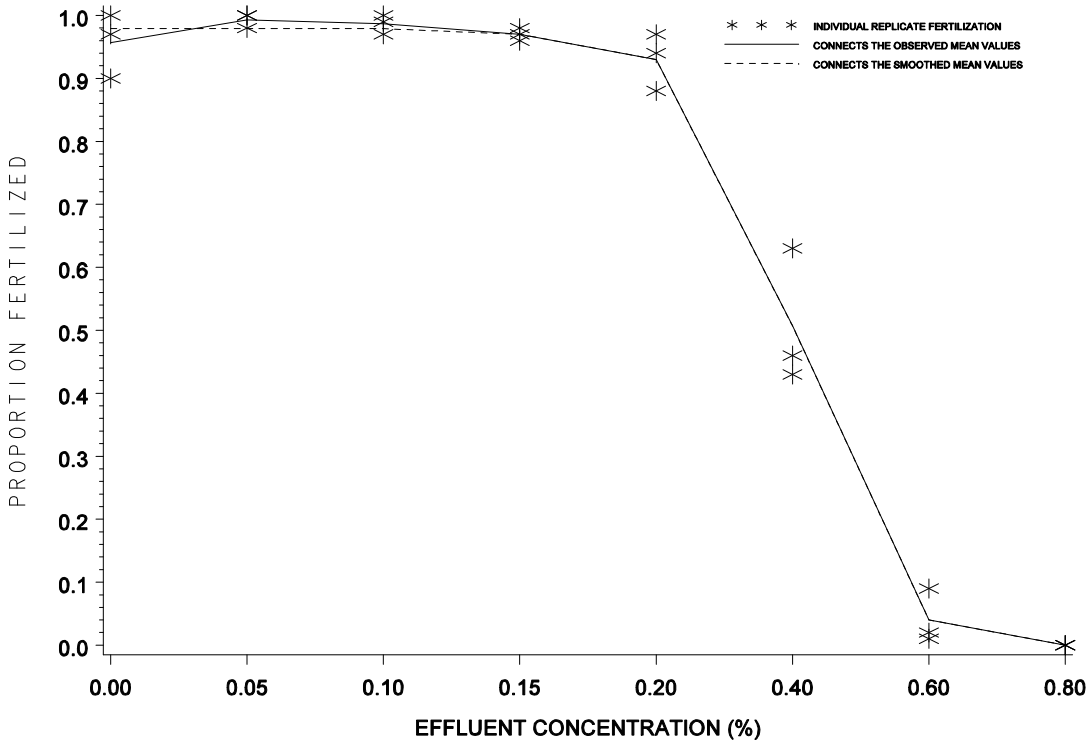


Figure 9. Plot of raw data, observed means, and smoothed means for the sea urchin, *Strongylocentrotus purpuratus*, eggs.

```
Conc. ID        1    2    3    4    5    6    7    8
-------------------------------------------------
Conc. Tested    0  .05  .10  .15  .20  .40  .60  .80
-------------------------------------------------
Response  1   .97 1.00 1.00  .98  .94  .43  .02    0
Response  2   .90 1.00  .97  .96  .88  .63  .01    0
Response  3  1.00  .98  .99  .97  .97  .46  .09    0
-------------------------------------------------
*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Effluent
Test Start Date:    Test Ending Date:
Test Species: Sea Urchin, Strongylocentrotus purpuratus
Test Duration:      40 minutes
DATA FILE: urchin.icp
OUTPUT FILE: urchin.i25
-------------------------------------------------------------------------
Conc.     Number     Concentration     Response      Std.       Pooled
 ID     Replicates                %       Means       Dev.   Response Means
-------------------------------------------------------------------------
  1          3             0.000          0.957      0.051      0.979
  2          3             0.050          0.993      0.012      0.979
  3          3             0.100          0.987      0.015      0.979
  4          3             0.150          0.970      0.010      0.970
  5          3             0.200          0.930      0.046      0.930
  6          3             0.400          0.507      0.108      0.507
  7          3             0.600          0.040      0.044      0.040
  8          3             0.800          0.000      0.000      0.000
-------------------------------------------------------------------------
The Linear Interpolation Estimate:     0.2925   Entered P Value: 25
-------------------------------------------------------------------------
Number of Resamplings:   80
The Bootstrap Estimates Mean:   0.2917 Standard Deviation:     0.0141
Original Confidence Limits:   Lower:     0.2739 Upper:     0.3241
Expanded Confidence Limits:   Lower:     0.2533 Upper:     0.3589
Resampling time in Seconds:     0.22  Random_Seed: -25579058
```

Figure 10. ICPIN program output for the IC25.

TABLE 17. SINGLE LABORATORY PRECISION OF THE SEA URCHIN, ***STRONGYLOCENTROTUS PURPURATUS*** FERTILIZATION TEST PERFORMED IN SEAWATER USING GAMETES FROM ADULTS MAINTAINED IN SEAWATER AFTER BEING COLLECTED FROM NATURAL POPULATIONS WITH COPPER (CU µG/L) SULFATE AS THE REFERENCE TOXICANT

Test Number	NOEC (µg/L)	EC25 (µg/L)	EC50 (µg/L)
1	6.9	9.7	14.3
2	23.0	26.2	30.9
3	11.2	19.6	25.8
4	16.0	16.4	31.1
5	15.3	17.8	24.6
6	10.8	18.6	28.3
Mean CV(%)		18.1 29.0	25.8 24.0

Tests performed by Sally Noack, AScI, at EPA's Pacific Ecosystems Branch of ERL-Narragansett, Newport, OR.
Copper concentrations were measured and within 10% of nominal; nominal concentrations were 5, 8, 12, 17, 25, 35, and 50 µg/L.
These tests used only three replicates per concentration.

TABLE 18. SINGLE LABORATORY PRECISION OF THE SAND DOLLAR, ***DENDRASTER EXCENTRICUS*** FERTILIZATION TEST PERFORMED IN SEAWATER USING GAMETES FROM ADULTS MAINTAINED IN SEAWATER AFTER BEING COLLECTED FROM NATURAL POPULATIONS WITH COPPER (CU µG/L) SULFATE AS THE REFERENCE TOXICANT

Test Date	Test Number	NOEC (µg/L)	EC25 (µg/L)	EC50 (µg/L)
7/11/94	1* 2** 3***	5.0 5.0 –	9.4 14.6 16.0	12.6 17.5 18.6
7/14/94	1* 2** 3***	12.0 <5.0 17.0	16.7 19.6 23.0	20.9 25.8 30.5
7/17/94	1* 2** 3***	8.0 5.0 12.0	15.3 13.5 13.4	17.7 16.4 17.0
7/19/94	1* 2** 3***	12.0 17.0 12.0	12.8 18.6 13.3	15.6 22.1 16.0
Mean	1 2 3 overall		13.5 16.6 16.4	16.7 20.5 20.5
SD	1 2 3 overall		3.2 3.0 4.6	3.5 4.3 6.7
CV(%)	1 2 3 overall		24% 18% 28%	21% 21% 33%

Tests performed at National Council of the Paper Industry for Air and Stream Improvement, Inc. Anacortes, WA.
Copper concentrations were nominal; nominal concentrations were 5, 8, 12, 17, 25, 35, and 50 µg/L.

* Tests conducted with nominal S:E ratio of 147:1
** Tests conducted with nominal S:E ratio of 166:1
*** Tests conducted with nominal S:E ratio of 224:1

TABLE 19. SINGLE LABORATORY PRECISION OF THE SAND DOLLAR, ***DENDRASTER EXCENTRICUS*** FERTILIZATION TEST PERFORMED IN SEAWATER USING GAMETES FROM ADULTS MAINTAINED IN SEAWATER AFTER BEING COLLECTED FROM NATURAL POPULATIONS WITH COPPER (CU µG/L) SULFATE AS THE REFERENCE TOXICANT.

Test Number	NOEC (µg/L)	EC25 (µg/L)	EC50 (µg/L)
1	17.0	25.8	31.0
2	25.0	34.3	41.8
3	12.0	31.1	43.7
4	8.0	14.2	19.8
5	25.0	27.2	30.5
Mean CV(%)		26.5 29.0	33.4 29.0

Tests performed by Gary Chapman and Debra Denton at EPA's Pacific Ecosystems Branch of ERL-Narragansett, Newport, OR.

Copper concentrations were nominal; nominal concentrations were 5, 8, 12, 17, 25, 35, and 50 µg/L.

TABLE 20. MULTIPLE LABORATORY PRECISION OF THE SEA URCHIN, ***STONGYLOCENTROTUS PURPURATUS***, FERTILIZATION TEST PERFORMED WITH COPPER (CU µG/L) SULFATE AS A REFERENCE TOXICANT

Lab	# of Tests	Statistic	EC25 (µg/L)
A	3	Mean SD CV(%)	7.8 3.0 38%
B	2	Mean SD CV(%)	4.0 - -
C	6	Mean SD CV(%)	18.0 5.4 30%
D	2	Mean NA CV(%)	14.9 - -
E	6	Mean SD CV(%)	19.3 10.5 54%

# of Lab Means	Statistic	EC25
5	Mean SD CV(%)	12.8 6.6 52%

Tests performed as part of a methods evaluation effort organized by the US EPA laboratory in Newport, Oregon; tests were conducted in 1991 by volunteer laboratories in California and Washington.

APPENDIX I. PURPLE URCHIN AND SAND DOLLAR TEST: STEP-BY-STEP SUMMARY

PREPARATION OF TEST SOLUTIONS

A. Determine test concentrations and appropriate dilution water based on NPDES permit conditions and guidance from the appropriate regulatory agency.

B. Prepare effluent test solutions by diluting well mixed unfiltered effluent using volumetric flasks and pipettes. Use hypersaline brine where necessary to maintain all test solutions at 34 ± 2‰. Include brine controls in tests that use brine.

C. Prepare a copper reference toxicant stock solution (2,000 mg/L) by adding 5.366 g of copper chloride ($CuCl_2$o2H_2O) to 1 liter of reagent water. For each reference toxicant test prepare a copper sub-stock of 3 mg/L by diluting 1.5 mL of stock to one liter with reagent water.

D. Prepare a control (0 Fg/L) plus at least five consecutive copper reference toxicant solutions (e.g., from the series 3.0, 4.4, 6.5, 9.5, 13.9, 20.4, and 30.0 Fg/L, by adding 0.10, 0.15, 0.22, 0.32, 0.46, 0.68, and 1.00 mL of sub-stock solution, respectively, to 100-L volumetric flasks and filling to 100-mL with dilution water).

E. Randomize numbers for test chambers and record the chamber numbers with their respective test concentrations on a randomization data sheet. Store the data sheet safely until after the test samples have been analyzed.

F. Sample effluent and reference toxicant solutions for physical/chemical analysis. Measure salinity, pH and dissolved oxygen from each test concentration.

G. Place test chambers in a water bath or environmental chamber set to 12EC and allow temperature to equilibrate.

H. Measure the temperature in several temperature blanks during the course of the test.

PREPARATION AND ANALYSIS OF TEST ORGANISMS

A. Obtain test organisms and hold or condition as necessary for spawning.

B. On day of test, spawn organisms, examine gametes, pool good eggs, pool good sperm.

C. Determine egg and sperm densities and adjust as necessary.

D. Run trial sperm:egg fertilization test (optional).

E. Adjust sperm density for definitive test.

F. Inject sperm into test solutions.

G. 20 minutes later inject eggs into test solutions.

H. 20 minutes after egg addition, stop the test by the addition of preservative.

I. Confirm sperm density in definitive test by hemacytometer counts.

J. Count at least 100 eggs in each test tube.

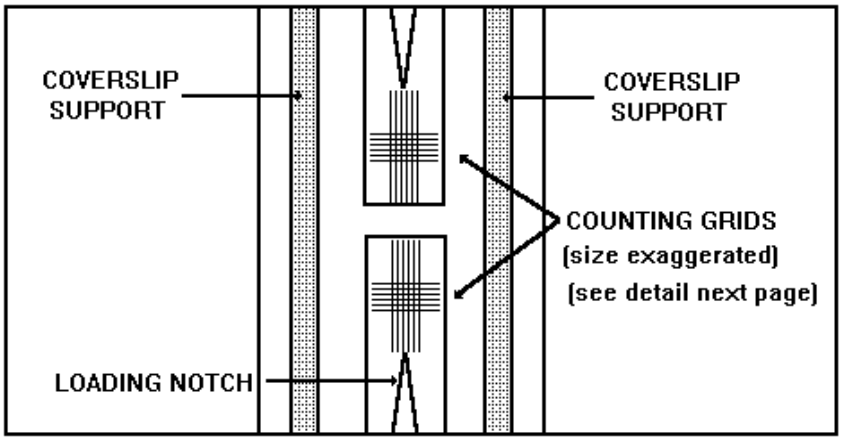
K. Analyze the data.

L. Include standard reference toxicant point estimate values in the standard quality control charts.

APPENDIX II. USING THE NEUBAUER HEMACYTOMETER TO ENUMERATE SEA URCHIN SPERM

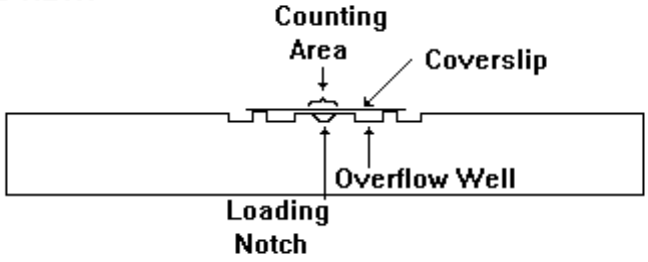
The Neubauer hemacytometer is a specialized microscope slide with two counting grids and a coverslip.

TOP VIEW:

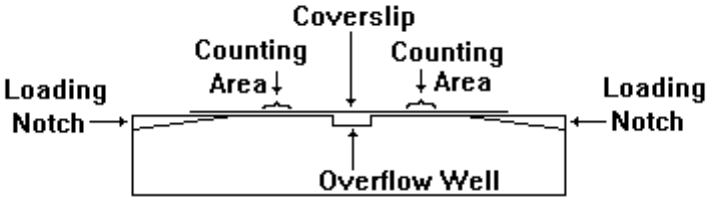


Together, the total area of each grid (1 mm^2) and the vertical distance between the grid and the coverslip (0.1 mm), provide space for a specific microvolume of aqueous sample (0.1 mm^3).

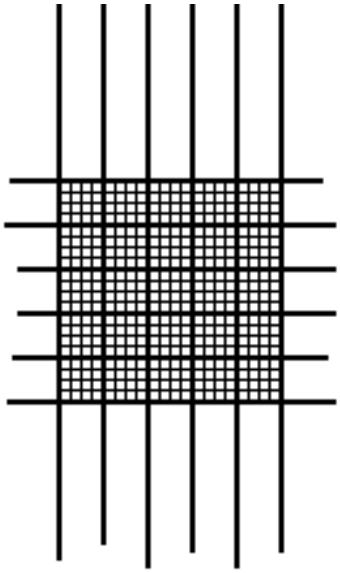
SIDE VIEW:



END VIEW THROUGH MID-CROSS SECTION:



This volume of liquid and the cells suspended therein (e.g., blood cells or sperm cells) represent 1/10,000th of the liquid volume and cell numbers of a full milliliter (cm^3) of the sampled material.



If the full 400-squares of each grid are counted, this represents the number of sperm in 0.1 mm^3. Multiplying this value times 10 yields the sperm per mm^3 (and is the source of the hemacytometer factor of 4,000 squares/mm^3). If this product is multiplied by 1,000 mm^3/cm^3, the answer is the number of sperm in one milliliter of the sample. If the counted sample represents a dilution of a more concentrated original sample, the above answer is multiplied by the dilution factor to yield the cell density in the original sample. If the cells are sufficiently dense, it is not necessary to count the entire 400-square field, and the final calculation takes into account the number of squares actually counted:

$$\text{cells/mL} = \frac{\text{(dilution) (4,000 squares/mm}^3\text{) (1,000 mm}^3\text{/cm}^3\text{) (cell count)}}{\text{(number of squares counted)}}$$

Thus, with a dilution of 4000 (0.025 mL of semen in 100 mL of dilution water), 80 squares counted, and a count of 100, the calculation becomes:

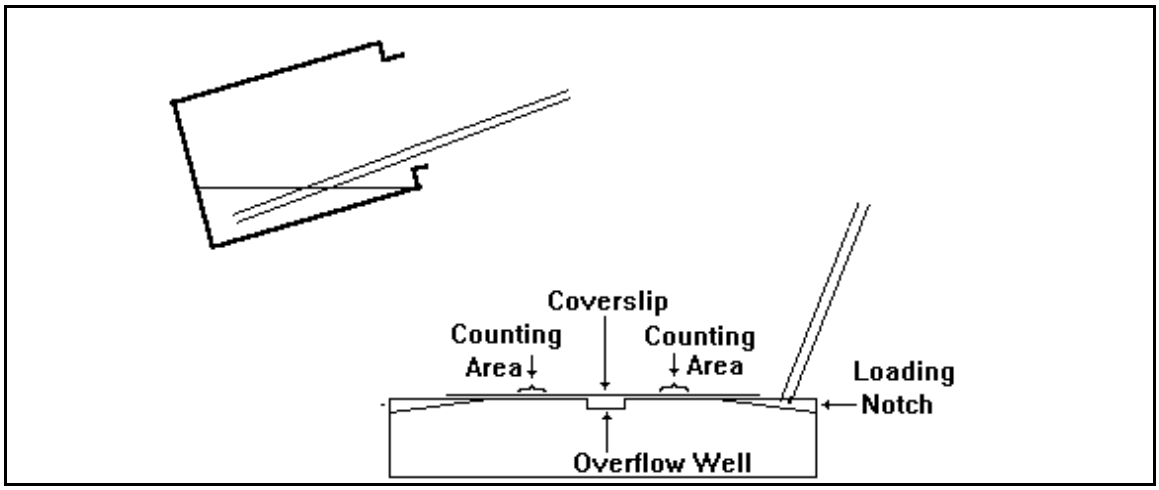
$$\text{cells/mL} = \frac{(4{,}000)\ (4{,}000)\ (1{,}000)\ (100)}{80}$$

$$= 20{,}000{,}000{,}000 \text{ cells/mL}$$

There are several procedures that are necessary for counts to be consistent within and between laboratories. These include mixing the sample, loading and emptying the hematocrit tube, cleaning the hemacytometer and cover slip, and actual counting procedures.

Obviously, if the sample is not homogeneous, subsamples can vary in sperm density. A few extra seconds in mixing can save a lot of wasted minutes in subsequent counting procedures. A full hematocrit tube empties more easily than one with just a little liquid, so withdraw a full sample. This can be expedited by tipping the sample vial.

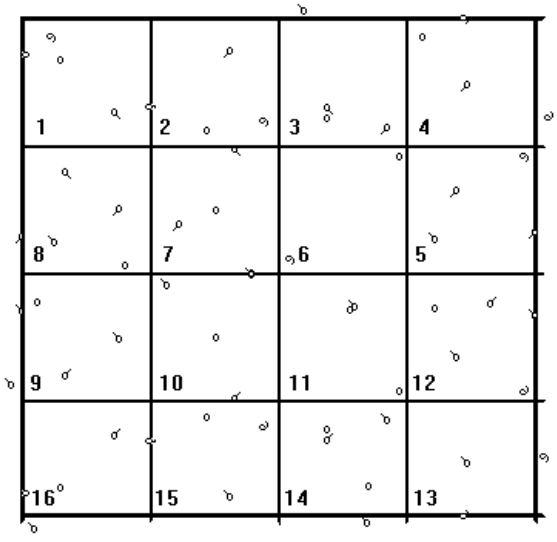
Because the sperm are killed prior to sampling, they will slowly settle. For this reason, the sample in the hematocrit tube should be loaded onto the hemacytometer as rapidly as possible. Two replicate samples are withdrawn in fresh hematocrit tubes and loaded onto opposite sides of a hemacytometer.



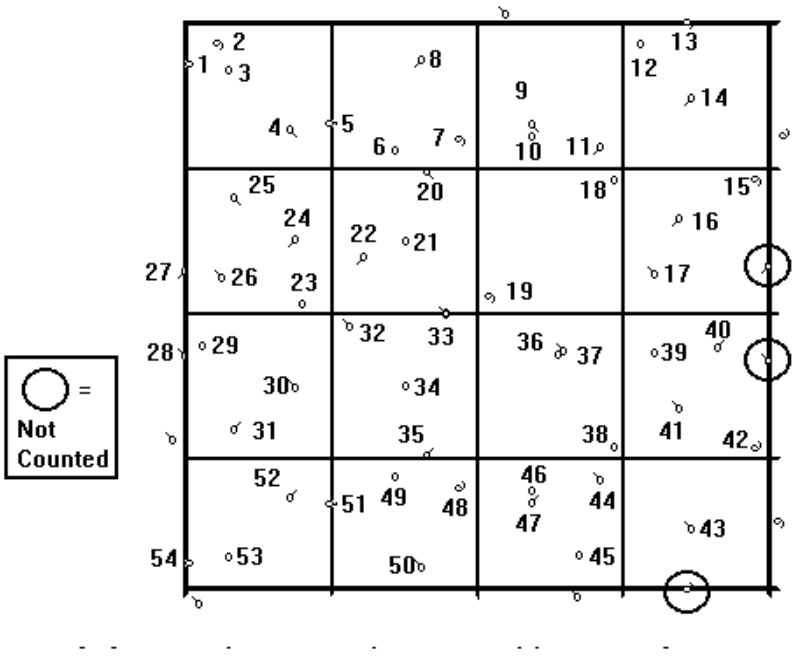
The loaded hemacytometer is left for 15 minutes to allow the sperm to settle onto the counting field. If the coverslip is moved after the samples are loaded, the hemacytometer should be rinsed and refilled with fresh sample. After 15 minutes, the hemacytometer is placed under a microscope and the counting grid located at 100x. Once the grid is properly positioned, the microscope is adjusted to 200x or 400x, and one of the corner squares is positioned for counting (any one of the four corners is appropriate). For consistency, use the same procedure each time (Many prefer to start in the upper left corner of the optical field, and this procedure will be used in the examples given below).

Examine the first large square in the selected corner. If no sperm are visible, or if the sperm are so dense or clumped to preclude accurate counting, count a sample with a more appropriate dilution.

In making counts of sperm, it is necessary to adopt a consistent method of scanning the smaller squares and counting sperm that fall upon the lines separating the squares. Count the sperm in the small squares by beginning in the upper left hand corner (square 1) and proceding right to square 4, down to square 5, left to square 8, etc. until all 16 squares are counted.



Because sperm that appear <u>on</u> lines might be counted as being in either square, it is important to avoid double counting or non-counting. For this reason a convention is decided upon and used consistently: paraphrasing the instructions received with one (Hausser Scientific) counting chamber "to avoid counting (sperm) twice, the best practice is to count all touching the top and left, and none touching the lower and right, boundary lines." Whatever convention is chosen, it must be adhered to. The example below shows a sperm count based upon a selected convention of counting sperm that fall on the upper and left lines, but not on the lower or right lines:



In the above illustration, sperm falling on the lower and right lines are not counted. The count begins at the upper left as illustrated in the preceding figure. A typical count sequence is demonstrated by the numbers next to each sperm illustrated. Sperm identified as numbers 1, 5, 13, 20, 27, 28, 33, 51 and 54 touch lines and are counted as being in the square below them or to their right. The circled sperm are not counted as being in this field of 16 small squares (but they would be included in any counts of adjacent squares in which they would be on upper or left hand lines).

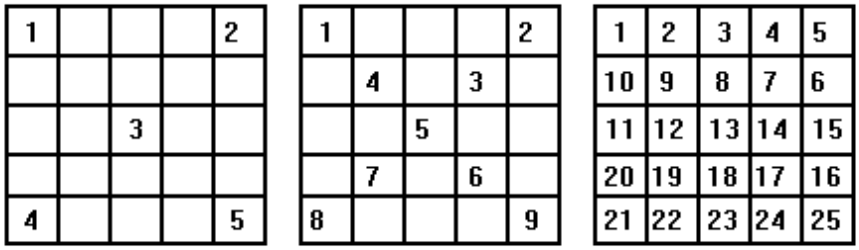
Once these counting conventions have been selected, it is advisable to follow another strict protocol outlining the number and sequence of large squares to be counted. Because the sperm may not be randomly distributed across the counting grid, it is recommended to count an array of squares covering the entire grid. The following procedure is recommended:

Count the number of sperm in the first large square.

1. If the number is less than 10, count all 25 squares using the same scanning pattern outlined above (left to right through squares 1 to 5, down to square 6, left through square 10, down to 11, etc.). See pattern no. 3.

2. If the number is between 10 and 19, count 9 large squares using pattern no. 2.

3. If the number is 20 or greater, count 5 large squares using pattern no. 1.



Pattern no. 1 Pattern no. 2 Pattern no. 3

The final consideration in achieving good replicate counts is keeping the hemacytometers and coverslips clean. They should be rinsed in distilled water soon after use. The coverslips should be stored in a good biocleanser such as hemasol. For an hour or so prior to use, the hemacytometer slides should also be soaked in the solution. Both slides and coverslips should then be rinsed off with reagent water, blotted dry with a lint-free tissue, and wiped with lens paper.

SECTION 17

GIANT KELP, *Macrocystis pyrifera* GERMINATION AND GERM-TUBE GROWTH TEST METHOD

Adapted from a method developed by
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SECTION 17

GIANT KELP, *MACROCYSTIS PYRIFERA* GERMINATION AND GROWTH TEST

17.1 SCOPE AND APPLICATION

17.1.1 This method estimates the chronic toxicity of effluents and receiving water to zoospores and embryonic gametophytes of giant kelp, ***Macrocystis pyrifera*** during a 48-h static non-renewal exposure. The effects include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms.

17.1.2 Detection limits of the toxicity of an effluent or chemical substance are organism dependent.

17.1.3 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling and because the test chambers are not sealed, highly volatile and highly degradable toxicants in the source may not be detected in the test.

17.1.4 This method is commonly used in one of two forms: (1) a definitive test, consisting of minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

17.1.5 This method should be restricted to use by, or under the supervision of, professionals experienced in aquatic toxicity testing. Specific experience with any toxicity test is usually needed before acceptable results become routine.

17.2 SUMMARY OF METHOD

17.2.1 This method provides step-by-step instructions for performing a 48-h day static non-renewal toxicity test using giant kelp to determine the toxicity of substances in marine and

estuarine waters. The test endpoints are germination of gameophyte spores and length of embryonic gametophyte germination tubes.

17.3 **INTERFERENCES**

17.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities and Equipment, and Supplies).

17.3.2 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests).

17.4 **SAFETY**

17.4.1 See Section 3, Health and Safety.

17.5 **APPARATUS AND EQUIPMENT**

17.5.1 Tanks, trays, or aquaria -- for holding and acclimating giant kelp, e.g., standard salt water aquarium or Instant Ocean Aquarium (capable of maintaining seawater at 10-20EC), with appropriate filtration and aeration system.

17.5.2 Air pump, air lines, and air stones -- for aerating water containing broodstock or for supplying air to test solutions with low dissolved oxygen.

17.5.3 Constant temperature chambers or water baths -- for maintaining test solution temperature and keeping dilution water supply, gametes, and embryo stock suspensions at test temperature (15EC) prior to the test.

17.5.4 Water purification system -- Millipore Super-Q, Deionized water (DI) or equivalent.

17.5.5 Refractometer -- for determining salinity.

17.5.6 Hydrometer(s) -- for calibrating refractometer.

17.5.7 Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

17.5.8 Thermometer, National Bureau of Standards Certified (see USEPA METHOD l70.l, USEPA, l979) -- to calibrate laboratory thermometers.

17.5.9 pH and DO meters -- for routine physical and chemical measurements.

17.5.10 Standard or micro-Winkler apparatus -- for determining DO (optional) and calibrating the DO meter.

17.5.11 Winkler bottles -- for dissolved oxygen determinations.

17.5.12 Balance -- Analytical, capable of accurately weighing to 0.0001 g.

17.5.13 Fume hood -- to protect the analyst from effluent or formaldehyde fumes.

17.5.14 Glass stirring rods -- for mixing test solutions.

17.5.15 Graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 50-l000 mL for making test solutions. (Note: not to be used interchangeably for gametes or embryos and test solutions).

17.5.16 Volumetric flasks -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making test solutions.

17.5.17 Pipets, automatic -- adjustable, to cover a range of delivery volumes from 0.010 to 1.000 mL.

17.5.18 Pipet bulbs and fillers -- PROPIPET® or equivalent.

17.5.19 Wash bottles -- for reagent water, for topping off graduated cylinders, for rinsing small glassware and instrument electrodes and probes.

17.5.20 Wash bottles -- for dilution water.

17.5.21 20-liter cubitainers or polycarbonate water cooler jugs -- for making hypersaline brine.

17.5.22 Cubitainers, beakers, or similar chambers of non-toxic composition for holding, mixing, and dispensing dilution water and other general non-effluent, non-toxicant contact uses. These should be clearly labeled and not used for other purposes.

17.5.23 Beakers, 250 borosilicate glass -- for mixing test solutions.

17.5.24 Beakers, 1,000 mL borosilicate glass -- for holding sporophyll blades.

17.5.25 Inverted or compound microscope -- for inspecting zoopspores and embryonic gametophytes.

17.5.26 Hemacytometer (bright-line rbc) -- for measuring zoospore density.

17.5.27 Counter, two unit, 0-999 -- for recording counts of zoopspores.

17.5.28 Light meter (irradiance meter w/cosine corrected sensor) -- for measuring light intensity.

17.5.29 Cool white fluorescent lights -- for providing light during incubation of developing gametophytes.

17.5.30 60 µm NITEX® filter -- for filtering receiving water.

17.6 **REAGENTS AND SUPPLIES**

17.6.1 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests).

17.6.2 Data sheets (one set per test) -- for data recording (Figures 1 and 2).

17.6.3 Tape, colored -- for labelling test chambers and containers.

17.6.4 Markers, water-proof -- for marking containers, etc.

17.6.5 Parafilm -- to cover graduated cylinders and vessels.

17.6.6 Gloves, disposable -- for personal protection from contamination.

17.6.7 Pipets, serological -- 1-10 mL, graduated.

17.6.8 Pipet tips -- for automatic pipets.

17.6.9 Coverslips -- for microscope slides.

17.6.10 Lens paper -- for cleaning microscope optics.

17.6.11 Laboratory tissue wipes -- for cleaning and drying electrodes, microscope slides, etc.

17.6.12 Disposable countertop covering -- for protection of work surfaces and minimizing spills and contamination.

17.6.13 pH buffers 4, 7, and 10 (or as per instructions of instrument manufacturer) -- for standards and calibration check (see USEPA Method 150.1, USEPA, 1979).

17.6.14 Membranes and filling solutions -- for dissolved oxygen probe (see USEPA Method 360.1, USEPA, 1979), or reagents for modified Winkler analysis.

17.6.15 Laboratory quality assurance samples and standards -- for the above methods.

17.6.16 Test chambers -- 600 mL, five chambers per concentration. The chambers should be borosilicate glass (for effluents) or nontoxic disposable plastic labware (for reference toxicants). To avoid contamination from the air and excessive evaporation of test solutions during the test, the chambers should be covered during the test with safety glass plates or a plastic sheet (6 mm thick).

17.6.17 Glutaraldehyde -- for specimen preservation - optional; (see Section 17.10.8.2).

17.6.18 Microscope slide (flat) -- for each test chamber to serve as the substratum upon which the zoospores will settle.

17.6.19 Reference toxicant solutions (see Section 17.10.2.4 and see Section 4, Quality Assurance).

17.6.20 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms (see Section 5, Facilities, Equipment, and Supplies and Section 7, Dilution Water).

17.6.21 Effluent and receiving water -- see Section 8, Effluent and Surface Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests.

17.6.22 Dilution water and hypersaline brine -- see Section 7, Dilution Water and Section 17.6.24, Hypersaline Brines. The dilution water should be uncontaminated 1-µm-filtered natural seawater. Hypersaline brine should be prepared from dilution water.

17.6.23 HYPERSALINE BRINES

17.6.23.1 Most industrial and sewage treatment effluents entering marine and estuarine systems have little measurable salinity. Exposure of larvae to these effluents will usually require increasing the salinity of the test solutions. It is important to maintain an essentially constant salinity across all treatments. In some applications it may be desirable to match the test salinity with that of the receiving water (See Section 7.1). Two salt sources are available to adjust salinities -- artificial sea salts and hypersaline brine (HSB) derived from natural seawater. Use of artificial sea salts is necessary only when high effluent concentrations preclude salinity adjustment by HSB alone.

17.6.23.2 Hypersaline brine (HSB) can be made by concentrating natural seawater by freezing or evaporation. HSB should be made from high quality, filtered seawater, and can be added to the effluent or to reagent water to increase salinity. HSB has several desirable characteristics for use in effluent toxicity testing. Brine derived from natural seawater contains the necessary trace metals, biogenic colloids, and some of the

microbial components necessary for adequate growth, survival, and/or reproduction of marine and estuarine organisms, and it can be stored for prolonged periods without any apparent degradation. However, even if the maximum salinity HSB (100‰) is used as a diluent, the maximum concentration of effluent (0‰) that can be tested is 66% effluent at 34‰ salinity (see Table 1).

TABLE 1. MAXIMUM EFFLUENT CONCENTRATION (%) THAT CAN BE TESTED AT 34‰ WITHOUT THE ADDITION OF DRY SALTS GIVEN THE INDICATED EFFLUENT AND BRINE SALINITIES.

Effluent Salinity ‰	Brine 60 ‰	Brine 70 ‰	Brine 80 ‰	Brine 90 ‰	Brine 100 ‰
0	43.33	51.43	57.50	62.22	66.00
1	44.07	52.17	58.23	62.92	66.67
2	44.83	52.94	58.97	63.64	67.35
3	45.61	53.73	59.74	64.37	68.04
4	46.43	54.55	60.53	65.12	68.75
5	47.27	55.38	61.33	65.88	69.47
10	52.00	60.00	65.71	70.00	73.33
15	57.78	65.45	70.77	74.67	77.65
20	65.00	72.00	76.67	80.00	82.50
25	74.29	80.00	83.64	86.15	88.00

17.6.23.3 High quality (and preferably high salinity) seawater should be filtered to at least 10 µm before placing into the freezer or the brine generator. Water should be collected on an incoming tide to minimize the possibility of contamination.

17.6.23.4 Freeze Preparation of Brine

17.6.23.4.1 A convenient container for making HSB by freezing is one that has a bottom drain. One liter of brine can be made from

four liters of seawater. Brine may be collected by partially freezing seawater at -10 to -20EC until the remaining liquid has reached the target salinity. Freeze for approximately six hours, then separate the ice (composed mainly of fresh water) from the remaining liquid (which has now become hypersaline).

17.6.23.4.2 It is preferable to monitor the water until the target salinity is achieved rather than allowing total freezing followed by partial thawing. Brine salinity should never exceed 100‰. It is advisable not to exceed about 70‰ brine salinity unless it is necessary to test effluent concentrations greater than 50%.

17.6.23.4.3 After the required salinity is attained, the HSB should be filtered through a 1 µm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4EC (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

17.6.23.5 Heat Preparation of Brine

17.6.23.5.1 The ideal container for making brine using heat-assisted evaporation of natural seawater is one that (1) has a high surface to volume ratio, (2) is made of a non-corrosive material, and (3) is easily cleaned (fiberglass containers are ideal). Special care should be used to prevent any toxic materials from coming in contact with the seawater being used to generate the brine. If a heater is immersed directly into the seawater, ensure that the heater materials do not corrode or leach any substances that would contaminate the brine. One successful method is to use a thermostatically controlled heat exchanger made from fiberglass. If aeration is needed, use only oil-free air compressors to prevent contamination.

17.6.23.5.2 Before adding seawater to the brine generator, thoroughly clean the generator, aeration supply tube, heater, and any other materials that will be in direct contact with the brine. A good quality biodegradable detergent should be used, followed by several (at least three) thorough reagent water rinses.

17.6.23.5.3 Seawater should be filtered to at least 10 µm before being put into the brine generator. The temperature of the seawater is increased slowly to 40EC. The water should be aerated to prevent temperature stratification and to increase water evaporation. The brine should be checked daily (depending on the volume being generated) to ensure that the salinity does not exceed 100% and that the temperature does not exceed 40EC. Additional seawater may be added to the brine to obtain the volume of brine required.

17.6.23.5.4 After the required salinity is attained, the HSB should be filtered through a 1 µm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4EC (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

17.6.23.6 Artificial Sea Salts

17.6.23.6.1 No data from giant kelp tests using sea salts or artificial seawater (e.g., GP2) are available for evaluation at this time, and their use must be considered provisional.

17.6.23.7 Dilution Water Preparation from Brine

17.6.23.7.1 Although salinity adjustment with brine is the preferred method, the use of high salinity brines and/or reagent water has sometimes been associated with discernible adverse effects on test organisms. For this reason, it is recommended that only the minimum necessary volume of brine and reagent water be used to offset the low salinity of the effluent, and that brine controls be included in the test. The remaining dilution water should be natural seawater. Salinity may be adjusted in one of two ways. First, the salinity of the highest effluent test concentration may be adjusted to an acceptable salinity, and then serially diluted. Alternatively, each effluent concentration can be prepared individually with appropriate volumes of effluent and brine.

17.6.23.7.2 When HSB and reagent water are used, thoroughly mix together the reagent water and HSB before mixing in the

effluent. Divide the salinity of the HSB by the expected test salinity to determine the proportion of reagent water to brine. For example, if the salinity of the brine is 100‰ and the test is to be conducted at 34‰, 100‰ divided by 34‰ = 2.94. The proportion of brine is 1 part, plus 1.94 parts reagent water. To make 1 L of dilution water at 34‰ salinity from a HSB of 100‰, 340 mL of brine and 660 mL of reagent water are required. Verify the salinity of the resulting mixture using a refractometer.

17.6.23.8 Test Solution Salinity Adjustment

17.6.23.8.1 Table 2 illustrates the preparation of test solutions (up to 50% effluent) at 34‰ by combining effluent, HSB, and dilution water. Note: if the highest effluent concentration does not exceed 50% effluent, it is convenient to prepare brine so that the sum of the effluent salinity and brine salinity equals 68‰; the required brine volume is then always equal to the effluent volume needed for each effluent concentration as in the example in Table 2.

17.6.23.8.2 Check the pH of all test solutions and adjust to within 0.2 units of dilution water pH by adding, dropwise, dilute hydrochloric acid or sodium hydroxide (see Section 8.8.9, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

17.6.23.8.3 To calculate the amount of brine to add to each effluent dilution, determine the following quantities: salinity of the brine (SB, in ‰), the salinity of the effluent (SE, in ‰), and volume of the effluent to be added (VE, in mL). Then use the following formula to calculate the volume of brine (VB, in mL) to be added:

$$VB = VE \times (34 - SE)/(SB - 34)$$

17.6.23.8.4 This calculation assumes that dilution water salinity is 34 ± 2‰.

17.6.23.9 Preparing Test Solutions

17.6.23.9.1 Two hundred mL of test solution are needed for each test chamber. To prepare test solutions at low effluent concentrations (<6%), effluents may be added directly to dilution

water. For example, to prepare 1% effluent, add 10 mL of effluent to a 1-liter volumetric flask using a volumetric pipet or calibrated automatic pipet. Fill the volumetric flask to the 1-Liter mark with dilution water, stopper it, and shake to mix. Distribute equal volumes into the replicate test chambers.

17.6.23.9.2 To prepare a test solution at higher effluent concentrations, hypersaline brine must usually be used. For example, to prepare 40% effluent, add 400 mL of effluent to a 1-liter volumetric flask. Then, assuming an effluent salinity of 2‰ and a brine salinity of 66‰, add 400 mL of brine (see equation above and Table 2) and top off the flask with dilution water. Stopper the flask and shake well. Distribute equal volumes into the replicate test chambers.

17.6.23.10 Brine Controls

17.6.23.10.1 Use brine controls in all tests where brine is used. Brine controls contain the same volume of brine as does the highest effluent concentration using brine, plus the volume of reagent water needed to reproduce the hyposalinity of the effluent in the highest concentration, plus dilution water. Calculate the amount of reagent water to add to brine controls by rearranging the above equation, (See, 17.6.23.8.3) setting SE = 0, and solving for VE.

$$VE = VB \times (SB - 34)/(34 - SE)$$

If effluent salinity is essentially 0‰, the reagent water volume needed in the brine control will equal the effluent volume at the highest test concentration. However, as effluent salinity and effluent concentration increase, less reagent water volume is needed.

17.6.24 TEST ORGANISMS

17.6.24.1 The test organisms for this method are the zoospores of the giant kelp, ***Macrocystis pyrifera***. ***Macrocystis*** is the dominant canopy forming Laminarian alga in southern and central California and forms extensive subtidal forests along the coast. Giant kelp forests support a rich diversity of marine life and provide habitat and food for hundreds of invertebrate and vertebrate species (North, 1971; Foster and Schiel, 1985). It

TABLE 2. EXAMPLES OF EFFLUENT DILUTION SHOWING VOLUMES OF EFFLUENT (x‰), BRINE, AND DILUTION WATER NEEDED FOR ONE LITER OF EACH TEST SOLUTION.

FIRST STEP: Combine brine with reagent water or natural seawater to achieve a brine of 68-x‰ and, unless natural seawater is used for dilution water, also a brine-based dilution water of 34‰.

SERIAL DILUTION:

Step 1. Prepare the highest effluent concentration to be tested by adding equal volumes of effluent and brine to the appropriate volume of dilution water. An example using 40% is shown.

Effluent Conc. (%)	Effluent x‰	Brine (68-x)‰	Dilution Water* 34‰
40	800 mL	800 mL	400 mL

Step 2. Use either serially prepared dilutions of the highest test concentration or individual dilutions of 100% effluent.

Effluent Conc. (%)	Effluent Source	Dilution Water* (34‰)
20	1000 mL of 40%	1000 mL
10	1000 mL of 20%	1000 mL
5	1000 mL of 10%	1000 mL
2.5	1000 mL of 5%	1000 mL
Control	none	1000 mL

INDIVIDUAL PREPARATION

Effluent Conc. (%)	Effluent x‰	Brine (68-x)‰	Dilution Water* 34‰
40	400 mL	400 mL	200 mL
20	200 mL	200 mL	600 mL
10	100 mL	100 mL	800 mL
5	50 mL	50 mL	900 mL
2.5	25 mL	25 mL	950 mL
Control	none	none	1000 mL

*May be natural seawater or brine-reagent water equivalent.

is an appropriate toxicity test species because of its availability, economic and ecological importance, history of successful laboratory culture (North, 1976; Luning, 1980; Kuwabara, 1981; Deysher and Dean, 1984; Linfield, 1985), and previous use in toxicity testing (Smith and Harrison, 1978; James et al., 1987; Anderson and Hunt, 1988; Hunt et al., 1989; Anderson et al., 1990). Other Laminarian alga species have proven to be useful for laboratory toxicity testing (Chung and Brinkhuis, 1986; Thompson and Burrows, 1984; Hopkin and Kain, 1978; see Thursby et al., 1993 for review).

17.6.24.2 Like all kelps, ***Macrocystis*** has a life cycle that alternates between a microscopic gametophyte stage and a macroscopic sporophyte stage. It is the sporophyte stage that forms kelp forests. These plants produce reproductive blades (sporophylls) at their base. The sporophylls develop patches (sori) in which biflagellate, haploid zoospores are produced. The zoospores are released into the water column where they swim and eventually settle onto the bottom and germinate. The dioecious spores develop into either male or female gametophytes. The male gametophytes produce flagellated gametes which may fertilize eggs produced by the female gametophytes. Fertilized eggs develop into sporophytes within 12- 15 days, completing the lifecycle.

17.6.24.3 The method described here focuses on germination of the zoospores and the initial growth of the developing gametophytes. It involves the controlled release of zoospores from the sporophyll blades, followed by the introduction of a spore suspension of known density into the test containers. The zoospores swim through the test solution and eventually settle onto glass microscope slides. The settled spores germinate by extruding the cytoplasm of the spore through the germ-tube into the first gametophytic cell. This stage is often referred to as the "dumbell" stage. The two endpoints measured after 48 hours are germination success and growth of the embryonic gametophytes (germ-tube length).

17.6.24.4 Species Identification

17.6.24.4.1 Although there is some debate over the taxonomy of the genus ***Macrocystis***, Abbott and Hollenberg (1976) consider only two species in California: ***M. pyrifera***, and ***M. integrifolia***. The

two are distinguished from each other based on habitat and the morphology of their holdfasts. ***Macrocystis pyrifera*** occurs subtidally while ***M. integrifolia*** occurs in the low intertidal and shallow subtidal zones. ***Macrocystis pyrifera*** has a conical holdfast while ***M. integrifolia*** has a more flattened, creeping holdfast. Consult Abbott and Hollenberg (1976) for a more detailed taxonomic discussion of the two species.

17.6.24.5 Obtaining Zoospores

17.6.24.5.1 ***Macrocystis*** zoospores are obtained from the reproductive blades (sporophylls) of the adult plant. The sporophylls are located near the base of the plant just above its conical holdfast. Sporophylls must be collected subtidally and should be collected from at least five different plants in any one location to give a good genetic representation of the population. The sporophylls should be collected from areas free of point and non-point source pollution to minimize the possibility of genetic or physiological adaptation to pollutants. In situations where a thermocline is present at the collection site, the sporophylls should be collected from below the thermocline to ensure adequate spore release. Sporophylls are identified in the field by the presence of darkened patches called sori. The zoospores develop within the sori. In addition, the sporophylls are distinguished from vegetative blades by their thinner width, basal location on the adult plant, and general lack of pneumatocysts (air bladders). Collection of algae is regulated by California law. Collectors must obtain a scientific collector's permit from the California Department of Fish and Game and observe any regulations regarding collection and transport of kelp. For further information regarding sporophyll collection, contact the Marine Pollution Studies Laboratory, 34500 Coast Route 1, Granite Canyon, Monterey CA, 93940, (408) 624-0947.

17.6.24.6 Broodstock Culture and Handling

17.6.24.6.1 After collection, the sporophylls should be kept damp and not exposed to direct sunlight. Avoid immersing the blades in seawater, however, to prevent premature spore release. The sporophylls should be <u>rinsed thoroughly</u> in 0.2 µm filtered seawater to remove diatoms and other epiphytic organisms. The individual blades can be gently rubbed between fingers under

running filtered seawater or brushed with a soft bristled brush. The blades are stored between moist paper towels (lasagna style so that the sporophylls do not overlap each other, and each layer of sporophylls are separated by a layer of paper towels) at approximately 9-12EC until needed. The zoospores must be released within 24 hours of collection to insure their viability. Preliminary data indicate that prolonged storage times may affect test results (Bottomley et al., 1991); however as long as germination rates meet control acceptability criteria this should not affect test results. Sporophylls should be kept shaded to prevent damage to the spores. For holding or transport times longer than approximately six hours, the sporophylls should be placed in an ice chest with blue ice. The blue ice should be wrapped in newspaper (10 layers) for insulation, then plastic to prevent leaking.

17.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

17.7.1 See Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

17.8 CALIBRATION AND STANDARDIZATION

17.8.1 See Section 4, Quality Assurance.

17.9 QUALITY CONTROL

17.9.1 See Section 4, Quality Assurance.

17.10 TEST PROCEDURES

17.10.1 TEST DESIGN

17.10.1.1 The test consists of at least five effluent concentrations plus a dilution water control. Tests that use brine to adjust salinity must also contain five replicates of a brine control.

17.10.1.2 Effluent concentrations are expressed as percent effluent.

17.10.2 TEST SOLUTIONS

17.10.2.1 Receiving waters

17.10.2.1.1 The sampling point is determined by the objectives of the test. At estuarine and marine sites, samples are usually collected at mid-depth. Receiving water toxicity is determined with samples used directly as collected or with samples passed through a 60 µm NITEX® filter and compared without dilution, against a control. Using five replicate chambers per test, each containing 200 mL, analysis would require approximately 1 L of sample per test.

17.10.2.2 Effluents

17.10.2.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of at least 0.5 is commonly used. A dilution factor of 0.5 provides hypothesis test discrimination of ± 100%, and testing of a 16 fold range of concentrations. Hypothesis test discrimination shows little improvement as dilution factors are increased beyond 0.5 and declines rapidly if smaller dilution factors are used. **USEPA recommends that one of the five effluent treatments must be a concentration of effluent mixed with dilution water which corresponds to the permittee's instream waste concentration (IWC).** At least two of the effluent treatments must be of lesser effluent concentration than the IWC, with one being at least one-half the concentration of the IWC. If 100‰ HSB is used as a diluent, the maximum concentration of effluent that can be tested will be 66% at 34‰ salinity.

17.10.2.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12% and 1.56%).

17.10.2.2.3 The volume in each test chamber is 200 mL.

17.10.2.2.4 Effluent dilutions should be prepared for all replicates in each treatment in one container to minimize variability among the replicates. Dispense into the appropriate effluent test chambers.

17.10.2.3 Dilution Water

17.10.2.3.1 Dilution water should be uncontaminated 1-µm-

filtered natural seawater or hypersaline brine prepared from uncontaminated natural seawater plus reagent water (see Section 7, Dilution Water). Natural seawater may be uncontaminated receiving water. This water is used in all dilution steps and as the control water.

17.10.2.4 Reference Toxicant Test

17.10.2.4.1 Reference toxicant tests should be conducted as described in Quality Assurance (see Section 4.7).

17.10.2.4.2 The preferred reference toxicant for giant kelp is copper chloride ($CuCl_2$º2H_2O). Reference toxicant tests provide an indication of the sensitivity of the test organisms and the suitability of the testing laboratory (see Section 4 Quality Assurance). Another toxicant may be specified by the appropriate regulatory agency. Prepare a 10,000 µg/L copper stock solution by adding 0.0268 g of copper chloride ($CuCl_2$º2H_2O) to one liter of reagent water in a polyethylene volumetric flask. Alternatively, certified standard solutions can be ordered from commercial companies.

17.10.2.4.3 Reference toxicant solutions should be five replicates each of 0 (control), 5.6, 10, 18, 32, 100, and 180 µg/L total copper. Prepare one liter of each concentration by adding 0, 0.56, 1.0, 1.8, 3.2, 5.6, 10.0, and 18.0 mL of stock solution, respectively, to one-liter volumetric flasks and fill with dilution water. Start with control solutions and progress to the highest concentration to minimize contamination.

17.10.2.4.4 If the effluent and reference toxicant tests are to be run concurrently, then the tests must use zoospores from the same release. The tests must be handled in the same way and test solutions delivered to the test chambers at the same time. Reference toxicant tests must be conducted at 34 ± 2‰.

17.10.3 RELEASE OF ZOOSPORES FOR THE TEST

17.10.3.1 Zoospores are released by slightly desiccating the sporophyll blades, and then placing them in filtered seawater. To desiccate the sporophylls, blot the blades with paper towels and expose them to air for 1 hour.

17.10.3.2 The number of sporophyll blades needed depends upon their maturity; usually 25-30 blades (~ 100 grams wet weight) are sufficient. After 1 hour the blades should be rinsed again thoroughly using 0.2 µm-filtered seawater, then placed in a one L glass or plastic beaker filled with 0.2 µm filtered seawater at 15-16EC. The release water should never exceed 18EC.

17.10.3.3 After one hour, a sufficient number of zoospores should be present to conduct the test. The presence of zoospores is indicated by a slight cloudiness in the water. To verify whether zoospores are present, periodically sample the solution and observe the sample microscopically (100x).

17.10.3.4 To insure that the zoospores are viable and have not begun to germinate before they are exposed to the toxicant, the zoospore release process should not be longer than two hours. If it takes longer than two hours to get an adequate density of zoospores (~7,500 zoospores/mL of test solution), repeat the release process with a new batch of sporophylls.

17.10.3.5 After the zoospores are released, remove the sporophylls and let the spore mixture settle for 30 minutes. After 30 minutes, decant 250 mLs from the top of the spore solution into a separate clean glass beaker. Sample the spore solution and determine the spore density using a bright-line hemacytometer (100x). Spores may be counted directly, or to obtain a more accurate count, fix a sample of spores by mixing nine milliliters of spore solution with 1-mL of 37% buffered formalin (or acetic acid) in a test tube. Shake the sample well before placing it on the hemacytometer.

17.10.3.6 After counting, the density is multiplied by 1.111 to correct for the dilution caused by adding 1 mL of formalin to the sample. Use at least five replicate counts. After the density is determined, calculate the volume of zoospores necessary to give approximately 7,500 spores/mL of test solution. To prevent over-dilution of the test solution, this volume should not exceed 1% of the test solution volume. If this volume exceeds 1% of the test solution volume, it should be noted in the results.

17.10.3.7 Test solutions must be prepared while the zoospores are releasing from the sporophylls. Test solutions must be mixed, sampled, and temperature equilibrated in time to receive

the swimming zoospores as soon as they are counted. Zoospore release and counting should be done in a room separate from that used for toxicant preparation, and care should be taken to avoid contaminating the zoospores prior to testing.

17.10.4 START OF THE TEST

17.10.4.1 Prior to Beginning the Test

17.10.4.1.1 The test should begin as soon as possible, preferably within 24 h of sample collection. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity tests unless permission is granted by the permitting authority. In no case should the sample be used in a test more than 72 h after sample collection (see Section 8 Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Test).

17.10.4.1.2 Just prior to test initiation (approximately 1 h), the temperature of a sufficient quantity of the sample to make the test solutions should be adjusted to the test temperature (15 ± 1EC) and maintained at that temperature during the addition of dilution water.

17.10.4.1.3 Increase the temperature of the water bath, room, or incubator to the required test temperature (15 ± 1EC).

17.10.4.1.4 Randomize the placement of test chambers in the temperature-controlled water bath, room, or incubator at the beginning of the test, using a position chart. Assign numbers for the position of each test chamber using a random numbers or similar process (see Appendix A, for an example of randomization). Maintain the chambers in this configuration throughout the test, using a position chart. Record these numbers on a separate data sheet together with the concentration and replicate numbers to which they correspond. Identify this sheet with the date, test organism, test number, laboratory, and investigator's name, and safely store it away until after the gametophyte spores have been examined at the end of the test.

17.10.4.1.5 Note: Loss of the randomization sheet would invalidate the test by making it impossible to analyze the data afterwards. Make a copy of the randomization sheet and store

separately. Take care to follow the numbering system exactly while filling chambers with the test solutions.

17.10.4.1.6 Arrange the test chambers randomly in the water bath or controlled temperature room. Once chambers have been labeled randomly and filled with test solutions, they can be arranged in

numerical order for convenience, since this will also ensure random placement of treatments.

17.10.4.2 Estimation of Zoospore Density

17.10.4.2.1 After determining the zoospore density and calculating the volume yielding 7,500 zoospores/mL test solution, add this volume to each test chamber (this is the start time of the test). Observe a sample of zoospores microscopically to verify that they are swimming before adding them to the test chambers.

17.10.4.2.2 Incubate the developing gametophytes for 48 hours in the test chambers at 15EC under 50 µE/m^2/s. The zoospores germinate and develop to the "dumbell" gametophyte stage during the exposure period.

17.10.5 LIGHT, PHOTOPERIOD, SALINITY AND TEMPERATURE

17.10.5.1 The lights used in this method are cool white fluorescent lights adjusted to give 50 µE/m^2/s at the top of each test chamber. Each test chamber must receive the same quanta of light (50 ± 10 µE/m^2/s). Areas of increased light can be eliminated by taping the outside of the light diffuser or wrapping the fluorescent bulbs with aluminum foil.

17.10.5.2 The water temperature in the test chambers should be maintained at 15 ± 1EC. If a water bath is used to maintain the test temperature, the water depth surrounding the test cups should be as deep as possible without floating the chambers.

15.10.5.3 The test salinity should be in the range of 34 ± 2‰. The salinity should vary by no more than ±2‰ among the chambers on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.

15.10.5.4 Rooms or incubators with high volume ventilation should be used with caution because the volatilization of the test solutions and evaporation of dilution water may cause wide fluctuations in salinity. Covering the test chambers with clean polyethylene plastic may help prevent volatilization and evaporation of the test solutions.

17.10.6 DISSOLVED OXYGEN (DO) CONCENTRATION

17.10.6.1 Aeration may affect the toxicity of effluent and should be used only as a last resort to maintain a satisfactory DO. The DO concentration should be measured on new solutions at the start of the test (Day 0). The DO should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). If it is necessary to aerate, all treatments and the control should be aerated. The aeration rate should not exceed that necessary to maintain a minimum acceptable DO and under no circumstances should it exceed 100 bubbles/minute, using a pipet with a 1-2 mm orifice, such as a 1 mL KIMAX® serological pipet No. 37033, or equivalent.

17.10.7 OBSERVATIONS DURING THE TEST

17.10.7.1 Routine Chemical and Physical Observations

17.10.7.1.1 DO is measured at the beginning of the exposure period in one test chamber at each test concentration and in the control.

17.10.7.1.2 Temperature, pH, and salinity are measured at the beginning of the exposure period in one test chamber at each concentration and in the control. Temperature should also be monitored continuously or observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be measured in a sufficient number of test chambers at the end of the test to determine temperature variation in the environmental chamber.

17.10.7.1.3 Record all the measurements on the data sheet.

17.10.8 TERMINATION OF THE TEST

17.10.8.1 Ending the Test

17.10.8.1.1 Record the time the test is terminated.

17.10.8.1.2 Temperature, pH, dissolved oxygen, and salinity are measured at the end of the exposure period in one test chamber at each concentration and in the control.

17.10.8.2 Sample preservation

17.10.8.2.1 In some cases it may be convenient to preserve the kelp cultures for later analysis. Preliminary work by Anderson and Hunt (Marine Pollution Studies Laboratory unpublished data) indicates that cultures can be preserved in 0.1% glutaraldehyde (final concentration) and that preservation has no significant effect on germination or germ-tube growth. Other researchers have used higher glutaraldehyde concentrations and found adequate preservation with no effect on spore germination or gametophyte growth (K. Goodwin, Calif. Inst. of Tech., unpublished data).

17.10.8.2.2 Because data on the effects of preservation are preliminary, it is recommended that anyone interested in preserving kelp cultures for later analysis first demonstrate that preservation does not affect test results. This can be accomplished by comparing germination and germ-tube growth in preserved vs non-preserved kelp cultures. We also recommend that if it is necessary to preserve kelp cultures for later analysis, a complete test should be preserved so that if any replicates are read preserved, all of the replicates should be read preserved. In the case where concurrent reference toxicant and complex effluent tests are conducted, it may be convenient to fix one test in glutaraldehyde and read the other test immediately.

17.10.8.2.3 When fixing kelp cultures, it is important to minimize disturbance to the gametophytes. Make sure that the culture slides are fixed and stored horizontally. We have used disposable petri dishes for preservation chambers; these allow individual replicate slides to be labelled and preserved separately to avoid mixing replicates. **Note:** Glutaraldehyde is toxic. If you intend to use this material as a preservative, study the material data safety sheets from the supplier and

follow strict safety precautions. Make sure test chambers and solutions contaminated with this material are disposed of properly.

17.10.8.3 Counting

17.10.8.3.1 After 48 hours, the test is terminated. Because it takes a considerable amount of time to read the test, reading can begin after 45 hours and must be completed within six hours. Remove the slide without decanting the test solution. The test slide can be lifted from the bottom of the test chamber with a separate clean microscope slide. Blot the bottom on a paper towel and place an 18-mm square cover slip on the slide. Blot the excess water around the edge of the cover slip to eliminate the flow of water under the cover slip.

17.10.8.4 Endpoints

17.10.8.4.1 The endpoints measured for the 48 hour ***Macrocystis*** method are percent germination success and germination tube length. Germination is considered successful if a germ-tube is present on the settled zoospore. Germination is considered to be unsuccessful if no germination tube is visible. To differentiate between a germinated and non-germinated zoospore, observe the settled zoospores at 400x magnification and determine whether they are circular (non-germinated) or have a protuberance that extends at least one spore diameter (about 3.0 µm) from the edge of the spore (germinated). Spores with a germination tubes less than one spore diameter are considered non-germinated.

17.10.8.4.2 The first 100 spores encountered while moving across the microscope slide are counted for each replicate of each treatment. **Note:** Sewage effluents may contain certain objects, such as ciliates, which look similar to non-germinated kelp spores. It is important to ensure that only kelp spores are counted for this endpoint. Kelp spores are green-brown in color, spherical, and lack mobility. Also, components of the cytoplasm of kelp spores appear to fluoresce a light green color when the spore is slightly out of focus. If a particular object cannot be identified, it should not be counted.

17.10.8.4.3 The growth endpoint is the measurement of the total length of the germination tube from the edge of the original

spore membrane. Only germinated spores with straight germination tubes and within the same focal plane are measured; if a spore is not completely in focus from tip to tip it should not be measured. The spores to be measured are randomly selected by moving the microscope stage to a new field of view without looking through the ocular lens.

17.10.8.4.4 Measure the germination-tube length of the spore whose spore case center is nearest the micrometer in each field; the spores case can be distinguished from the growing tip because it is usually clear (empty) at 48 hours, and it is more circular than the growing tip. If more than one spore case is touching the micrometer, both (or all) germinated spores are measured. A total of 10 spores for each replicate of each treatment are measured. It is easier to measure germ-tube length with a micrometer having a 10 mm linear scale (0.1 mm subdivisions); measure lengths to the nearest micron (typically to the nearest half micrometer unit; see Section 10200E, Standard Methods 17th edition, for micrometer/microscope calibration procedures). In situations where germination is significantly inhibited it may be difficult to find germinated spores for germ-tube growth measurement using the random search technique.

17.10.8.4.5 To expedite reading, the slide can be scanned to find germinated spores if germination is 30% or less. In this situation the first 10 spores encountered are measured for germ-tube length.

17.11 **SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA**

17.11.1 A summary of test conditions and test acceptability criteria is listed in Table 3.

17.12 **ACCEPTABILITY OF TEST RESULTS**

17.12.1 For tests to be considered acceptable, the following requirements must be met:

(1) Mean control germination must be at least 70% in the controls.

(2) Mean germination-tube length in the controls must be at least 10 µm in the controls.

(3) The germination-tube growth NOEC must be below 35 µg/liter in the reference toxicant test.

(4) The minimum significant difference (%MSD) is <20% relative to the control for both germination and germ-tube length in the reference toxicant test.

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR GIANT KELP, ***MACROCYSTIS PYRIFERA***, GERMINATION AND GERM-TUBE LENGTH TEST WITH EFFLUENTS AND RECEIVING WATERS

1. Test type:	Static non-renewal
2. Salinity:	34 ± 2‰
3. Temperature:	15 ± 1EC
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	50 ± 10 µE/m^2/s
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	600 mL
8. Test solution volume:	200 mL/replicate
9. Spore density per test chamber:	7500/mL of test solution
10. No. replicate chambers per concentration:	5
11. Dilution water:	Uncontaminated 1-µm-filtered natural seawater or hypersaline brine prepared from natural seawater
12. Test concentrations:	Effluents: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
13. Dilution factor:	Effluents: $0.5 Receiving waters: None or $0.5
14. Test duration:	48 h

15. Endpoints:	Germination and germ-tube length
16. Test acceptability criteria:	$70% germination in the controls; $10 µm germ-tube length in the controls and the NOEC must be below 35 µg/L in the reference toxicant test; must achieve a %MSD of <20 for both germination and germ-tube length in the reference toxicant.
17. Sampling requirements:	One sample collected at test initiation, and preferably used within 24 h of the time it is removed from the sampling device (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests)
18. Sample volume required:	2 L per test

17.13 **DATA ANALYSIS**

17.13.1 GENERAL

17.13.1.1 Tabulate and summarize the data. Table 4 presents a sample set of germination and growth data.

17.13.1.2 The endpoints of the giant kelp 48-hour chronic test are based on the adverse effects on germination and growth. The IC25 endpoints are calculated using point estimation techniques (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). LOEC and NOEC values for germination and growth are obtained using a hypothesis testing approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981) (see Section 9). Separate analyses are performed for the estimation of the LOEC and NOEC endpoints and for the estimation of the IC25 endpoints. Concentrations at which there is no germination in any of the test chambers are excluded from the statistical analysis of the NOEC and LOEC for germination and growth, but included in the estimation of the

IC25. See the Appendices for examples of the manual computations, and examples of data input and program output.

17.13.1.3 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

17.13.2 EXAMPLE OF ANALYSIS OF GIANT KELP, ***MACROCYSTIS PYRIFERA***, GERMINATION DATA

17.13.2.1 Formal statistical analysis of the germination data is outlined in Figure 1. The response used in the analysis is the proportion of germinated spores in each test or control chamber. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the IC25 endpoint. Concentrations at which there is no germination in any of the test chambers are excluded from statistical analysis of the NOEC and LOEC, but included in the estimation of the IC endpoints.

17.13.2.2 For the case of equal numbers of replicates across all concentrations and the control, the evaluation of the NOEC and LOEC endpoints is made via a parametric test, Dunnett's Procedure, or a nonparametric test, Steel's Many-one Rank Test, on the arc sine square root transformed data. Underlying assumptions of Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test, and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are estimated by the parametric procedure.

17.13.2.3 If unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a ***t*** test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative.

TABLE 4. DATA FROM GIANT KELP, ***MACTOCYSTIS PYRIFERA*** GERMINATION AND GROWTH TEST

Copper Conc. (µg/L)	Replicate Chamber	Number Counted	Number Germinated	Proportion Germinated	Mean Length
Control	1	100	89	0.89	19.58
	2	100	88	0.88	18.75
	3	100	85	0.85	19.14
	4	100	89	0.89	16.50
	5	100	91	0.91	17.93
5.6	1	100	82	0.82	18.26
	2	100	55	0.55	16.25
	3	100	84	0.84	16.39
	4	100	96	0.96	18.70
	5	100	85	0.85	15.62
10.0	1	100	90	0.90	13.31
	2	100	90	0.90	18.92
	3	100	70	0.70	15.62
	4	100	83	0.83	14.30
	5	100	87	0.87	15.29
18.0	1	100	88	0.88	18.59
	2	100	52	0.52	12.88
	3	100	83	0.83	16.28
	4	100	54	0.54	15.38
	5	100	49	0.49	19.75
32.0	1	100	71	0.71	12.54
	2	100	82	0.82	10.67
	3	100	86	0.86	15.95
	4	100	81	0.81	12.54
	5	100	82	0.82	11.66
56.0	1	100	84	0.84	11.44
	2	100	68	0.68	11.88
	3	100	62	0.62	11.88
	4	100	80	0.80	11.00
	5	100	83	0.83	11.55
100.0	1	100	66	0.66	7.92
	2	100	72	0.72	7.59
	3	100	63	0.63	8.25
	4	100	72	0.72	9.13
	5	100	71	0.71	8.80
180.0	1	100	37	0.37	6.49
	2	100	69	0.69	7.25
	3	100	0	0.00	--
	4	100	32	0.32	7.63
	5	100	48	0.48	8.13

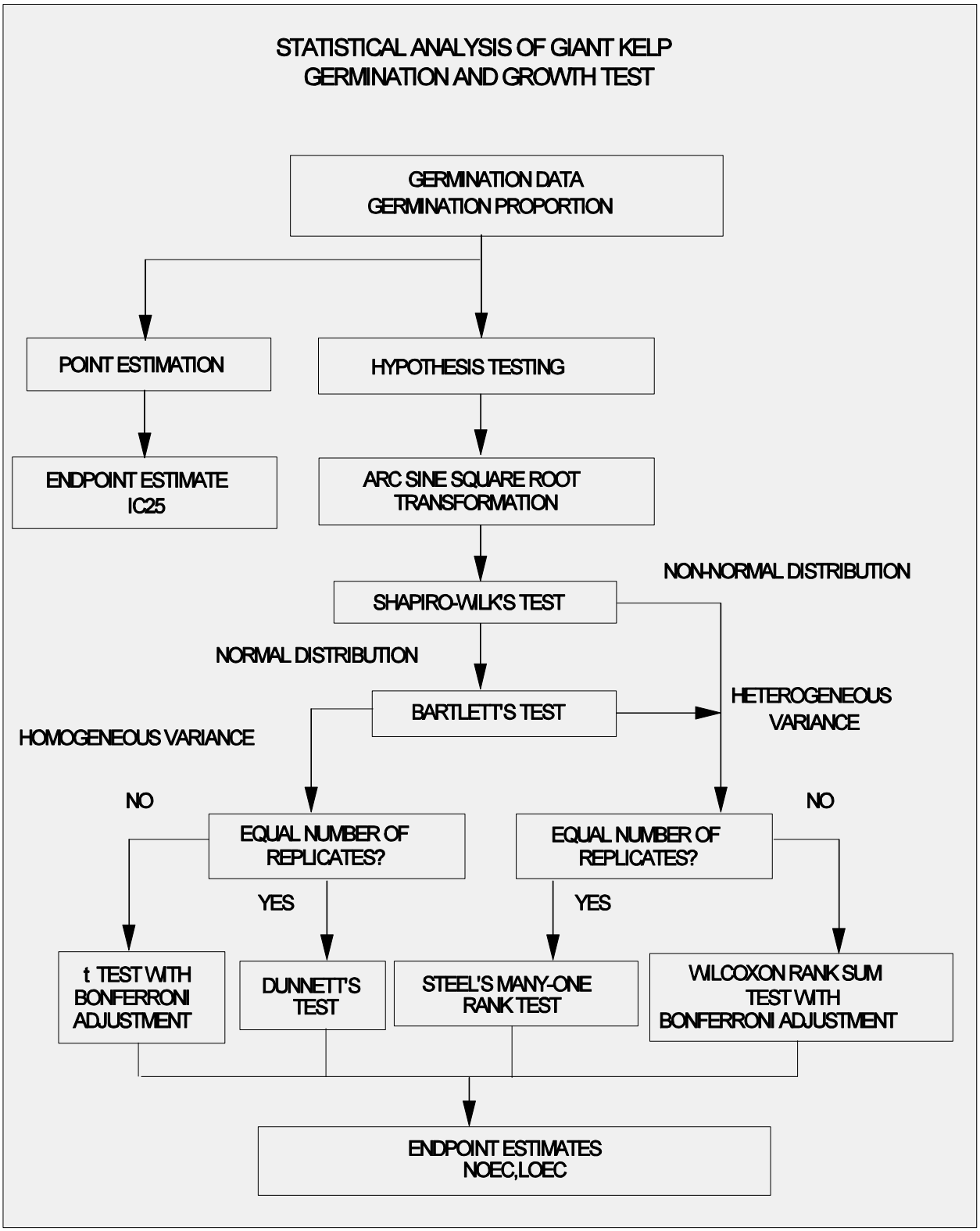

Figure 1. Flowchart for statistical analysis of giant kelp, *Macrocystis pyrifera*, germination data.

17.13.2.4 Example of Analysis of Germination Data

17.12.2.4.1 This example used toxicity data from a giant kelp, Macrocystis pyrifera, germination and growth test performed with copper. The response of interest is the proportion of germinated spores, thus each replicate must be transformed by the arc sine square root transformation procedure described in Appendix B. The raw and transformed data, means and variances of the transformed observations at each concentration including the control are listed in Table 5. A plot of the survival data is provided in Figure 2.

17.13.2.5 Test for Normality

17.13.2.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are listed in Table 6.

17.13.2.5.2 Calculate the denominator, D, of the test statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: δ_i = the ith centered observation

δ = the overall mean of the centered observations

n = the total number of centered observations.

17.13.2.5.3 For this set of data, n = 40

$$\delta = \frac{1}{40}(-0.002) = 0.000$$

D = 0.9281

17.13.2.5.4 Order the centered observations from smallest to largest:

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

TABLE 5. GIANT KELP, ***MACTOCYSTIS PYRIFERA*** GERMINATION DATA

COPPER CONCENTRATION (µg/L)	REPLICATE CHAMBER	RAW DATA	ARC SINE SQUARE ROOT TRANSFORMED	i	MEAN &	S_i^2
Control	1	0.89	1.233	1	1.224	0.00114
	2	0.88	1.217			
	3	0.85	1.173			
	4	0.89	1.233			
	5	0.91	1.266			
5.6	1	0.82	1.133	2	1.134	0.03670
	2	0.55	0.835			
	3	0.84	1.159			
	4	0.96	1.369			
	5	0.85	1.173			
10.0	1	0.90	1.249	3	1.167	0.01152
	2	0.90	1.249			
	3	0.70	0.991			
	4	0.83	1.146			
	5	0.87	1.202			
18.0	1	0.88	1.217	4	0.954	0.04423
	2	0.52	0.805			
	3	0.83	1.146			
	4	0.54	0.825			
	5	0.49	0.775			
32.0	1	0.71	1.002	5	1.115	0.00466
	2	0.82	1.133			
	3	0.86	1.187			
	4	0.81	1.120			
	5	0.82	1.133			
56.0	1	0.84	1.159	6	1.058	0.01272
	2	0.68	0.970			
	3	0.62	0.907			
	4	0.80	1.107			
	5	0.83	1.146			
100.0	1	0.66	0.948	7	0.979	0.00191
	2	0.72	1.013			
	3	0.63	0.917			
	4	0.72	1.013			
	5	0.71	1.002			
180.0	1	0.37	0.654	8	0.610	0.11914
	2	0.69	0.980			
	3	0.00	0.050			
	4	0.32	0.601			
	5	0.48	0.765			

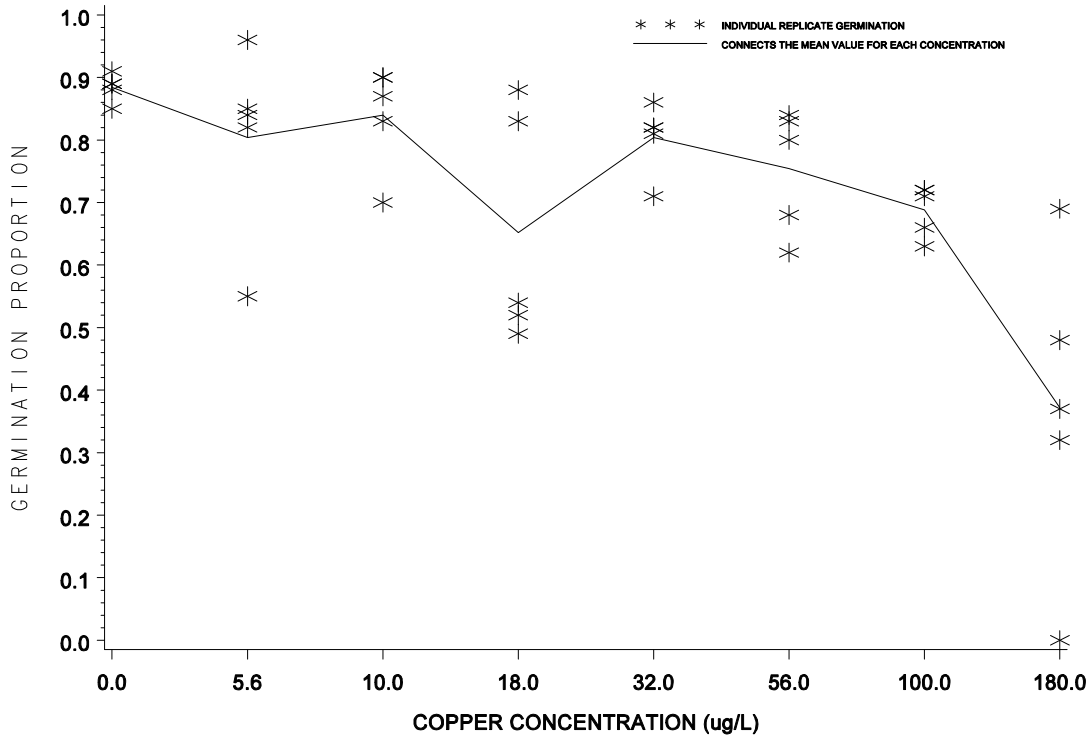

Figure 2. Plot of germination proportions of the giant kelp, *Macrocystis pyrifera*, at each treatment level.

TABLE 6. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

	Copper Concentration (µg/L)							
Rep	Control	5.6	10.0	18.0	32.0	56.0	100.0	180.0
1	0.009	-0.001	0.082	0.263	-0.113	0.101	-0.031	0.044
2	-0.007	-0.299	0.082	-0.149	0.018	-0.088	0.034	0.370
3	-0.051	0.025	-0.176	0.192	0.072	-0.151	-0.062	-0.560
4	0.009	0.235	-0.021	-0.129	0.005	0.049	0.034	-0.009
5	0.042	0.039	0.035	-0.179	0.018	0.088	0.023	0.155

TABLE 7. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.560	21	0.018
2	-0.299	22	0.023
3	-0.179	23	0.025
4	-0.176	24	0.034
5	-0.151	25	0.034
6	-0.149	26	0.035
7	-0.129	27	0.039
8	-0.113	28	0.042
9	-0.088	29	0.044
10	-0.062	30	0.049
11	-0.051	31	0.072
12	-0.031	32	0.082
13	-0.021	33	0.082
14	-0.009	34	0.088
15	-0.007	35	0.101
16	-0.001	36	0.155
17	0.005	37	0.192
18	0.009	38	0.235
19	0.009	39	0.263
20	0.018	40	0.370

Where $X^{(i)}$ is the ith ordered observation. These ordered observations are listed in Table 7.

17.13.2.5.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2,...., a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 40 and k = 20. The a_i values are listed in Table 8.

17.13.2.5.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i\left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 8. For this data in this example:

$$W = \frac{1}{0.9281}(0.9230)^2 = 0.918$$

TABLE 8. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.3964	0.930	$X^{(40)} - X^{(1)}$
2	0.2737	0.562	$X^{(39)} - X^{(2)}$
3	0.2368	0.414	$X^{(38)} - X^{(3)}$
4	0.2098	0.368	$X^{(37)} - X^{(4)}$
5	0.1878	0.306	$X^{(36)} - X^{(5)}$
6	0.1691	0.250	$X^{(35)} - X^{(6)}$
7	0.1526	0.217	$X^{(34)} - X^{(7)}$
8	0.1376	0.195	$X^{(33)} - X^{(8)}$
9	0.1237	0.170	$X^{(32)} - X^{(9)}$
10	0.1108	0.134	$X^{(31)} - X^{(10)}$
11	0.0986	0.100	$X^{(30)} - X^{(11)}$
12	0.0870	0.075	$X^{(29)} - X^{(12)}$
13	0.0759	0.063	$X^{(28)} - X^{(13)}$
14	0.0651	0.048	$X^{(27)} - X^{(14)}$
15	0.0546	0.042	$X^{(26)} - X^{(15)}$
16	0.0444	0.035	$X^{(25)} - X^{(16)}$
17	0.0343	0.029	$X^{(24)} - X^{(17)}$
18	0.0244	0.016	$X^{(23)} - X^{(18)}$
19	0.0146	0.014	$X^{(22)} - X^{(19)}$
20	0.0049	0.000	$X^{(21)} - X^{(20)}$

TABLE 9. ASSIGNING RANKS TO THE CONTROL AND 5.6 µg/L CONCENTRATION LEVEL FOR STEEL'S MANY-ONE RANK TEST

Rank	Transformed Proportion Germinated	Concentration
1	0.835	5.6 µg/L
2	1.133	5.6 µg/L
3	1.159	5.6 µg/L
4.5	1.173	5.6 µg/L
4.5	1.173	Control
6	1.217	Control
7.5	1.233	Control
7.5	1.233	Control
9	1.266	Control
10	1.369	5.6 µg/L

TABLE 10. TABLE OF RANKS[1]

Rep.	Control	Concentration (µg/L) 5.6	10.0
1	1.233(7.5,6.5,8.5,8.5,8.5,8.5,8.5)	1.133(2)	1.249(8.5)
2	1.217(6,5,6.5,7,7,7,7)	0.835(1)	1.249(8.5)
3	1.173(4.5,3,5,5,6,6,6)	1.159(3)	0.991(1)
4	1.233(7.5,6.5,8.5,8.5,8.5,8.5,8.5)	1.369(10)	1.146(2)
5	1.266(9,10,10,10,10,10,10)	1.173(4.5)	1.202(4)

Concentration (µg/L) (Continued)

Rep.	18.0	32.0	56.0	100.0	180.0
1	1.217(6.5)	1.002(1)	1.159(5)	0.948(2)	0.654(3)
2	0.805(2)	1.133(3.5)	0.970(2)	1.013(4.5)	0.980(5)
3	1.146(4)	1.187(6)	0.907(1)	0.917(1)	0.050(1)
4	0.825(3)	1.120(2)	1.107(3)	1.013(4.5)	0.601(2)
5	0.775(1)	1.133(3.5)	1.146(4)	1.002(3)	0.765(4)

[1]Control ranks are given in the order of the concentration with which they were ranked.

17.13.2.5.7 The decision rule for this test is to compare W as calculated in Subsection 5.6 with the critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For this set of data, the critical value at a significance level of 0.01 and n = 40 observations is 0.919. Since W = 0.918 is less than the critical value, conclude that the data are not normally distributed.

17.13.2.5.8 Since the data do not meet the assumption of normality, Steel's Many-one Rank Test will be used to analyze the germination data.

17.13.2.6 Steel's Many-one Rank Test

17.13.2.6.1 For each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign the ranks (1, 2, ... , 10) to the ordered observations with a rank of 1 assigned to the smallest observation, rank of 2 assigned to the next larger observation, etc. If ties occur when ranking, assign the average rank to each tied observation.

17.13.2.6.2 An example of assigning ranks to the combined data for the control and 5.6 µg/L copper concentration is given in Table 9. This ranking procedure is repeated for each control/concentration combination. The complete set of rankings is summarized in Table 10. The ranks are then summed for each concentration level, as shown in Table 11.

17.13.2.6.3 For this example, determine if the survival in any of the concentrations is significantly lower than the survival in the control. If this occurs, the rank sum at that concentration would be significantly lower than the rank sum of the control. Thus compare the rank sums for the survival at each of the various concentration levels with some "minimum" or critical rank sum, at or below which the survival would be considered significantly lower than the control. At a significance level of 0.05, the minimum rank sum in a test with seven concentrations (excluding the control) and five replicates is 16 (See Table 5, Appendix E).

17.13.2.6.4 Since the rank sum for the 32.0 µg/L concentration is equal to the critical value and the rank sums for the 56.0, 100.0 and 180.0 µg/L concentrations are less than the critical value, the germination proportions in those concentrations are considered significantly less than that in the control. Hence, the NOEC and the LOEC are considered to be 18.0 µg/L and 32.0 µg/L, respectively.

TABLE 11. RANK SUMS

Concentration	Rank Sum
5.6	20.5
10.0	24.0
18.0	16.5
32.0	16.0
56.0	15.0
100.0	15.0
180.0	15.0

17.13.2.7 Calculation of the ICp

17.13.2.7.1 The germination data from Table 4 and Figure 2 are utilized in this example. As can be seen from the figure, the observed means are not monotonically non-increasing with respect to concentration. Therefore, the means must be smoothed prior to calculating the IC.

17.13.2.7.2 Starting with the observed control mean, $\overline{Y}_1 = 0.884$ is less than the observed mean for the lowest effluent concentration, $\overline{Y}_2 = 0.804$, so set $M_1 = 0.884$.

17.13.2.7.3 Comparing $\overline{Y}_2$ to $\overline{Y}_3 = 0.840$, we see that $\overline{Y}_2$ is less than $\overline{Y}_3$.

17.13.2.7.4 Calculate the smoothed means:

$$M_2 = M_3 = (\overline{Y}_2 + \overline{Y}_3)/2 = 0.822$$

17.13.2.7.5 Since M_3 is larger than $\overline{Y}_4 = 0.652$, set $M_4 = 0.652$. Since $\overline{Y}_5 = 0.804$ is larger than M_4, these means must be smoothed.

17.13.2.7.6 Calculate the smoothed means:

$$M_4 = M_5 = (M_4 + \overline{Y}_5)/2 = 0.728.$$

17.13.2.7.7 Since $\overline{Y}_6 = 0.754$ is larger than M_5, average $\overline{Y}_6$ with the two previous concentrations:

$$M_4 = M_5 = M_6 = (M_4 + M_5 + \overline{Y}_6)/3 = 0.737.$$

17.13.2.7.8 Since $M_6 > \overline{Y}_7 = 0.688 > \overline{Y}_8 = 0.372$, set $M_7 = 0.688$ and $M_8 = 0.372$. Table 12 contains the smoothed means and Figure 3 gives a plot of the smoothed means and the interpolated response curve.

17.13.2.7.9 An IC25 can be estimated using the Linear Interpolation Method. A 25% reduction in germination, compared to the controls, would result in a mean germination of 0.663, where $M_1(1-p/100) = 0.884(1-25/100)$. Examining the smoothed means and their associated concentrations (Table 12), the response, 0.663, is bracketed by $C_7 = 100.0$ µg/L and $C_8 = 180.0$ µg/L.

17.13.2.7.10 Using the equation in Section 4.2 from Appendix L, the estimate of the IC25 is calculated as follows:

$$ICp \text{ ' } C_j\%[M_1(1\&p/100)\&M_j]\frac{(C_{(j\%1)}\&C_j)}{(M_{(j\%1)}\&M_j)}$$

$$IC25 = 100.0 + [0.884(1 - 25/100) - 0.688] \left(\frac{(180.0 - 100.0)}{(0.372 - 0.688)}\right)$$

$$= 106.3 \text{ µg/L}.$$

17.13.2.7.11 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 106.3291 µg/L. The empirical 95.0% confidence interval for the true mean was 94.6667 µg/L to 117.0588 µg/L. The computer program output for the IC25 for this data set is shown in Figure 4.

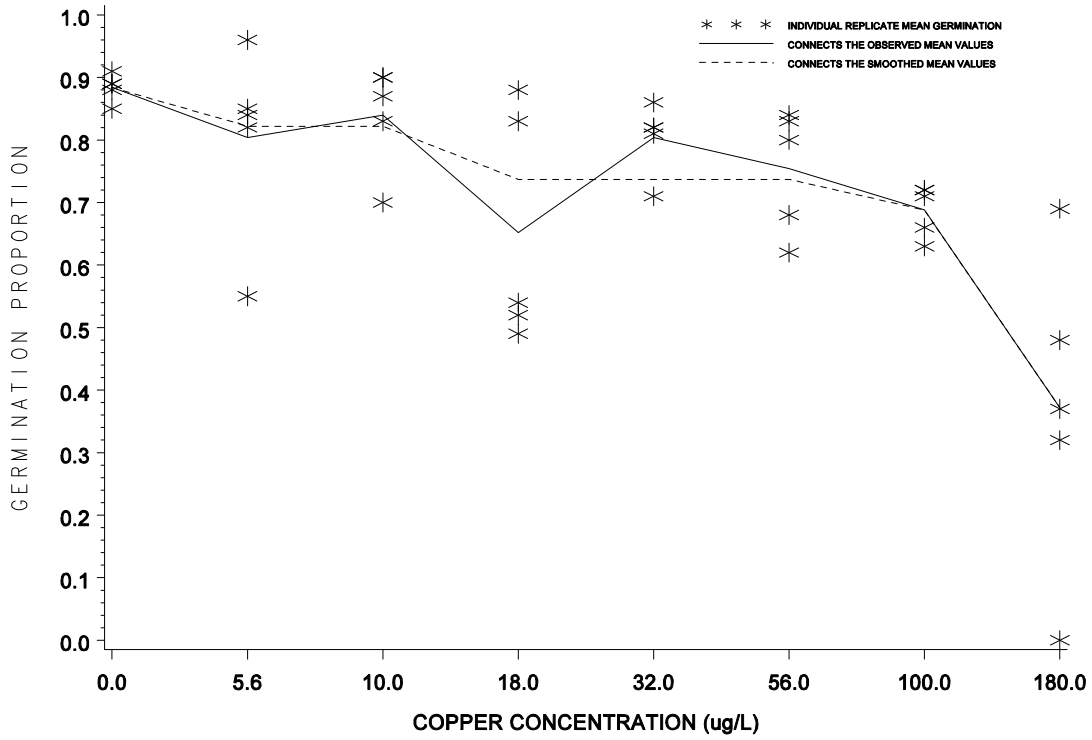

Figure 3. Plot of raw data, observed means, and smoothed means for the giant kelp, *Macrocystis pyrifera*, germination data from Tables 4 and 13.

```
Conc. ID        1    2    3    4    5    6    7    8
-------------------------------------------------------
Conc. Tested    0  5.6   10   18   32   56  100  180
-------------------------------------------------------
Response  1   .89  .82  .90  .88  .71  .84  .66  .37
Response  2   .88  .55  .90  .52  .82  .68  .72  .69
Response  3   .85  .84  .70  .83  .86  .62  .63    0
Response  4   .89  .96  .83  .54  .81  .80  .72  .32
Response  5   .91  .85  .87  .49  .82  .83  .71  .48
-------------------------------------------------------
*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Copper
Test Start Date:    Test Ending Date:
Test Species: Giant Kelp, Macrocystis pyrifera
Test Duration:        48 hours
DATA FILE: kelpgerm.icp
OUTPUT FILE: kelpgerm.i25
-------------------------------------------------------------------------
Conc.     Number     Concentration     Response      Std.       Pooled
 ID     Replicates            ug/L       Means       Dev.   Response Means
-------------------------------------------------------------------------
  1          5             0.000          0.884      0.022      0.884
  2          5             5.600          0.804      0.152      0.822
  3          5            10.000          0.840      0.083      0.822
  4          5            18.000          0.652      0.187      0.737
  5          5            32.000          0.804      0.056      0.737
  6          5            56.000          0.754      0.098      0.737
  7          5           100.000          0.688      0.041      0.688
  8          5           180.000          0.372      0.252      0.372
-------------------------------------------------------------------------
The Linear Interpolation Estimate:   106.3291   Entered P Value: 25
-------------------------------------------------------------------------
Number of Resamplings:   80
The Bootstrap Estimates Mean: 105.8680 Standard Deviation:     5.6981
Original Confidence Limits:   Lower:    94.6667 Upper:   117.0588
Expanded Confidence Limits:   Lower:    88.8354 Upper:   122.4237
Resampling time in Seconds:     0.28  Random_Seed: 390692880
```

Figure 4. ICPIN program output for the IC25.

17.13.3 EXAMPLE OF ANALYSIS OF GIANT KELP, ***MACROCYSTIS PYRIFERA***, GROWTH DATA

17.13.3.1 Formal statistical analysis of the growth data is outlined in Figure 5. The response used in the statistical analysis is mean germ-tube length per replicate. An IC25 can be calculated for the growth data via a point estimation technique (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). Hypothesis testing can be used to obtain the NOEC and LOEC for growth.

17.13.3.2 The statistical analysis using hypothesis tests consists of a parametric test, Dunnett's Procedure, and a nonparametric test, Steel's Many-one Rank Test. The underlying assumptions of the Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are determined by the parametric test.

17.13.3.3 Additionally, if unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a ***t*** test with the Bonferroni adjustment. The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative. For detailed information on the Bonferroni adjustment, see Appendix D.

17.13.3.4 The data, mean and variance of the observations at each concentration including the control for this example are listed in Table 13. A plot of the data is provided in Figure 6.

17.13.3.5 Test for Normality

17.13.3.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are listed in Table 14.

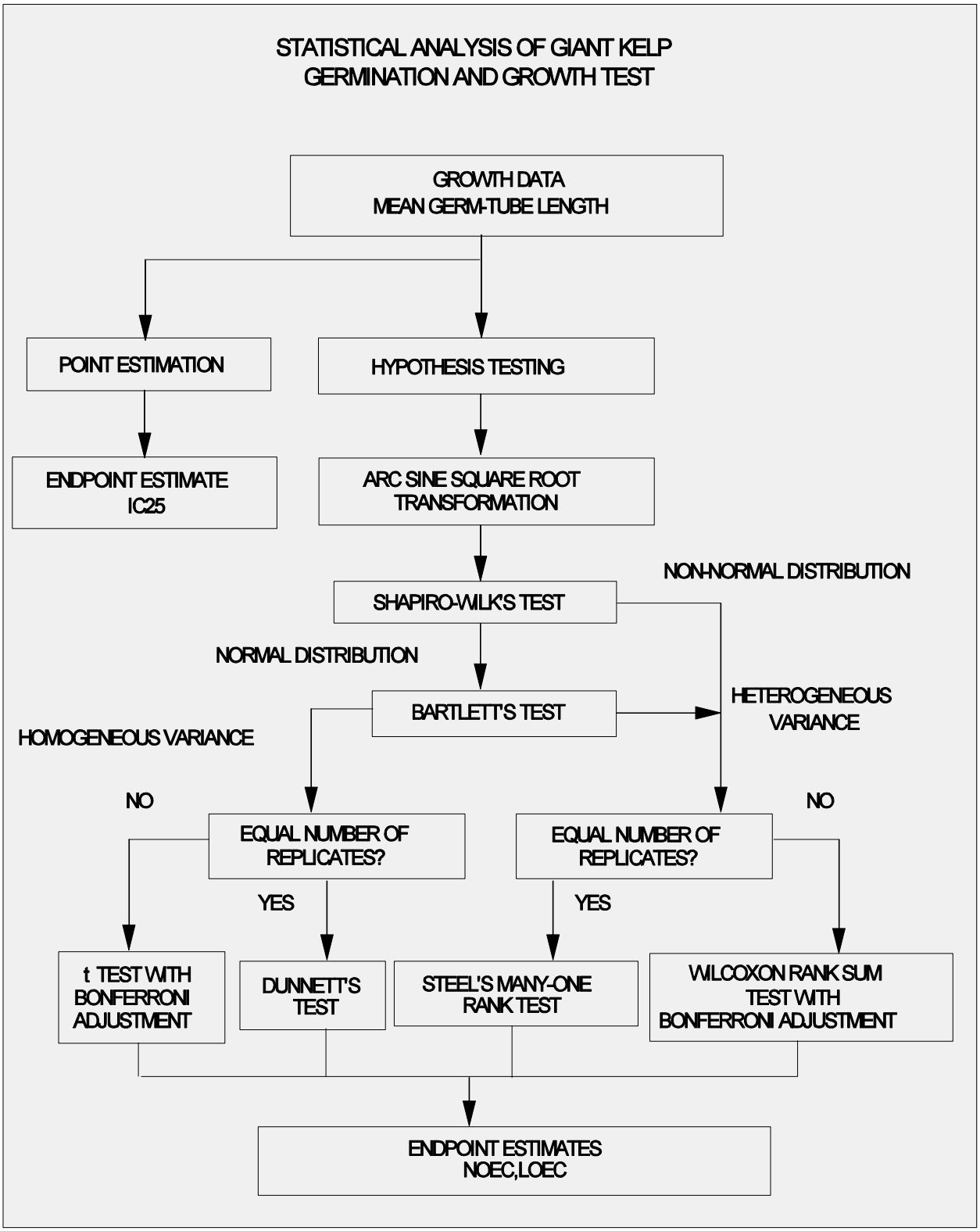

Figure 5. Flowchart for statistical analysis of giant kelp, *Macrocystis pyrifera*, growth data.

TABLE 13. GIANT KELP, ***MACROCYSTIS PYRIFERA***, GROWTH DATA

	Copper Concentration (µg/L)							
Rep	Control	5.60	10.0	18.0	32.0	56.0	100.0	180.0
1	19.58	18.26	13.31	18.59	12.54	11.44	7.92	6.49
2	18.75	16.25	18.92	12.88	10.67	11.88	7.59	7.25
3	19.14	16.39	15.62	16.28	15.95	11.88	8.25	--
4	16.50	18.70	14.30	15.38	12.54	11.00	9.13	7.63
5	17.93	15.62	15.29	19.75	11.66	11.55	8.80	8.13
Mean($\bar{X}_i$)	18.38	17.04	15.49	16.58	12.67	11.55	8.34	7.38
S_i^2	1.473	1.827	4.498	7.327	3.953	0.133	0.396	0.478
i	1	2	3	4	5	6	7	8

TABLE 14. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

	Copper Concentration (µg/L)							
Rep	Control	5.6	10.0	18.0	32.0	56.0	100.0	180.0
1	1.20	1.22	-2.18	2.01	-0.13	-0.11	-0.42	-0.89
2	0.37	-0.79	3.43	-3.70	-2.00	0.33	-0.75	-0.13
3	0.76	-0.65	0.13	-0.30	3.28	0.33	-0.09	--
4	-1.88	1.66	-1.19	-1.20	-0.13	-0.55	0.79	0.25
5	-0.45	-1.42	-0.20	3.17	-1.01	0.00	0.46	0.75

17.13.3.5.2 Calculate the denominator, D, of the statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

$\bar{X}$ = the overall mean of the centered observations

n = the total number of centered observations

17.13.3.5.3 For this set of data, n = 39

$$\& = \frac{1}{39}(-0.03) = 0.000$$

D = 79.8591

17.13.3.5.4 Order the centered observations from smallest to largest

$$X^{(1)} \# X^{(2)} \# \ldots \# X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 15.

TABLE 15. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-3.70	21	-0.11
2	-2.18	22	-0.09
3	-2.00	23	0.00
4	-1.88	24	0.13
5	-1.42	25	0.25
6	-1.20	26	0.33
7	-1.19	27	0.33
8	-1.01	28	0.37
9	-0.89	29	0.46
10	-0.79	30	0.75
11	-0.75	31	0.76
12	-0.65	32	0.79
13	-0.55	33	1.20
14	-0.45	34	1.22
15	-0.42	35	1.66
16	-0.30	36	2.01
17	-0.20	37	3.17
18	-0.13	38	3.28
19	-0.13	39	3.43
20	-0.13		

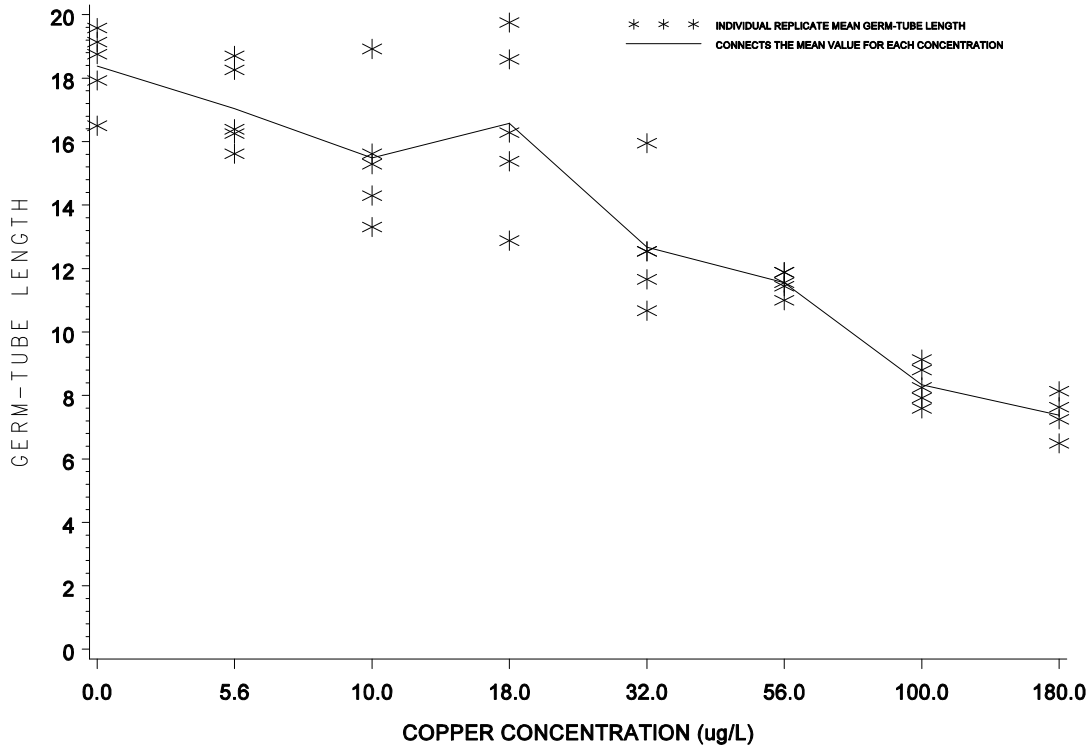

Figure 6. Plot of mean growth data for the giant kelp, *Macrocystis pyrifera*, tests.

17.13.3.5.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2, ... a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 39 and k = 19. The a_i values are listed in Table 16.

17.13.3.5.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i\left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 16. For this set of data:

$$W = \frac{1}{79.8591}(8.7403)^2 = 0.957$$

TABLE 16. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.3989	7.13	$X^{(39)} - X^{(1)}$
2	0.2755	5.46	$X^{(38)} - X^{(2)}$
3	0.2380	5.17	$X^{(37)} - X^{(3)}$
4	0.2104	3.89	$X^{(36)} - X^{(4)}$
5	0.1880	3.08	$X^{(35)} - X^{(5)}$
6	0.1689	2.42	$X^{(34)} - X^{(6)}$
7	0.1520	2.39	$X^{(33)} - X^{(7)}$
8	0.1366	1.80	$X^{(32)} - X^{(8)}$
9	0.1225	1.65	$X^{(31)} - X^{(9)}$
10	0.1092	1.54	$X^{(30)} - X^{(10)}$
11	0.0967	1.21	$X^{(29)} - X^{(11)}$
12	0.0848	1.02	$X^{(28)} - X^{(12)}$
13	0.0733	0.88	$X^{(27)} - X^{(13)}$
14	0.0622	0.78	$X^{(26)} - X^{(14)}$
15	0.0515	0.67	$X^{(25)} - X^{(15)}$
16	0.0409	0.43	$X^{(24)} - X^{(16)}$
17	0.0305	0.20	$X^{(23)} - X^{(17)}$
18	0.0203	0.04	$X^{(22)} - X^{(18)}$
19	0.0101	0.02	$X^{(21)} - X^{(19)}$

17.13.3.5.7 The decision rule for this test is to compare W as calculated in Subsection 5.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For this set of data, the critical value at a significance level of 0.01 and n = 39 observations is 0.917. Since W = 0.957 is greater than the critical value, conclude that the data are normally distributed.

17.13.3.6 Test for Homogeneity of Variance

17.13.3.6.1 The test used to examine whether the variation in mean weight of the mysids is the same across all concentration levels including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{[(\sum_{i=1}^{P} V_i)\ 1n\ \bar{S}^2 - \sum_{i=1}^{P} V_i\ 1n\ S_i^2]}{C}$$

Where: V_i = degrees of freedom for each copper concentration and control, $V_i = (n_i - 1)$

p = number of concentration levels including the control

ln = $\log_e$

i = 1, 2, ..., p where p is the number of concentrations including the control

n_i = the number of replicates for concentration i.

$$\bar{S}^2 = \frac{(\sum_{i=1}^{P} V_i S_i^2)}{\sum_{i=1}^{P} V_i}$$

$$C = 1 + [3(p-1)]^{-1}[\sum_{i=1}^{P} 1/V_i - (\sum_{i=1}^{P} V_i)^{-1}]$$

17.13.3.6.2 For the data in this example (See Table 13), all concentrations including the control have five replicates except the 180 µg/L concentration which has four replicates (n_i = 5 for i = 1 - 7; n_8 = 4). Thus, V_i = 4 for i = 1 - 7 and V_8 = 3.

17.13.3.6.3 Bartlett's statistic is therefore:

$$B = [(31)\ln(2.5761) - \sum_{i=1}^{P} V_i \ln(S_i^2)] / 1.0977$$

= [31(0.9463) - [4ln(1.4729) + ... + 3ln(0.4780)] / 1.0977

= [29.3353 - 9.4481] / 1.0977

= 18.12

17.13.3.6.4 B is approximately distributed as chi-square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with seven degrees of freedom, is 18.48. Since B = 18.12 is less than the critical value, conclude that the variances are not different.

17.13.3.7 ***t*** Test with Bonferroni's Adjustment

17.13.3.7.1 To obtain an estimate of the pooled variance for the ***t*** test with Bonferroni's adjustment, construct an ANOVA table as described in Table 17.

TABLE 17. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2 = SSW/(N-p)
Total	N - 1	SST	

Where: p = number of concentration levels including the control

N = total number of observations $n_1 + n_2 \ldots + n_p$

n_i = number of observations in concentration i

$$SSB = \sum_{i=1}^{P} T_i^2/n_i - G^2/N$$ Between Sum of Squares

$$SST = \sum_{i=1}^{P} \sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$ Total Sum of Squares

$$SSW = SST - SSB$$ Within Sum of Squares

G = the grand total of all sample observations,

$$G = \sum_{i=1}^{P} T_i$$

T_i = the total of the replicate measurements for concentration i

Y_{ij} = the jth observation for concentration i (represents the mean length of the germ-tubes for concentration i in test chamber j)

17.13.3.7.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = n_6 = n_7 = 5$; $n_8 = 4$

N = 39

$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} + Y_{15} = 91.90$
$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} + Y_{25} = 85.22$
$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} + Y_{35} = 77.44$
$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} + Y_{45} = 82.88$
$T_5 = Y_{51} + Y_{52} + Y_{53} + Y_{54} + Y_{55} = 63.36$
$T_6 = Y_{61} + Y_{62} + Y_{63} + Y_{64} + Y_{65} = 57.75$
$T_7 = Y_{71} + Y_{72} + Y_{73} + Y_{74} + Y_{75} = 41.69$
$T_8 = Y_{81} + Y_{82} + Y_{83} + Y_{84} = 29.50$

$G = T_1 + T_2 + T_3 + T_4 + T_5 + T_6 + T_7 + T_8 = 529.74$

$$SSB = \sum_{i=1}^{P} T_i^2/n_i - G^2/N$$

$$= 7749.905 - \frac{(529.74)^2}{39} = 554.406$$

$$SST = \sum_{i=1}^{P} \sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$

$$= 7829.764 - \frac{(529.74)^2}{39} = 634.265$$

$$SSW = SST - SSB = 634.265 - 554.406 = 79.859$$

S_B^2 = SSB/(p-1) = 554.406/(8-1) = 79.201

S_W^2 = SSW/(N-p) = 79.859/(39-8) = 2.576

17.13.3.7.3 Summarize these calculations in the ANOVA table (Table 18).

TABLE 18. ANOVA TABLE FOR THE *t* TEST WITH BONFERRONI'S ADJUSTMENT EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	7	554.406	79.201
Within	31	79.859	2.576
Total	38	634.265	

17.13.3.7.4 To perform the individual comparisons, calculate the *t* statistic for each concentration, and control combination as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_w \sqrt{(1/n_1) + (1/n_i)}}$$

Where: $\bar{x}_i$ = mean length for concentration i

$\bar{x}_1$ = mean length for the control

S_W = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i

17.13.3.7.5 Table 19 includes the calculated t values for each concentration and control combination. In this example, comparing the 5.6 µg/L concentration with the control, the calculation is as follows:

$$t_2 = \frac{(18.38 - 17.04)}{[1.605\sqrt{(1/5)+(1/5)}]}$$

$$= 1.320$$

TABLE 19. CALCULATED t VALUES

Concentration (µg/L)	i	t_i
5.6	2	1.320
10.0	3	2.847
18.0	4	1.773
32.0	5	5.625
56.0	6	6.728
100.0	7	9.891
180.0	8	10.836

17.13.3.7.6 Since the purpose of this test is to detect a significant reduction in mean length, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix D. For an overall alpha level of 0.05, 31 degrees of freedom for error and seven concentrations (excluding the control) the approximate critical value is 2.597. The mean weight for concentration "i" is considered significantly less than the mean weight for the control if t_i is greater than the critical value. Therefore, the 10.0 µg/L, 32 µg/L, 56.0 µg/L,

100.0 µg/L, 180.0 µg/L concentrations have significantly lower mean length than the control. Because the 10.0 µg/L concentration shows signigicantly lower mean length than the control while the higher 18.0 µg/L concentration does not, these test results are considered to have an anomalous dose-response relationship and it is recommended that the test be repeated. If an NOEC and LOEC must be determined for this test, the lowest concentration with significant growth impairment versus the control is considered to the LOEC for growth. Thus, for this test, the NOEC and LOEC would be 5.6 µg/L and 10.0 µg/L, respectively.

17.13.3.8 Calculation of the ICp

17.13.3.8.1 The growth data from Table 13 and Figure 3 are utilized in this example. As can be seen in the figure, the observed means are not monotonically non-increasing with respect to concentration. Therefore, the means must be smoothed prior to calculating the IC

17.13.3.8.2 Starting with the observed control mean, $\overline{Y}_1 = 18.38$ is greater than the observed mean for the lowest copper concentration, $\overline{Y}_2 = 17.044$, so set $M_1 = 18.38$. Likewise, $\overline{Y}_2$ is greater than the observed mean for the next copper concentration, $\overline{Y}_3 = 15.488$, so set $M_2 = 17.044$.

17.13.3.8.3 Comparing $\overline{Y}_3$ to $\overline{Y}_4 = 16.576$, we see that $\overline{Y}_3$ is less than $\overline{Y}_4$.

17.13.3.8.4 Calculate the smoothed means:

$$M_3 = M_4 = (\overline{Y}_3 + \overline{Y}_4)/2 = 16.032$$

17.13.3.8.5 Since $M_4 > \overline{Y}_5 = 12.672 > \overline{Y}_6 = 11.550 > \overline{Y}_7 = 8.338 > \overline{Y}_8 = 7.375$, set $M_5 = 12.672$, $M_6 = 11.550$, $M_7 = 8.338$ and $M_8 = 7.375$. Table 20 contains the smoothed means and Figure 7 gives a plot of the smoothed response curve.

TABLE 20. GIANT KELP, ***MACROCYSTIS PYRIFERA***, MEAN GERM-TUBE LENGTHS AFTER SMOOTHING

Copper Conc. (µg/L)	i	Response Means $\bar{x}_i$ (mm)	Smoothed Means M_i (mm)
Control	1	18.380	18.380
5.6	2	17.044	17.044
10.0	3	15.488	16.032
18.0	4	16.576	16.032
32.0	5	12.672	12.672
56.0	6	11.550	11.550
100.0	7	8.338	8.338
180.0	8	7.375	7.375

17.13.3.8.7 Using the equation in Section 4.2 from Appendix L, the estimate of the IC25 is calculated as follows:

$$ICp = C_j \% [M_1(1 \& p/100) \& M_j] \frac{(C_{(j\%1)} \& C_j)}{(M_{(j\%1)} \& M_j)}$$

$$IC25 = 18.0 + [18.380(1 - 25/100) - 16.032] \frac{(32.0 - 18.0)}{(12.672 - 16.032)}$$

$$= 27.36 \text{ µg/L.}$$

17.13.3.8.6 An IC25 can be estimated using the Linear Interpolation Method. A 25% reduction in length, compared to the controls, would result in a mean length of 13.785 mm, where $M_1(1-p/100) = 18.380(1-25/100)$. Examining the smoothed means and their associated concentrations (Table 20), the response, 13.785 mm, is bracketed by C_4 = 18.0 µg/L and C_5 = 32.0 µg/L.

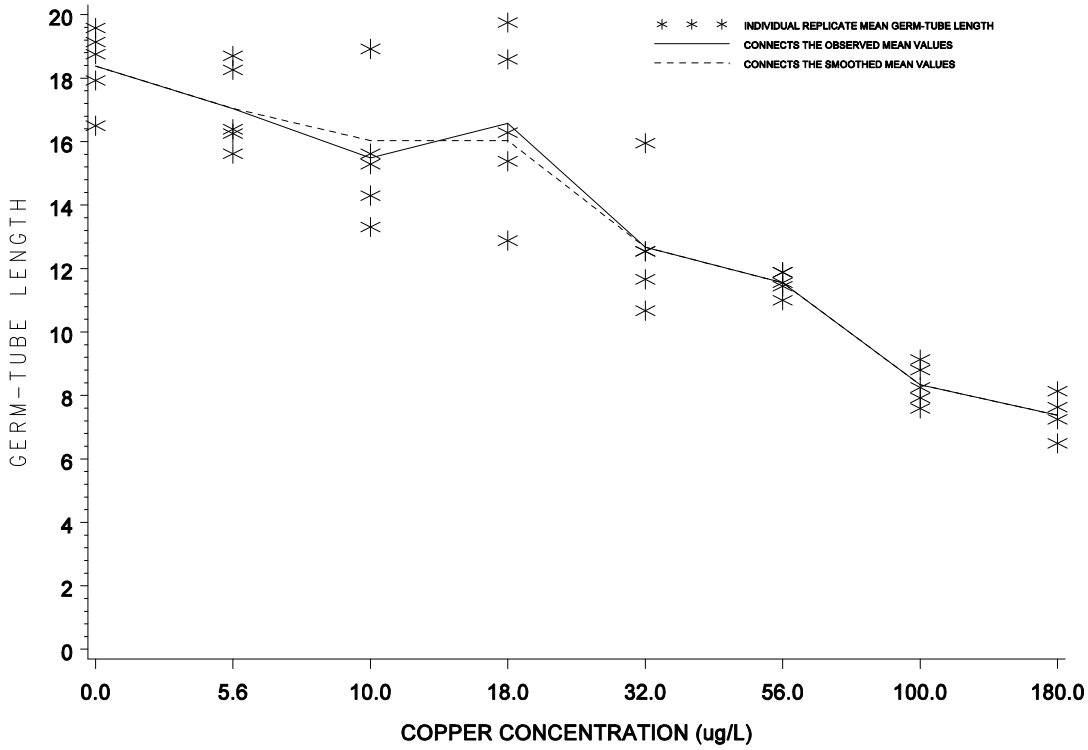

Figure 7. Plot of raw data, observed means, and smoothed means for the giant Kelp, *Macrocystis pyrifera*, growth data from Tables 13 and 20.

17.13.3.8.8 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 27.3625 µg/L. The empirical 95.0% confidence interval for the true mean was 20.8734 µg/L to 42.3270 µg/L. The computer program output for the IC25 for this data set is shown in Figure 8.

17.14 **PRECISION AND ACCURACY**

17.14.1 PRECISION

17.14.1.1 Single-Laboratory Precision

17.14.1.1.1 Single-laboratory precision data for the giant kelp 48-hour test method with the reference toxicants copper chloride and sodium azide with natural seawater are provided in Tables 21-22. The coefficient of variation (CV) of the germination EC50s using copper was 38.8%; the CV of the germ-tube length IC40s using copper was 32.9% (Table 21). The coefficient of variation (CV) of the germination EC50s using azide was 36.7%; the CV of the germ-tube length IC25s using azide was 30.8%, the CV of the germ-tube length IC50s using azide was 28.4% (Table 22).

17.14.1.2 Multi-laboratory Precision

17.14.1.2.1 Multi-laboratory precision data for the kelp 48-hour test method with the reference toxicant copper chloride are provided in Table 23. The coefficient of variation of the IC50s for the germ-tube length endpoint ranged between 8.4% and 55.5% using copper chloride. The coefficient of variation of the IC50s for the germination endpoint ranged between >1.1% and 67.6% using copper chloride.

17.14.2 ACCURACY

17.14.2.1 The accuracy of toxicity tests cannot be determined.

```
Conc. ID        1    2    3    4    5    6    7    8
-------------------------------------------------------
Conc. Tested    0  5.6   10   18   32   56  100  180
-------------------------------------------------------
Response  1 19.5818.2613.3118.5912.5411.44 7.92 6.49
Response  2 18.7516.2518.9212.8810.6711.88 7.59 7.25
Response  3 19.1416.3915.6216.2815.9511.88 8.25
Response  4 16.5018.7014.3015.3812.5411.00 9.13 7.63
Response  5 17.9315.6215.2919.7511.6611.55 8.80
-------------------------------------------------------
*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Copper
Test Start Date:    Test Ending Date:
Test Species: Giant kelp, Macrocystis pyrifera
Test Duration:        48 hours
DATA FILE: kelpgrow.icp
OUTPUT FILE: kelpgrow.i25
-------------------------------------------------------------------------
Conc.     Number     Concentration     Response      Std.       Pooled
 ID     Replicates            ug/L       Means       Dev.   Response Means
-------------------------------------------------------------------------
  1          5             0.000         18.380      1.214     18.380
  2          5             5.600         17.044      1.352     17.044
  3          5            10.000         15.488      2.121     16.032
  4          5            18.000         16.576      2.707     16.032
  5          5            32.000         12.672      1.988     12.672
  6          5            56.000         11.550      0.365     11.550
  7          5           100.000          8.338      0.629      8.338
  8          4           180.000          7.375      0.691      7.375
-------------------------------------------------------------------------
The Linear Interpolation Estimate:    27.3625   Entered P Value: 25
-------------------------------------------------------------------------
Number of Resamplings:   80
The Bootstrap Estimates Mean:  27.5292 Standard Deviation:     4.7812
Original Confidence Limits:   Lower:    20.8734 Upper:    42.3270
Expanded Confidence Limits:   Lower:    17.6289 Upper:    49.8093
Resampling time in Seconds:     0.28  Random_Seed: -35158431
```

Figure 8. ICPIN program output for the IC25.

TABLE 21. SINGLE LABORATORY PRECISION OF THE GIANT KELP, ***MACROCYSTIS PYRIFERA*** GERMINATION AND GERM-TUBE LENGTH TEST WITH COPPER (CU FG/L) CHLORIDE AS THE REFERENCE TOXICANT

Test Number	Germ-Tube Length		Germination	
	NOEC	IC40	NOEC	EC50
1	<5.6	122.7	10.0	67.5
2	10.0	43.1	18.0	73.5
3	18.0	70.7	18.0	124.3
4	5.6	88.0	56.0	101.6
5	32.0	124.7	56.0	122.9
Mean	89.8		90.7	
CV	38.8%		32.9%	

Data from Anderson et al., 1994

TABLE 22. SINGLE LABORATORY PRECISION OF THE GIANT KELP, ***MACROCYSTIS PYRIFERA*** GERMINATION AND GERM-TUBE LENGTH TEST WITH SODIUM AZIDE (MG/L) AS THE REFERENCE TOXICANT

Test Date	Germ-Tube Length			Germination	
	NOEC	IC25	IC50	NOEC	EC50
2/11/92	18.0	39.5	133.7	18.0	52.3
2/18/92	18.0	34.1	96.5	32.0	72.6
6/29/92	32.0	57.5	142.2	32.0	132.1
7/07/92	10.0	33.1	92.5	10.0	79.2
7/15/92	18.0	42.8	138.9	18.0	117.8
7/16/92	5.6	25.0	68.4	10.0	48.3
7/22/92	10.0	30.2	80.6	18.0	62.4
10/09/92	5.6	25.1	80.0	5.6	60.3
7/02/92	10.0	24.8	80.1	18.0	84.0
Mean	34.7		101.4	78.8	
CV	30.8%		28.4%	36.7%	

Data from Hunt et al., 1991

TABLE 23. MULTI-LABORATORY PRECISION OF THE GIANT KELP, ***MACROCYSTIS PYRIFERA*** GERMINATION AND GERM-TUBE LENGTH TEST PERFORMED WITH COPPER CHLORIDE (µG/L) AS THE REFERENCE TOXICANT

	Lab	Germ-tube length NOEC	IC40	Germination NOEC	EC50	CV Germ-tube	CV Germination
March 1990	1	5.6	122.7	10.0	46.9	8.4%	>1.1%
	2	32.0	117.8	32.0	46.2		
	3	18.0	104.1	32.0	*		
May 1990	1	10.0	43.1	18.0	112.0	39.9%	59.3%
	2	<5.6	99.1	32.0	164.2		
	3	18.0	68.7	18.0	67.9		
May 1990	1	18.0	70.7	18.0	112.0	45.3%	nc
	2	18.0	91.3	56.0	64.5		
	3	32.0	134.2	32.0	158.0		
December 1990	1	5.6	88.0	56.0	77.7	45.3%	nc
	2	5.6	45.3	18.0	*		
September 1990	1	32.0	124.7	56.0	127.4	55.5%	7.4%
	2	18.0	54.4	56.0	114.8		
September 1989	1	<10.0	89.3**	56.0	115.5	44.5%	67.6%
	2	<10.0	171.8**	56.0	327.7		
November 1989	1	32.0	>180.0	<10.0	>180.0	nc	nc
	2	10.0	>180.0	18.0	>180.0		
May 1988	1	<56.0	232.0***	<56.0	211.0	nc	50.0%
	2	<56.0	*	56.0	100.7		

* No EC50 calculated because response was less than 50%.
** Only concentration means available, therefore no IC40 values were calculated.
nc Not calculated (Insufficient numbers to calculate the coefficient of variation).
*** IC50 value, not IC40

Data from Hunt et al., 1991

APPENDIX I. MACROCYSTIS TEST: STEP-BY-STEP SUMMARY

PREPARATION OF TEST SOLUTIONS

A. Determine test concentrations and appropriate dilution water based on NPDES permit conditions and guidance from the appropriate regulatory agency.

B. Prepare effluent test solutions by diluting well mixed unfiltered effluent using volumetric flasks and pipettes. Use hypersaline brine where necessary to maintain all test solutions at 34 ± 2‰. Include brine controls in tests that use brine.

C. Prepare a copper reference toxicant stock solution (10,000 µg/L) by adding 0.0268 of copper chloride ($CuCl_2$º2H_2O) to 1 liter of reagent water.

D. Prepare copper reference toxicant solution of 0 (control) 5.6, 10, 18, 32, 100 and 180 µg/L by adding 0, 0.56, 1.0 1.8, 3.2, 10.0 and 18.0 mL of stock solution, respectively, to a 1-L volumetric flask and filling to 1-L with dilution water.

E. Sample effluent and reference toxicant solutions for physical/chemical analysis. Measure salinity, pH and dissolved oxygen from each test concentration.

F. Randomize numbers for test chambers and record the chamber numbers with their respective test concentrations on a randomization data sheet. Store the data sheet safely until after the test samples have been analyzed.

G. Place test chambers in a water bath or environmental chamber set to 15EC and allow temperature to equilibrate.

H. Measure the temperature daily in one random replicate (or separate chamber) of each test concentration. Monitor the temperature of the water bath or environmental chamber continuously.

I. At the end of the test, measure salinity, pH, and dissolved oxygen concentration from each test concentration.

PREPARATION AND ANALYSIS OF TEST ORGANISMS

A. Collect sporophylls and rinse in 0.2 µm filtered seawater. Store at 9-12EC for no more than 24 hours before zoospore release.

B. Blot sporophylls and leave exposed to air for one hour.

C. Place 25-30 sporophylls one liter of 0.2 µm filtered seawater for no more than two hours. The presence of zoospores is indicated by a slight cloudiness in the water.

D. Take a sample of the zoospore solution from the top 5 centimeters of the beaker and determine the spore density using a hemacytometer. Determine the volume of water necessary to give 7,500 spores/mL of test solution. This volume should not exceed one percent of the test solution volume.

E. Verify that the zoospores are swimming, then pipet the volume of water necessary to give 7,500 spores/mL into each of the test chambers. Take zoospores from the top 5 centimeters of the release beaker so that only swimming zoospores are used.

F. At 48 ± 3 hours, count the number of germinated and non-germinated spores of the first 100 spores encountered in each replicate of each concentration. Measure the length of 10 randomly selected germination tubes (or preserve with 0.1% glutaraldehyde for later examination).

G. Analyze the data.

H. Include standard reference toxicant point estimate values in the standard quality control charts.

Data Sheet for Kelp Toxicity Test

Test Start Date: Start Time: Kelp Species :
Test End Date: End Time: Collection/Arrival Date:
Reference Toxicant: Kelp Source:

Sample Source: Microscope Model:
Sample Type: Solid Elutriate Pore Water Water Effluent Ref Tox
Micrometer Conversion Factor:

Test Cont. #	Station Code	Number of Spores Germ.	Number of Spores Not Germ.	Length Measurements (in ocular micrometer units)										Notes
				L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	
1														
2														
3														
4														
5														
6														
7														
8														
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Computer Data Storage
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CITED REFERENCES

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APPENDIX A

INDEPENDENCE, RANDOMIZATION, AND OUTLIERS

1. STATISTICAL INDEPENDENCE

1.1 Dunnett's Procedure and the *t* test with Bonferroni's adjustment are parametric procedures based on the assumptions that (1) the observations within treatments are independent and normally distributed, and (2) that the variance of the observations is homogeneous across all toxicant concentrations and the control. Of the three possible departures from the assumptions, non-normality, heterogeneity of variance, and lack of independence, those caused by lack of independence are the most difficult to resolve (see Scheffe, 1959). For toxicity data, statistical independence means that given knowledge of the true mean for a given concentration or control, knowledge of the error in any one actual observation would provide no information about the error in any other observation. Lack of independence is difficult to assess and difficult to test for statistically. It may also have serious effects on the true alpha or beta level. Therefore, it is of utmost importance to be aware of the need for statistical independence between observations and to be constantly vigilant in avoiding any patterned experimental procedure that might compromise independence. One of the best ways to help insure independence is to follow proper randomization procedures throughout the test.

2. RANDOMIZATION

2.1 Randomization of the distribution of test organisms among test chambers, and the arrangement of treatments and replicate chambers is an important part of conducting a valid test. The purpose of randomization is to avoid situations where test organisms are placed serially into test chambers, or where all replicates for a test concentration are located adjacent to one another, which could introduce bias into the test results.

2.2 An example of randomization of the distribution of test organisms among test chambers, and an example of randomization of arrangement of treatments and replicate chambers are described using the topsmelt, *Atherinops affinis*, Survival and Growth test. For the purpose of the example, the test design is as follows:

Five effluent concentrations are tested in addition to the control. The effluent concentrations are as follows: 6.25%, 12.5%, 25.0%, 50.0%, and 100.0%. There are five replicate chambers per treatment. Each replicate chamber contains five larvae.

2.3 RANDOMIZATION OF FISH TO REPLICATE CHAMBERS EXAMPLE

2.3.1 Consider first the random assignment of the fish to the replicate chambers. The first step is to label each of the replicate chambers with the control or effluent concentration and the replicate number. The next step is to assign each replicate chamber three double-digit numbers. An example of this assignment is provided in Table A.1. Note that the double digits 00 and 91 through 99 were not used.

2.3.2 The random numbers used to carry out the random assignment of fish to replicate chambers are provided in Table A.2. The third step is to choose a starting position in Table A.2, and read the first double digit number. The first number read identifies the replicate chamber for the first fish taken from the tank. For the example, the first entry in row 2 was chosen as the starting position. The first number in this row is 37. According to Table A.1, this number corresponds to replicate chamber 2 of the 6.25% effluent concentration. Thus, the first fish taken from the tank is to be placed in replicate chamber 2 of the 6.25% effluent concentration.

2.3.3 The next step is to read the double digit number to the right of the first one. The second number identifies the replicate chamber for the second fish taken from the tank. Continuing the example, the second number read in row 2 of Table A.2 is 54. According to Table A.1, this number corresponds to replicate chamber 4 of the 50.0% effluent concentration. Thus, the second fish taken from the tank is to be placed in replicate chamber 4 of the 50.0% effluent concentration.

2.3.4 Continue in this fashion until all the fish have been randomly assigned to a replicate chamber. In order to fill each replicate chamber with ten fish, the assigned numbers will be used more than once. If a number is read from the table that was not assigned to a replicate chamber, then ignore it and continue to the next number. If a replicate chamber becomes filled and a

number is read from the table that corresponds to it, then ignore that value and continue to the next number. The first ten random summarized in Table A.3.2.3.5 Three double-digit numbers were assigned to each replicate chamber (instead of one or two double-digit numbers) in order to make efficient use of the random number table (Table A.2). To illustrate, consider the assignment of only one double-digit number to each replicate chamber: the first column of assigned numbers in Table A.1. Whenever the numbers 00 and 31 through 99 are read from Table A.2, they will be disregarded and the next number will be read.

TABLE A.1. RANDOM ASSIGNMENT OF FISH TO REPLICATE CHAMBERS EXAMPLE ASSIGNED NUMBERS FOR EACH REPLICATE CHAMBER

Assigned Numbers	Replicate Chamber
01, 31, 61	Control, replicate chamber 1
02, 32, 62	Control, replicate chamber 2
03, 33, 63	Control, replicate chamber 3
04, 34, 64	Control, replicate chamber 4
05, 35, 65	Control, replicate chamber 5
06, 36, 66	6.25% effluent, replicate chamber 1
07, 37, 67	6.25% effluent, replicate chamber 2
08, 38, 68	6.25% effluent, replicate chamber 3
09, 39, 69	6.25% effluent, replicate chamber 4
10, 40, 70	6.25% effluent, replicate chamber 5
11, 41, 71	12.5% effluent, replicate chamber 1
12, 42, 72	12.5% effluent, replicate chamber 2
13, 43, 73	12.5% effluent, replicate chamber 3
14, 44, 74	12.5% effluent, replicate chamber 4
15, 45, 75	12.5% effluent, replicate chamber 5
16, 46, 76	25.0% effluent, replicate chamber 1
17, 47, 77	25.0% effluent, replicate chamber 2
18, 48, 78	25.0% effluent, replicate chamber 3
19, 49, 79	25.0% effluent, replicate chamber 4
20, 50, 80	25.0% effluent, replicate chamber 5
21, 51, 81	50.0% effluent, replicate chamber 1
22, 52, 82	50.0% effluent, replicate chamber 2
23, 53, 83	50.0% effluent, replicate chamber 3
24, 54, 84	50.0% effluent, replicate chamber 4
25, 55, 85	50.0% effluent, replicate chamber 5
26, 56, 86	100.0% effluent, replicate chamber 1
27, 57, 87	100.0% effluent, replicate chamber 2
28, 58, 88	100.0% effluent, replicate chamber 3
29, 59, 89	100.0% effluent, replicate chamber 4
30, 60, 90	100.0% effluent, replicate chamber 5

TABLE A.2. TABLE OF RANDOM NUMBERS (Dixon and Massey, 1983)

10 09 73 25 33	76 52 01 35 86	34 67 35 43 76	80 95 90 91 17	39 29 27 49 45
37 54 20 48 05	64 89 47 42 96	24 80 52 40 37	20 63 61 04 02	00 82 29 16 65
08 42 26 89 53	19 64 50 93 03	23 20 90 25 60	15 95 33 47 64	35 08 03 36 06
99 01 90 25 29	09 37 67 07 15	38 31 13 11 65	88 67 67 43 97	04 43 62 76 59
12 80 79 99 70	80 15 73 61 47	64 03 23 66 53	98 95 11 68 77	12 27 17 68 33
66 06 57 47 17	34 07 27 68 50	36 69 73 61 70	65 81 33 98 85	11 19 92 91 70
31 06 01 08 05	45 57 18 24 06	35 30 34 26 14	86 79 90 74 39	23 40 30 97 32
85 26 97 76 02	02 05 16 56 92	68 66 57 48 18	73 05 38 52 47	18 62 38 85 79
63 57 33 21 35	05 32 54 70 48	90 55 35 75 48	28 46 82 87 09	83 49 12 56 24
73 79 64 57 53	03 52 96 47 78	35 80 83 42 82	60 93 52 03 44	35 27 38 84 35
98 52 01 77 67	14 90 56 86 07	22 10 94 05 58	60 97 09 34 33	50 50 07 39 98
11 80 50 54 31	39 80 82 77 32	50 72 56 82 48	29 40 52 42 01	52 77 56 78 51
83 45 29 96 34	06 28 89 80 83	13 74 67 00 78	18 47 54 06 10	68 71 17 78 17
88 68 54 02 00	86 50 75 84 01	36 76 66 79 51	90 36 47 64 93	29 60 91 10 62
99 59 46 73 48	87 51 76 49 69	91 82 60 89 28	93 78 56 13 68	23 47 83 41 13
65 48 11 76 74	17 46 85 09 50	58 04 77 69 74	73 03 95 71 86	40 21 81 65 44
80 12 43 56 35	17 72 70 80 15	45 31 82 23 74	21 11 57 82 53	14 38 55 37 63
74 35 09 98 17	77 40 27 72 14	43 23 60 02 10	45 52 16 42 37	96 28 60 26 55
69 91 62 68 03	66 25 22 91 48	36 93 68 72 03	76 62 11 39 90	94 40 05 64 18
09 89 32 05 05	14 22 56 85 14	46 42 75 67 88	96 29 77 88 22	54 38 21 45 98
91 49 91 45 23	68 47 92 76 86	46 16 28 35 54	94 75 08 99 23	37 08 92 00 48
80 33 69 45 98	26 94 03 68 58	70 29 73 41 35	53 14 03 33 40	42 05 08 23 41
44 10 48 19 49	85 15 74 79 54	32 97 92 65 75	57 60 04 08 81	22 22 20 64 13
12 55 07 37 42	11 10 00 20 40	12 86 07 46 97	96 64 48 94 39	28 70 72 58 15
63 60 64 93 29	16 50 53 44 84	40 21 95 25 63	43 65 17 70 82	07 20 73 17 90
61 19 69 04 46	26 45 74 77 74	51 92 43 37 29	65 39 45 95 93	42 58 26 05 27
15 47 44 52 66	95 27 07 99 53	59 36 78 38 48	82 39 61 01 18	33 21 15 94 66
94 55 72 85 73	67 89 75 43 87	54 62 24 44 31	91 19 04 25 92	92 92 74 59 73
42 48 11 62 13	97 34 40 87 21	16 86 84 87 67	03 07 11 20 59	25 70 14 66 70
23 52 37 83 17	73 20 88 98 37	68 93 59 14 16	26 25 22 96 63	05 52 28 25 62
04 49 35 24 94	75 24 63 38 24	45 86 25 10 25	61 96 27 93 35	65 33 71 24 72
00 54 99 76 54	64 05 18 81 59	96 11 96 38 96	54 69 28 23 91	23 28 72 95 29
35 96 31 53 07	26 89 80 93 45	33 35 13 54 62	77 97 45 00 24	90 10 33 93 33
59 80 80 83 91	45 42 72 68 42	83 60 94 97 00	13 02 12 48 92	78 56 52 01 06
46 05 88 52 36	01 39 09 22 86	77 28 14 40 77	93 91 08 36 47	70 61 74 29 41
32 17 90 05 97	87 37 92 52 41	05 56 70 70 07	86 74 31 71 57	85 39 41 18 38
69 23 46 14 06	20 11 74 52 04	15 95 66 00 00	18 74 39 24 23	97 11 89 63 38
19 56 54 14 30	01 75 87 53 79	40 41 92 15 85	66 67 43 68 06	84 96 28 52 07
45 15 51 49 38	19 47 60 72 46	43 66 79 45 43	59 04 79 00 33	20 82 66 95 41
94 86 43 19 94	36 16 81 08 51	34 88 88 15 53	01 54 03 54 56	05 01 45 11 76
98 08 62 48 26	45 24 02 84 04	44 99 90 88 96	39 09 47 34 07	35 44 13 18 80
33 18 51 62 32	41 94 15 09 49	89 43 54 85 81	88 69 54 19 94	37 54 87 30 43
80 95 10 04 06	96 38 27 07 74	20 15 12 33 87	25 01 62 52 98	94 62 46 11 71
79 75 24 91 40	71 96 12 82 96	69 86 10 25 91	74 85 22 05 39	00 38 75 95 79
18 63 33 25 37	98 14 50 65 71	31 01 02 46 74	05 45 56 14 27	77 93 89 19 36
74 02 94 39 02	77 55 73 22 70	97 79 01 71 19	52 52 75 80 21	80 81 45 17 48
54 17 84 56 11	80 99 33 71 43	05 33 51 29 69	56 12 71 92 55	36 04 09 03 24
11 66 44 98 83	52 07 98 48 27	59 38 17 15 39	09 97 33 34 40	88 46 12 33 56
48 32 47 79 28	31 24 96 47 10	02 29 53 68 70	32 30 75 75 46	15 02 00 99 94
69 07 49 41 38	87 63 79 19 76	35 58 40 44 01	10 51 82 16 15	01 84 87 69 38

2.4 RANDOMIZATION OF REPLICATE CHAMBERS TO POSITIONS EXAMPLE

2.4.1 Next consider the random assignment of the 30 replicate chambers to positions within the water bath (or equivalent). Assume that the replicate chambers are to be positioned in a five row by six column rectangular array. The first step is to label the positions in the water bath. Table A.4 provides an example layout. assignments of fish to replicate chambers for the example are

TABLE A.3. EXAMPLE OF RANDOM ASSIGNMENT OF FIRST TEN FISH TO REPLICATE CHAMBERS

Fish	Assignment
First fish taken from tank	6.25% effluent, replicate chamber 2
Second fish taken from tank	50.0% effluent, replicate chamber 4
Third fish taken from tank	25.0% effluent, replicate chamber 5
Fourth fish taken from tank	25.0% effluent, replicate chamber 3
Fifth fish taken from tank	Control, replicate chamber 5
Sixth fish taken from tank	Control, replicate chamber 4
Seventh fish taken from tank	100.0% effluent, replicate chamber 4
Eighth fish taken from tank	25.0% effluent, replicate chamber 2
Ninth fish taken from tank	12.5% effluent, replicate chamber 2
Tenth fish taken from tank	50.0% effluent, replicate chamber 4

TABLE A.4. RANDOM ASSIGNMENT OF REPLICATE CHAMBERS TO POSITIONS: EXAMPLE LABELLING THE POSITIONS WITHIN THE WATER BATH

1	2	3	4	5	6
7	8	9	10	11	12
13	14	15	16	17	18
19	20	21	22	23	24
25	26	27	28	29	30

2.4.2 The second step is to assign each of the 30 positions three double-digit numbers. An example of this assignment is provided in Table A.5. Note that the double digits 00 and 91 through 99 were not used.

2.4.3 The random numbers used to carry out the random assignment of replicate chambers to positions are provided in Table A.2. The third step is to choose a starting position in Table A.2, and read the first double-digit number. The first number read identifies the position for the first replicate chamber of the control. For the example, the first entry in row 10 of Table A.2 was chosen as the starting position. The first number in this row was 73. According to Table A.5, this number corresponds to position 13. Thus, the first replicate chamber for the control will be placed in position 13.

2.4.4 The next step is to read the double-digit number to the right of the first one. The second number identifies the position for the second replicate chamber of the control. Continuing the example, the second number read in row 10 of Table A.2 is 79. According to Table A.5, this number corresponds to position 19. Thus, the second replicate chamber for the control will be placed in position 19.

2.4.5 Continue in this fashion until all the replicate chambers have been assigned to a position. The first five numbers read will identify the positions for the control replicate chambers, the second five numbers read will identify the positions for the lowest effluent concentration replicate chambers, and so on. If a number is read from the table that was not assigned to a position, then ignore that value and continue to the next number. If a number is repeated in Table A.2, then ignore the repeats and continue to the next number. The complete randomization of replicate chambers to positions for the example is displayed in Table A.6.

2.4.6 Three double-digit numbers were assigned to each position (instead of one or two) in order to make efficient use of the random number table (Table A.2). To illustrate, consider the assignment of only one double-digit number to each position: the first column of assigned numbers in Table A.5. Whenever the numbers 00 and 31 through 99 are read from Table A.2, they will be disregarded and the next number will be read.

3. **OUTLIERS**

3.1 An outlier is an inconsistent or questionable data point that appears unrepresentative of the general trend exhibited by the majority of the data. Outliers may be detected by tabulation of the data, plotting, and by an analysis of the residuals. An explanation should be sought for any questionable data points. Without an explanation, data points should be discarded only with extreme caution. If there is no explanation, the analysis should be performed both with and without the outlier, and the results of both analyses should be reported.

3.2 Gentleman-Wilk's A statistic gives a test for the condition that the extreme observation may be considered an outlier. For a discussion of this, and other techniques for evaluating outliers, see Draper and John (1981).

TABLE *A.5.* *RANDOM ASSIGNMENT OF REPLICATE CHAMBERS TO POSITIONS: EXAMPLE ASSIGNED NUMBERS FOR EACH POSITION*

Assigned Numbers	Position
01, 31, 61	1
02, 32, 62	2
03, 33, 63	3
04, 34, 64	4
05, 35, 65	5
06, 36, 66	6
07, 37, 67	7
08, 38, 68	8
09, 39, 69	9
10, 40, 70	10
11, 41, 71	11
12, 42, 72	12
13, 43, 73	13
14, 44, 74	14
15, 45, 75	15
16, 46, 76	16
17, 47, 77	17
18, 48, 78	18
19, 49, 79	19
20, 50, 80	20
21, 51, 81	21
22, 52, 82	22
23, 53, 83	23
24, 54, 84	24
25, 55, 85	25
26, 56, 86	26
27, 57, 87	27
28, 58, 88	28
29, 59, 89	29
30, 60, 90	30

TABLE A.6. EXAMPLE OF RANDOM ASSIGNMENT OF REPLICATE CHAMBERS TO POSITIONS:

ASSIGNMENT OF ALL 30 POSITIONS					
25.0%	50.0%	6.25%	Control	6.25%	100.0%
25.0%	12.5%	50.0%	25.0%	50.0%	12.5%
Control	12.5%	100.0%	100.0%	6.25%	6.25%
Control	12.5%	100.0%	6.25%	Control	25.0%
100.0%	25.0%	Control	50.0%	50.0%	12.5%

APPENDIX B

VALIDATING NORMALITY AND HOMOGENEITY OF VARIANCE ASSUMPTIONS

1. INTRODUCTION

1.1 Dunnett's Procedure and the *t* test with Bonferroni's adjustment are parametric procedures based on the assumptions that the observations within treatments are independent and normally distributed, and that the variance of the observations is homogeneous across all toxicant concentrations and the control. These assumptions should be checked prior to using these tests, to determine if they have been met. Tests for validating the assumptions are provided in the following discussion. If the tests fail (if the data do not meet the assumptions), a nonparametric procedure such as Steel's Many-one Rank Test may be more appropriate. However, the decision on whether to use parametric or nonparametric tests may be a judgement call, and a statistician should be consulted in selecting the analysis.

2. TEST FOR NORMAL DISTRIBUTION OF DATA

2.1 SHAPIRO-WILK'S TEST

2.1.1 One formal test for normality is the Shapiro-Wilk's Test (Conover, 1980). The test statistic is obtained by dividing the square of an appropriate linear combination of the sample order statistics by the usual symmetric estimate of variance. The calculated W must be greater than zero and less than or equal to one. This test is recommended for a sample size of 50 or less. If the sample size is greater than 50, the Kolmogorov "D" statistic (Stephens, 1974) is recommended. An example of the Shapiro-Wilk's test is provided below.

2.2 The example uses growth data from the Mysid Larval Survival and Growth Test. The same data are used later in the discussions of the homogeneity of variance determination in Section 3 of this appendix and Dunnett's Procedure in Appendix C. The data, the mean and variance of the observations at each concentration, including the control, are listed in Table B.1.

TABLE B.1. MYSID, ***HOLMESIMYSIS COSTATA***, GROWTH DATA

Replicate	Control	Concentration (%) 1.80	3.20	5.60
1	0.048	0.055	0.057	0.041
2	0.058	0.048	0.050	0.040
3	0.047	0.042	0.046	0.041
4	0.058	0.041	0.043	0.043
5	0.051	0.052	0.045	0.040
Mean($\bar{X}_i$)	0.052	0.048	0.048	0.041
S_i^2	0.0000283	0.0000373	0.0000307	0.0000015
i	1	2	3	4

2.3 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are listed in Table B.2.

TABLE B.2. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

Replicate	Control	Concentration (%) 1.80	3.20	5.60
1	-0.004	0.007	0.009	0.000
2	0.006	0.000	0.002	-0.001
3	-0.005	-0.006	-0.002	0.000
4	0.006	-0.007	-0.005	0.002
5	-0.001	0.004	-0.003	-0.001

2.4 Calculate the denominator, D, of the statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

& = the overall mean of the centered observations

n = the total number of centered observations

2.4.1 For this set of data, n = 20

$$\text{\&} = \frac{1}{20}(0.001) = 0.000$$

D = 0.000393

2.5 Order the centered observations from smallest to largest

$$X^{(1)} \# X^{(2)} \# \ldots \# X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table B.3.

TABLE B.3. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.007	11	0.000
2	-0.006	12	0.000
3	-0.005	13	0.000
4	-0.005	14	0.002
5	-0.004	15	0.002
6	-0.003	16	0.004
7	-0.002	17	0.006
8	-0.001	18	0.006
9	-0.001	19	0.007
10	-0.001	20	0.009

2.6 From Table B.4, for the number of observations, n, obtain the coefficients a_1, a_2, ... a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 20 and k = 10. The a_i values are listed in Table B.5.

TABLE B.4. COEFFICIENTS FOR THE SHAPIRO-WILK'S TEST (Conover, 1980)

	Number of Observations								
$i \pm^n$	2	3	4	5	6	7	8	9	10
1	0.7071	0.7071	0.6872	0.6646	0.6431	0.6233	0.6052	0.5888	0.5739
2	-	0.0000	0.1667	0.2413	0.2806	0.3031	0.3164	0.3244	0.3291
3	-	-	-	0.0000	0.0875	0.1401	0.1743	0.1976	0.2141
4	-	-	-	-	-	0.0000	0.0561	0.0947	0.1224
5	-	-	-	-	-	-	-	0.0000	0.0399

	Number of Observations									
$i \pm^n$	11	12	13	14	15	16	17	18	19	20
1	0.5601	0.5475	0.5359	0.5251	0.5150	0.5056	0.4968	0.4886	0.4808	0.4734
2	0.3315	0.3325	0.3325	0.3318	0.3306	0.3290	0.3273	0.3253	0.3232	0.3211
3	0.2260	0.2347	0.2412	0.2460	0.2495	0.2521	0.2540	0.2553	0.2561	0.2565
4	0.1429	0.1586	0.1707	0.1802	0.1878	0.1939	0.1988	0.2027	0.2059	0.2085
5	0.0695	0.0922	0.1099	0.1240	0.1353	0.1447	0.1524	0.1587	0.1641	0.1686
6	0.0000	0.0303	0.0539	0.0727	0.0880	0.1005	0.1109	0.1197	0.1271	0.1334
7	-	-	0.0000	0.0240	0.0433	0.0593	0.0725	0.0837	0.0932	0.1013
8	-	-	-	-	0.0000	0.0196	0.0359	0.0496	0.0612	0.0711
9	-	-	-	-	-	-	0.0000	0.0163	0.0303	0.0422
10	-	-	-	-	-	-	-	-	0.0000	0.0140

	Number of Observations									
$i \pm^n$	21	22	23	24	25	26	27	28	29	30
1	0.4643	0.4590	0.4542	0.4493	0.4450	0.4407	0.4366	0.4328	0.4291	0.4254
2	0.3185	0.3156	0.3126	0.3098	0.3069	0.3043	0.3018	0.2992	0.2968	0.2944
3	0.2578	0.2571	0.2563	0.2554	0.2543	0.2533	0.2522	0.2510	0.2499	0.2487
4	0.2119	0.2131	0.2139	0.2145	0.2148	0.2151	0.2152	0.2151	0.2150	0.2148
5	0.1736	0.1764	0.1787	0.1807	0.1822	0.1836	0.1848	0.1857	0.1864	0.1870
6	0.1399	0.1443	0.1480	0.1512	0.1539	0.1563	0.1584	0.1601	0.1616	0.1630
7	0.1092	0.1150	0.1201	0.1245	0.1283	0.1316	0.1346	0.1372	0.1395	0.1415
8	0.0804	0.0878	0.0941	0.0997	0.1046	0.1089	0.1128	0.1162	0.1192	0.1219
9	0.0530	0.0618	0.0696	0.0764	0.0823	0.0876	0.0923	0.0965	0.1002	0.1036
10	0.0263	0.0368	0.0459	0.0539	0.0610	0.0672	0.0728	0.0778	0.0822	0.0862
11	0.0000	0.0122	0.0228	0.0321	0.0403	0.0476	0.0540	0.0598	0.0650	0.0697
12	-	-	0.0000	0.0107	0.0200	0.0284	0.0358	0.0424	0.0483	0.0537
13	-	-	-	-	0.0000	0.0094	0.0178	0.0253	0.0320	0.0381
14	-	-	-	-	-	-	0.0000	0.0084	0.0159	0.0227
15	-	-	-	-	-	-	-	-	0.0000	0.0076

TABLE B.4. COEFFICIENTS FOR THE SHAPIRO-WILK'S TEST (CONTINUED)

	Number of Observations									
$i \pm^n$	31	32	33	34	35	36	37	38	39	40
1	0.4220	0.4188	0.4156	0.4127	0.4096	0.4068	0.4040	0.4015	0.3989	0.3964
2	0.2921	0.2898	0.2876	0.2854	0.2834	0.2813	0.2794	0.2774	0.2755	0.2737
3	0.2475	0.2462	0.2451	0.2439	0.2427	0.2415	0.2403	0.2391	0.2380	0.2368
4	0.2145	0.2141	0.2137	0.2132	0.2127	0.2121	0.2116	0.2110	0.2104	0.2098
5	0.1874	0.1878	0.1880	0.1882	0.1883	0.1883	0.1883	0.1881	0.1880	0.1878
6	0.1641	0.1651	0.1660	0.1667	0.1673	0.1678	0.1683	0.1686	0.1689	0.1691
7	0.1433	0.1449	0.1463	0.1475	0.1487	0.1496	0.1505	0.1513	0.1520	0.1526
8	0.1243	0.1265	0.1284	0.1301	0.1317	0.1331	0.1344	0.1356	0.1366	0.1376
9	0.1066	0.1093	0.1118	0.1140	0.1160	0.1179	0.1196	0.1211	0.1225	0.1237
10	0.0899	0.0931	0.0961	0.0988	0.1013	0.1036	0.1056	0.1075	0.1092	0.1108
11	0.0739	0.0777	0.0812	0.0844	0.0873	0.0900	0.0924	0.0947	0.0967	0.0986
12	0.0585	0.0629	0.0669	0.0706	0.0739	0.0770	0.0798	0.0824	0.0848	0.0870
13	0.0435	0.0485	0.0530	0.0572	0.0610	0.0645	0.0677	0.0706	0.0733	0.0759
14	0.0289	0.0344	0.0395	0.0441	0.0484	0.0523	0.0559	0.0592	0.0622	0.0651
15	0.0144	0.0206	0.0262	0.0314	0.0361	0.0404	0.0444	0.0481	0.0515	0.0546
16	0.0000	0.0068	0.0131	0.0187	0.0239	0.0287	0.0331	0.0372	0.0409	0.0444
17	-	-	0.0000	0.0062	0.0119	0.0172	0.0220	0.0264	0.0305	0.0343
18	-	-	-	-	0.0000	0.0057	0.0110	0.0158	0.0203	0.0244
19	-	-	-	-	-	-	0.0000	0.0053	0.0101	0.0146
20	-	-	-	-	-	-	-	-	0.0000	0.0049

	Number of Observations									
$i \pm^n$	41	42	43	44	45	46	47	48	49	50
1	0.3940	0.3917	0.3894	0.3872	0.3850	0.3830	0.3808	0.3789	0.3770	0.3751
2	0.2719	0.2701	0.2684	0.2667	0.2651	0.2635	0.2620	0.2604	0.2589	0.2574
3	0.2357	0.2345	0.2334	0.2323	0.2313	0.2302	0.2291	0.2281	0.2271	0.2260
4	0.2091	0.2085	0.2078	0.2072	0.2065	0.2058	0.2052	0.2045	0.2038	0.2032
5	0.1876	0.1874	0.1871	0.1868	0.1865	0.1862	0.1859	0.1855	0.1851	0.1847
6	0.1693	0.1694	0.1695	0.1695	0.1695	0.1695	0.1695	0.1693	0.1692	0.1691
7	0.1531	0.1535	0.1539	0.1542	0.1545	0.1548	0.1550	0.1551	0.1553	0.1554
8	0.1384	0.1392	0.1398	0.1405	0.1410	0.1415	0.1420	0.1423	0.1427	0.1430
9	0.1249	0.1259	0.1269	0.1278	0.1286	0.1293	0.1300	0.1306	0.1312	0.1317
10	0.1123	0.1136	0.1149	0.1160	0.1170	0.1180	0.1189	0.1197	0.1205	0.1212
11	0.1004	0.1020	0.1035	0.1049	0.1062	0.1073	0.1085	0.1095	0.1105	0.1113
12	0.0891	0.0909	0.0927	0.0943	0.0959	0.0972	0.0986	0.0998	0.1010	0.1020
13	0.0782	0.0804	0.0824	0.0842	0.0860	0.0876	0.0892	0.0906	0.0919	0.0932
14	0.0677	0.0701	0.0724	0.0745	0.0765	0.0783	0.0801	0.0817	0.0832	0.0846
15	0.0575	0.0602	0.0628	0.0651	0.0673	0.0694	0.0713	0.0731	0.0748	0.0764
16	0.0476	0.0506	0.0534	0.0560	0.0584	0.0607	0.0628	0.0648	0.0667	0.0685
17	0.0379	0.0411	0.0442	0.0471	0.0497	0.0522	0.0546	0.0568	0.0588	0.0608
18	0.0283	0.0318	0.0352	0.0383	0.0412	0.0439	0.0465	0.0489	0.0511	0.0532
19	0.0188	0.0227	0.0263	0.0296	0.0328	0.0357	0.0385	0.0411	0.0436	0.0459
20	0.0094	0.0136	0.0175	0.0211	0.0245	0.0277	0.0307	0.0335	0.0361	0.0386
21	0.0000	0.0045	0.0087	0.0126	0.0163	0.0197	0.0229	0.0259	0.0288	0.0314
22	-	-	0.0000	0.0042	0.0081	0.0118	0.0153	0.0185	0.0215	0.0244
23	-	-	-	-	0.0000	0.0039	0.0076	0.0111	0.0143	0.0174
24	-	-	-	-	-	-	0.0000	0.0037	0.0071	0.0104
25	-	-	-	-	-	-	-	-	0.0000	0.0035

2.7 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i\left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table B.5. For this set of data:

$$W = \frac{1}{0.000393}(0.0194)^2 = 0.958$$

TABLE B.5. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4734	0.016	$X^{(20)} - X^{(1)}$
2	0.3211	0.013	$X^{(19)} - X^{(2)}$
3	0.2565	0.011	$X^{(18)} - X^{(3)}$
4	0.2085	0.011	$X^{(17)} - X^{(4)}$
5	0.1686	0.008	$X^{(16)} - X^{(5)}$
6	0.1334	0.005	$X^{(15)} - X^{(6)}$
7	0.1013	0.004	$X^{(14)} - X^{(7)}$
8	0.0711	0.001	$X^{(13)} - X^{(8)}$
9	0.0422	0.001	$X^{(12)} - X^{(9)}$
10	0.0140	0.001	$X^{(11)} - X^{(10)}$

2.8 The decision rule for this test is to compare the computed W to the critical value found in Table B.6. If the computed W is less than the critical value, conclude that the data are not normally distributed. For this set of data, the critical value at a significance level of 0.01 and n = 20 observations is 0.868. Since W = 0.958 is greater than the critical value, conclude that the data are normally distributed.

2.9 In general, if the data fail the test for normality, a transformation such as to log values may normalize the data. After transforming the data, repeat the Shapiro Wilk's Test for normality.

TABLE B.6. QUANTILES OF THE SHAPIRO WILK'S TEST STATISTIC (Conover, 1980)

n	0.01	0.02	0.05	0.10	0.50	0.90	0.95	0.98	0.99
3	0.753	0.756	0.767	0.789	0.959	0.998	0.999	1.000	1.000
4	0.687	0.707	0.748	0.792	0.935	0.987	0.992	0.996	0.997
5	0.686	0.715	0.762	0.806	0.927	0.979	0.986	0.991	0.993
6	0.713	0.743	0.788	0.826	0.927	0.974	0.981	0.986	0.989
7	0.730	0.760	0.803	0.838	0.928	0.972	0.979	0.985	0.988
8	0.749	0.778	0.818	0.851	0.932	0.972	0.978	0.984	0.987
9	0.764	0.791	0.829	0.859	0.935	0.972	0.978	0.984	0.986
10	0.781	0.806	0.842	0.869	0.938	0.972	0.978	0.983	0.986
11	0.792	0.817	0.850	0.876	0.940	0.973	0.979	0.984	0.986
12	0.805	0.828	0.859	0.883	0.943	0.973	0.979	0.984	0.986
13	0.814	0.837	0.866	0.889	0.945	0.974	0.979	0.984	0.986
14	0.825	0.846	0.874	0.895	0.947	0.975	0.980	0.984	0.986
15	0.835	0.855	0.881	0.901	0.950	0.975	0.980	0.984	0.987
16	0.844	0.863	0.887	0.906	0.952	0.976	0.981	0.985	0.987
17	0.851	0.869	0.892	0.910	0.954	0.977	0.981	0.985	0.987
18	0.858	0.874	0.897	0.914	0.956	0.978	0.982	0.986	0.988
19	0.863	0.879	0.901	0.917	0.957	0.978	0.982	0.986	0.988
20	0.868	0.884	0.905	0.920	0.959	0.979	0.983	0.986	0.988
21	0.873	0.888	0.908	0.923	0.960	0.980	0.983	0.987	0.989
22	0.878	0.892	0.911	0.926	0.961	0.980	0.984	0.987	0.989
23	0.881	0.895	0.914	0.928	0.962	0.981	0.984	0.987	0.989
24	0.884	0.898	0.916	0.930	0.963	0.981	0.984	0.987	0.989
25	0.888	0.901	0.918	0.931	0.964	0.981	0.985	0.988	0.989
26	0.891	0.904	0.920	0.933	0.965	0.982	0.985	0.988	0.989
27	0.894	0.906	0.923	0.935	0.965	0.982	0.985	0.988	0.990
28	0.896	0.908	0.924	0.936	0.966	0.982	0.985	0.988	0.990
29	0.898	0.910	0.926	0.937	0.966	0.982	0.985	0.988	0.990
30	0.900	0.912	0.927	0.939	0.967	0.983	0.985	0.988	0.990
31	0.902	0.914	0.929	0.940	0.967	0.983	0.986	0.988	0.990
32	0.904	0.915	0.930	0.941	0.968	0.983	0.986	0.988	0.990
33	0.906	0.917	0.931	0.942	0.968	0.983	0.986	0.989	0.990
34	0.908	0.919	0.933	0.943	0.969	0.983	0.986	0.989	0.990
35	0.910	0.920	0.934	0.944	0.969	0.984	0.986	0.989	0.990
36	0.912	0.922	0.935	0.945	0.970	0.984	0.986	0.989	0.990
37	0.914	0.924	0.936	0.946	0.970	0.984	0.987	0.989	0.990
38	0.916	0.925	0.938	0.947	0.971	0.984	0.987	0.989	0.990
39	0.917	0.927	0.939	0.948	0.971	0.984	0.987	0.989	0.991
40	0.919	0.928	0.940	0.949	0.972	0.985	0.987	0.989	0.991
41	0.920	0.929	0.941	0.950	0.972	0.985	0.987	0.989	0.991
42	0.922	0.930	0.942	0.951	0.972	0.985	0.987	0.989	0.991
43	0.923	0.932	0.943	0.951	0.973	0.985	0.987	0.990	0.991
44	0.924	0.933	0.944	0.952	0.973	0.985	0.987	0.990	0.991
45	0.926	0.934	0.945	0.953	0.973	0.985	0.988	0.990	0.991
46	0.927	0.935	0.945	0.953	0.974	0.985	0.988	0.990	0.991
47	0.928	0.936	0.946	0.954	0.974	0.985	0.988	0.990	0.991
48	0.929	0.937	0.947	0.954	0.974	0.985	0.988	0.990	0.991
49	0.929	0.937	0.947	0.955	0.974	0.985	0.988	0.990	0.991
50	0.930	0.938	0.947	0.955	0.974	0.985	0.988	0.990	0.991

3. **TEST FOR HOMOGENEITY OF VARIANCE**

3.1 For Dunnett's Procedure and the t test with Bonferroni's adjustment, the variances of the data obtained from each toxicant concentration and the control are assumed to be equal. Bartlett's Test is a formal test of this assumption. In using this test, it is assumed that the data are normally distributed.

3.2 The data used in this example are growth data from a Mysid Survival and Growth Test, and are the same data used in Appendix C. These data are listed in Table B.7, together with the calculated variance for the control and each toxicant concentration.

TABLE B.7. MYSID, ***HOLMESIMYSIS COSTATA***, GROWTH DATA

	Concentration (%)			
Replicate	Control	1.80	3.20	5.60
1	0.048	0.055	0.057	0.041
2	0.058	0.048	0.050	0.040
3	0.047	0.042	0.046	0.041
4	0.058	0.041	0.043	0.043
5	0.051	0.052	0.045	0.040
Mean($\bar{x}_i$)	0.052	0.048	0.048	0.041
S_i^2	0.0000283	0.0000373	0.0000307	0.0000015
i	1	2	3	4

3.3 The test statistic for Bartlett's Test (Snedecor and Cochran, 1980) is as follows:

$$B = \frac{[(\sum_{i=1}^{p} V_i) \ln \bar{S}^2 - \sum_{i=1}^{p} V_i \ln S_i^2]}{C}$$

Where: V_i = degrees of freedom for each effluent concentration and control, ($V_i = n_i - 1$)

p = number of levels of toxicant concentration including the control

ln = $\log_e$

i = 1, 2, ..., p where p is the number of concentrations including the control

n_i =the number of replicates for concentration i.

$$\overline{S}^2 = \frac{\left(\sum_{i=1}^{p} V_i S_i^2\right)}{\sum_{i=1}^{p} V_i}$$

$$C = 1 + [3(p-1)]^{-1}\left[\sum_{i=1}^{p} 1/V_i - \left(\sum_{i=1}^{p} V_i\right)^{-1}\right]$$

3.4 Since B is approximately distributed as chi-square with p - 1 degrees of freedom when the variances are equal, the appropriate critical value is obtained from a table of the chi-square distribution for p - 1 degrees of freedom and a significance level of 0.01. If B is less than the critical value then the variances are assumed to be equal.

3.5 For the data in this example, all concentrations including the control have the same number of replicates (n_i - 5 for all i). Thus, V_i = 4 for all i. For this data, p = 4, $\overline{S}^2$ = 0.0000245, and C = 1.104. Bartlett's statistic is therefore:

$$B = [(16)\ln(0.0000245) - 4\sum_{i=1}^{p} \ln(S_i^2)]/1.104$$

$$= [16(-10.617) - 4(-44.470)]/1.104$$

$$= [-169.872 - (-177.880)]/1.104$$

$$= 7.254$$

3.6 Since B is approximately distributed as chi-square with p - 1 degrees of freedom when the variances are equal, the appropriate critical value for the test is 9.21 for a significance level of 0.01. Since B = 7.254 is less than 9.21, conclude that the variances are not different.

4. **TRANSFORMATIONS OF THE DATA**

4.1 When the assumptions of normality and/or homogeneity of variance are not met, transformations of the data may remedy the problem, so that the data can be analyzed by parametric procedures, rather than nonparametric technique such as Steel's Many-one Rank Test or Wilcoxon's Rank Sum Test. Examples of transformations include log, square root, arc sine square root, and reciprocals. After the data have been transformed, the Shapiro-Wilk's and Bartlett's tests should be performed on the transformed observations to determine whether the assumptions of normality and/or homogeneity of variance are met.

4.2 ARC SINE SQUARE ROOT TRANSFORMATION (USEPA, 1993).

4.2.1 For data consisting of proportions from a binomial (response/no response; live/dead) response variable, the variance within the ith treatment is proportional to P_i (1 - P_i), where P_i is the expected proportion for the treatment. This clearly violates the homogeneity of variance assumption required by parametric procedures such as Dunnett's Procedure or the ***t*** test with Bonferroni's adjustment, since the existence of a treatment effect implies different values of P_i for different treatments, i. Also, when the observed proportions are based on small samples, or when P_i is close to zero or one, the normality assumption may be invalid. The arc sine square root (arc sine **%&&&**) transformation is commonly used for such data to stabilize the variance and satisfy the normality requirement.

4.2.2 Arc sine transformation consists of determining the angle (in radians) represented by a sine value. In the case of arc sine square root transformation of mortality data, the organism response proportion (proportion dead or affected; proportion surviving) is taken as the sine value, the square root of the sine value is determined, and the angle (in radians) for the square root of the sine value is determined. Whenever the response proportion is 0 or 1, a special modification of the arc sine square root transformation must be used (Bartlett, 1937). An explanation of the arc sine square root transformation and the modification is provided below.

4.2.3 Calculate the response proportion (RP) at each effluent concentration, in this case proportion surviving where:

RP = (number of surviving or unaffected organisms)/(number exposed).

Example: If 12 of 20 animals in a given treatment replicate survive:

RP = 12/20

= 0.60

4.2.4 Transform each RP to its arc sine square root, as follows:

4.2.4.1 For RPs greater than zero or less than one:

Angle (radians) = $\sqrt{RP}$

Example: If RP = 0.60:

Angle = arc sine $\sqrt{0.60}$

= arc sine 0.7746

= 0.8861 radians

4.2.4.2 Modification of the arc sine square root when RP = 0.

Angle (in radians) = arc sine $\sqrt{1/4N}$

Where: N = Number of animals/treatment replicate

Example: If 20 animals are used:

Angle = arc sine $\sqrt{1/80}$

= arc sine 0.1118

= 0.1120 radians

4.2.4.3 Modification of the arc sine square root when RP = 0

Angle = 1.5708 radians - (radians for RP = 0)

Example: Using above value:

Angle = 1.5708 - 0.1120

= 1.4588 radians

APPENDIX C

DUNNETT'S PROCEDURE

1. MANUAL CALCULATIONS

1.1 Dunnett's Procedure (Dunnett, 1955; Dunnett, 1964) is used to compare each concentration mean with the control mean to decide if any of the concentrations differ from the control. This test has an overall error rate of alpha, which accounts for the multiple comparisons with the control. It is based on the assumptions that the observations are independent and normally distributed and that the variance of the observations is homogeneous across all concentrations and control. (See Appendix B for a discussion on validating the assumptions). Dunnett's Procedure uses a pooled estimate of the variance, which is equal to the error value calculated in an analysis of variance. Dunnett's Procedure can only be used when the same number of replicate test vessels have been used at each concentration and the control. When this condition is not met, the *t* test with Bonferroni's adjustment is used (see Appendix D).

1.2 The data used in this example are growth data from a Mysid Survival and Growth Test, and are the same data used in Appendix B. These data are listed in Table C.1.

TABLE C.1. MYSID, ***HOLMESIMYSIS COSTATA***, GROWTH DATA

	Concentration (%)			
Replicate	Control	1.80	3.20	5.60
1	0.048	0.055	0.057	0.041
2	0.058	0.048	0.050	0.040
3	0.047	0.042	0.046	0.041
4	0.058	0.041	0.043	0.043
5	0.051	0.052	0.045	0.040
Mean ($\bar{x}_i$)	0.052	0.048	0.048	0.041
Total (T_i)	0.262	0.238	0.241	0.205
i	1	2	3	4

1.3 One way to obtain an estimate of the pooled variance is to construct an ANOVA table including all sums of squares, as described in Table C.2:

TABLE C.2. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2 = SSW/(N-p)
Total	N - 1	SST	

Where: p = number of effluent concentrations including the control:

N = the total sample size; $N = \sum_i n_i$

n_i = the number of replicates for concentration "i"

$SST = \sum_{ij} Y_{ij}^2 - G^2/N$ Total Sum of Squares

$SSB = \sum_i T_i^2/n_i - G^2/N$ Between Sum of Squares

$SSW = SST - SSB$ Within Sum of Squares

G = the grand total of all sample observations; $G = \sum_{i=1}^{p} T_i$

T_i = the total of the replicate measurements for concentration i

N = the total sample size; $N = \sum_i n_i$

n_i = the number of replicates for concentration i

Y_{ij} = the jth observation for concentration i

1.4 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = 5$

$N = 20$

$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} + Y_{15} = 0.262$
$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} + Y_{25} = 0.238$
$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} + Y_{35} = 0.241$
$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} + Y_{45} = 0.205$

$G = T_1 + T_2 + T_3 + T_4 = 0.946$

$$SSB = \sum_{i=1}^{p} T_i^2/n_i - G^2/N$$

$$= \frac{1}{5}(0.225) - \frac{(0.946)^2}{20} = 0.000254$$

$$SST = \sum_{i=1}^{p}\sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$

$$= 0.0455 - \frac{(0.946)^2}{20} = 0.000754$$

$SSW = SST - SSB = 0.000754 - 0.000254 = 0.000500$

$S_B^2 = SSB/(p-1) = 0.000254/(4-1) = 0.0000847$

$S_W^2 = SSW/(N-p) = 0.000500/(20-4) = 0.0000313$

1.5 Summarize these data in the ANOVA table, as shown in Table C.3:

TABLE C.3. COMPLETED ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	3	0.000254	0.0000847
Within	16	0.000500	0.0000313
Total	19	0.000754	

1.6 To perform the individual comparisons, calculate the t statistic for each concentration and control combination, as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_w\sqrt{(1/n_1) + (1/n_i)}}$$

Where: $\bar{Y}_1$ = mean for the control

$\bar{Y}_i$ = mean for each concentratioon i

S_w = square root of the within mean square

n_1 = number of replicates in the control

n_i = number of replicates for concentration i.

1.7 Table C.4 includes the calculated t values for each concentration and control combination.

TABLE C.4. CALCULATED t VALUES

Concentration (ppb)	i	t_i
1.80	2	1.131
3.20	3	1.131
5.60	4	3.111

1.8 Since the purpose of the test is only to detect a decrease in growth from the control, a one-sided test is appropriate. The critical value for the one-sided comparison is read from the table of Dunnett's "t" values (Table C.5; this table assumes an equal number of replicates in all treatment concentrations and the control). For this set of data, with an overall alpha level of 0.05, 16 degrees of freedom and three concentrations excluding the control, the critical value is 2.23. The mean weight for concentration "i" is considered significantly less than the mean weight for the control if t_i is greater than the critical value. Comparing each of the calculated t values in Table C.4 with the critical value, a significant decrease in growth from the control is detected in the 5.60% concentration. Therefore, the NOEC and the LOEC for growth are 3.20% and 5.60%, respectively.

TABLE C.5. DUNNETT'S "T" VALUES (Miller, 1981)

(One-tailed) d^{a}_{k}																		
v k	a = .05									a = 0.1								
	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9
5	2.02	2.44	2.68	2.85	2.98	3.08	3.16	3.24	3.30	3.37	3.90	4.21	4.43	4.60	4.73	4.85	4.94	5.03
6	1.94	2.34	2.56	2.71	2.83	2.92	3.00	3.07	3.12	3.14	3.61	3.88	4.07	4.21	4.33	4.43	4.51	4.59
7	1.89	2.27	2.48	2.62	2.73	2.82	2.89	2.95	3.01	3.00	3.42	3.56	3.83	3.96	4.07	4.15	4.23	4.30
8	1.86	2.22	2.42	2.55	2.66	2.74	2.81	2.87	2.92	2.90	3.29	3.51	3.67	3.79	3.88	3.96	4.03	4.09
9	1.83	2.18	2.37	2.50	2.60	2.68	2.75	2.81	2.86	2.82	3.19	3.40	3.55	3.66	3.75	3.82	3.89	3.94
10	1.81	2.15	2.34	2.47	2.56	2.64	2.70	2.76	2.81	2.76	3.11	3.31	3.45	3.56	3.64	3.71	3.78	3.83
11	1.80	2.13	2.31	2.44	2.53	2.60	2.67	2.72	2.77	2.72	3.06	3.25	3.38	3.48	3.56	3.63	3.69	3.74
12	1.78	2.11	2.29	2.41	2.50	2.58	2.64	2.69	2.74	2.68	3.01	3.19	3.32	3.42	3.50	3.56	3.62	3.67
13	1.77	2.09	2.27	2.39	2.48	2.55	2.61	2.66	2.71	2.65	2.97	3.15	3.27	3.37	3.44	3.91	3.56	3.61
14	1.76	2.08	2.25	2.37	2.46	2.53	2.59	2.64	2.69	2.62	2.94	3.11	3.23	3.32	3.40	3.46	3.51	3.56
15	1.75	2.07	2.24	2.36	2.44	2.51	2.57	2.62	2.67	2.60	2.91	3.08	3.20	3.29	3.36	3.42	3.47	3.52
16	1.75	2.06	2.23	2.34	2.43	2.50	2.56	2.61	2.65	2.58	2.88	3.05	3.17	3.26	3.33	3.39	3.44	3.48
17	1.74	2.05	2.22	2.33	2.42	2.49	2.54	2.59	2.64	2.57	2.86	3.03	3.14	3.23	3.30	3.36	3.41	3.45
18	1.73	2.04	2.21	2.32	2.41	2.48	2.53	2.58	2.62	2.55	2.84	3.01	3.12	3.21	3.27	3.33	3.38	3.42
19	1.73	2.03	2.20	2.31	2.40	2.47	2.52	2.57	2.61	2.54	2.83	2.99	3.10	3.18	3.25	3.31	3.36	3.40
20	1.72	2.03	2.19	2.30	2.39	2.46	2.51	2.56	2.60	2.53	2.81	2.97	3.08	3.17	3.23	3.29	3.34	3.38
24	1.71	2.01	2.17	2.28	2.36	2.43	2.48	2.53	2.57	2.49	2.77	2.92	3.03	3.11	3.17	3.22	3.27	3.31
30	1.70	1.99	2.15	2.25	2.33	2.40	2.45	2.50	2.54	2.46	2.72	2.87	2.97	3.05	3.11	3.16	3.21	3.24
40	1.68	1.97	2.13	2.23	2.31	2.37	2.42	2.47	2.51	2.42	2.68	2.82	2.92	2.99	3.05	3.10	3.14	3.18
60	1.67	1.95	2.10	2.21	2.28	2.35	2.39	2.44	2.48	2.39	2.64	2.78	2.87	2.94	3.00	3.04	3.08	3.12
120	1.66	1.93	2.08	2.18	2.26	2.32	2.37	2.41	2.45	2.36	2.60	2.73	2.82	2.90	2.94	2.99	3.03	3.06

1.9 To quantify the sensitivity of the test, the minimum significant difference (MSD) may be calculated. The formula is as follows:

$$MSD = d\ S_w\sqrt{(1/n_1)+(1/n)}$$

Where: d = critical value for the Dunnett's Procedure

S_w = the square root of the within mean square

n = the number of replicates at each concentration, assuming an equal number of replicates at all treatment concentrations

n_1 = number of replicates in the control

For example:

$$MSD = 2.23(0.00559)\sqrt{(1/5)+(1/5)}$$

$$= 2.23\ (0.00559)(0.632)$$

$$= 0.00788$$

1.10 Therefore, for this set of data, the minimum difference between the control mean and a concentration mean that can be detected as statistically significant is 0.00788 mg. This represents a 15.2% reduction in mean weight from the control.

1.11 If the data have not been transformed, the MSD (and the percent decrease from the control mean that it represents) can be reported as is.

1.11.1 In the case where the data have been transformed, the MSD would be in transformed units. In this case carry out the following conversion to determine the MSD in untransformed units.

1.11.2 Subtract the MSD from the transformed control mean. Call this difference D. Next, obtain untransformed values for the control mean and the difference, D. Finally, compute the untransformed MSD as follows:

$$MSD_u = control_u - D_u$$

Where: MSD_u = the minimum significant difference for untransformed data

$Control_u$ = the untransformed control mean

D_u = the untransformed difference

1.11.3 Calculate the percent reduction from the control that MSD_u represents as:

$$\text{Percent Reduction} = \frac{MSD_u}{Control_u} \text{ X } 100$$

1.11.3.1 An example of a conversion of the MSD to untransformed units, when the arc sine square root transformation was used on the data, follows.

Step 1. Subtract the MSD from the transformed control mean. As an example, assume the data in Table C.1 were transformed by the arc sine square root transformation. Thus:

$$0.052 - 0.00788 = 0.04412$$

Step 2. Obtain untransformed values for the control mean (0.052) and the difference (0.04412) obtained in Step 1, above.

$$[\text{ Sine } (0.052)]^2 = 0.00270$$

$$[\text{ Sine } (0.04412)]^2 = 0.00195$$

Step 3. The untransformed MSD (MSD_u) is determined by subtracting the untransformed values obtained in Step 2.

$$MSD_u = 0.00270 - 0.00195 = 0.00075$$

In this case, the MSD would represent a 1.4% decrease in survival from the control [(0.00075/0.052)(100)].

2. **COMPUTER CALCULATIONS**

2.1 This computer program incorporates two analyses: an analysis of variance (ANOVA), and a multiple comparison of treatment means with the control mean (Dunnett's Procedure). The ANOVA is used to obtain the error value. Dunnett's Procedure indicates which toxicant concentration means (if any) are statistically different from the control mean at the 5% level of significance. The program also provides the minimum difference between the control and treatment means that could be detected as statistically significant, and tests the validity of the homogeneity of variance assumption by Bartlett's Test. The multiple comparison is performed based on procedures described by Dunnett (1955).

2.2 The source code for the Dunnett's program is structured into a series of subroutines, controlled by a driver routine. Each subroutine has a specific function in the Dunnett's Procedure, such as data input, transforming the data, testing for equality of variances, computing p values, and calculating the one-way analysis of variance.

2.3 The program compares up to seven toxicant concentrations against the control, and can accommodate up to 50 replicates per concentration.

2.4 If the number of replicates at each toxicant concentration and control are not equal, a *t* test with the Bonferroni adjustment is performed instead of Dunnett's Procedure (see Appendix D).

2.5 The program was written in IBM-PC FORTRAN by Computer Sciences Corporation, 26 W. Martin Luther King Drive, Cincinnati, OH 45268. A compiled version of the program can be obtained from EMSL-Cincinnati by sending a diskette with a written request.

2.6 DATA INPUT AND OUTPUT

2.6.1 The mysid growth data from Table C.1 are used to illustrate the data input and output for this program.

2.6.2 Data Input

2.6.2.1 When the program is entered, the user is asked to select the type of data to be analyzed:

1. Response proportions, like survival or fertilization proportions data.
2. Counts and measurements, like offspring counts, cystocarp and algal cell counts, weights, chlorophyll measurements or turbidity measurements.

2.6.2.2 After the type of analysis for the data is chosen, the user has the following options:

1. Create a data file
2. Edit a data file
3. Perform analysis on existing data set
4. Stop

2.6.2.3 When Option 1 (Create a data file) is selected for response proportions, the program prompts the user for the following information:

1. Number of concentrations, including control
2. For each concentration and replicate:
   - number of organisms exposed per replicate
   - number of organisms responding per replicate (e.g., surviving organisms, fertilized eggs, etc.)

2.6.2.4 After the data have been entered, the user may save the file on a disk, and the program returns to the main menu (see below).

2.6.2.5 Sample data input is shown in Figure C.1.

2.6.3. Program Output

2.6.3.1 When Option 3 (perform analysis on existing data set) is selected from the menu, the user is asked to select the transformation desired, and indicate whether they expect the means of the test groups to be less or greater than the mean for the control group (see Figure C.2).

```
                    EMSL Cincinnati Dunnett Software
                              Version 1.5


What type of data do you wish to analyze?

     1)  response proportions
         (like survival data or fertility proportion data)
         Note:  The program calculates a proportion after prompting for
                number of exposed organisms and number of responding
                organisms.

     2)  counts and measurements
         (like offspring counts, cystocarps and algal cell counts,
         weights, chlorophyll measurements, or turbidity measurements)


 Enter "1", "2", (or "q" to quit program): 2


Title ? Appendix C, Dunnett's Procedure Example - Mysid Data

Output to printer or disk file ? P

 1) Create a data file
 2) Edit a data file
 3) Analyze an existing data set
 4) Stop

Your choice ? 1

Number of concentrations, including control ? 4


Number of observations for conc. 1 (the control) ? 5

Enter the data for conc. 1 (the control) one observation at a time.

NO.  1? 0.048

NO.  2? 0.058

NO.  3? 0.047

NO.  4? 0.058

NO.  5? 0.051
```

Figure C.1. Sample Data Input for Dunnett's Program for Survival Data from Table C.1.

```
Enter the data for conc.  2 one observation at a time.
NO.  1? 0.055
NO.  2? 0.048
NO.  3? 0.042
NO.  4? 0.041
NO.  5? 0.052

Number of observations for conc.  3 ? 5
Enter the data for conc.  3 one observation at a time.
NO.  1? 0.057
NO.  2? 0.050
NO.  3? 0.046
NO.  4? 0.043
NO.  5? 0.045

Number of observations for conc.  4 ? 5
Enter the data for conc.  4 one observation at a time.
NO.  1? 0.041
NO.  2? 0.040
NO.  3? 0.041
NO.  4? 0.043
NO.  5? 0.040

Do you wish to save the data on disk ? Y
Disk file for output ? c:\mysid.dat
```

Figure C.1. Sample Data Input for Dunnett's Program for Survival Data from Table C.1. (Continued)

```
                    EMSL Cincinnati Dunnett Software
                             Version 1.5



 1) Create a data file
 2) Edit a data file
 3) Analyze an existing data set
 4) Stop

Your choice ? 3


File name ? c:\mysid.dat



 Available Transformations
     1)  no transform
     2)  square root
     3)  log10

Your choice ? 1



 Dunnett's test as implemented in this program is
 a one-sided test. You must specify the direction
 the test is to be run; that is, do you expect the
 means for the test concentrations to be less than
 or greater than the mean for the control
 concentration.

Direction for Dunnetts test : L=less than, G=greater than ? L
```

Figure C.2. Example of Choosing Option 3 from the Main Menu of the Dunnett Program.

2.6.3.2 Summary statistics (Figure C.3) for the raw and transformed data, if applicable, the ANOVA table, results of Bartlett's Test, the results of the multiple comparison procedure, and the minimum detectable difference are included in the program output.

EMSL Cincinnati Dunnett Software
Version 1.5

Appendix C, Dunnett's Procedure Example - Mysid Data

Summary Statistics and ANOVA

Transformation = None

Conc.	n	Mean	s.d.	cv%
1 = control	5	.0524	.0053	10.2
2	5	.0476	.0061	12.8
3	5	.0482	.0055	11.5
4*	5	.0410	.0012	3.0

*) the mean for this conc. is significantly less than
the control mean at alpha = 0.05 (1-sided) by Dunnett's test

Minimum detectable difference for Dunnett's test = -.006974
This difference corresponds to -13.31 percent of control

Between concentrations
sum of squares = .000333 with 3 degrees of freedom.

Error mean square = .000024 with 16 degrees of freedom.

Bartlett's test p-value for equality of variances = .060

Do you wish to restart the program ?

Figure C.3. Example of Program Output for the Dunnett's Program Using the Data in Table C.1.

APPENDIX D

t TEST WITH BONFERRONI'S ADJUSTMENT

1. The *t* test with Bonferroni's adjustment is used as an alternative to Dunnett's Procedure when the number of replicates is not the same for all concentrations. This test sets an upper bound of alpha on the overall error rate, in contrast to Dunnett's Procedure, for which the overall error rate is fixed at alpha. Thus, Dunnett's Procedure is a more powerful test.

2. The *t* test with Bonferroni's adjustment is based on the same assumptions of normality of distribution and homogeneity of variance as Dunnett's Procedure (See Appendix B for testing these assumptions), and, like Dunnett's Procedure, uses a pooled estimate of the variance, which is equal to the error value calculated in an analysis of variance.

3. An example of the use of the *t* test with Bonferroni's adjustment is provided below. The data used in the example are a set of red abalone growth data. Because there are only four replicates in the highest concentration, Dunnett's Procedure cannot be used. The length data are presented in Table D.1.

TABLE D.1. GIANT KELP, ***MACROCYSTIS PYRIFERA***, GROWTH DATA

	Copper Concentration (µg/L)							
Rep	Control	5.60	10.0	18.0	32.0	56.0	100.0	180.0
1	19.58	18.26	13.31	18.59	12.54	11.44	7.92	6.49
2	18.75	16.25	18.92	12.88	10.67	11.88	7.59	7.25
3	19.14	16.39	15.62	16.28	15.95	11.88	8.25	--
4	16.50	18.70	14.30	15.38	12.54	11.00	9.13	7.63
5	17.93	15.62	15.29	19.75	11.66	11.55	8.80	8.13
$\&_i$	18.38	17.04	15.49	16.58	12.67	11.55	8.34	7.38
S_i^2	1.473	1.827	4.498	7.327	3.953	0.133	0.396	0.478
i	1	2	3	4	5	6	7	8

3.1 One way to obtain an estimate of the pooled variance is to construct an ANOVA table including all sums of squares, as described in Table D.2:

TABLE D.2. ANOVA TABLE

Source	*df*	*Sum of Squares (SS)*	*Mean Square(MS) (SS/df)*
Between	*p - 1*	*SSB*	$S_B^2 = SSB/(p-1)$
Within	*N - p*	*SSW*	$S_W^2 = SSW/(N-p)$

Where: p = number of effluent concentrations including the control

N = the total sample size; $N = \sum_i n_i$

n_i = the number of replicates for concentration i

$$SST = \sum_{ij} Y_{ij}^2 - G^2/N$$

Total Sum of Squares

$$SSB = \sum_i T_i^2/n_i - G^2/N$$ Between Sum of Squares

$$SSW = SST - SSB$$ Within Sum of Squares

Where: G = The grand total of all sample observations; $G = \sum_{i=1}^{p} T_i$

T_i = The total of the replicate measurements for concentration i

Y_{ij} = The jth observation for concentration i

3.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = n_6 = n_7 = 5; \quad n_8 = 4$

$N = 39$

$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} + Y_{15} = 91.90$
$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} + Y_{25} = 85.22$
$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} + Y_{35} = 77.44$
$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} + Y_{45} = 82.88$
$T_5 = Y_{51} + Y_{52} + Y_{53} + Y_{54} + Y_{55} = 63.36$
$T_6 = Y_{61} + Y_{62} + Y_{63} + Y_{64} + Y_{65} = 57.75$
$T_7 = Y_{71} + Y_{72} + Y_{73} + Y_{74} + Y_{75} = 41.69$
$T_8 = Y_{81} + Y_{82} + Y_{83} + Y_{84} = 29.50$

$G = T_1 + T_2 + T_3 + T_4 + T_5 + T_6 + T_7 + T_8 = 529.74$

$$SSB = \sum_{i=1}^{p} T_i^2/n_i - G^2/N$$

$$= 7749.905 - \frac{(529.74)^2}{39} = 554.406$$

$$SST = \sum_{i=1}^{p} \sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$

$$= 7829.764 - \frac{(529.74)^2}{39} = 634.265$$

$SSW = SST - SSB = 634.265 - 554.406 = 79.859$

$S_B^2 = SSB/(p-1) = 554.406/(8-1) = 79.201$

$S_W^2 = SSW/(N-p) = 79.859/(39-8) = 2.576$

3.3 Summarize these calculations in the ANOVA table (Table D.3):

TABLE D.3. COMPLETED ANOVA TABLE FOR THE *t* TEST WITH BONFERRONI'S ADJUSTMENT EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	7	554.406	79.201
Within	31	79.859	2.576
Total	38	634.265	

3.4 To perform the individual comparisons, calculate the *t* statistic for each concentration and control combination, as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_w\sqrt{(1/n_1) + (1/n_i)}}$$

Where: $\bar{Y}_i$ = mean for concentration i

$\bar{Y}_1$ = mean for the control

S_w = square root of the within mean square

n_1 = number of replicates in the control.

n_i = number of replicates for concentration i.

3.5 Table D.4 includes the calculated *t* values for each concentration and control combination.

TABLE D.4. CALCULATED t VALUES

Concentration (µg/L)	i	t_i
5.6	2	1.320
10.0	3	2.847
18.0	4	1.773
32.0	5	5.625
56.0	6	6.728
100.0	7	9.891
180.0	8	10.217

3.6 Since the purpose of this test is to detect a significant reduction in mean length, a one-sided test is appropriate. The critical value for this one-sided test is found in Table D.5. For an overall alpha level of 0.05, 31 degrees of freedom for error and seven concentrations (excluding the control) the approximate critical value is 2.597. The mean length for concentration "i" is considered significantly less than the mean length for the control if t_i is greater than the critical value. Comparing each of the calculated t values in Table D.4 with the critical value, the 10.0 µg/L, 32 µg/L, 56.0 µg/L, 100.0 µg/L, 180.0 µg/L concentrations have significantly lower mean length than the control. Because the 10.0 µg/L concentration shows signigicantly lower mean length that the control while the higher 18.0 µg/L concentration does not, these test results are considered to have an anomalous dose-response relationship and it is recommended that the test be repeated. If an NOEC and LOEC must be determined for this test, the lowest concentration with significant growth impairment versus the control is considered to the LOEC for growth. Thus, for this test, the NOEC and LOEC would be 5.6 µg/L and 10.0 µg/L, respectively.

TABLE D.5. CRITICAL VALUES FOR "t" FOR THE t TEST WITH BONFERRONI'S ADJUSTMENT P = 0.05
CRITICAL LEVEL, ONE TAILED

d.f.	K = 1	K = 2	K = 3	K = 4	K = 5	K = 6	K = 7	K = 8	K = 9	K = 10
1	6.314	12.707	19.002	25.452	31.821	38.189	44.556	50.924	57.290	63.657
2	2.920	4.303	5.340	6.206	6.965	7.649	8.277	8.861	9.408	9.925
3	2.354	3.183	3.741	4.177	4.541	4.857	5.138	5.392	5.626	5.841
4	2.132	2.777	3.187	3.496	3.747	3.961	4.148	4.315	4.466	4.605
5	2.016	2.571	2.912	3.164	3.365	3.535	3.681	3.811	3.927	4.033
6	1.944	2.447	2.750	2.969	3.143	3.288	3.412	3.522	3.619	3.708
7	1.895	2.365	2.642	2.842	2.998	3.128	3.239	3.336	3.422	3.500
8	1.860	2.307	2.567	2.752	2.897	3.016	3.118	3.206	3.285	3.356
9	1.834	2.263	2.510	2.686	2.822	2.934	3.029	3.111	3.185	3.250
10	1.813	2.229	2.406	2.634	2.764	2.871	2.961	3.039	3.108	3.170
11	1.796	2.201	2.432	2.594	2.719	2.821	2.907	2.981	3.047	3.106
12	1.783	2.179	2.404	2.561	2.681	2.778	2.863	2.935	2.998	3.055
13	1.771	2.161	2.380	2.533	2.651	2.746	2.827	2.897	2.958	3.013
14	1.762	2.145	2.360	2.510	2.625	2.718	2.797	2.864	2.924	2.977
15	1.754	2.132	2.343	2.490	2.603	2.694	2.771	2.837	2.895	2.947
16	1.746	2.120	2.329	2.473	2.584	2.674	2.749	2.814	2.871	2.921
17	1.740	2.110	2.316	2.459	2.567	2.655	2.729	2.793	2.849	2.899
18	1.735	2.101	2.305	2.446	2.553	2.640	2.712	2.775	2.830	2.879
19	1.730	2.094	2.295	2.434	2.540	2.626	2.697	2.759	2.813	2.861
20	1.725	2.086	2.206	2.424	2.528	2.613	2.684	2.745	2.798	2.846
21	1.721	2.080	2.278	2.414	2.518	2.602	2.672	2.732	2.785	2.832
22	1.718	2.074	2.271	2.406	2.509	2.592	2.661	2.721	2.773	2.819
23	1.714	2.069	2.264	2.398	2.500	2.583	2.651	2.710	2.762	2.808
24	1.711	2.064	2.258	2.391	2.493	2.574	2.642	2.701	2.752	2.797
25	1.709	2.060	2.253	2.385	2.486	2.566	2.634	2.692	2.743	2.788

TABLE D.5. CRITICAL VALUES FOR "t" FOR THE t TEST WITH BONFERRONI'S ADJUSTMENT
P = 0.05 CRITICAL LEVEL, ONE TAILED (CONTINUED)

df	K = 1	K = 2	K = 3	K = 4	K = 5	K = 6	K = 7	K = 8	K = 9	K = 10
29	1.700	2.046	2.235	2.364	2.463	2.541	2.607	2.664	2.713	2.757
30	1.698	2.043	2.231	2.360	2.458	2.536	2.602	2.658	2.707	2.750
31	1.696	2.040	2.228	2.356	2.453	2.531	2.597	2.652	2.701	2.745
32	1.694	2.037	2.224	2.352	2.449	2.527	2.592	2.647	2.696	2.739
33	1.693	2.035	2.221	2.349	2.445	2.523	2.587	2.643	2.691	2.734
34	1.691	2.033	2.219	2.346	2.442	2.519	2.583	2.638	2.686	2.729
35	1.690	2.031	2.216	2.342	2.438	2.515	2.579	2.634	2.682	2.724
36	1.689	2.029	2.213	2.340	2.435	2.512	2.575	2.630	2.678	2.720
37	1.688	2.027	2.211	2.337	2.432	2.508	2.572	2.626	2.674	2.716
38	1.686	2.025	2.209	2.334	2.429	2.505	2.568	2.623	2.670	2.712
39	1.685	2.023	2.207	2.332	2.426	2.502	2.565	2.619	2.667	2.708
40	1.684	2.022	2.205	2.329	2.424	2.499	2.562	2.616	2.663	2.705
50	1.676	2.009	2.189	2.311	2.404	2.478	2.539	2.592	2.638	2.678
60	1.671	2.001	2.179	2.300	2.391	2.463	2.324	2.576	2.621	2.661
70	1.667	1.995	2.171	2.291	2.381	2.453	2.513	2.564	2.609	2.648
80	1.665	1.991	2.166	2.285	2.374	2.446	2.505	2.556	2.600	2.639
90	1.662	1.987	2.162	2.280	2.369	2.440	2.499	2.549	2.593	2.632
100	1.661	1.984	2.158	2.276	2.365	2.435	2.494	2.544	2.588	2.626
110	1.659	1.982	2.156	2.273	2.361	2.432	2.490	2.540	2.583	2.622
120	1.658	1.980	2.153	2.270	2.358	2.429	2.487	2.536	2.580	2.618
Infinite	1.645	1.960	2.129	2.242	2.327	2.394	2.450	2.498	2.540	2.576

d.f. = Degrees of freedom for MSE (Mean Square Error) from ANOVA.
K = Number of concentrations to be compared to the control.

APPENDIX E

STEEL'S MANY-ONE RANK TEST

1. Steel's Many-one Rank Test is a nonparametric test for comparing treatments with a control. This test is an alternative to Dunnett's Procedure, and may be applied to data when the normality assumption has not been met. Steel's Test requires equal variances across the treatments and the control, but it is thought to be fairly insensitive to deviations from this condition (Steel, 1959). The tables for Steel's Test require an equal number of replicates at each concentration. If this is not the case, use Wilcoxon's Rank Sum Test, with Bonferroni's adjustment (See Appendix F).

2. For an analysis using Steel's Test, for each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign the ranks to the ordered observations (1 to the smallest, 2 to the next smallest, etc.). If ties occur in the ranking, assign the average rank to the observation. (Extensive ties would invalidate this procedure). The sum of the ranks within each concentration is then calculated. To determine if the response in a concentration is significantly less than the response in the control, the rank sum for each concentration is compared to the significant values of rank sums given later in the section. In this table, k equals the number of treatments excluding the control and n equals the number of replicates for each concentration and the control.

3. An example of the use of this test is provided below. The test employs embryo-larval development data from a bivalve 48-hour chronic test. The data are listed in Table E.1.

4. For each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign the ranks (1, 2, 3, ..., 8) to the ordered observations (1 to the smallest, 2 to the next smallest, etc.). If ties occur in the ranking, assign the average rank to each tied observation.

5. An example of assigning ranks to the combined data for the control and 0.13 µg/L copper concentration is given in Table E.2.

This ranking procedure is repeated for each control and concentration combination. The complete set of rankings is listed in Table E.3. The ranks are then summed for each toxicant concentration, as shown in Table E.4.

6. For this set of data, determine if the development in any of the effluent concentrations is significantly lower than the development of the control organisms. If this occurs, the rank sum at that concentration would be significantly lower than the rank sum of the control. Thus, compare the rank sums for the development at each of the various effluent concentrations with some "minimum" or critical rank sum, at or below which the survival would be considered to be significantly lower than the control. At a probability level of 0.05, the critical rank sum in a test with five concentrations and four replicates per concentration, is 10 (see Table F.4).

7. Since the rank sums for the 0.50 µg/L and 1.00 µg/L concentration levels are equal to the critical value, the proportions of normal development in those concentrations are considered significantly less than that in the control. Since no other rank sum is less than or equal to the critical value, no other concentration has a significantly lower proportion normal than the control. Because the 0.50 µg/L concentration shows signigicantly lower normal development than the control while the higher 2.00 µg/L concentration does not, these test results are considered to have an anomalous dose-response relationship and it is recommended that the test be repeated. If an NOEC and LOEC must be determined for this test, the lowest concentration with significant impairment versus the control is considered to the LOEC for growth. Thus, for this test, the NOEC and LOEC would be 0.25 µg/L and 0.50 µg/L, respectively.

TABLE E.1. BIVALVE EMBRYO-LARVAL DEVELOPMENT DATA

		Copper Concentration (µg/L)					
	Replicate	Control	0.13	0.25	0.50	1.00	2.00
	A	1.00	0.96	0.92	0.91	0.88	1.00
RAW	B	0.96	0.97	0.95	0.93	0.83	0.67
	C	1.00	1.00	0.90	0.88	0.88	0.75
	D	0.97	0.96	0.96	0.93	0.82	0.60
ARC SINE	A	1.571	1.369	1.284	1.266	1.217	1.571
SQUARE ROOT	B	1.369	1.397	1.345	1.303	1.146	0.959
TRANSFORMED	C	1.571	1.571	1.249	1.217	1.217	1.047
	D	1.397	1.369	1.369	1.303	1.133	0.886
Mean ($\bar{x}_i$)		1.477	1.427	1.312	1.272	1.178	1.116
S_i^2		0.01191	0.00945	0.00303	0.00166	0.00203	0.09644
i		1	2	3	4	5	6

TABLE E.2. ASSIGNING RANKS TO THE CONTROL AND 0.13 µg/L CONCENTRATION LEVEL FOR STEEL'S MANY-ONE RANK TEST

Rank	Transformed Proportion Normal	Concentration
2	1.369	0.13 µg/L
2	1.369	0.13 µg/L
2	1.369	Control
4.5	1.397	0.13 µg/L
4.5	1.397	Control
7	1.571	0.13 µg/L
7	1.571	Control
7	1.571	Control

TABLE E.3. TABLE OF RANKS[1]

Replicate	Control	Copper Concentration (µg/L) 0.13	0.25
1	1.571(7,7.5,7.5,7.5,7)	1.369(2)	1.284(2)
2	1.369(2,4.5,5,5,4)	1.397(4.5)	1.345(3)
3	1.571(7,7.5,7.5,7.5,7)	1.571(7)	1.249(1)
4	1.397(4.5,6,6,6,5)	1.369(2)	1.369(4.5)

Replicate	Copper Concentration (µg/L) (Continued) 0.50	1.00	2.00
1	1.266(2)	1.217(3.5)	1.571(7)
2	1.303(3.5)	1.146(2)	0.959(2)
3	1.217(1)	1.217(3.5)	1.047(3)
4	1.303(3.5)	1.133(1)	0.886(1)

[1]*Control ranks are given in the order of the concentration with which they were ranked.*

TABLE E.4. RANK SUMS

Concentration µg/L Copper)	Rank Sum
0.13	15.5
0.25	10.5
0.50	10.0
1.00	10.0
2.00	13.0

TABLE E.5. SIGNIFICANT VALUES OF RANK SUMS: JOINT CONFIDENCE COEFFICIENTS OF 0.95 (UPPER) and 0.99 (LOWER) FOR ONE-SIDED ALTERNATIVES (Steel, 1959)

		k = number of treatments (excluding control)							
n	%	2	3	4	5	6	7	8	9
	%								
4	%	11	10	10	10	10	--	--	--
	%	--	--	--	--	--	--	--	--
5	%	18	17	17	16	16	16	16	15
	%	15	--	--	--	--	--	--	--
6	%	27	26	25	25	24	24	24	23
	%	23	22	21	21	--	--	--	--
7	%	37	36	35	35	34	34	33	33
	%	32	31	30	30	29	29	29	29
8	%	49	48	47	46	46	45	45	44
	%	43	42	41	40	40	40	39	39
9	%	63	62	61	60	59	59	58	58
	%	56	55	54	53	52	52	51	51
10	%	79	77	76	75	74	74	73	72
	%	71	69	68	67	66	66	65	65
11	%	97	95	93	92	91	90	90	89
	%	87	85	84	83	82	81	81	80
12	%	116	114	112	111	110	109	108	108
	%	105	103	102	100	99	99	98	98
13	%	138	135	133	132	130	129	129	128
	%	125	123	121	120	119	118	117	117
14	%	161	158	155	154	153	152	151	150
	%	147	144	142	141	140	139	138	137
15	%	186	182	180	178	177	176	175	174
	%	170	167	165	164	162	161	160	160
16	%	213	209	206	204	203	201	200	199
	%	196	192	190	188	187	186	185	184
17	%	241	237	234	232	231	229	228	227
	%	223	219	217	215	213	212	211	210
18	%	272	267	264	262	260	259	257	256
	%	252	248	245	243	241	240	239	238
19	%	304	299	296	294	292	290	288	287
	%	282	278	275	273	271	270	268	267
20	%	339	333	330	327	325	323	322	320
	%	315	310	307	305	303	301	300	299

APPENDIX F

WILCOXON RANK SUM TEST

1. Wilcoxon's Rank Sum Test is a nonparametric test, to be used as an alternative to Steel's Many-one Rank Test when the number of replicates are not the same at each concentration. A Bonferroni's adjustment of the pairwise error rate for comparison of each concentration versus the control is used to set an upper bound of alpha on the overall error rate, in contrast to Steel's Many-one Rank Test, for which the overall error rate is fixed at alpha. Thus, Steel's Test is a more powerful test.

2. The use of this test may be illustrated with devlopment data from the red abalone test in Table F.1. The control group has four replicates while each of the concentration levels has five replicates. Since there is 100% abnormality in all replicates for the 5.6% and 10.0% concentrations, they are not included in the statistical analysis and are considered qualitative abnormality effects.

3. For each concentration and control combination, combine the data and arrange the values in order of size, from smallest to largest. Assign ranks to the ordered observations (a rank of 1 to the smallest, 2 to the next smallest, etc.). If ties in rank occur, assign the average rank to each tied observation.

4. An example of assigning ranks to the combined data for the control and effluent concentration 0.56% is given in Table F.2. This ranking procedure is repeated for each of the three remaining control versus test concentration combinations. The complete set of ranks is listed in Table F.3. The ranks are then summed for each effluent concentration, as shown in Table F.4.

5. For this set of data, determine if the development in any of the test concentrations is significantly lower than the development in the control. If this occurs, the rank sum at that concentration would be significantly lower than the rank sum of the control. Thus, compare the rank sums for fecundity of each of the various effluent concentrations with some "minimum" or critical rank sum, at or below which the fecundity would be considered to be significantly lower than the control. At a

probability level of 0.05, the critical rank in a test with four concentrations (excluding the control), four control replicates, and five concentration replicates is 15 (see Table F.5, for K = 4).

TABLE F.1. RED ABALONE, HALIOTUS RUFESCENS, SHELL DEVELOPMENT DATA

			Effluent Concentration (%)					
	Dilution							
	Replicate	Control	0.56	1.00	1.80	3.20	5.6	10.0
RAW	A	0.99	0.99	0.99	0.99	0.39	0	0
	B	0.99	0.99	1.00	0.99	0.57	0	0
	C	0.99	0.98	0.99	0.99	0.61	0	0
	D	1.00	1.00	0.99	0.98	0.65	0	0
	E		1.00	1.00	0.97	0.80	0	0
ARC SINE SQUARE ROOT TRANSFORMED	A	1.471	1.471	1.471	1.471	0.674	-	-
	B	1.471	1.471	1.521	1.471	0.856	-	-
	C	1.471	1.429	1.471	1.471	0.896	-	-
	D	1.521	1.521	1.471	1.429	0.938	-	-
	E		1.521	1.521	1.397	1.107	-	-
Mean ($\bar{x}_i$)		1.484	1.483	1.491	1.448	0.894	-	-
S_i^2		0.000625	0.001523	0.000750	0.001137	0.024288	-	-
i		1	2	3	4	5	6	7

TABLE F.2. ASSIGNING RANKS TO THE CONTROL AND 0.56% CONCENTRATION LEVEL FOR THE WILCOXON RANK SUM TEST WITH THE BONFERRONI ADJUSTMENT

Rank	Transformed Proportion Normal	Concentration
1	1.429	0.56 %
4	1.471	0.56 %
4	1.471	0.56 %
4	1.471	Control
4	1.471	Control
4	1.471	Control
8	1.521	0.56 %
8	1.521	0.56 %
8	1.521	Control

TABLE F.3. TABLE OF RANKS[1]

Replicate	Control	Effluent Concentration (%) 0.56	1.00	1.80	3.20
1	1.471(4,3.5,5.5,7)	1.471(4)	1.471(3.5)	1.471(5.5)	0.674(1)
2	1.471(4,3.5,5.5,7)	1.471(4)	1.521(8)	1.471(5.5)	0.856(2)
3	1.471(4,3.5,5.5,7)	1.429(1)	1.471(3.5)	1.471(5.5)	0.896(3)
4	1.521(8,8,9,9)	1.521(8)	1.471(3.5)	1.429(2)	0.938(4)
5		1.521(8)	1.521(8)	1.397(1)	1.107(5)

[1]*Control ranks are given in the order of the concentration with which they were ranked.*

6. Comparing the rank sums in Table F.4 to the appropriate critical rank, the rank sum for the 3.20% concentration level is equal to the critical value, so the proportion normal in that concentration is considered significantly less than that in the control. Since no other rank sum is less than or equal to the critical value, no other concentration has a significantly lower proportion normal than the control. Hence, the NOEC and the LOEC are 1.80% and 3.20%, respectively.

TABLE F.4. RANK SUMS

Concentration (% Effluent)	Rank Sum
0.56	25.0
1.00	26.5
1.80	19.5
3.20	15.0

TABLE F.5. CRITICAL VALUES FOR WILCOXON'S RANK SUM TEST WITH BONFERRONI'S ADJUSTMENT OF ERROR RATE FOR COMPARISON OF "K" TREATMENTS VERSUS A CONTROL FIVE PERCENT CRITICAL LEVEL (ONE-SIDED ALTERNATIVE: TREATMENT CONTROL)

K	No. Replicates in Control	No. of Replicates Per Effluent Concentration							
		3	4	5	6	7	8	9	10
1	3	6	10	16	23	30	39	49	59
	4	6	11	17	24	32	41	51	62
	5	7	12	19	26	34	44	54	66
	6	8	13	20	28	36	46	57	69
	7	8	14	21	29	39	49	60	72
	8	9	15	23	31	41	51	63	72
	9	10	16	24	33	43	54	66	79
	10	10	17	26	35	45	56	69	82
2	3	--	--	15	22	29	38	47	58
	4	--	10	16	23	31	40	49	60
	5	6	11	17	24	33	42	52	63
	6	7	12	18	26	34	44	55	66
	7	7	13	20	27	36	46	57	69
	8	8	14	21	29	38	49	60	72
	9	8	14	22	31	40	51	62	75
	10	9	15	23	32	42	53	65	78
3	3	--	--	--	21	29	37	46	57
	4	--	10	16	22	30	39	48	59
	5	--	11	17	24	32	41	51	62
	6	6	11	18	25	33	43	53	65
	7	7	12	19	26	35	45	56	68
	8	7	13	20	28	37	47	58	70
	9	7	13	21	29	39	49	61	73
	10	8	14	22	31	41	51	63	76
4	3	--	--	--	21	28	37	46	56
	4	--	--	15	22	30	38	48	59
	5	--	10	16	23	31	40	50	61
	6	6	11	17	24	33	42	52	64
	7	6	12	18	26	34	44	55	67
	8	7	12	19	27	36	46	57	69
	9	7	13	20	28	38	48	60	72
	10	7	14	21	30	40	50	62	75

TABLE F.5. CRITICAL VALUES FOR WILCOXON'S RANK SUM TEST WITH BONFERRONI'S ADJUSTMENT OF ERROR RATE FOR COMPARISON OF "K" TREATMENTS VERSUS A CONTROL FIVE PERCENT CRITICAL LEVEL (ONE-SIDED ALTERNATIVE: TREATMENT CONTROL) (CONTINUED)

K	No. Replicates in Control	No. of Replicates Per Effluent Concentration							
		3	4	5	6	7	8	9	10
5	3	--	--	--	--	28	36	46	56
	4	--	--	15	22	29	38	48	58
	5	--	10	16	23	31	40	50	61
	6	--	11	17	24	32	42	52	63
	7	6	11	18	25	34	43	54	66
	8	6	12	19	27	35	45	56	68
	9	7	13	20	28	37	47	59	71
	10	7	13	21	29	39	49	61	74
6	3	--	--	--	--	28	36	45	56
	4	--	--	15	21	29	38	47	58
	5	--	10	16	22	30	39	49	60
	6	--	11	16	24	32	41	51	63
	7	6	11	17	25	33	43	54	65
	8	6	12	18	26	35	45	56	68
	9	6	12	19	27	37	47	58	70
	10	7	13	20	29	38	49	60	73
7	3	--	--	--	--	--	36	45	56
	4	--	--	--	21	29	37	47	58
	5	--	--	15	22	30	39	49	60
	6	--	10	16	23	32	41	51	62
	7	--	11	17	25	33	43	53	65
	8	6	11	18	26	35	44	55	67
	9	6	12	19	27	36	46	58	70
	10	7	13	20	28	38	48	60	72
8	3	--	--	--	--	--	36	45	55
	4	--	--	--	21	29	37	47	57
	5	--	--	15	22	30	39	49	59
	6	--	10	16	23	31	40	51	62
	7	--	11	17	24	33	42	53	64
	8	6	11	18	25	34	44	55	67
	9	6	12	19	27	36	46	57	69
	10	6	12	19	28	37	48	59	72

TABLE F.5. CRITICAL VALUES FOR WILCOXON'S RANK SUM TEST WITH BONFERRONI'S ADJUSTMENT OF ERROR RATE FOR COMPARISON OF "K" TREATMENTS VERSUS A CONTROL FIVE PERCENT CRITICAL LEVEL (ONE-SIDED ALTERNATIVE: TREATMENT CONTROL) (CONTINUED)

K	No. Replicates in Control	No. of Replicates Per Effluent Concentration							
		3	4	5	6	7	8	9	10
9	3	--	--	--	--	--	--	45	55
	4	--	--	--	21	28	37	46	57
	5	--	--	15	22	30	39	48	59
	6	--	10	16	23	31	40	50	62
	7	--	10	17	24	33	42	52	64
	8	--	11	18	25	34	44	55	66
	9	6	11	18	26	35	46	57	69
	10	6	12	19	28	37	47	59	71
10	3	--	--	--	--	--	--	45	55
	4	--	--	--	21	28	37	46	57
	5	--	--	15	22	29	38	48	59
	6	--	10	16	23	31	40	50	61
	7	--	10	16	24	32	42	52	64
	8	--	11	17	25	34	43	54	66
	9	6	11	18	26	35	45	56	68
	10	6	12	19	27	37	47	58	71

APPENDIX G

SINGLE CONCENTRATION TOXICITY TEST - COMPARISON OF CONTROL WITH 100% EFFLUENT OR RECEIVING WATER OR COMPARISON OF DILUTION AND BRINE CONTROLS

1. To statistically compare a control with one concentration, such as 100% effluent or the instream waste concentration, a ***t*** test is the recommended analysis. The ***t*** test is based on the assumptions that the observations are independent and normally distributed and that the variances of the observations are equal between the two groups.

2. Shapiro-Wilk's test may be used to test the normality assumption (See Appendix B for details). For the two sample case, the datasets must be tested for normality separately. If either set of data does not meet the normality assumption, the nonparametric test, Wilcoxon's Rank Sum Test, may be used to analyze the data. An example of this test is given in Appendix F. Since a control and one concentration are being compared, the K = 1 section of Table F.5 contains the needed critical values for one-sided tests. An additional reference, such as Snedecor and Cochran (1980) must be used to determine critical values for two-sided tests, such as comparing brine and dilution controls.

3. The ***F*** test for equality of variances is used to test the homogeneity of variance assumption. When conducting the ***F*** test, the alternative hypothesis of interest is that the variances are not equal.

4. To make the two-tailed ***F*** test at the 0.01 level of significance, put the larger of the two variances in the numerator of ***F***.

$$F = \frac{S_1^2}{S_2^2} \quad \text{where } S_1^2 > S_2^2$$

5. Compare ***F*** with the 0.005 level of a tabled ***F*** value with $n_1 - 1$ and $n_2 - 1$ degrees of freedom, where n_1 and n_2 are the number of replicates for each of the two groups.

6. A set of mysid growth data from a single-concentration effluent test will be used to illustrate the ***F*** test. The raw data, mean and variance for the two controls are given in Table G.1. The data from each concentration meets the assumption of normality.

TABLE G.1. MYSID, HOLMESIMYSIS COSTATA, GROWTH DATA FROM A SINGLE-CONCENTATION EFFLUENT TEST

	Replicate	Control	Effluent
RAW	A	0.048	0.041
	B	0.058	0.033
	C	0.047	0.044
	D	0.055	0.040
	E	0.051	0.043
Mean ($\bar{x}_i$)		0.052	0.040
S_i^2		0.0000217	0.0000187
i		1	2

7. Since the variability of the control is greater than the variability of the effluent concentration, S^2 for the control is placed in the numerator of the F statistic and S^2 for the effluent concentration control is placed in the denominator.

$$F = \frac{0.0000217}{0.0000187} = 1.160$$

8. There are 5 replicates for the each groups, so the numerator and denominator degrees of freedom, $n_i - 1$, are both 4. For a two-tailed test at the 0.01 level of significance, the critical F value is obtained from a table of the ***F*** distribution (Snedecor and Cochran, 1980). The critical ***F*** value for this test is 23.16. Since 2.41 is not greater than 23.16, conclude that the variances of the brine and dilution controls are homogeneous.

9. Equal Variance ***t*** Test.

9.1 To perform the ***t*** test, calculate the following test statistic:

$$t = \frac{\bar{Y}_1 - \bar{Y}_2}{S_p \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

Where: μ_1 = mean for the control

μ_2 = mean for the effluent concentration

$$S_p = \sqrt{\frac{(n_1 - 1)S_1^2 + (n_2 - 1)S_2^2}{n_1 + n_2 - 2}}$$

S_1^2 = estimate of the variance for the control

S_2^2 = estimate of the variance for the effluent concentration

n_1 = number of replicates for the control

n_2 = number of replicates for the effluent concentration

9.2 Since we are concerned here with a decrease in response from the control, a one-tailed test is appropriate. Thus, we will compare the calculated ***t*** with a critical ***t***, where the critical ***t*** is at the 5% level of significance with $n_1 + n_2 - 2$ degrees of freedom. If the calculated ***t*** exceeds the critical ***t***, the mean responses are declared different.

9.3 When comparing brine and dilution controls, the concern is for any difference between the two control groups, and a two-tailed test is appropriate. In that case, the calculated ***t*** would be compared with a critical ***t***, where the critical ***t*** is a two-tailed value at the 5% level of significance with $n_1 + n_2 - 2$ degrees of freedom. If the absolute value of the calculated ***t*** exceeds the critical ***t***, the mean responses are declared different.

9.4 Using the data from Table G.1 to illustrate the *t* test, the calculation of *t* is as follows:

$$t = \frac{0.052 - 0.040}{0.00449\sqrt{\frac{1}{5} + \frac{1}{5}}} = 4.226$$

$$S_p = \sqrt{\frac{(5-1)0.0000217 + (5-1)0.0000187}{5 + 5 - 2}} = 0.00449$$

Where:

9.5 For a one-tailed test at the 0.05 level of significance and 8 degrees of freedom, the appropriate critical *t* value is 1.860. Note: Table D.5 for K = 1 includes the critical *t* values for comparing two groups in a one-tailed test. Since t = 4.226 is greater than 1.860, conclude that the growth in the effluent concentration is significantly less than the control group growth.

9.6 Critical *t* values for two-tailed tests, such as those needed in comparing a brine control and a dilution control, can be found in a table of the *t* distribution, such as the one in Snedecor and Cochran, 1980. Note that the critical *t* for a two-tailed test is the upper-tail value at the a/2 level of significance.

10. UNEQUAL VARIANCE *t* TEST.

10.1 If the *F* test for equality of variance fails, the *t* test is still a valid test. However, the denominator of the *t* statistic is adjusted as follows:

$$t = \frac{\bar{Y}_1 - \bar{Y}_2}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}}$$

Where: $\&_1$ = mean for the control

$\bar{x}_2$ = mean for the effluent concentration

S_1^2 = estimate of the variance for the control

S_2^2 = estimate of the variance for the effluent concentration

n_1 = number of replicates for the control

n_2 = number of replicates for the effluent concentration

10.2 Additionally, the degrees of freedom for the test are adjusted using the following formula:

$$df' = \frac{(n_1 - 1)(n_2 - 1)}{(n_2 - 1)C^2 + (1 - C)^2(n_1 - 1)}$$

Where:

$$C = \frac{\frac{S_1^2}{n_1}}{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}$$

10.3 The modified degrees of freedom is usually not an integer. Common practice is to round down to the nearest integer.

10.4 The t test is then conducted as the equal variance t test. The calculated t is compared to the critical t at the 0.05 significance level with the modified degrees of freedom. If the calculated t exceeds the critical t, the mean responses are found to be statistically different.

APPENDIX H

PROBIT ANALYSIS

1. This program calculates the EC1 and EC50 (or LC1 and LC50), and the associated 95% confidence intervals.

2. The program is written in IBM PC Basic for the IBM compatible PC by Computer Sciences Corporation, 26 W. Martin Luther King Drive, Cincinnati, OH 45268. A compiled, executable version of the program and supporting documentation can be obtained from EMSL-Cincinnati by sending a written request to EMSL at 3411 Church Street, Cincinnati, OH 45244.

2.1 A set of mortality data from a mysid survival and growth test is given in Table H.1. The program's data input routine is illustrated with this data in Figure H.1. The program begins with a request for the following information:

1. Desired output of abbreviated (A) or full (F) output? (Note: only abbreviated output is shown below.)
2. Output designation (P = printer, D = disk file).
3. Title for the output.
4. The number of exposure concentrations.
5. Toxicant concentration data.2.2 The program output for the abbreviated output options, shown in Figure H.2, includes the following:

TABLE H.1. DATA FOR PROBIT ANALYSIS

		Concentration (%)				
	Control	1.80	3.20	5.60	10.0	18.0
No. Dead	1	0	3	9	24	25
No. Exposed	25	25	25	25	25	25

2.2 The program output for the abbreviated output options, shown in figure H.2, includes the following:

1. A table of the observed proportion responding and the proportion responding adjusted for the controls.
2. The calculated chi-square statistic for heterogeneity and the tabular value. This test is one indicator of how well the data fit the model. The program will issue a warning when the test indicates that the data do not fit the model.
3. The estimated LC1 and LC50 values and associated 95% confidence intervals.

```
$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$
$                                                                       $
$                    EPA PROBIT ANALYSIS PROGRAM                        $
$                 USED FOR CALCULATING LC/EC VALUES                     $
$                            Version 1.5                                $
$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$


Do you wish abbreviated (A) or full (F) input/output? A
Output to printer (P) or disk file (D)? P
Title ? Example of Probit Analysis for Appendix H

Number responding in the control group = ? 1
Number of animals exposed in the concurrent control group = ? 25
Number of exposure concentrations, exclusive of controls ? 5

Input data starting with the lowest exposure concentration

Concentration = ? 1.80
Number responding = ? 0
Number exposed = ? 25

Concentration = ? 3.20
Number responding = ? 3
Number exposed = ? 25

Concentration = ? 5.60
Number responding = ? 9
Number exposed = ? 25

Concentration = ? 10.0
Number responding = ? 24
Number exposed = ? 25

Concentration = ? 18.0
Number responding = ? 25
Number exposed = ? 25


                          Number      Number
    Number       Conc.    Resp.      Exposed
    ______     _______    ______     _______
       1        1.8000       0          25
       2        3.2000       3          25
       3        5.6000       9          25
       4       10.0000      24          25
       5       18.0000      25          25

Do you wish to modify your data ? N

The number of control animals which responded =  1
The number of control animals exposed  =  25
Do you wish to modify these values ? N
```

Example of Probit Analysis for Appendix H

Conc.	Number Exposed	Number Resp.	Observed Proportion Responding	Proportion Responding Adjusted for Controls
Control	25	1	0.0400	0.0000
1.8000	25	0	0.0000	-.0306
3.2000	25	3	0.1200	0.0930
5.6000	25	9	0.3600	0.3404
10.0000	25	24	0.9600	0.9588
18.0000	25	25	1.0000	1.0000

Chi - Square for Heterogeneity (calculated) = 3.004
Chi - Square for Heterogeneity (tabular value at 0.05 level) = 7.815

Example of Probit Analysis for Appendix H

Estimated LC/EC Values and Confidence Limits

Point	Exposure Conc.	95% Confidence Limits Lower	Upper
LC/EC 1.00	2.642	1.384	3.519
LC/EC 50.00	5.973	4.998	6.920

Figure H.2. USEPA Probit Analysis Program used for Calculating LC/EC Values, Version 1.5.

APPENDIX I

SPEARMAN-KARBER METHOD

1. The Spearman-Karber Method is a nonparametric statistical procedure for estimating the LC50 and the associated 95% confidence interval (Finney, 1978). The Spearman-Karber Method estimates the mean of the distribution of the $\log_{10}$ of the tolerance. If the log tolerance distribution is symmetric, this estimate of the mean is equivalent to an estimate of the median of the log tolerance distribution.

2. If the response proportions are not monotonically non-decreasing with increasing concentration (constant or steadily increasing with concentration), the data must be smoothed. Abbott's procedure is used to "adjust" the concentration response proportions for mortality occurring in the control replicates.

3. Use of the Spearman-Karber Method is recommended when partial mortalities occur in the test solutions, but the data do not fit the Probit model.

4. To calculate the LC50 using the Spearman-Karber Method, the following must be true: 1) the smoothed adjusted proportion mortality for the lowest effluent concentration (not including the control) must be zero, and 2) the smoothed adjusted proportion mortality for the highest effluent concentration must be one.

5. To calculate the 95% confidence interval for the LC50 estimate, one or more of the smoothed adjusted proportion mortalities must be between zero and one.

6. The Spearman-Karber Method is illustrated below using a set of mortality data from a Mysid Survival and Growth test. These data are listed in Table I.1.

TABLE I.1. EXAMPLE OF SPEARMAN-KARBER METHOD: MORTALITY DATA FROM A MYSID SURVIVAL AND GROWTH TEST (25 ORGANISMS PER CONCENTRATION)

Effluent Concentration %	Number of Mortalities	Mortality Proportion
Control	2	0.08
6.25	2	0.08
12.5	0	0.00
25.0	3	0.12
50.0	16	0.64
100.0	25	1.00

7. Let p_0, p_1, ..., p_k denote the observed response proportion mortalities for the control and k effluent concentrations. The first step is to smooth the p_i if they do not satisfy $p_0 \# p_1 \# \ldots \# p_k$. The smoothing process replaces any adjacent p_i's that do not conform to $p_0 \# p_1 \# \ldots \# p_k$ with their average. For example, if p_i is less than p_{i-1} then:

$$p^s_{i\&1} = p^s_i = (p_i + p_{i\&1})/2$$

Where: p^s_i = the smoothed observed proportion mortality for effluent concentration i.

7.1 For the data in this example, because the observed mortality proportions for the control and the 6.25% effluent concentration are greater than the observed response proportions for the 12.5% effluent concentration, the responses for these three groups must be averaged:

$$p^s_0 = p^s_1 = p^s_2 = \frac{0.08+0.08+0.00}{3} = \frac{0.16}{3} = 0.053$$

7.2 Since $p_3 = 0.12$ is larger than p_2^s, set $p_3^s = 0.12$. Similarly, $p_4 = 0.64$ is larger than p_3^s, so set $p_4^s = 0.64$. Finally, $p_5 = 1.00$ is larger than p_4^s, so set $p_5^s = 1.00$. Additional smoothing is not necessary. The smoothed observed proportion mortalities are shown in Table I.2.

TABLE I.2. EXAMPLE OF SPEARMAN-KARBER METHOD: SMOOTHED, ADJUSTED MORTALITY DATA FROM A MYSID SURVIVAL AND GROWTH TEST

Effluent Concentration %	*Mortality Proportion*	*Smoothed Mortality Proportion*	*Smoothed, Adjusted Mortality Proportion*
Control	*0.08*	*0.053*	*0.000*
6.25	*0.08*	*0.053*	*0.000*
12.5	*0.00*	*0.053*	*0.000*
25.0	*0.12*	*0.120*	*0.071*
50.0	*0.64*	*0.640*	*0.620*
100.0	*1.00*	*1.000*	*1.000*

8. Adjust the smoothed observed proportion mortality in each effluent concentration for mortality in the control group using Abbott's formula (Finney, 1971). The adjustment takes the form:

$$p_i^a = (p_i^s - p_0^s) / (1 - p_0^s)$$

Where: p_0^s = the smoothed observed proportion mortality for the control

p_i^s = the smoothed observed proportion mortality for effluent concentration i.

8.1 For the data in this example, the data for each effluent concentration must be adjusted for control mortality using Abbott's formula, as follows:

$$p_0^a \text{ , } p_1^a \text{ , } p_2^a \text{ , } \frac{p_1^s \,\&\, p_0^s}{1 \,\&\, p_0^s} \text{ , } \frac{0.053 \,\&\, 0.053}{1 \,\&\, 0.053} \text{ , } \frac{0.0}{0.947} \text{ , } 0.0$$

$$p_3^a = \frac{p_3^s - p_0^s}{1 - p_0^s} = \frac{0.120 - 0.053}{1 - 0.053} = \frac{0.067}{0.947} = 0.071$$

$$p_4^a = \frac{p_4^s - p_0^s}{1 - p_0^s} = \frac{0.640 - 0.053}{1 - 0.053} = \frac{0.587}{0.947} = 0.620$$

$$p_5^a = \frac{p_5^s - p_0^s}{1 - p_0^s} = \frac{1.000 - 0.053}{1 - 0.053} = \frac{0.947}{0.947} = 1.000$$

The smoothed, adjusted response proportions for the effluent concentrations are shown in Table I.2. A plot of the smoothed, adjusted data is shown in Figure I.1.

9. Calculate the $\log_{10}$ of the estimated LC50, m, as follows:

$$m = \sum_{i=1}^{k-1} \frac{(p_{i+1}^a - p_i^a)(X_i + X_{i+1})}{2}$$

Where: p_i^a = the smoothed adjusted proportion mortality at concentration i

X_i = the $\log_{10}$ of concentration i

k = the number of effluent concentrations tested, not including the control.

9.1 For this example, the $\log_{10}$ of the estimated LC50, m, is calculated as follows:

$$\begin{aligned} m = {} & [(0.000 - 0.000)(0.7959 + 1.0969)]/2 + \\ & [(0.071 - 0.000)(1.0969 + 1.3979)]/2 + \\ & [(0.620 - 0.071)(1.3979 + 1.6990)]/2 + \\ & [(1.000 - 0.620)(1.6990 + 2.0000)]/2 \end{aligned}$$

$$= 1.64147$$

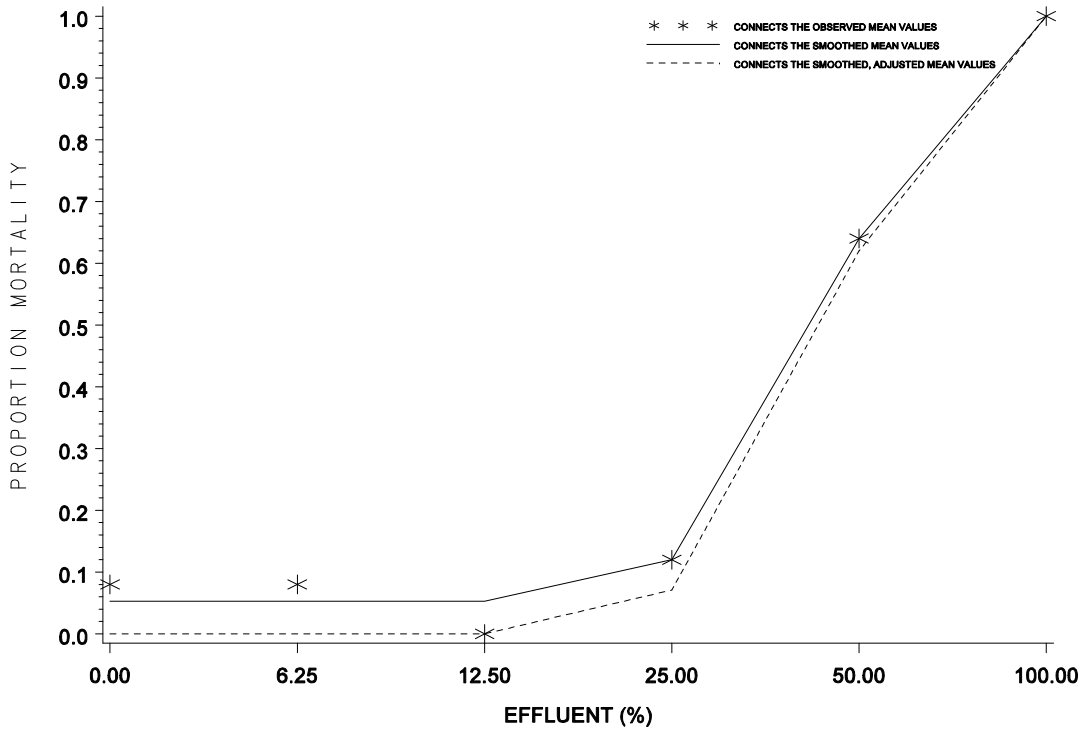

Figure I.1. Plot of observed, smoothed, and adjusted response proportions for mysid, Holmesimysis costata, survival data.

10. Calculate the estimated variance of m as follows:

$$V(m) = \sum_{i=2}^{k-1} \frac{p_i^a(1-p_i^a)(X_{i+1}-X_{i-1})^2}{4(n_i-1)}$$

Where: X_i = the $\log_{10}$ of concentration i

n_i = the number of organisms tested at effluent concentration i

p_i^a = the smoothed adjusted observed proportion mortality at effluent concentration i

k = the number of effluent concentrations tested, not including the control.

10.1 For this example, the estimated variance of m, V(m), is calculated as follows:

$$\begin{aligned} V(m) = {} & (0.000)(1.000)(1.3979 - 0.7959)^2/4(24) + \\ & (0.071)(0.929)(1.6990 - 1.0969)^2/4(24) + \\ & (0.620)(0.380)(2.0000 - 1.3979)^2/4(24) \end{aligned}$$

$$= 0.0011388$$

11. Calculate the 95% confidence interval for m:

$$m \pm 2.0\sqrt{V(m)}$$

11.1 For this example, the 95% confidence interval for m is calculated as follows:

$$1.64147 \pm 2\sqrt{0.0011388} = (1.57398, 1.70896)$$

12. The estimated LC50 and a 95% confidence interval for the estimated LC50 can be found by taking base_{10} antilogs of the above values.

12.1 For this example, the estimated LC50 is calculated as follows:

LC50 = antilog(m) = antilog(1.64147) = 43.8%.

12.2 The limits of the 95% confidence interval for the estimated LC50 are calculated by taking the antilogs of the upper and lower limits of the 95% confidence interval for m as follows:

lower limit: antilog(1.57398) = 37.5%

upper limit: antilog(1.70896) = 51.2%

APPENDIX J

TRIMMED SPEARMAN-KARBER METHOD

1. The Trimmed Spearman-Karber Method is a modification of the Spearman-Karber Method, a nonparametric statistical procedure for estimating the LC50 and the associated 95% confidence interval (Hamilton, et al, 1977). The Trimmed Spearman-Karber Method estimates the trimmed mean of the distribution of the $\log_{10}$ of the tolerance. If the log tolerance distribution is symmetric, this estimate of the trimmed mean is equivalent to an estimate of the median of the log tolerance distribution.

2. If the response proportions are not monotonically non-decreasing with increasing concentration (constant or steadily increasing with concentration), the data must be smoothed. Abbott's procedure is used to "adjust" the concentration response proportions for mortality occurring in the control replicates.

3. Use of the Trimmed Spearman-Karber Method is recommended only when the requirements for the Probit Analysis and the Spearman-Karber Method are not met.

4. To calculate the LC50 using the Trimmed Spearman-Karber Method, the smoothed, adjusted, observed proportion mortalities must bracket 0.5.

5. To calculate the 95% confidence interval for the LC50 estimate, one or more of the smoothed, adjusted, observed proportion mortalities must be between zero and one.

6. Let p_0, p_1, ..., p_k denote the observed proportion mortalities for the control and the k effluent concentrations. The first step is to smooth the p_i if they do not satisfy $p_0 \# p_1 \# \ldots \# p_k$. The smoothing process replaces any adjacent p_i's that do not conform to $p_0 \# p_1 \# \ldots \# p_k$, with their average. For example, if p_i is less than p_{i-1} then:

$$p^s_{i-1} = p^s_i = (p_i + p_{i-1})/2$$

Where: p^s_i = the smoothed observed proportion mortality for effluent concentration i.

7. Adjust the smoothed observed proportion mortality in each effluent concentration for mortality in the control group using Abbott's formula (Finney, 1971). The adjustment takes the form:

$$p_i^a = (p_i^s - p_0^s) / (1 - p_0^s)$$

Where: p_0^s = the smoothed observed proportion mortality for the control

p_i^s = the smoothed observed proportion mortality for effluent concentration i.

8. Calculate the amount of trim to use in the estimation of the LC50 as follows:

$$Trim = \max(p_1^a, 1-p_k^a)$$

Where: p_1^a = the smoothed, adjusted proportion mortality for the lowest effluent concentration, exclusive of the control

p_k^a = the smoothed, adjusted proportion mortality for the highest effluent concentration

k = the number of effluent concentrations, exclusive of the control.

The minimum trim should be calculated for each data set rather than using a fixed amount of trim for each data set.

9. Due to the intensive nature of the calculation for the estimated LC50 and the calculation of the associated 95% confidence interval using the Trimmed Spearman-Karber Method, it is recommended that the data be analyzed by computer.

10. A computer program which estimates the LC50 and associated 95% confidence interval using the Trimmed Spearman-Karber Method, can be obtained through EMSL, 3411 Church Street, Cincinnati, OH 45244. The program can be obtained from EMSL-Cincinnati by sending a written request to the above address.

11. The Trimmed Spearman-Karber program automatically performs the following functions:

a. Smoothing.
b. Adjustment for mortality in the control.
c. Calculation of the necessary trim.
d. Calculation of the LC50.
e. Calculation of the associated 95% confidence interval.

12. To illustrate the Trimmed Spearman-Karber method using the Trimmed Spearman-Karber computer program, a set of data from a Topsmelt Larval Survival and Growth test will be used. The data are listed in Table J.1.

TABLE J.1. EXAMPLE OF TRIMMED SPEARMAN-KARBER METHOD: MORTALITY DATA FROM A TOPSMELT LARVAL SURVIVAL AND GROWTH TEST (25 ORGANISMS PER CONCENTRATION)

Effluent Concentration %	Number of Mortalities	Mortality Proportion
Control	0	0.00
6.25	2	0.08
12.5	1	0.04
25.0	5	0.20
50.0	25	1.00
100.0	25	1.00

12.1 The program requests the following input (Figure J.1):
a. Output destination (D = disk file or P = printer).
b. Control data.
c. Data for each toxicant concentration.

12.2 The program output includes the following (Figure J.2):
a. A table of the concentrations tested, number of organisms exposed, and the mortalities.
b. The amount of trim used in the calculation.
c. The estimated LC50 and the associated 95% confidence interval.

```
A:>TSK

TRIMMED SPEARMAN-KARBER METHOD.  VERSION 1.5
ENTER DATE OF TEST:
1
 ENTER TEST NUMBER:
2
WHAT IS TO BE ESTIMATED?
(ENTER "L" FOR LC50 AND "E" FOR EC50)
L
ENTER TEST SPECIES NAME:
Topsmelt
ENTER TOXICANT  NAME:
effluent
ENTER UNITS FOR EXPOSURE CONCENTRATION OF TOXICANT :
%
ENTER THE NUMBER OF INDIVIDUALS IN THE CONTROL:
25
ENTER THE NUMBER OF MORTALITIES IN THE CONTROL:
0
ENTER THE NUMBER OF CONCENTRATIONS
(NOT INCLUDING THE CONTROL;  MAX = 10):
5
ENTER THE  5 EXPOSURE CONCENTRATIONS (IN INCREASING ORDER):
6.25  12.5  25  50  100
ARE THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION EQUAL(Y/N)?
y
ENTER THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION:
25
ENTER UNITS FOR DURATION OF EXPERIMENT
(ENTER "H" FOR HOURS, "D" FOR DAYS, ETC.):
Days
ENTER DURATION OF TEST:
7
ENTER THE NUMBER OF MORTALITIES AT EACH EXPOSURE CONCENTRATION:
2 1 5 25 25
WOULD YOU LIKE THE AUTOMATIC TRIM CALCULATION(Y/N)?
y
```

Figure J.1. Example input for Trimmed Spearman-Karber Method.

TRIMMED SPEARMAN-KARBER METHOD. VERSION 1.5

DATE: 1 TEST NUMBER: 2 DURATION: 7 Days
TOXICANT: effluent
SPECIES: Topsmelt

RAW DATA: Concentration (%)	Number Exposed	Mortalities
.00	25	0
6.25	25	2
12.50	25	1
25.00	25	5
50.00	25	25
100.00	25	25

SPEARMAN-KARBER TRIM: 6.00%

SPEARMAN-KARBER ESTIMATES: LC50: 30.98
95% LOWER CONFIDENCE: 27.17
95% UPPER CONFIDENCE: 35.32

NOTE: MORTALITY PROPORTIONS WERE NOT MONOTONICALLY INCREASING.
ADJUSTMENTS WERE MADE PRIOR TO SPEARMAN-KARBER ESTIMATION.

Figure J.2. Example output for Trimmed Spearman-Karber Method.

APPENDIX K

GRAPHICAL METHOD

1. The Graphical Method is used to calculate the LC50. It is a mathematical procedure which estimates the LC50 by linearly interpolating between points of a plot of observed percent mortality versus the base 10 logarithm (log_{10}) of percent effluent concentration. This method does not provide a confidence interval for the LC50 estimate and its use is only recommended when there are no partial mortalities after the data is smoothed and adjusted for control mortality. The only requirement for the Graphical Method is that the observed percent mortalities bracket 50%.

2. For an analysis using the Graphical Method the data must first be smoothed and adjusted for mortality in the control replicates. The procedure for smoothing and adjusting the data is detailed in the following steps.

3. The Graphical Method is illustrated below using a set of mortality data from a Topsmelt Larval Survival and Growth test. These data are listed in Table K.1.

TABLE K.1. EXAMPLE OF GRAPHICAL METHOD: MORTALITY DATA FROM A TOPSMELT LARVAL SURVIVAL AND GROWTH TEST (25 ORGANISMS PER CONCENTRATION)

Effluent Concentration %	Number of Mortalities	Mortality Proportion
Control	1	0.04
6.25	0	0.00
12.5	0	0.00
25.0	0	0.00
50.0	25	1.00
100.0	25	1.00

4. Let p_0, p_1, ..., p_k denote the observed proportion mortalities for the control and the k effluent concentrations. The first step is to smooth the p_i if they do not satisfy $p_0 \# p_1 \# \ldots \# p_k$. The smoothing process replaces any adjacent p_i's that do not conform to $p_0 \# p_1 \# \ldots \# p_k$ with their average. For example, if p_i is less than p_{i-1} then:

$$p_{i\&1}^{s} \leftarrow p_i^{s} \leftarrow (p_i \% p_{i\&1})/2$$

Where: p_i^s = the smoothed observed proportion mortality for effluent concentration i.

4.1 For the data in this example, because the observed mortality proportions for the 6.25%, 12.5%, and 25.0% effluent concentrations are less than the observed response proportion for the control, the values for these four groups must be averaged:

$$p_0^{s} \leftarrow p_1^{s} \leftarrow p_2^{s} \leftarrow p_3^{s} \leftarrow \frac{0.04 \% 0.00 \% 0.00 \% 0.00}{4} \leftarrow \frac{0.04}{4} \leftarrow 0.01$$

4.2 Since $p_4 = p_5 = 1.00$ are larger then 0.01, set $p_4^s = p_5^s = 1.00$. Additional smoothing is not necessary. The smoothed observed proportion mortalities are shown in Table K.2.

5. Adjust the smoothed observed proportion mortality in each effluent concentration for mortality in the control group using Abbott's formula (Finney, 1971). The adjustment takes the form:

$$p_i^{a} \leftarrow (p_i^{s} \& p_0^{s}) /(1 \& p_0^{s})$$

Where: p_0^s = the smoothed observed proportion mortality for the control

p_i^s = the smoothed observed proportion mortality for effluent concentration i.

5.1 Because the smoothed observed proportion mortality for the control group is greater than zero, the responses must be adjusted using Abbott's formula, as follows:

$$p_0^a \text{ , } p_1^a \text{ , } p_2^a \text{ , } p_3^a \text{ , } \frac{p_1^s \text{ \& } p_0^s}{1 \text{ \& } p_0^s} \text{ , } \frac{0.01 \text{ \& } 0.01}{1 \text{ \& } 0.0125} \text{ , } \frac{0.0}{0.99} \text{ , } 0.0$$

$$p_4^a \text{ , } p_5^a \text{ , } \frac{p_4^s \text{ \& } p_0^s}{1 \text{ \& } p_0^s} \text{ , } \frac{1.00 \text{ \& } 0.01}{1 \text{ \& } 0.01} \text{ , } \frac{0.99}{0.99} \text{ , } 1.00$$

A table of the smoothed, adjusted response proportions for the effluent concentrations is shown in Table K.2.

TABLE K.2. EXAMPLE OF GRAPHICAL METHOD: SMOOTHED, ADJUSTED MORTALITY DATA FROM A TOPSMELT LARVAL SURVIVAL AND GROWTH TEST

Effluent Concentration %	Mortality Proportion	Smoothed Mortality Proportion	Smoothed, Adjusted Mortality Proportion
Control	0.04	0.01	0.00
6.25	0.00	0.01	0.00
12.5	0.00	0.01	0.00
25.0	0.00	0.01	0.00
50.0	1.00	1.00	1.00
100.0	1.00	1.00	1.00

5.2 Plot the smoothed, adjusted data on 2-cycle semi-log graph paper with the logarithmic axis (the y axis) used for percent effluent concentration and the linear axis (the x axis) used for observed percent mortality. A plot of the smoothed, adjusted data is shown in Figure K.1.

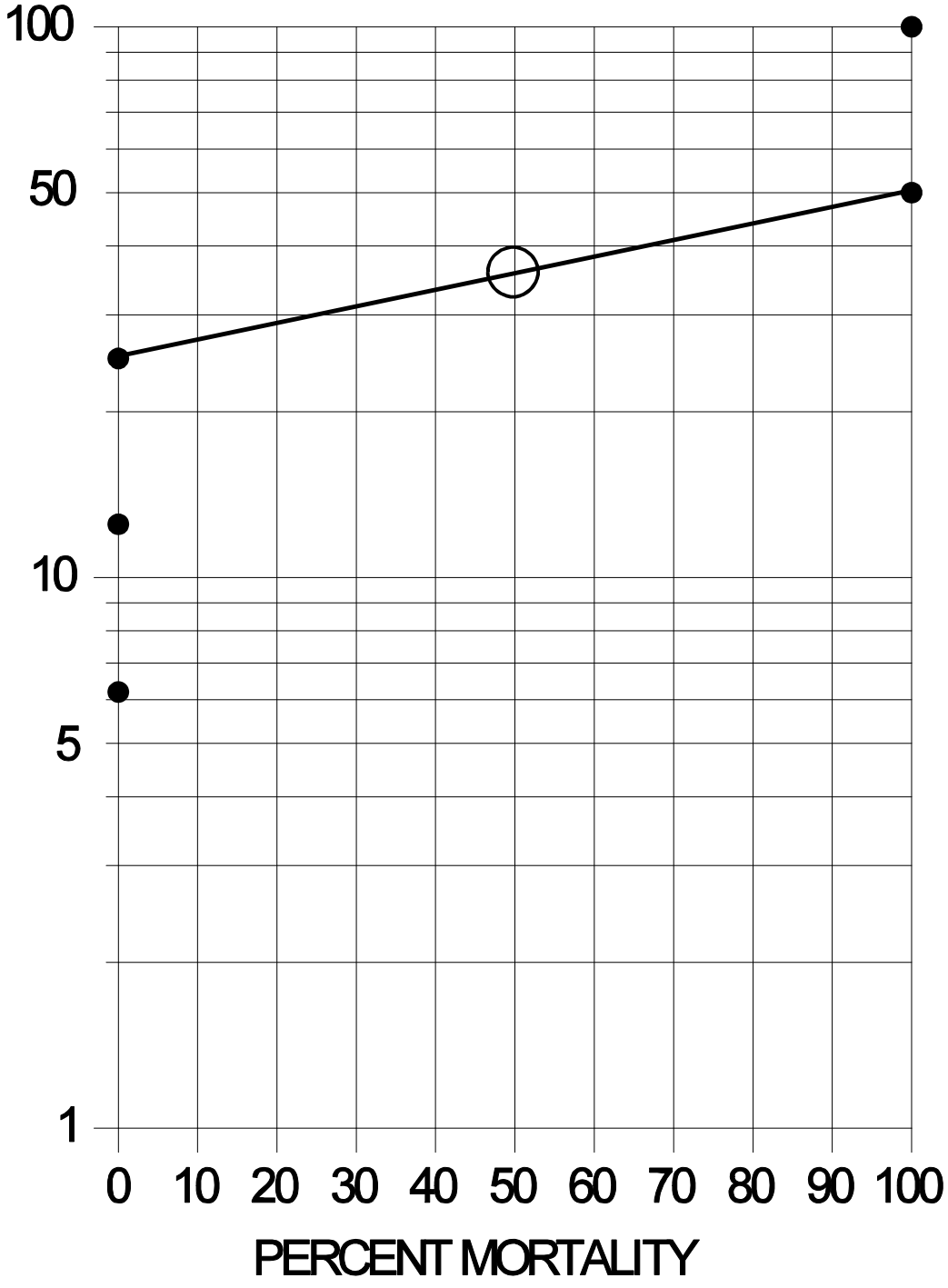

Figure K.1. Plot of the smoothed adjusted response proportions for topsmelt, Atherinops affinis, survival data.

6. Locate the two points on the graph which bracket 50% mortality and connect them with a straight line.

7. On the scale for percent effluent concentration, read the value for the point where the plotted line and the 50% mortality line intersect. This value is the estimated LC50 expressed as a percent effluent concentration.

7.1 For this example, the two points on the graph which bracket the 50% mortality line (0% mortality at 25% effluent, and 100% mortality at 50% effluent) are connected with a straight line. The point at which the plotted line intersects the 50% mortality line is the estimated LC50. The estimated LC50 = 35% effluent.

APPENDIX L

LINEAR INTERPOLATION METHOD

1. GENERAL PROCEDURE

1.1 The Linear Interpolation Method is used to calculate a point estimate of the effluent or other toxicant concentration that causes a given percent reduction (e.g., 25%, 50%, etc.) in the reproduction or growth of the test organisms (Inhibition Concentration, or IC). The procedure was designed for general applicability in the analysis of data from short-term chronic toxicity tests, and the generation of an endpoint from a continuous model that allows a traditional quantitative assessment of the precision of the endpoint, such as confidence limits for the endpoint of a single test, and a mean and coefficient of variation for the endpoints of multiple tests.

1.2 The Linear Interpolation Method assumes that the responses (1) are monotonically non-increasing, where the mean response for each higher concentration is less than or equal to the mean response for the previous concentration, (2) follow a piecewise linear response function, and (3) are from a random, independent, and representative sample of test data. If the data are not monotonically non-increasing, they are adjusted by smoothing (averaging). In cases where the responses at the low toxicant concentrations are much higher than in the controls, the smoothing process may result in a large upward adjustment in the control mean. Also, no assumption is made about the distribution of the data except that the data within a group being resampled are independent and identically distributed.

2. DATA SUMMARY AND PLOTS

2.1 Calculate the mean responses for the control and each toxicant concentration, construct a summary table, and plot the data.

3. MONOTONICITY

3.1 If the assumption of monotonicity of test results is met, the observed response means ($\&_i$) should stay the same or decrease

as the toxicant concentration increases. If the means do not decrease monotonically, the responses are "smoothed" by averaging (pooling) adjacent means.

3.2 Observed means at each concentration are considered in order of increasing concentration, starting with the control mean ($\&_1$). If the mean observed response at the lowest toxicant concentration ($\&_2$) is equal to or smaller than the control mean ($\&_1$), it is used as the response. If it is larger than the control mean, it is averaged with the control, and this average is used for both the control response (M_1) and the lowest toxicant concentration response (M_2). This mean is then compared to the mean observed response for the next higher toxicant concentration ($\&_3$). Again, if the mean observed response for the next higher toxicant concentration is smaller than the mean of the control and the lowest toxicant concentration, it is used as the response. If it is higher than the mean of the first two, it is averaged with the first two, and the mean is used as the response for the control and two lowest concentrations of toxicant. This process is continued for data from the remaining toxicant concentrations. A numerical example of smoothing the data is provided below. (Note: Unusual patterns in the deviations from monotonicity may require an additional step of smoothing). Where $\&_i$ decrease monotonically, the $\&_i$ become M_i without smoothing.

4. **LINEAR INTERPOLATION METHOD**

4.1 The method assumes a linear response from one concentration to the next. Thus, the ICp is estimated by linear interpolation between two concentrations whose responses bracket the response of interest, the (p) percent reduction from the control.

4.2 To obtain the estimate, determine the concentrations C_J and C_{J+1} which bracket the response M_1 (1 - p/100), where M_1 is the smoothed control mean response and p is the percent reduction in response relative to the control response. These calculations can easily be done by hand or with a computer program as described below. The linear interpolation estimate is calculated as follows:

$$ICp = C_J + [M_1(1 - p/100) - M_J] \frac{(C_{J+1} - C_J)}{(M_{J+1} - M_J)}$$

Where: C_J = tested concentration whose observed mean response is greater than $M_1(1 - p/100)$.

C_{J+1} = tested concentration whose observed mean response is less than $M_1(1 - p/100)$.

M_1 = smoothed mean response for the control.

M_J = smoothed mean response for concentration J.

M_{J+1} = smoothed mean response for concentration J + 1.

p = percent reduction in response relative to the control response.

IC***p*** = estimated concentration at which there is a percent reduction from the smoothed mean control response. The IC***p*** is reported for the test, together with the 95% confidence interval calculated by the ICPIN.EXE program described below.

4.3 If the C_J is the highest concentration tested, the IC***p*** would be specified as ***greater than*** C_J. If the response at the lowest concentration tested is used to extrapolate the ICp value, the ICp should be expressed as a ***less than the lowest test concentration.***

5. **CONFIDENCE INTERVALS**

5.1 Due to the use of a linear interpolation technique to calculate an estimate of the ICp, standard statistical methods for calculating confidence intervals are not applicable for the ICp. This limitation is avoided by use a technique known as the bootstrap method as proposed by Efron (1982) for deriving point estimates and confidence intervals.

5.2 In the Linear Interpolation Method, the smoothed response means are used to obtain the ICp estimate reported for the test. The bootstrap method is used to obtain the 95% confidence interval for the true mean. In the bootstrap method, the test data Y_{ji} is randomly resampled with replacement to produce a new set of data Y_{ji}*, that is statistically equivalent to the original data, but a new and slightly different estimate of the IC*p* (IC*p**) is obtained. This process is repeated at least 80 times (Marcus and Holtzman, 1988) resulting in multiple "data" sets, each with an associate ICp* estimate. The distribution of the ICp* estimates derived from the sets of resampled data approximates the sampling distribution of the ICp estimate. The standard error of the ICp is estimated by the standard deviation of the individual ICp* estimates. Empirical confidence intervals are derived from the quantiles of the ICp* empirical distribution. For example, if the test data are resampled a minimum of 80 times, the empirical 2.5% and the 97.5% confidence limits are approximately the second smallest and second largest ICp* estimates (Marcus and Holtzman, 1988).

5.3 The width of the confidence intervals calculated by the bootstrap method is related to the variability of the data. When confidence intervals are wide, the reliability of the IC estimate is in question. However, narrow intervals do not necessarily indicate that the estimate is highly reliable, because of undetected violations of assumptions and the fact that the confidence limits based on the empirical quantiles of a bootstrap distribution of 80 samples may be unstable.

5.4 The bootstrapping method of calculating confidence intervals is computationally intensive. For this reason, all of the calculations associated with determining the confidence intervals for the ICp estimate have been incorporated into a computer program. Computations are most easily done with a computer program such as the revision of the BOOTSTRP program (USEPA, 1988; USEPA, 1989) which is now called "ICPIN" and is described below in subsection 7.

6. **MANUAL CALCULATIONS**

6.1 DATA SUMMARY AND PLOTS

6.1.1 The data used in this example are the mysid growth data used in the example in Section 14. The data is presented as the mean weight per surviving organism. Table L.1 includes the raw data and the mean growth for each concentration. A plot of the data is provided in Figure L.1.

6.2 MONOTONICITY

6.2.1. As seen in the table, the observed means are monotonically non-increasing with respect to concentration. Therefore, the smoothed means will be simply the corresponding observed mean. The observed means are represented by $\hat{y}_i$ and the smoothed means by M_i. Table L.2 contains the smoothed means and Figure L.1 gives a plot of the smoothed response curve.

6.3 LINEAR INTERPOLATION

6.3.1 An estimates of the IC25 can be calculated using the Linear Interpolation Method. A 25% reduction in mean weight, compared to the controls, would result in a mean weight of 0.039, where $M_1(1-p/100) = 0.052(1-25/100)$. Examining the smoothed means and their associated concentrations (Table L.2), the response, 0.039 mg, is bracketed by $C_4 = 5.60\%$ and and $C_5 = 10.0\%$.

TABLE L.1. MYSID, HOLMESIMYSIS COSTATA, GROWTH DATA

	Concentration (%)				
Replicate	*Control*	*1.80*	*3.20*	*5.60*	*10.0*
1	*0.048*	*0.055*	*0.057*	*0.041*	*0.033*
2	*0.058*	*0.048*	*0.050*	*0.040*	*0.000*
3	*0.047*	*0.042*	*0.046*	*0.041*	*0.000*
4	*0.058*	*0.041*	*0.043*	*0.043*	*0.000*
5	*0.051*	*0.052*	*0.045*	*0.040*	*0.000*
Mean($\hat{y}_i$)	*0.052*	*0.048*	*0.048*	*0.041*	*0.007*
i	*1*	*2*	*3*	*4*	*5*

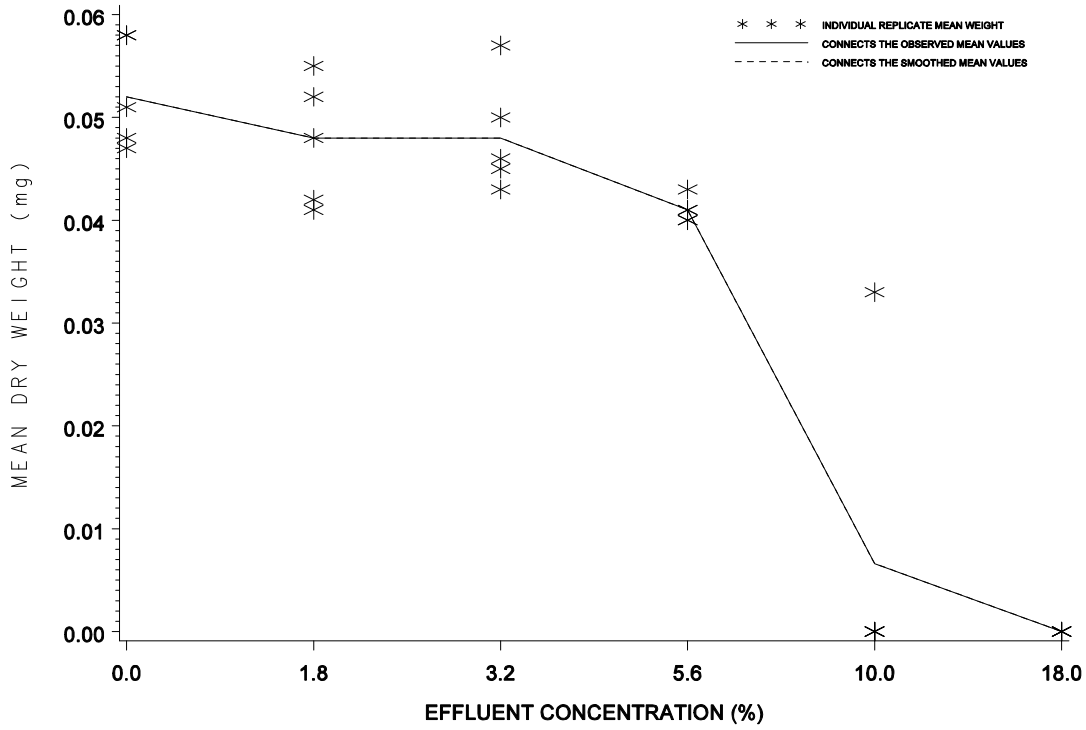

Figure L.1. Plot of raw data, observed means, and smoothed means for the mysid, Holmesimsys costata, growth data.

TABLE L.2. MYSID, HOLMESIMYSIS COSTATA, MEAN GROWTH RESPONSE AFTER SMOOTHING

Toxicant Conc. (%)	i	Response Means Y_i (mg)	Smoothed Means M_i (mg)
Control	1	0.052	0.052
1.80	2	0.048	0.048
3.20	3	0.048	0.048
5.60	4	0.041	0.041
10.00	5	0.007	0.007
18.00	6	0.000	0.000

6.3.2 Using the equation from section 4.2, the estimate of the IC25 is calculated as follows:

$$ICp = C_J \% [\, M_1 (1 - p/100) - M_J \,] \; \frac{(C_{J+1} - C_J)}{(M_{J+1} - M_J)}$$

$$IC25 = 5.60 \% [0.052 (1 - 25/100) - 0.041] \frac{(10.0 - 5.60)}{(0.007 - 0.041)}$$

$$= 5.86\%$$

6.4 CONFIDENCE INTERVALS

6.4.1 Confidence intervals for the ICp are derived using the bootstrap method. As described above, this method involves randomly resampling the individual observations and recalculating the ICp at least 80 times, and determining the mean ICp, standard deviation, and empirical 95% confidence intervals. For this reason, the confidence intervals are calculated using a computer program called ICPIN. This program is described below and is available to carry out all the calculations of both the interpolation estimate (ICp) and the confidence intervals.

7. **COMPUTER CALCULATIONS**

7.1 The computer program, ICPIN, prepared for the Linear Interpolation Methods was written in TURBO PASCAL for IBM compatible PCs. The program (version 2.0) has been modified by Computer Science Corporation, Duluth, MN with funding provided by the Environmental Research Laboratory, Duluth, MN (Norberg-King, 1993). The program was originally developed by Battelle Laboratories, Columbus, OH through a government contract supported by the Environmental Research Laboratory, Duluth, MN (USEPA, 1988). A compiled, executable version of the program and supporting documentation can be obtained by sending a written request to EMSL-Cincinnati, 3411 Church Street, Cincinnati, OH 45244.

7.2 The ICPIN.EXE program performs the following functions: 1) it calculates the observed response means ($\overline{Y}_i$) (response means); 2) it calculates the standard deviations; 3) checks the responses for monotonicity; 4) calculates smoothed means (M_i) (pooled response means) if necessary; 5) uses the means, M_i, to calculate the initial ICp of choice by linear interpolation; 6) performs a user-specified number of bootstrap resamples between 80 and 1000 (as multiples of 40); 7) calculates the mean and standard deviation of the bootstrapped ICp estimates; and 8) provides an original 95% confidence intervals to be used with the initial ICp when the number of replicates per concentration is over six and provides both original and expanded confidence intervals when the number of replicates per concentration are less than seven (Norberg-King, 1993).

7.3 For the IC***p*** calculation, up to twelve treatments can be input (which includes the control). There can be up to 40 replicates per concentration, and the program does not require an equal number of replicates per concentration. The value of p can range from 1% to 99%.

7.4 DATA INPUT

7.4.1 Data is entered directly into the program onscreen. A sample data entry screen in shown in Figure L.2. The program documentation provides guidance on the entering and analysis of data for the Linear Interpolation Method.

```
ICp Data Entry/Edit Screen                          Current File:
Conc. ID       *   1      *     2     *     3     *     4     *   5      *  6
Conc. Tested   *          *           *           *           *          *
Response  1    *          *           *           *           *          *
Response  2    *          *           *           *           *          *
Response  3    *          *           *           *           *          *
Response  4    *          *           *           *           *          *
Response  5    *          *           *           *           *          *
Response  6    *          *           *           *           *          *
Response  7    *          *           *           *           *          *
Response  8    *          *           *           *           *          *
Response  9    *          *           *           *           *          *
Response 10    *          *           *           *           *          *
Response 11    *          *           *           *           *          *
Response 12    *          *           *           *           *          *
Response 13    *          *           *           *           *          *
Response 14    *          *           *           *           *          *
Response 15    *          *           *           *           *          *
Response 16    *          *           *           *           *          *
Response 17    *          *           *           *           *          *
Response 18    *          *           *           *           *          *
Response 19    *          *           *           *           *          *
Response 20    *          *           *           *           *          *

444444444444444444444444444444444444444444444444444444444444444444444444444
F10 for Command Menu                    Use Arrow Keys to Switch Fields
```

Figure L.2. *ICp data entry/edit screen. Twelve concentration identifications can be used. Data for concentrations are entered in columns 1 through 6. For concentrations*

7.4.2 The user selects the ICp estimate desired (e.g., IC25 or IC50) and the number of resamples to be taken for the bootstrap method of calculating the confidence intervals. The program has the capability of performing any number of resamples from 80 to 1000 as multiples of 40. However, Marcus and Holtzman (1988) recommend a minimum of 80 resamples for the bootstrap method be used and at least 250 resamples are better (Norberg-King, 1993).

7.5 DATA OUTPUT

7.5.1 The program output includes the following (see Figure L.3)

1. A table of the concentration identification, the concentration tested and raw data response for each replicate and concentration.
2. A table of test concentrations, number of replicates, concentration (units), response means (Y_i), standard deviations for each response mean, and the pooled response means (smoothed means; M_i).
3. The linear interpolation estimate of the ICp using the means (M_i). ***Use this value for the ICp estimate.***
4. The mean ICp and standard deviation from the bootstrap resampling.
5. The confidence intervals calculated by the bootstrap method for the ICp. Provides an original 95% confidence intervals to be used with the initial ICp when the number of replicates per concentration is over six and provides both original and expanded confidence intervals when the number of replicates per concentration are less than seven.

7.6 ICPIN program output for the analysis of the mysid growth data in Table L.1 is provided in Figure L.3.

7.6.1 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 5.8174%. The empirical 95% confidence intervals for the true mean was 4.9440% to 6.2553%.

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	1.80	3.20	5.60	10.0	18.0
Response 1	.048	.055	.057	.041	.033	0
Response 2	.058	.048	.050	.040	0	0
Response 3	.047	.042	.046	.041	0	0
Response 4	.058	.041	.043	.043	0	0
Response 5	.051	.052	.045	.040	0	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Effluent
Test Start Date: Test Ending Date:
Test Species: mysid, Holmesimysis costata
Test Duration: 7 days
DATA FILE: mysid.icp
OUTPUT FILE: mysid.i25

Conc. ID	Number Replicates	Concentration %	Response Means	Std. Dev.	Pooled Response Means
1	5	0.000	0.052	0.005	0.052
2	5	1.800	0.048	0.006	0.048
3	5	3.200	0.048	0.006	0.048
4	5	5.600	0.041	0.001	0.041
5	5	10.000	0.007	0.015	0.007
6	5	18.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 5.8174 Entered P Value: 25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 5.8205 Standard Deviation: 0.2673
Original Confidence Limits: Lower: 4.9440 Upper: 6.2553
Expanded Confidence Limits: Lower: 4.5073 Upper: 6.4743
Resampling time in Seconds: 0.22 Random_Seed: 526805435

Figure L.3. Example of ICPIN program output for the IC25.

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