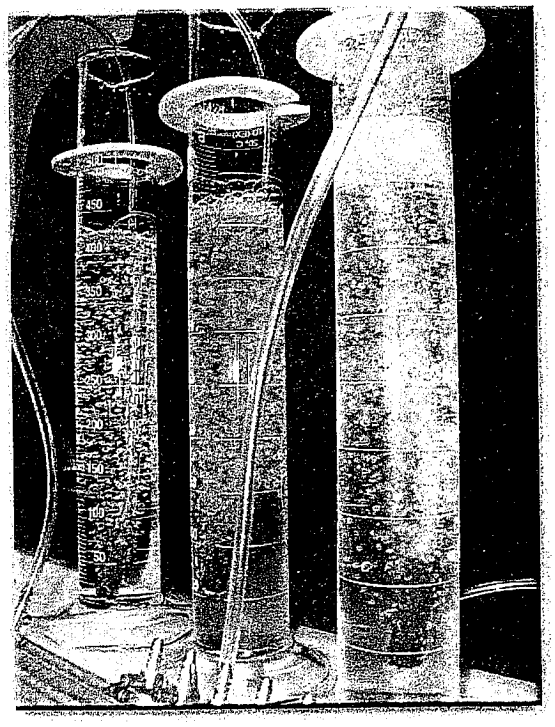
California Regional Water Quality Control Board, Colorado River Basin
Prosecution Team Evidence
on the matter of
Administrative Civil Liability Complaint R7-2014-0041
Exhibit 9

Toxicity Identification Evaluation (TIE)
City of Brawley WWTP
Spring 2002



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INTRODUCTION

A series of Toxicity Identification Evaluation (TIE) studies were performed on effluent samples collected from the City of Brawley wastewater treatment plant (WWTP) during the spring of 2002. TIE tests were conducted using the fathead minnow *Pimephales promelas* and freshwater Cladoceran *Ceriodaphnia dubia*. Testing was conducted at the AMEC Earth & Environmental, Inc. Bioassay Laboratory (AMEC) in San Diego, California.

Survival of *P. promelas and C. dubia* was evaluated at the end of exposures to effluent in accordance with standard U.S. Environmental Protection Agency (EPA) methods. Acute screening level tests using *P. promelas* and *C. dubia* were conducted to confirm toxicity prior to initiating TIE studies. The presence and magnitude of toxicity was then used as a decision-making tool to proceed from screening level analyses to TIE methods.

The TIE procedures developed by the EPA are divided into three sequential phases. Phase I methods are designed to characterize the chemical and physical nature of the constituents responsible for causing toxicity. During Phase I characterization procedures, the sample undergoes a number of physical and chemical manipulations. Each of these manipulated fractions is subsequently tested for toxicity to identify the property or class of contaminants associated with toxicity (e.g., filterable materials, trace metals, nonpolar organics, oxidants, volatile compounds). During Phase II identification procedures, additional manipulations and measurements are performed in an effort to identify specific compounds associated, in part or in full, with toxicity. Specific Phase II methods are dependent on results obtained during Phase I testing. Confirmation of suspected toxicants is performed using Phase II and Phase III TIE procedures.

This report includes results of the initial screening level analyses and subsequent Phase I, Phase II, and Phase III TIE procedures. All testing was performed on a single 24-hour composite sample collected on 8 April 2002.

Toxicity screening tests were initiated on 9 April 2002. Phase I procedures were initiated using both *C. dubia* and *P. promelas* on 12 April 2002 and Phase II/III procedures were initiated 19 April 2002 using *P. promelas*. Phase I results were very similar for both species, therefore, to conserve resources, Phase II/III TIE procedures were conducted with only the fathead minnow due to its greater sensitivity.

The TIE study results were compared to historical toxicity data and Phase I TIEs previously conducted for the Brawley WWTP in 1999 and 2001 using the fathead minnow *P. promelas*.

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MATERIALS AND METHODS

This study was performed using procedures published in the following documents:

(1) Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms (EPA 1993a)

(2) Methods for Aquatic Toxicity Identification Evaluations - Phase I Toxicity Characterization Procedures, Second Edition (EPA 1991)

(3) Methods for Aquatic Toxicity Identification Evaluations - Phase II Toxicity Identification Procedures for Samples Exhibiting Acute and Chronic Toxicity (EPA 1993b)

(4) Methods for Aquatic Toxicity Identification Evaluations (TIE) – Phase III Toxicity Confirmation Procedures for Samples Exhibiting Acute and Chronic Toxicity (EPA 1993c)

TEST MATERIALS

The test material consisted of a 24-hour composite effluent sample collected from the City of Brawley WWTP. Effluent sample collection and transport was coordinated under the supervision of Mr. Joe Delgado of the City of Brawley. Effluents were transported in 4-liter (L) and 10-L high-density polyethylene (HDPE) cubitainers. All samples were held in insulated ice chests containing blue ice during transport to the AMEC laboratory.

Immediately upon arrival at AMEC, an aliquot of each sample was drawn and the following water quality characteristics were measured and recorded in the laboratory sample check-in log:

- temperature
- pH
- dissolved oxygen (DO)
- conductivity
- alkalinity
- hardness
- free and total chlorine
- total ammonia

Results of check-in analyses are detailed in Table 1.

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Table 1. Chemical and Physical Parameters upon Sample Check-In

Temp. (ºC)	pH (units)	DO (mg/L)	Conduct. (µmhos/ cm)	Alkalinity (mg/L $CaCO_3$)	Hardness (mg/L $CaCO_3$)	Total Chlorine (mg/L)	Total Ammonia (mg/L)
15.0	7.98	4.2	1957	319	355	0.06	34.2

Temperature and conductivity were measured with an electrode using an Orion Model 135 meter. DO was measured using a YSI Model 57 meter. An Orion Model 250A meter was used to measure pH. Alkalinity and hardness were checked using a Hach titrimetric test kit. Free chlorine was tested using a Hach DPD colorimetric test kit (Hach Method 8021). Total ammonia was measured using the Hach colorimetric "Test 'N Tube" Salicylate Method 10031. The Hach method cited for ammonia analysis is an EPA-accepted procedure equivalent to EPA Method 350.2. The Hach DR2000 spectrophotometer was used for this procedure. Concentrations of unionized ammonia were calculated using methods described in Hampson (1977).

Appropriate chain-of-custody procedures were followed during all phases of this study. Samples were held at 4°C prior to testing. Copies of the chain-of-custody forms for this study are attached in Appendix G.

LABORATORY DILUTION WATER

Prior to test initiation, a batch of dilution water was prepared (eight parts Nanopure to two parts Perrier) and aerated overnight. The pH, hardness, and alkalinity were measured and recorded daily to ensure that they were within the ranges designated in the EPA manual (1993a) for conducting freshwater acute bioassays.

BIOASSAY TEST METHODS

Test Animals

Ceriodaphnia dubia (freshwater cladoceran)

Four to 5 days prior to test initiation, adult female daphnids were isolated from internal batch cultures and placed in individual holding cups. The number of daphnids isolated was equal to the number of neonates required to initiate testing. Each cup contained 15 milliliters (ml) of dilution water. A diet consisting of a vitamin-enriched mixture of yeast, Cerophyll®, and trout chow (YCT) and *Selenastrum* was added to each cup daily. Cups were held in a polypropylene holder and the entire board was placed in a temperature-controlled room maintained at 25°C. Isolated females were transferred to

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cups containing fresh laboratory water every 48 hours and again at 24 hours prior to test initiation. *C. dubia* (<24 hours old) were collected from gravid females producing more than 8 neonates and transferred to a 500-ml glass petri bowl. Neonates were collected using a 1-ml wide-bore polypropylene pipette with the end removed in order to obtain a wide bore. A mixture of vitamin-enriched YCT and *Selenastrum* was added to the holding bowl 2 hours prior to test initiation.

Pimephales promelas (fathead minnow)

Fathead minnow embryos were purchased from Aquatic Biosystems of Fort Collins, Colorado. Embryos were transported to the laboratory in plastic bags containing oxygen-saturated water held in a Styrofoam cooler. Animals were shipped by overnight delivery service. In the laboratory, *P. promelas* were acclimated to dilution water over a minimum 4-hour period. Embryos were fed freshly hatched brine shrimp during the holding period. All TIE bioassays were initiated with 24- to 48-hour-old (post-hatch) embryos.

EXPERIMENTAL DESIGN

The TIE treatments and experimental design used in this study are summarized in Table 2.

ACUTE 48-HOUR SCREENING ASSAYS

Prior to initiating TIE studies, acute screening tests were conducted using *P. promelas* and *C. dubia* to confirm toxicity.

Testing was conducted under static conditions in an environmental chamber maintained at 25 ± 1°C. The concentrations tested were 100, 50, and 25 percent effluent. A control consisting of untreated laboratory dilution water was initiated with the assay.

Test chambers for toxicity screening analyses consisted of 30-ml glass scintillation vials with a 20-ml sample volume. Tests were initiated by distributing test solution to each vial and acclimating the solutions to 25°C in a temperature-controlled room. Five organisms were then arbitrarily selected and distributed to each test chamber using a wide bore pipette. Test vials were placed in a 25°C environmental room and covered with clear Plexiglas covers. *C. dubia* were fed 200 microliter (µl) each of *Selenastrum* algae and YCT prior to test initiation. Fathead minnows were fed approximately 10 artemia nauplii per fish twice daily during the 48-hr exposure. Artemia were concentrated and added to each test chamber containing *P. promelas* in a single drop to minimize sample dilution due to feeding.

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Table 2. Toxicity Test Experimental Design

Scope of Testing:	Replicates	Dilution Series (Sample in %)
Toxicity Screens	4	Control, 25, 50, 100%
TIE Baseline Test	3	Control, 6.25, 12.5, 25, 50, 100%
Phase I Manipulations	3	Control, 25, 50, 100%
(STS addition, EDTA addition, PBO addition, particulate removal, C18 extraction, aeration, pH 6 and pH 9 adjustment)		
Phase II/III Manipulations	3	Control, 25, 50, 100%
(zeolite extraction, zeolite extraction + NH_4 spike, aeration, aeration at pH 11, aeration at pH 11 + EDTA, aeration at pH 11 + NH_4 spike, lab control + NH_4 spike)		
$CuCl_2$ Reference Toxicant Tests		
C. dubia	4	0, 3.13, 6.25, 12.5, 25, 50 µg/L Cu [a]
P. promelas	2	0, 18.8, 37.5, 75, 150, 300 µg/L Cu [a]

[a] Nominal concentration
Pp: *Pimephales promelas*
Cd: *Ceriodaphnia dubia*
mg/L: milligrams per liter
µg/L: micrograms per liter
EDTA: ethylenediaminetetraacetic acid
STS: sodium thiosulfate
PBO: piperonyl butoxide
STS: sodium thiosulfate
PBO: piperonyl butoxide

Observations of mortality were made daily during the assay. Dead animals were removed when observed to prevent degrading effects on water quality. Each test was monitored for DO, pH, conductivity, and temperature at the beginning and end of each exposure period. Total ammonia was measured at the beginning of each test. The concentration of unionized ammonia was derived based on total ammonia and pH values as described by Hampson (1977). Complete summaries of mortality and water quality characteristics for the screening studies are contained in reports included in Appendix B.

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BASELINE TOXICITY TESTS

Baseline testing was performed concurrent to all Phase I methods and served as a control to compare survival in untreated effluent to responses obtained after TIE manipulations. Methodology, other than a reduced number of replicates, was identical to that used for the screening test.

COPPER REFERENCE TOXICANT TESTS

Reference toxicant testing with copper (II) chloride was performed concurrent to the Phase I TIE tests. Nominal concentrations of 300, 150, 75, 50, 37.5, and 0 micrograms per liter (µg/L) copper (II) chloride (as copper) were prepared and tested for *P. promelas*. Nominal concentrations of 50, 25, 12.5, 6.25, 3.13, and 0 µg/L copper were prepared and tested for *C. dubia*. Reference toxicant testing is a quality assurance/quality control (QA/QC) procedure performed to confirm the health and toxicant susceptibility of test organisms as well as to demonstrate the use of proper and consistent test conditions and procedures. Results for reference toxicant tests performed during this study are located in Appendix F.

TIE PHASE I - TOXICITY CHARACTERIZATION

All Phase I adjustments and manipulations were carried out on both 100 percent effluent and laboratory dilution water. The term "method control" used throughout this report refers to clean laboratory dilution water that underwent manipulations identical to those performed on the effluents. The method control serves to assess whether sample manipulations result in adverse effects due to the procedures themselves. Procedures, with some modifications, follow methods described by EPA (1991). A single method control was tested for each manipulation.

Thirty-ml capacity glass scintillation vials were used as test containers for all TIE analyses. Each glass vessel contained 15-ml of test material for *C. dubia* and 20-ml test material for *P. promelas*. Five organisms were randomly assigned to each replicate test chamber. *Artemia* were concentrated and added to each test chamber containing *P. promelas* in a single drop to minimize sample dilution due to feeding. *C. dubia* were fed 200 µl each of *Selenastrum* algae and YCT prior to test initiation and at 48 hours.

Following TIE manipulations, 25 and 50 percent concentrations of effluent were prepared by diluting sample material with 8:2 (eight parts Nanopure to two parts Perrier) laboratory control water. A dilution series was created for each treatment so that median lethal concentration (LC_{50}) values could be calculated for comparison purposes.

A general schematic of Phase I TIE fractionation procedures employed for this study is outlined in Figure 1.

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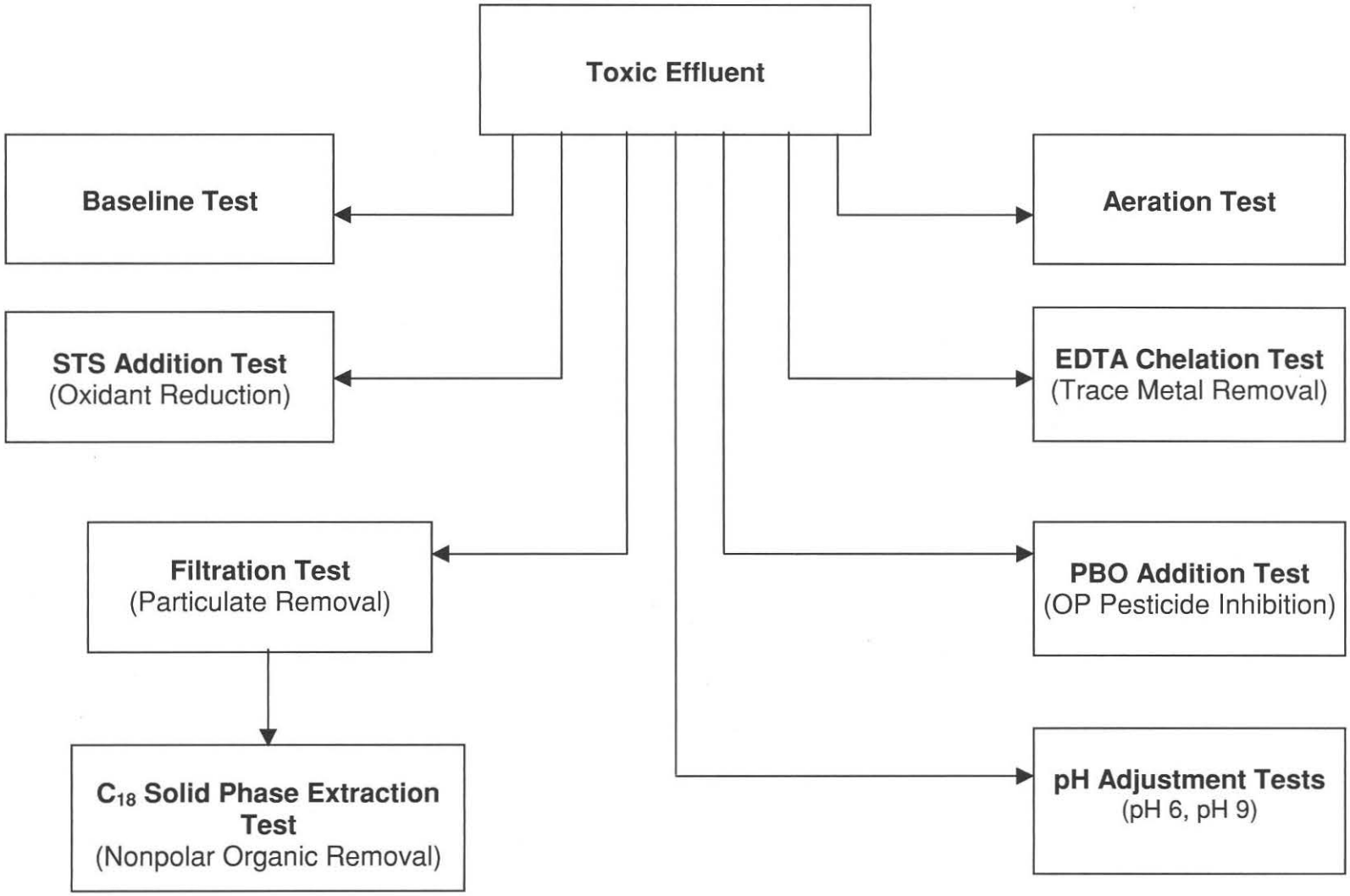

Figure 1. Overview of Phase I TIE fractionation procedures for City of Brawley WWTP effluent initiated 12 April 2002.

Oxidant Reduction

This test was performed to determine whether effluent constituents, reduced by the addition of sodium thiosulfate (STS), were associated with observed toxicity. This procedure provides valuable information about chlorine toxicity. The addition of STS may also neutralize the toxicity of other chemicals used as disinfection agents (such as ozone and chloride), as well as chemicals formed during chlorination (mono and dichloramines), bromine, iodine, manganous ions, and some electrophilic organic chemicals. The toxicity of some cationic metals may also be reduced by the addition of STS (EPA 1991).

STS was added to the method controls and all effluent dilutions at an exposure concentration of 50 milligrams per liter (mg/L) of STS.

EDTA Metal Chelation

The addition of ethylenediaminetetraacetic acid (EDTA) was used to determine the extent of toxicity attributable to cationic trace metals (EPA 1991). EDTA chelates cationic trace metals, thereby reducing their bioavailability.

EDTA was added to the method controls and all effluent dilutions at an exposure concentration of 60 mg/L of EDTA.

Organophosphorous Pesticide Inhibition (PBO Addition)

Piperonyl butoxide (PBO) has been found to be an effective metabolic inhibitor of organophosphorous pesticide metabolism (Ankley et al. 1991). Toxicity due to organophosphorous compounds is, consequently, blocked by the addition of PBO.

Due to its limited solubility in water, a 1,000-µg/L stock PBO solution was prepared in 100 percent methanol prior to addition to test solutions. Appropriate controls were tested to ensure that the methanol and PBO additions were below toxic levels. PBO was added to the method controls and each effluent dilution at a final exposure concentration of 50 µg/L of PBO.

Particulate Removal

This physical fractionation provided information on toxicants associated with suspended particulate material. Suspended particulates were removed by filtering effluent through a Gelman 0.45-micron (µm) glass-fiber filter. The same procedure was performed with control dilution water.

Subsamples of filtered control water and effluent were used for the C18 solid-phase extraction (SPE) column fractionations.

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C18 Solid-Phase Extraction

Solid-phase extraction with a C18 column was used to determine the extent of toxicity associated with nonpolar organic compounds. It has been found that C18 columns also have the ability to remove some metal chelates (EPA 1991). A 5-ml capacity Baker brand column was used for this procedure.

A 1,000-ml subsample of the filtered effluent and control dilution water was passed through C18 SPE columns as described in the test protocol.

Post-filtered SPE columns were labeled, wrapped in an airtight resealable bag, and placed in a freezer at -10°C for potential subsequent Phase II testing.

Aeration Treatment

The aeration test is designed to determine the degree of effluent toxicity attributable to volatile, sublatable, or oxidizable compounds (EPA 1991). Sublatable compounds include surface-active compounds such as resin acids, soaps, detergents, charged stabilization polymers, and coagulation polymers used in chemical manufacturing processes. The same procedure was conducted using clean laboratory seawater as a method control.

To perform this treatment, a 400-ml sample of 100 percent effluent was vigorously aerated with a glass air stone in a 1-L glass graduated cylinder for a period of 60 minutes.

Graduated pH Adjustment

The toxicity of many compounds found in effluents are substantially affected by pH. Changes in pH may affect the solubility, stability, volatility, polarity, and speciation of many compounds (EPA 1991). Ammonia is a common toxicant of concern that increases in toxicity as pH increases.

Three pH treatments were tested: (1) the initial sample pH (baseline test); (2) pH 6 adjusted sample; and (3) pH 9 adjusted sample. Both effluent and laboratory dilution water were pH adjusted with 0.1N solutions of HCl and NaOH as described in the test protocol.

Ammonia Analyses

Subsamples of effluent were collected upon receipt for the analysis of total and unionized ammonia. Samples were also collected for analysis of total and unionized ammonia at the beginning and end of the Phase I test series in all 100 percent treatments tested for both test species. In addition, ammonia was also analyzed at the

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end of the test in the 25 and 50 percent test concentrations for the baseline tests, aeration tests, pH adjustment tests, and zeolite extraction tests.

Surfactant Analyses

Due to their prevalence in runoff leading to wastewater treatment plants, surfactants were measured by analyzing methylene blue activated substances (MBAS). MBAS includes a common group of surfactants known as linear alkyl sulfonates (LAS). Surfactants were analyzed by Calscience following EPA Method 425.1. Analytical reports of MBAS analyses are included in Appendix E.

Diazinon and Chlorpyrifos Analyses

Organophosphorous pesticides diazinon and chlorpyrifos have been identified as primary chemicals responsible for toxicity to *Ceriodaphnia dubia* in a variety of habitats in southern California (Ogden 1999; Lee et al. 1999). Based on this information and the presence of widespread agricultural activities in the Imperial Valley, the Brawley WWTP effluent was also analyzed for these two pesticides. Analyses of diazinon and chlorpyrifos were conducted by Aqua-Science Environmental Toxicology Consultants located in Davis, California using an enzyme-linked immunosorbant assay (ELISA).

Trace Metal Analyses

A complete scan of trace metals, excluding mercury, was performed in the original Brawley WWTP effluent collected on 8 April 2002. Analysis was conducted by Calscience Analytical Laboratories, Inc. using inductively coupled plasma spectroscopy (ICP) in accordance with EPA Method 6010B. The analytical report of trace metal analyses is included in Appendix E.

TIE Phase II - Toxicity Identification and Phase III – Toxicant Confirmation

The following Phase II and Phase III TIE procedures were performed to confirm toxicity due to ammonia and to further investigate the potential for other contributing toxicants that may be masked by ammonia. In each case, as with Phase I procedures, treatments were compared to a concurrent baseline test to evaluate their effectiveness. A method control was also performed concurrent to all treatments to ensure these procedures had no negative impact on the test organisms. A general schematic of Phase I TIE fractionation procedures employed for this study is outlined in Figure 2.

pH 11/Aeration

In an effort to reduce the level of total ammonia in the effluents, the samples were adjusted to pH11 with NaOH and vigorously aerated in 1-L glass graduated cylinders for 1 hour. The pH was monitored closely during the aeration procedure and readjusted as

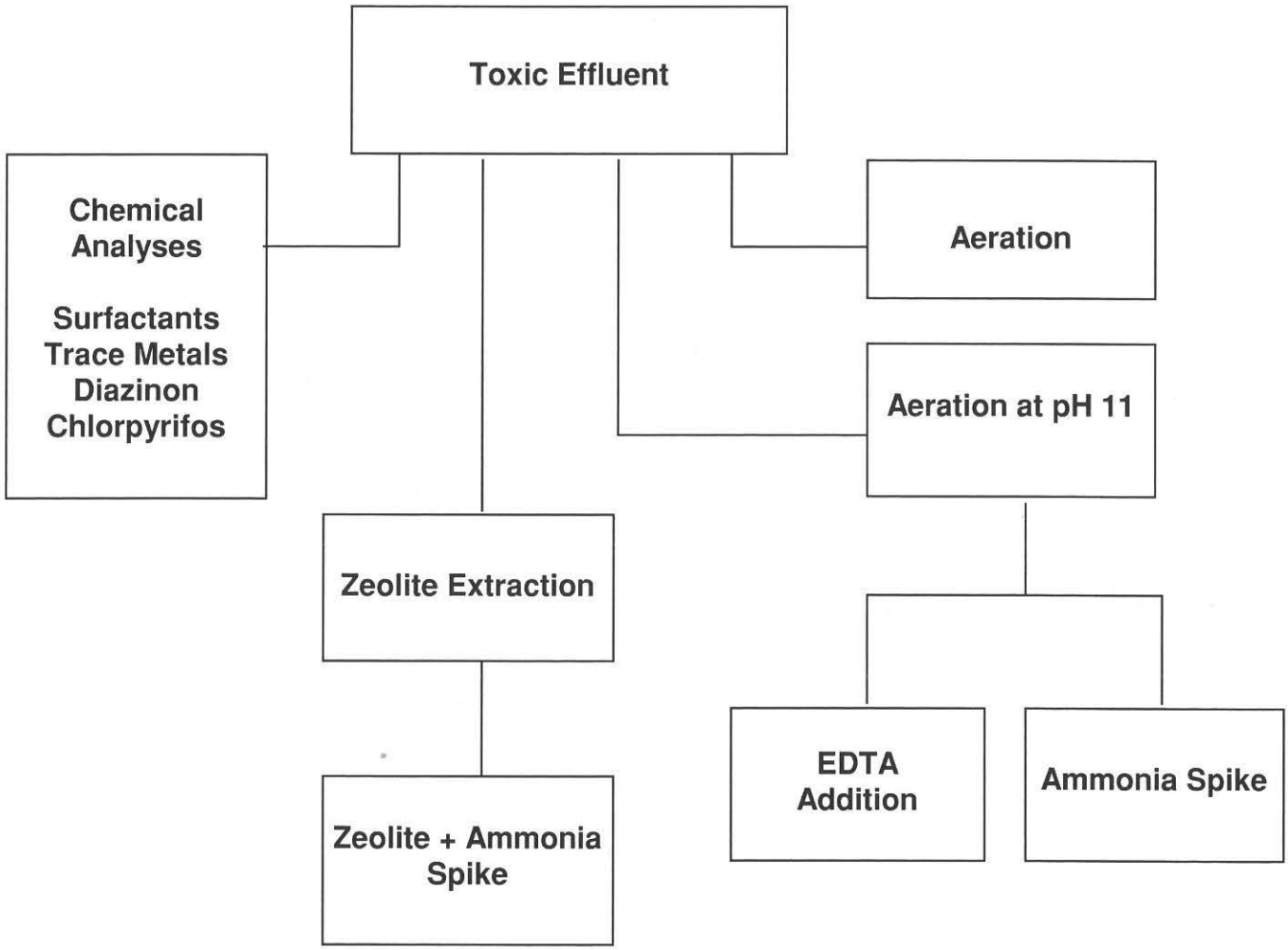

Figure 2. Overview of Phase II/III TIE fractionation procedures for Brawley WWTP effluent initiated 19 April 2002.

necessary. The sample pH was readjusted to the baseline value with HCl prior to testing. Increased pH with aeration is a method described in EPA (1993b) that has successfully reduced ammonia levels in previous studies at AMEC.

pH 11/Aeration + EDTA Addition

In an effort to investigate potential toxicity due to trace metals, EDTA was added to a subsample of effluent treated with the pH 11/aeration manipulation. The final concentration of EDTA in all dilutions was 60 mg/L.

pH 11/Aeration + Ammonia Spike

This treatment was performed to further evaluate and confirm the fraction of toxicity attributable to ammonia. Ammonia reduced by the pH 11/aeration treatment was added back to its original concentration in the baseline effluent.

Aeration – Original sample (no pH adjustment)

This treatment was performed to serve as a comparison for all other Phase II aeration treatments. Methods were identical to those performed in Phase I.

Zeolite Extraction

This procedure was also employed to reduce the level of ammonia in the effluent. The samples were gravity filtered through a Pyrex glass column filled with aquarium-grade zeolite. Samples (200 ml) were filtered through the column at a rate of approximately 10 ml per minute. The process was repeated four times prior to testing. In addition to ammonia removal, zeolite also has the ability to remove particulates and some cationic trace metals (EPA 1993b).

Zeolite Extraction + Ammonia Spike

As an additional confirmation step, an attempt to recover toxicity following the zeolite treatment was performed by adding ammonia back to the post-zeolite effluent to the level measured in the original baseline sample.

Phase II Ammonia Analyses

Samples of all initial control and 100 percent dilutions tested during Phase II studies were collected and measured for total ammonia. The initial concentration of ammonia in the 25 and 50 percent dilutions was calculated based on levels measured in the 100 percent test concentration. Total ammonia was also measured at the end of the exposure period in all Phase II dilutions tested. The concentration of unionized ammonia is highly dependent on pH, and to a lesser extent, temperature. Mean values

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of unionized ammonia were subsequently calculated from initial and final measurements in each dilution.

STATISTICAL ANALYSES

Toxicity Comparisons

Survival of both species was evaluated at 48 hours in screening studies. During the Phase I TIE survival was evaluated at 96 and 72 hours for *P. promelas* and *C. dubia*, respectively. Survival of *C. dubia* was evaluated at 72 hours due to reduced control performance at 96 hours in several treatments. Decreased survival of *C. dubia* in the controls at 96 hours appeared to be related to a lack of sufficient food. Due to strong dose response curves at 24 hours and variable control survival thereafter in several treatments, Phase II baseline TIE tests with *P. promelas* were evaluated at 24 hrs.

Mean survival data for each *P. promelas* and *C. dubia* replicate was transformed into a percentage. Percentage data were arcsine square-root transformed prior to analysis. Normality of the data was checked with the Shapiro-Wilks Test. Median lethal concentration (LC50) values were calculated for all baseline tests, TIE manipulations, and reference toxicant tests that exhibited a dose-response curve. These endpoints were calculated with Probit, Trimmed Spearman-Karber, or Linear Interpolation methods using ToxCalc Comprehensive Toxicity Data Analysis and Database Software, Version 5.0. Dunnett's Test was used to identify significant differences between concentrations in each baseline and reference toxicant test. The choice of statistical method was dependent upon specific model assumptions met or not met by the data as addressed in EPA (1993a). Statistical data summaries are contained in Appendix C

Correlation Studies

Linear regression analyses were performed to evaluate relationships between survival and mean unionized ammonia levels measured in all Phase I and Phase II/III treatments. Regression analyses were performed using GraphPad Statistical Software Version 3.0. Non-detect data were included for analysis using half of the value of the reporting limit.

Ammonia LC50 Calculations

LC50 values for the mean level of unionized ammonia in each treatment were calculated for all TIE manipulations that exhibited a dose-response curve. These endpoints were calculated with Probit, Trimmed Spearman-Karber, or Linear Interpolation methods using ToxCalc Comprehensive Toxicity Data Analysis and Database Software, Version 5.0. Complete statistical summaries for ammonia results are located in Appendix D.

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RESULTS AND DISCUSSION

TOXICITY SCREENS

Results of the toxicity screens are detailed in Table 3. A toxicity screen was performed prior to initiation of TIE procedures to ensure an adequate dose response. Successful TIE methodology typically requires a 30 to 40 percent reduction from control survival in the baseline "unmanipulated" material. For comparison purposes, mean survival is shown in the following sections. A complete summary of survival results in all test concentrations is located in Appendix B

Mean survival of *C. dubia* and *P. promelas* was 0 percent in the 100 percent test concentration. The LC50 values for these two species were 71 and 45 percent effluent, respectively. The level of unionized ammonia (1.72 mg/L) at the beginning of the exposure period exceeded levels expected to cause toxicity to both test species based on internal experiments performed at AMEC and published literature values.

Phase I TIEs were subsequently initiated on this sample using both *C. dubia* and *P. promelas* due to the observed toxicity for both of these two species.

Table 3. Acute Pre-TIE Screening Study Using *C. dubia* and *P. promelas*

Sample Collected 8 April 2002

	C. dubia		*P. promelas*		Initial Ammonia (mg/L)	
Sample ID	**% Surv**	**LC50**	**% Surv**	**LC50**	**Total**	**Unionized**
Control	100	---	100	---	< 0.1	< 0.01
Brawley 100%	0	71	0	45	34.2	1.72

Mean percent survival at 96 hrs in the 100% sample material is displayed
--- Not applicable

PHASE I TIE TOXICITY RESULTS

Mean survival in 100 percent material is summarized in Figures 3a and 3b. LC50 values for Phase I tests are summarized in Figures 4a and 4b. The mean concentration of unionized ammonia in each 100 percent treatment is also displayed in each figure. A complete summary of mean survival, LC50 values, and ammonia concentrations is located in Appendix A. Water quality, survival, and statistical summaries for Phase I testing are included in Appendix C.

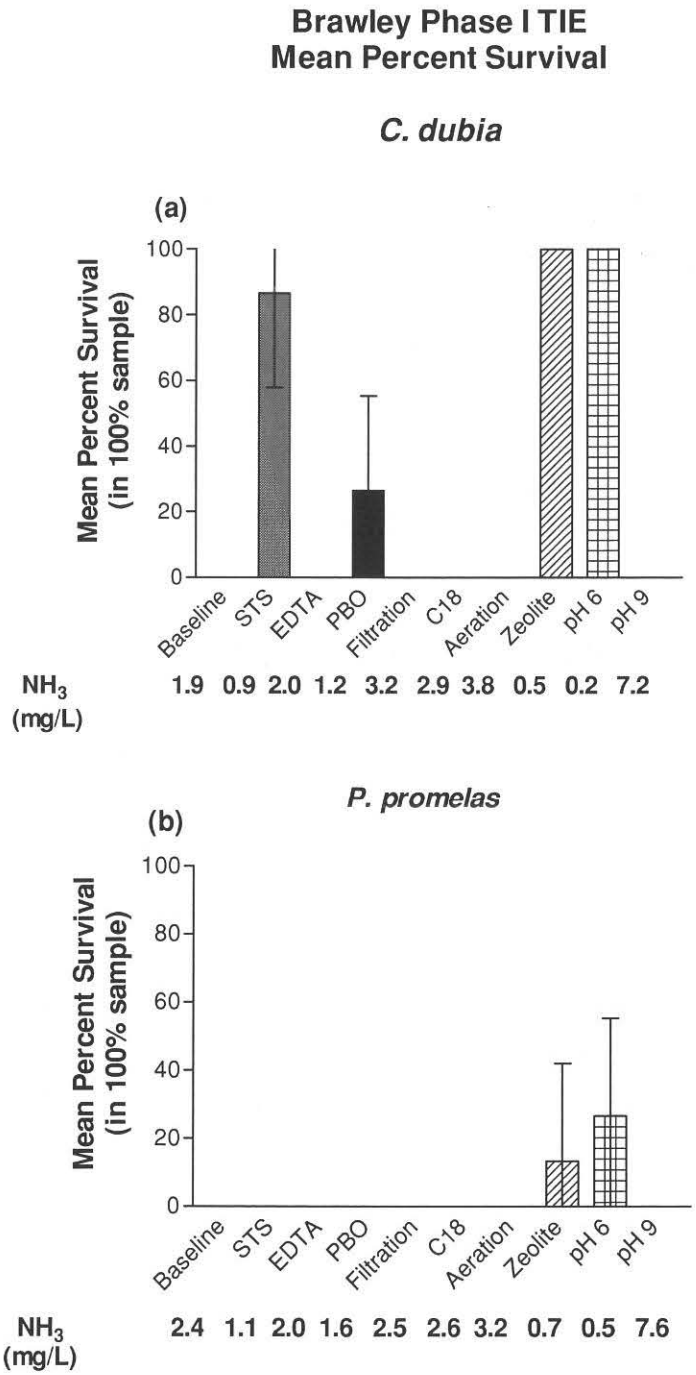

Figure 3. Summary of Phase I TIE results initiated on 12 April 2002. Mean percent survival in 100% sample material for *C. dubia* (a), and *P. promelas* (b). Concentrations of unionized ammonia (mg/L) are depicted at the base of each treatment.

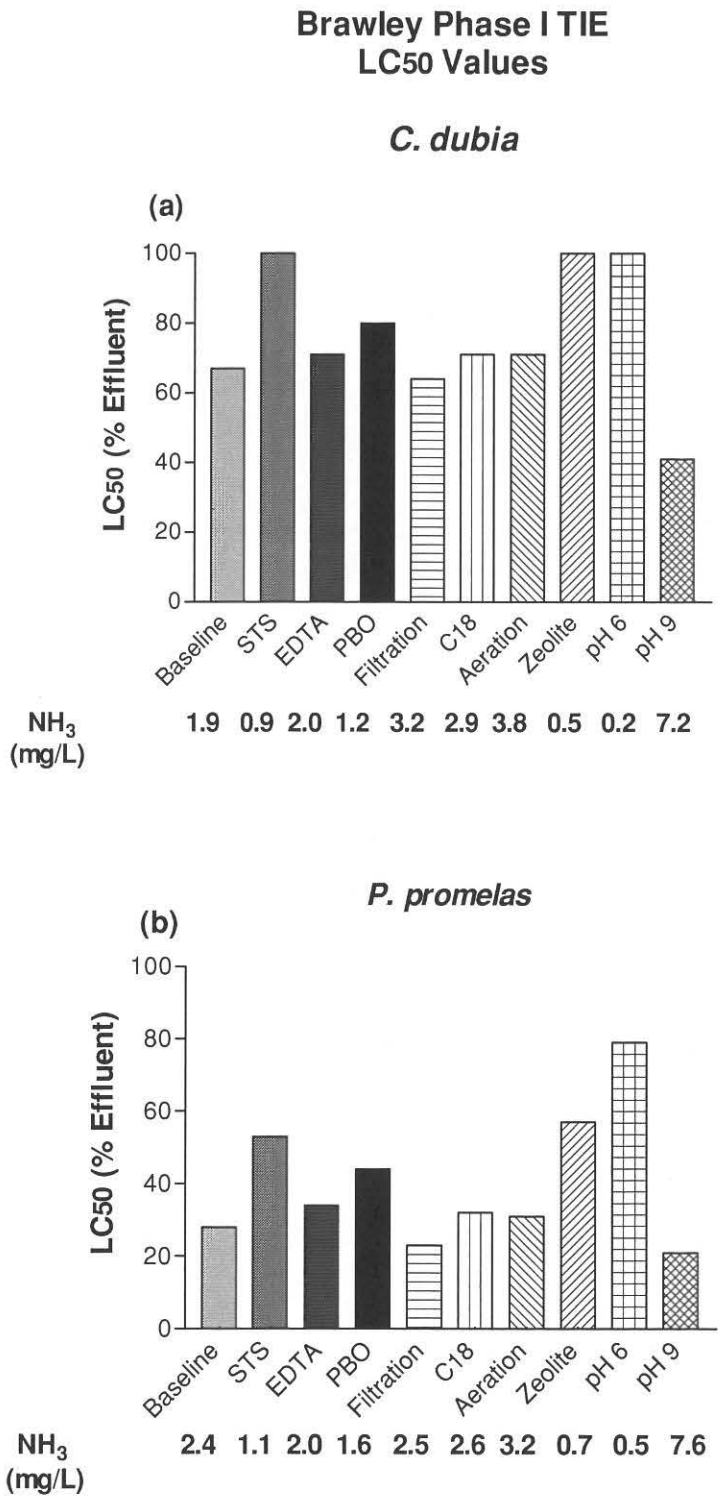

Figure 4. Summary of Phase I TIE results initiated 12 April 2002. LC50 values for *C. dubia* (a), and *P. promelas* (b). Concentrations of unionized ammonia (mg/L) are depicted at the base of each treatment.

Toxicity of the baseline effluent was similar in magnitude to that obtained during the screening test initiated eight days earlier. Mean survival in 100 percent sample was 0 percent for both species at the end of a 96-hour exposure. The LC_{50} values for the baseline treatment were 68 and 28 percent effluent for *C. dubia* and *P. promelas*, respectively.

Several treatments reduced the toxicity of the original effluent for both *C. dubia* and *P. promelas*. Results were strikingly similar for both species. A distinct observation was the change in toxicity following both pH adjustment and extraction of the sample through zeolite. These are the only treatments that resulted in survival of fathead minnows (a mean of 13 and 27 percent, respectively) in the 100 percent test concentrations. Survival of *C. dubia* increased to 100 percent in these two treatments.

Toxicity to both species was substantially enhanced in one treatment, the pH 9 adjusted sample. Survival of *P. promelas* was zero percent in the 50 and 100 percent test concentrations at 24 hours for this treatment. Survival of *C. dubia* was zero percent in the 100 percent test concentrations at 24 hours for this treatment. The rate of mortality in the pH 9 adjusted sample for both species was also more rapid than all other manipulations.

The relationship between pH and toxicity was evaluated further by performing a regression analysis between LC_{50} values for each Phase I treatment and the mean pH in the 100 percent sample. This relationship was statistically significant for both species with p values of 0.012 for *C. dubia* and 0.002 for *P. promelas* (Figure 5). Such pH-dependent results are highly indicative of toxicity due to ammonia (EPA 1993a). As expected, the concentration of unionized ammonia at the end of the exposure period was lower in the pH 6 treatment and higher in the pH 9 treatment relative to that in the baseline effluent.

Toxicity was also reduced, to a lesser extent for both species, following addition of STS and PBO.

STS reduces toxicity due to oxidants such as chlorine or other disinfection agents. STS can also reduce toxicity due to some cationic metals. The addition of STS during this test also reduced the level of unionized ammonia by slightly lowering the pH.

PBO inhibits metabolism and toxicity of organophosphate pesticides. The reduction in toxicity following this treatment, however, was very slight and also appears to be related to pH. The C18 extraction treatment failed to reduce toxicity to the extent that PBO did. If PBO did remove a fraction of toxicity attributable to organophosphate compounds, the C18 column should have also reduced toxicity by at least the same amount.

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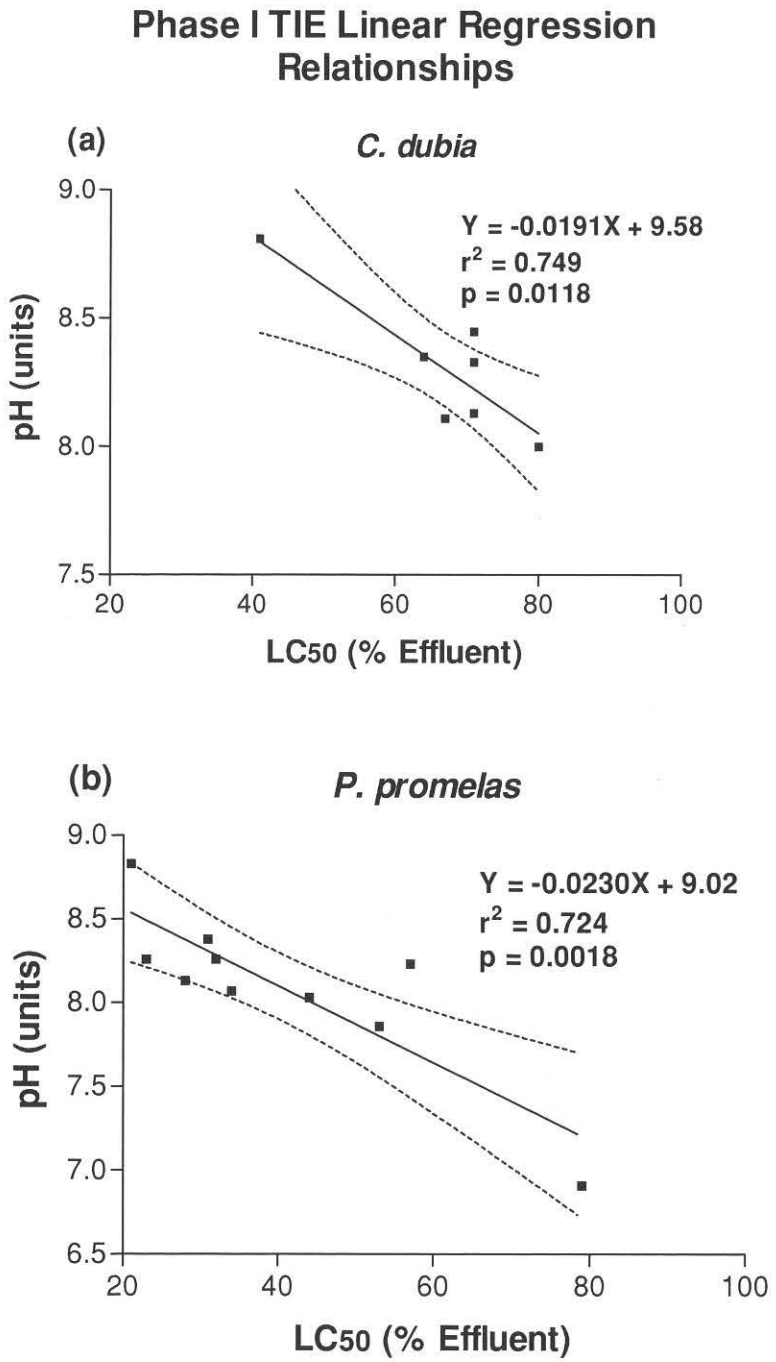

Figure 5. Linear regression relationships (± 95% CI) between effluent LC50 values and pH in 100% test concentrations for *C. dubia* (n=7) (a), and *P. promelas* (n=10) (b). Phase I TIE testing.

SUMMARY OF PHASE I TOXICITY RESULTS

The reduction of toxicity following removal of ammonia and pH-dependent results during Phase I testing implicates this constituent as a primary potential toxicant of concern for both *C. dubia* and *P. promelas*. Although a number of ionic compounds can theoretically be more toxic at high pH values than low pH values, ammonia is an extremely common toxicant that exhibits this property in aquatic samples (EPA 1993b; Ankley et al. 1990a). An additional observation supporting ammonia toxicity is the greater magnitude of the toxic response observed for *P. promelas* than *C. dubia* exposed to Brawley WWTP effluent. It is well documented that fathead minnows are more sensitive to ammonia than *Ceriodaphnia*.

The toxicity of aqueous ammonia in solutions to aquatic organisms is primarily due to the unionized fraction (Thurston et al. 1983). The equilibrium distribution of ammonia is determined primarily by pH and, to a lesser extent, temperature and salinity. Toxicity of ammonia will, therefore, depend on these factors as well as total ammonia concentration. The toxicity of a given total ammonia concentration will increase with increasing pH and temperature due to a shift in equilibrium toward the toxic unionized species.

AMMONIA ANALYSES

The concentration of total and unionized ammonia in City of Brawley effluent at the beginning and end of the Phase I TIE tests is summarized in Appendix A. Acute unionized ammonia LC_{50} values derived in a number of studies for *C. dubia* and *P. promelas* are summarized in Table 4.

The concentration of total ammonia upon receipt was 34.2 mg/L. Corresponding unionized ammonia based on the initial pH upon receipt (7.98) was 1.72 mg/L. The mean concentration of total and unionized ammonia at the end of the 96-hour exposure for fathead minnows in the baseline test was 29.9 and 3.74 mg/L, respectively. The concentration of total and unionized ammonia at the end of the 72-hour exposure for *C. dubia* in the baseline test was 22.7 and 2.62 mg/L, respectively. The observed increases in unionized ammonia during the test were due to slight increases in pH during the exposure periods.

The concentration of total ammonia in the baseline samples was well above the mean LC_{50} values derived at AMEC for both *P. promelas and C.* dubia (Table 4). These levels of ammonia would, therefore, be expected to cause toxicity to both test species.

Table 4. Acute Unionized Ammonia 96 hr LC50 Values for *P. promelas* (Pp) and *C. dubia* (Cd) - AMEC Internal Reference Toxicant Data and Published Literature Values

			Acute LC50 (mg/L) [a]	
Source	**Species**	**N**	**Mean**	**Range**
AMEC Internal Ref Tox	**Pp**	**4**	**0.62**	**0.64 - 1.27**
Mayes et al., 1986	Pp	15	1.6	1.1 - 2.8
Nimmo et al., 1989	Pp	3	0.87	0.56 - 1.12
Thurston et al., 1983	Pp	28	1.64	0.75 - 3.44
Thurston and Russo, 1981	Pp	2	1.02	0.65 – 1.38 [b]
Reinbold and Pescitelli, 1982	Pp	4	1.11	0.73 - 1.65
Swigert and Spacie, 1983	Pp	2	1.81	1.75 - 1.87
West, 1985	Pp	4	2.19	1.83 - 2.55
AMEC Internal Ref Tox	**Cd**	**2**	**1.08**	**0.66 - 1.50**
Andersen and Buckley, 1998	Cd	5	1.18	1.03 - 1.31

[a] Acute values for *P. promelas* are derived from 48 to 96-hr exposures. Acute values for *C. dubia* are derived from 48-hr exposures.

[b] 0.65 mg/L at a pH of 7.5, 1.38 mg/L at a pH of 8.5.

PHASE I AMMONIA CORRELATION ANALYSES

The relationships between the concentration of unionized ammonia and effluent LC50 values for both *C. dubia* and *P. promelas* were evaluated by performing linear regression analyses. Results for these comparisons are displayed in Figure 6. Data obtained from all baseline and Phase I TIE treatments were included for analysis. Statistically significant relationships were obtained for both species. This information provides strong supporting evidence of a cause and effect relationship for this constituent for both test species. As would be expected for ammonia, toxicity to the fathead minnow was also greater than that of *C. dubia* across all TIE treatments.

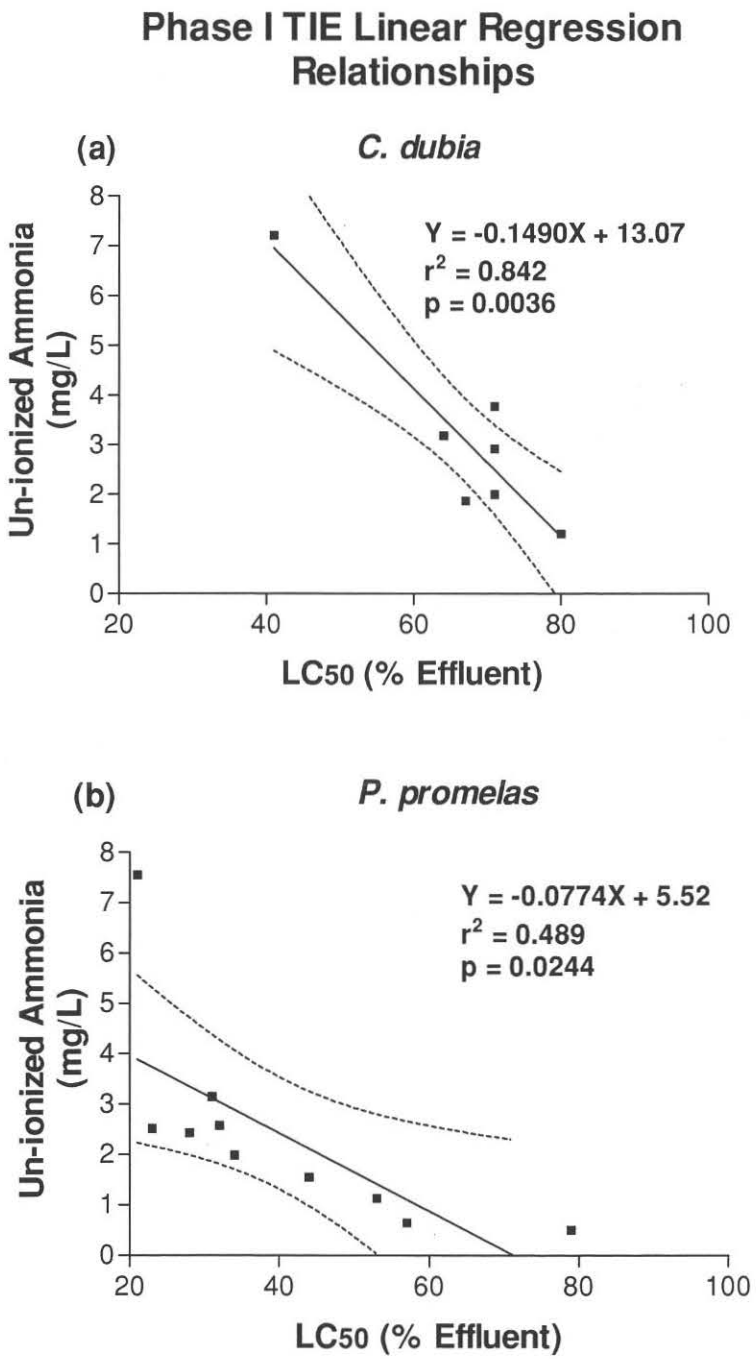

Figure 6. Linear regression relationships (± 95% CI) between effluent LC50 values and un-ionized ammonia concentrations in 100% test concentrations for *C. dubia* (n=7) (a), and *P. promelas* (n=10) (b). Phase I TIE testing.

ADDITIONAL CHEMICAL ANALYSES

Chlorine

The concentration of total chlorine upon receipt was 0.06 mg/L. This level is well below concentrations expected to cause acute toxicity to *P. promelas* (AMEC 2001b; Taylor 1993). Internal experiments performed at AMEC have derived acute 96-hour LC_{50} values of 0.05 mg/L total chlorine for *C. dubia* and 0.27 mg/L for *P. promelas*. These results minimize any concern for toxicity to *P. promelas* due to chlorine in this sample.

There is some concern with this level of chlorine for *C. dubia,* however, as internal observations and measurements indicate that chlorine is much more toxic in clean lab water than wastewater effluent. This appears to be due to the numerous binding and chemical reaction processes chlorine may go through in a complex effluent.

Surfactants (MBAS)

Concentrations of surfactants, measured as MBAS, was 0.73 mg/L in the sample collected for this study. A level of 3.0 mg/L was measured previously in a sample collected on 24 May 2001. These concentrations are above the lowest LC_{50} values derived for several specific classes of surfactants for both *P. promelas* and *C. dubia* and (Tables 6 and 7). The range of LC_{50} values, however, is great (up to 43 mg/L for *C. dubia* and 28 mg/L for *P. promelas*) depending on the specific type of surfactant tested. Surfactants with low water solubility and low ethylene oxide molar ratios have been found to be the most toxic of those tested in a study by Hall et al. (1989).

Trace Metals

Based on literature values and internal data derived at AMEC, the concentrations of all trace metals with the exception of copper were well below levels expected to cause toxicity to both *C. dubia* and *P. promelas*. Complete trace metal results are contained in Appendix E. The concentration of total copper (12.5 μg/L) was within the range of acute LC_{50} values derived for *C. dubia* but well below levels of concern for *P. promelas* (Tables 6 and 7). TIE confirmation studies failed to identify toxicity due to trace metals and, therefore, it is believed this level of copper was not a contributor to observed toxicity. It is likely that a large portion of copper in the Brawley WWTP effluent is bound and in a biologically unavailable fraction (Lee and Jones-Lee 1997).

Diazinon and Chlorpyrifos

Based on their prevalence and toxicological concern in southern California watersheds, the organophosphorous pesticides diazinon and chlorpyrifos were analyzed in both effluent samples tested (AMEC 1999; Lee et al. 1999). To achieve the low detection

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limits required for these analyses to be evaluated for toxicological concern, concentrations of these two compounds were derived using an antibody-specific method referred to as an enzyme-linked immuno-sorbent assay, or ELISA. Results are displayed in Table 5. Acute LC_{50} values for *P. promelas* and *C. dubia* are displayed in Tables 6 and 7.

Based on literature values the concentration of diazinon (0.055 µg/L) and chlorpyrifos (0.019 µg/L) were well below levels expected to cause toxicity to both *P. promelas* and *C. dubia*. (Bailey et al. 1997). Concentrations of diazinon (0.145 µg/L) and chlorpyrifos 0.039) previously measured on 24 May 2001 were also below levels expected to cause toxicity to either species.

Table 5. Summary of Surfactant, Copper, Diazinon, and Chlorpyrifos Concentrations in Baseline Effluent

Sample ID	Date Collected	MBAS [a] (mg/L)	Copper (µg/L)	Diazinon [b] (µg/L)	Chlorpyrifos [b] (µg/L)
Brawley	5/24/01	**0.73**	**12.5**	0.055	0.019

Bold MBAS value indicates a concentration exceeding lowest-derived LC_{50} values for *C. dubia* and *P. promelas* (Tables 6 and 7)
Bold copper value indicates a concentration exceeding a lowest-derived LC_{50} value for *C. dubia* (Table 7)
The sample was analyzed on 5/25 and 6/13 for MBAS and OP pesticides, respectively.
[a] EPA Method 425.1
[b] ELISA Analysis
mg/L = milligrams per liter
µg/L = micrograms per liter

A Note on the Use of Toxicity Values for Comparison Purposes

LC_{50} values are used as a general guideline to evaluate concentrations likely to exceed levels expected to cause toxicity, although toxicity can be expected at concentrations below derived LC_{50} values. Comparisons between laboratory-derived values and concentrations in "real-world" samples, however, are difficult to interpret because of numerous chemical, biological, and physical factors and interactions that may alter the toxicity of any given constituent in field collected material. Relative to clean laboratory dilution water, effluents contain a large fraction of dissolved and particulate organic matter which has the capability of greatly reducing the bioavailability and toxicity of trace metals and other potential toxicants (Lee and Jones-Lee 1997). These interactions should always be considered when comparing toxicity values for chemicals to environmental concentrations.

Table 6. Selected Toxicity Data (LC50 and EC50 values) Published for Metals, OP Pesticides, and Surfactants of Potential Concern for *P. promelas*

Constituent	Test Duration (hr)	Mean LC/EC50 Values	Reference
Trace Metals		**LC50 (µg/L)**	
Copper	96	52	AMEC Internal Database (n=20)
	96	15 to > 200 [a]	Schubauer-Berigan et al. (1993a)
Zinc	96	330 to > 780 [a]	Schubauer-Berigan et al. (1993a)
OP Pesticides		**LC50 (µg/L)**	
Diazinon	96	6900 - 9350	Siepmann and Finlayson (2000)
Chlorpyrifos	96	140 - 249	Siepmann and Finlayson (2000)
Surfactants		**EC50 (mg/L)**	
Anionic LAS	28 days[b]	0.10 - 28.0	Lewis (1991)
Nonionic LAS	28 days[b]	0.13 - 0.32	Lewis (1991)
Cationic	28 days[c]	0.05 - 0.45	Lewis (1991)

[a] pH-dependent
[b] Survival/ hatching/ and growth endpoints
[c] Hatching and growth endpoints
µg/L - micrograms per liter
mg/L - milligrams per liter

Table 7. Selected Toxicity Data (LC50 values) Published for Metals, OP Pesticides, and Surfactants of Potential Concern for *C. dubia*

Constituent	Test Duration (hr)	Mean LC50 Values	Reference
Trace Metals		(µg/L)	
Chromium	96	45	EPA Aquire Database, 2001
Copper	48	17	AMEC Internal Database (n=20)
	48	11	Bitton et al. (1996)
	48	9.5 to 200 [a]	Schubauer-Berigan et al. (1993a)
Zinc	48	60	Bitton et al. (1996)
	48	95 to > 530 [a]	Schubauer-Berigan et al. (1993a)
OP Pesticides		(µg/L)	
Diazinon	96	0.41 - 0.47	Bailey et al. (1997)
Chlorpyrifos	96	0.06	Bailey et al. (1997)
Surfactants		(mg/L)	
Dowfax 2EP	48	2.3	Cowgill and Milazzo (1991)
LAS	48	4.21 - 4.83	Ankley et al. (1990b)
LAE	48	1.23 - 2.14	Ankley et al. (1990b)
NP	48	0.34 - 0.71	Ankley et al. (1990b)
Nonionic	48	0.90 - 14.7	Ankley et al. (1990b)
Cationic	48	0.25 - 0.81	Ankley et al. (1990b)
Brand Detergents	48	12.3 - 42.9	Ankley et al. (1990b)

[a] pH-dependent
LAS - Linear alkyl sulfonates
LAE - Linear alkyl ethoxylates
NP - Nonylphenols
µg/L - micrograms per liter
mg/L - milligrams per liter

PHASE II TIE TOXICITY RESULTS

Subsequent Phase II and Phase III TIE procedures were initiated using the fathead minnow *P. promelas* to: (1) further identify and confirm toxicity due to ammonia toxicity and; (2) identify any secondary potential contaminants of concern. Phase II and III survival and LC50 values are displayed in Figure 7. The mean concentration of unionized ammonia is displayed for each treatment for comparison purposes. A complete summary of survival, LC50 values, and ammonia concentrations is located in Appendix A. Results for each treatment are summarized below.

Baseline Test

Toxicity of the sample remained substantial but reduced from that in the original baseline test performed seven days earlier. The LC50 increased (indicating lessening toxicity) from 28 to 54 percent effluent during this time frame. Correspondingly, both total and unionized ammonia levels in the effluent also dropped during this seven-day storage period. Between the two baseline initiation dates, total ammonia decreased from 29.7 to 23.8 mg/L and unionized ammonia dropped from 2.4 to 1.8 mg/L.

Zeolite Extraction

Similar to Phase I results, zeolite removed almost all observed toxicity. Mean survival was 93 percent in the 100 percent test concentration. The zeolite treatment successfully reduced the level of unionized ammonia from 1.8 to 0.3 mg/L.

Zeolite Extraction + Ammonia Spike

Toxicity was recovered after adding ammonia back to the zeolite extract. The degree of toxicity and level of ammonia was very similar to that in the baseline test. This treatment provides further supporting evidence that ammonia is responsible for most, if not all, of the observed toxicity observed in the Brawley WWTP effluent.

Aeration

Similar to Phase I results, aeration slightly decreased toxicity and ammonia levels relative to the baseline test.

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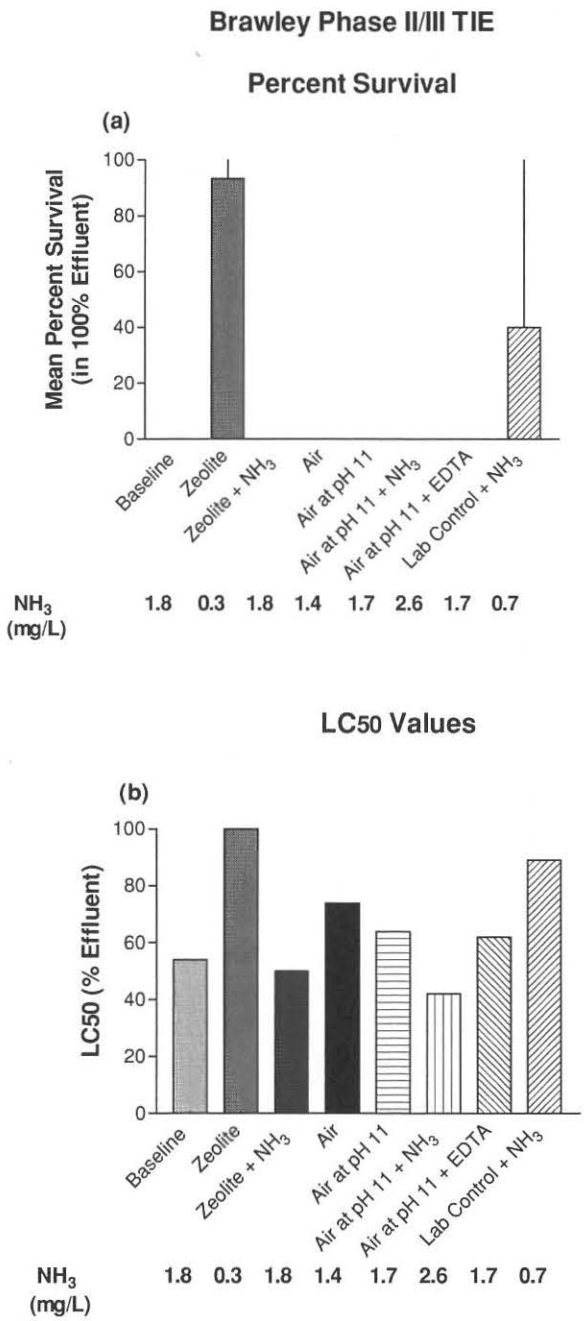

Figure 7. Summary of Phase II/III TIE results initiated on 19 April 2002. Mean percent survival in 100% sample material (a), and LC50 values (b) for *P. promelas*. Concentrations of unionized ammonia (mg/L) are depicted at the base of each treatment.

pH 11/Aeration

As expected aeration of the sample at pH 11 reduced total ammonia levels. Relative to the baseline test, total ammonia decreased from 23.8 to 17.4 mg/L Toxicity was also reduced relative to the baseline test but similar to that in the aeration treatment with unadjusted pH. The mean concentration of unionized ammonia (1.66 mg/L) was only slightly reduced compared to the baseline test (1.76 mg/L) due to an upward pH drift in the manipulated samples.

pH 11/Aeration + Ammonia Spike

Toxicity was recovered to a level that was slightly greater than that observed in the baseline test after adding ammonia back to the pH 11/aeration treatment. The addition of ammonia to this treatment also resulted in a slightly greater concentration (31.7 mg/L total ammonia) than that in the baseline test. This treatment provides further supporting evidence that ammonia is responsible for most, if not all, of the observed toxicity in the Brawley WWTP effluent.

pH 11/Aeration + EDTA Addition

Addition of EDTA to the pH/aeration treated samples failed to reduce toxicity of the sample. This result provides additional evidence that trace metals are not contributing to observed toxicity in this sample.

Lab Control + Ammonia Spike

Addition of an ammonia spike to clean laboratory water recovered toxicity to the level expected based on its concentration and survival results in the unadjusted Brawley effluent. Toxicity was less than that in the baseline sample as was the level of unionized ammonia due to a lower mean pH during this test.

PHASE II/III pH AND AMMONIA CORRELATION ANALYSES

The relationship between Phase II LC_{50} values and both pH and unionized ammonia is displayed in Figures 8a and 8b. Data obtained from the baseline and all Phase II treatments were included for analysis. Good relationships were obtained for both of these comparisons.

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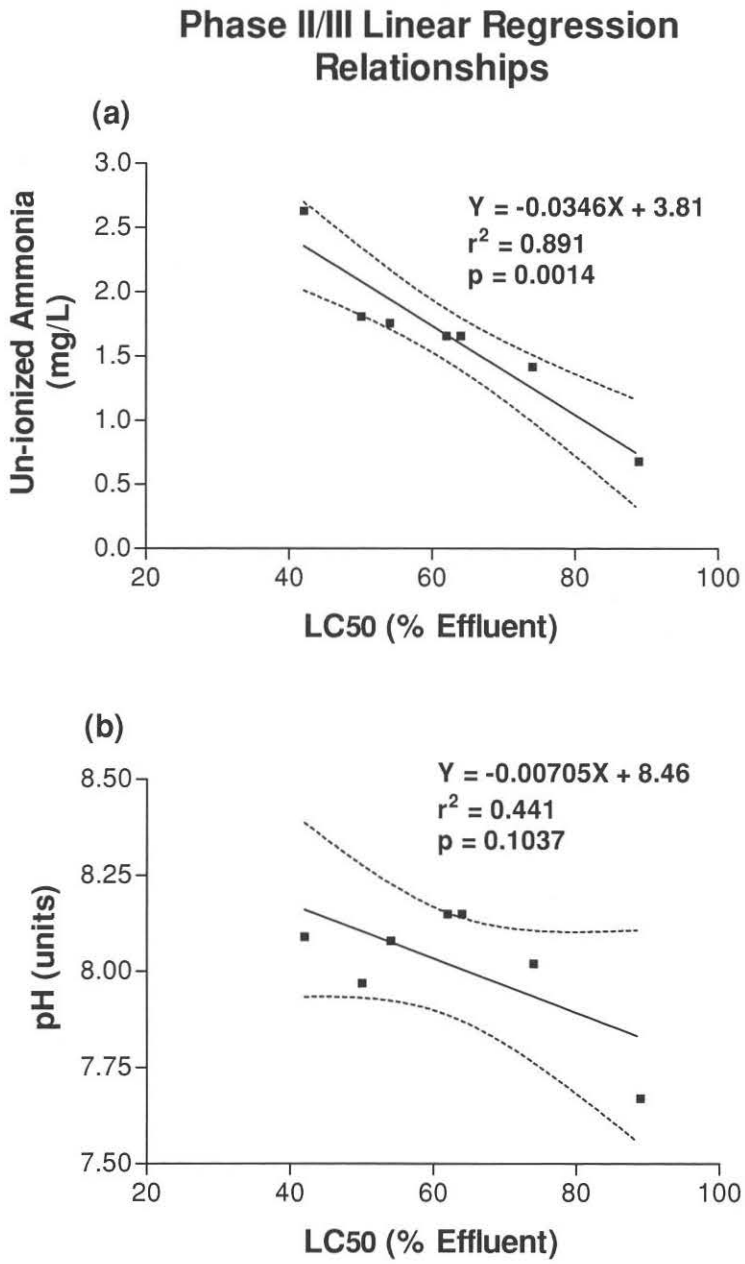

Figure 8. Linear regression relationships (± 95% CI) between effluent LC50 values and un-ionized ammonia concentrations (a), and pH (b) in 100% test concentrations for *P. promelas* (n=7). Phase II/III test series.

AMMONIA LC50 CALCULATIONS

To further evaluate the relationship between ammonia and toxicity, acute LC50 values were derived for the concentration of the unionized fraction in all TIE treatments. Results are summarized in Table 8.

LC50 values for fathead minnows (0.43 to 1.24 mg/L unionized ammonia) were similar to one another and to values previously derived for this constituent using *P. promelas*. (see Table 4). LC50 values for *C. dubia* (0.97 to 2.06 mg/L unionized ammonia excluding the pH 9 treatment) were also similar to one another and close to values previously derived for this constituent using *C. dubia*. It appears that unionized ammonia is less toxic at pH 9 relative to its toxicity at lower pH values. This observation is supported by an elevated LC50 value of 2.41 mg/L unionized ammonia obtained for this treatment. These comparisons provide further supporting evidence for toxicity due to ammonia for both test species.

HISTORICAL DATA EVALUATIONS

An important component of a TIE study is a review of historical data to evaluate trends and help identify the cause of toxicity when it is observed. Results for historical ammonia measurements and chronic toxicity to *C. dubia* and *P. promelas* follow.

Historical Ammonia

Concentrations of total and unionized ammonia measured in effluent collected during the past two years are displayed in Figure 9. Not all samples tested for toxicity are displayed since ammonia was not routinely analyzed in Brawley effluent samples prior to December 2001. LC50 values for *C. dubia* and *P. promelas* are shown for comparison purposes. Results clearly show that levels of ammonia routinely, but not always, exceed LC50 values for these two species.

Historical Toxicity

Toxicity of Brawley WWTP effluent to *C. dubia* and *P. promelas* over the past two years is displayed in Figure 10. For comparison purposes, survival in 100 percent effluent is shown. Survival of fathead minnows was less than 80 percent in 17 of the last 23 tests from June 2000 to May 2002. Survival of *C. dubia* was less than 80 percent in 8 of the last 23 samples. Fathead minnows were more sensitive than *C. dubia* in 91 percent (21 of 23) of these samples. As discussed earlier, ammonia is more toxic to fathead minnows than *C. dubia*, therefore, this observation provides strong supporting evidence that ammonia is likely a consistent historical primary toxicant of concern. Events where toxicity is greater to *C. dubia* than *P. promelas*, however, indicate that toxicants other than ammonia are causing toxicity on some sampling dates.

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Table 8. Summary of Unionized Ammonia LC50 Value Derivations for TIE Tests

Phase I TIE Test	LC50 (mg/L unionized ammonia) *C. dubia*	*P. promelas*
Baseline	1.18	0.43
STS Addition	> 0.88 [a]	0.60
EDTA Addition	1.5	0.69
PBO Addition	0.97	0.67
Aeration	1.93	0.49
Filtration	2.06	0.59
C18 Extraction	2.06	0.83
Zeolite Extraction	> 0.47 [a]	0.48
pH 6 Adjustment	> 0.24 [a]	0.35
pH 9 Adjustment	2.41	1.24
Mean	**1.73**	**0.64**

Phase II/III TIE Test	LC50 (mg/L unionized ammonia) *P. promelas*
Baseline	0.57
Zeolite extraction	> 0.32 [a]
Zeolite + Ammonia Spike	0.61
Aeration	0.89
Aeration at pH 11	0.73
Aeration at pH 11 + Ammonia Spike	0.72
Aeration at pH 11 + EDTA	0.69
Lab Control + Ammonia Spike	0.62
Mean	**0.69**

[a] Value not used for mean determination due to minimal observed toxicity.
NA - Not applicable due to no dose response.

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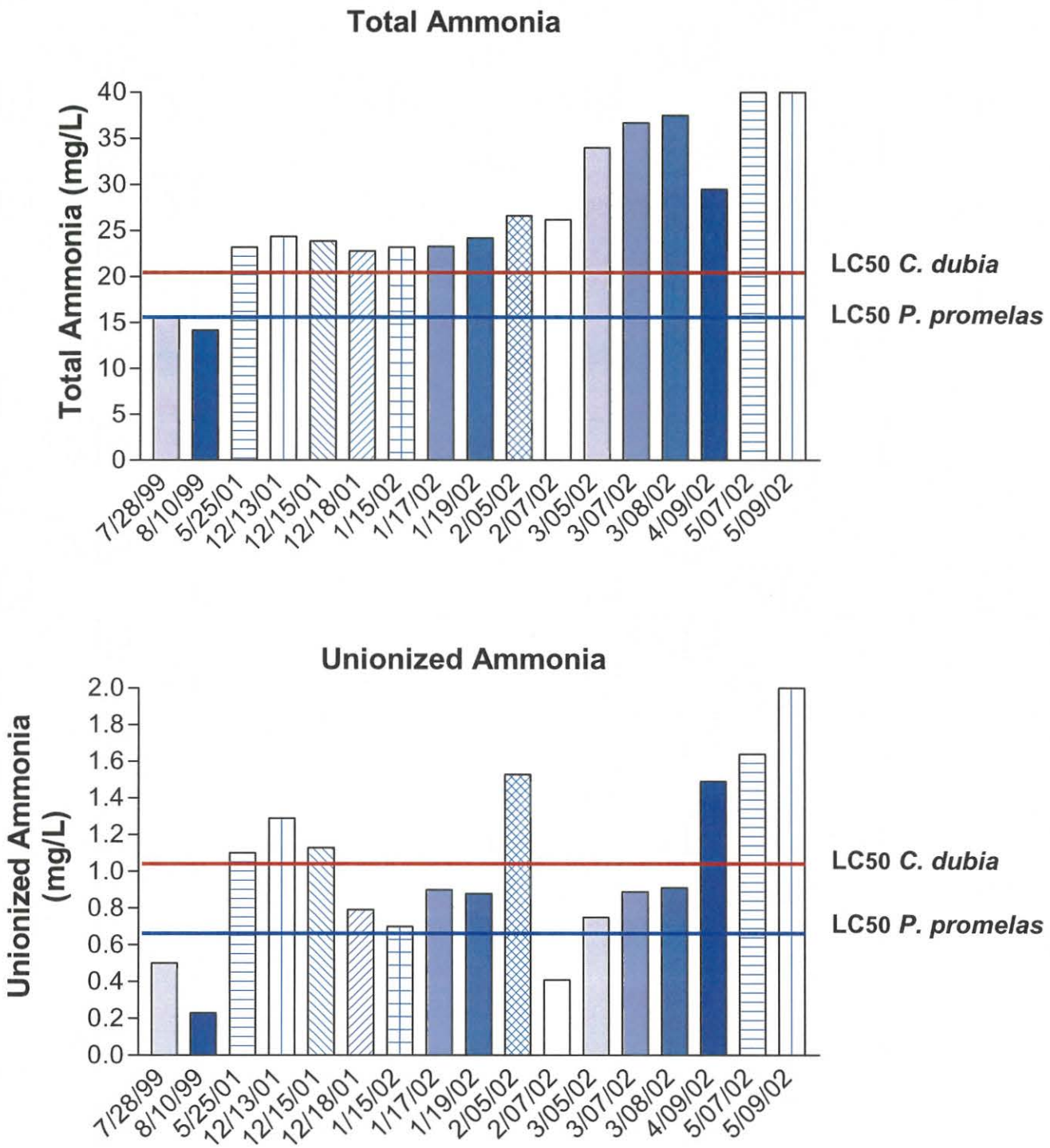

Figure 9. Historical concentrations of total and unionized ammonia in Brawley WWTP effluent. Mean LC50 values derived at AMEC for *C. dubia* and *P. promelas* are displayed for comparison purposes.

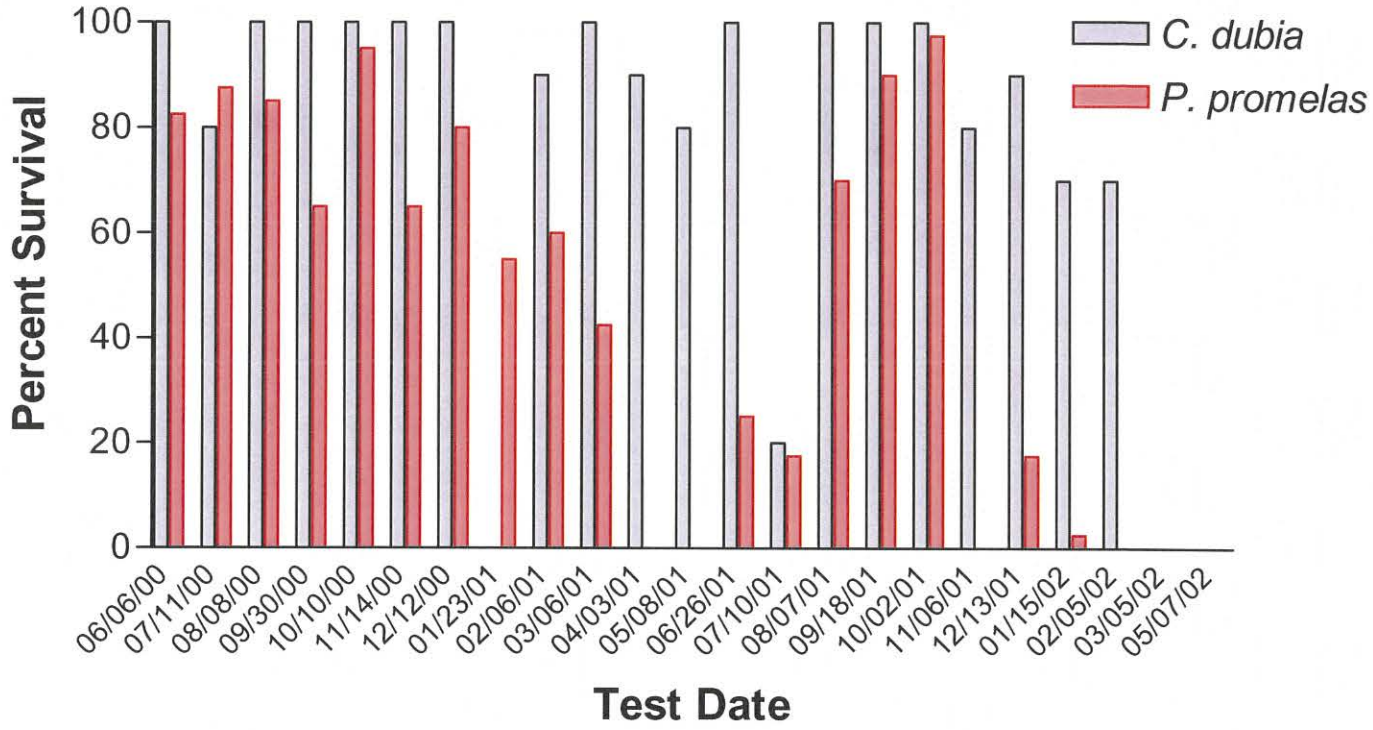

Figure 10. Historical toxicity over the last two years for Brawley WWTP effluent. Mean 7-day survival in 100 percent effluent is displayed for *C. dubia* and *P. promelas*.

RESULTS SUMMARY

The following points summarize important findings and observations of this study.

1. pH-dependent toxicity observed in all tests (increased toxicity at higher pH values) is highly indicative of toxicity due to ammonia (EPA 1993b; Thurston and Russo 1981).

2. Total and unionized concentrations of ammonia during the exposure periods were consistently above levels found to cause toxicity to both *C. dubia* and *P. promelas.*

3. Strong relationships between the concentration of unionized ammonia and survival were found for both test species.

4. Toxicity was completely recovered after removal and re-addition of ammonia to its original concentration.

5. LC_{50} values based on the concentration of unionized ammonia in all TIE treatments were within ranges expected to cause toxicity to both *C. dubia* and *P. promelas.*

6. Despite levels of surfactants and copper at or above potential levels of concern, confirmatory TIE studies failed to relate toxicity to these constituents.

7. A number of studies have shown that *P. promelas* is more sensitive to ammonia than *C. dubia* (AMEC 2001a; EPA 1993b; Ankley et al. 1990a; Thurston and Russo 1981). Likewise, chronic toxicity in 21 of the last 23 tests performed for the City of Brawley WWTP over the past two years has been greater to *P. promelas* than *C. dubia.*

8. An historical evaluation of total and unionized ammonia levels in Brawley WWTP effluent found concentrations that routinely exceed levels expected to cause toxicity to both *C. dubia* and *P. promelas.*

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CONCLUSIONS AND RECOMMENDATIONS

In summary, accumulated evidence from the combination of Phase I characterization, Phase II identification, and Phase III confirmation TIE studies performed on the City of Brawley WWTP effluent during this study concludes that all observed toxicity can be attributed to ammonia in the sample collected on 8 April 2002. It is recommended that analysis of total ammonia be continued to evaluate its relationship to toxicity in future samples.

It is possible that the contribution of toxicity due to ammonia may, at times, mask effects due to less toxic constituents. It is important to note that wastewater treatment plant effluent consists of a complex mixture of compounds that may change in composition over time. A single dominant compound may control the majority of observed toxicity; however, it is also possible that a number of different compounds may contribute to and influence toxicity to varying degrees over time. Although ammonia was found to be responsible for all observed toxicity in this study it does not mean that other contaminants might not contribute or be responsible for toxicity at other times.

Monthly and quarterly toxicity screens historically performed at AMEC for the City of Brawley have, in several cases, shown greater sensitivity of *C. dubia* than *P. promelas*. This type of result implicates toxicants of concern other than ammonia at these time periods. Chemistry results to date indicate that copper and surfactants may be other chemicals of potential concern in Brawley WWTP effluent. The historical frequency of high ammonia levels and greater toxicity to fathead minnows than *C. dubia*, however, indicates that ammonia is routinely the primary toxicant of concern in the City of Brawley WWTP effluent.

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