A Workshop on Toxicity Identification Evaluation (TIE's) in the San Francisco Bay Region: Lessons Learned

1993

San Francisco Bay - Delta
Aquatic Habitat Institute

PREFACE

This proceedings is the result of a workshop held on September 30 and October 1, 1993 at the University of California at Berkeley Field Station in Richmond, California. This is the second workshop on the subject of Toxicity Identification Evaluations sponsored by discharging agencies in the San Francisco Bay Region. The first Workshop, held in March of 1992, focused on case studies presented by nationally recognized experts in the field of chronic toxicity identification. At that time, many area dischargers were just beginning to implement chronic toxicity testing in their own facilities.

This second workshop, held 18 months after the first, focused on the experiences of dischargers in the San Francisco Bay Region in conducting both chronic and acute toxicity identification evaluations. Thirteen papers were presented, of which eight were submitted to be published in these proceedings. For information on the studies for which no paper was presented, interested parties should contact the presenter for information.

On behalf of the Aquatic Habitat Institute, I wish to thank all of our workshop sponsors and participants for making the event possible. Special thanks go to Bhupinder Dhawliwal of the Laboratory Committee of the Bay Area Dischargers Association, Tom Mumley and Lila Tang of San Francisco Bay Regional Water Quality Control Board, and Bruce Thompson, Gabriele Marek, and Elizabeth Hartman of the Aquatic Habitat Institute for their efforts in making the event a success.

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Session #3 - New Developments in Toxicity Evaluation Procedures

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KEYNOTE ADDRESS FOR AHI WORKSHOP ON TIE'S
STATUS OF TIE'S AS A REGULATORY TOOL IN S.F. BAY REGION

We have come a long way with using bioassays and toxicity requirements as a regulatory tool in this Region. In the late seventies, we started with static bioassays with one species: the faithful 3-spined sticklebacks. In the mid-eighties we embarked on flow-through bioassays with trout, and chronic toxicity tests using a wide range of organisms. Today, acute toxicity limits have been tightened and chronic toxicity limits are in place for the largest dischargers to the Bay.

Each step has resulted in significant improvements to water quality. An example is treatment of oil refinery wastewater with advanced biological treatment as a result of the first set of acute limits; and because of the recent tighter acute limits, activated carbon treatment has been added. thus far, all of the improvements have been from TIE;s and TRE;s for acute toxicity.

We are beginning to find that the causes of toxicity, especially of chronic toxicity do not lend themselves to end of pipe treatment or quick fixes. For instance, acute toxicity at Central San. was found to be caused by a common type of pesticide, and chronic toxicity at Sunnyvale and Palo Alto are caused by zinc and hardness. The most reasonable solution for these cases is pollution prevention programs. This entails a long term, continuous effort on the part of the dischargers because success achieved can be quickly lost if the effort is not sustained.

These findings are not expected. As we become more sophisticated in toxicity testing, we are going find the subtle adverse effects for which there are no easy solutions. The future need is for the dischargers to have more complete understanding and control of not only the treatment plant, but also of what is upstream of the treatment plant because future improvements will mostly come from pollution prevention efforts.

After six years of the Effluent Toxicity Characterization Program, we are confident of our ability to detect chronic toxicity, and of the quality and validity of the test results. We are also confident that the detected toxicity is an indication of real effects in the Bay. With this knowledge, we cannot back off of toxicity limits.

Toxicity is the best measure we have to evaluate whether the discharge is safe, in other words whether the chemical specific limits are adequate. Toxicity will continue to be an important tool in the future to ensure that problems which may be caused by changes, such as changes in the type of industrial users, do not go undetected. Acute toxicity with younger, and more

sensitive species, such as those being used in the "chronic" tests, will drive future efforts on this front.

The challenge is to develop and refine TIE procedures, both acute and chronic for the full range of test species, fresh water and marine. Without these TIE tools, we can only know that there is a problem. With these tools, we can determine what are the causes of the problem and begin to focus our efforts towards the solutions.

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REPORT ON THE ACUTE TOXICITY IDENTIFICATION EVALUATION (TIE) ON THE EAST BAY MUNICIPAL UTILITY DISTRICT EFFLUENT

The East Bay Municipal Utility District (EBMUD) conducted an acute Toxicity Identification Evaluation (TIE) from March 1992 to April 1993. Effluent samples were evaluated for sources of consistent acute toxicity to the cladoceran, *Ceriodaphnia dubia,* and occasional acute toxicity to larvae of the estuarine fish, *Menidia beryllina.*

Results indicated that ammonia is a constituent in the District's effluent that regularly contributes to observed acute toxicity to *Ceriodaphnia dubia* and is the major toxicant to *Menidia beryllina.* Silver may contribute to the acute toxic response of *Ceriodaphnia* to the District's effluent, but there was no evidence that other metals contribute to this response. The toxic response of *Menidia* to the District's effluent was not attributable to metals. Variable dissolved constituents in the effluent (Cl^- in particular) may contribute to the observed toxicity to *Ceriodaphnia.* For *Menidia* there may be inorganic constituents other than ammonia contributing to observed toxicity, but those constituents could not be identified. Acute toxicity of the District's effluent is not caused by organic constituents. Other factors (i.e. low dissolved oxygen), which are often artifacts of the toxicity tests, may heighten the sensitivity of *Menidia* to other toxicants by stressing the organisms.

INTRODUCTION

The East Bay Municipal Utility District conducted an Effluent Toxicity Characterization Study (ETCS) from 1989-1990. Results of this study showed that the District's effluent was consistently acutely toxic to *Ceriodaphnia dubia* and occasionally acutely toxic to *Menidia beryllina*.

The Regional Board required the District to perform an acute Toxicity Identification Evaluation (TIE). A study plan was submitted and approved, and the TIE was initiated in March 1992. This final report presents results and conclusions from the TIE. *Ceriodaphnia* and *Menidia* results are presented independently.

CONCLUSIONS

The following conclusions are based on EPA Phase I TIE methods (EPA-600/3-88/034), Walsh and Garnas (1983) fractionation procedures, draft EPA marine TIE methods, Mount and Mount (1992) modifications of TIE procedures and in-house procedures:

1. The toxic responses of *Ceriodaphnia* and *Menidia* are linked to undissociated ammonia concentrations in the District's effluent.

2. Inorganic constituents, other than ammonia, contribute to the toxic response of *Ceriodaphnia* and *Menidia*. For *Ceriodaphnia*, silver and chloride are possible toxicants; for *Menidia* the constituents are unidentified.

3. The toxic responses of *Ceriodaphnia* and *Menidia* are not attributable to organic compounds in the District's effluent.

CERIODAPHNIA DUBIA RESULTS

Phase I tests

Effluent fractionations were performed on 3/31, 4/16, and 4/29/92 according to EPA Phase I procedures (EPA-600/3-88/034). Toxicity tests were conducted using effluent fractions of 24-hour, flow-weighted composites. *Ceriodaphnia* were exposed to 100, 80, 64, and 51% concentrations for 96 hours under static conditions (51% was discontinued after the first two tests). The remainder of this section reviews the toxicity test results of the fractionations. The biological responses for each test are summarized in Appendix A.

Table I summarizes effluent water quality characteristics for the four composited samples. Measurements were made immediately after the sample was composited. The parameter measures are generally consistent between samples.

Table I. Effluent Water Quality for Phase I Characterizations

Date	3/30/92	4/16/92	4/29/92	8/10/93	avg.	s.d.
pH	6.9	6.5	6.7	6.9	6.8	0.2
Alkalinity mg/L $CaCO_3$	180	150	180	140	162	20.6
Hardness mg/L $CaCO_3$	160	160	170	160	163	1.8
Conductivity umhos/cm	1460	1700	1300	1780	1560	220
Cl Residual total, mg/L	<0.1	<0.1	<0.1	<0.1	<0.1	NA
Ammonia total, mg/L	30.5	27.4	33.0	35.1	31.5	3.3

Baseline toxicity tests

Although variability within treatment replicates makes it difficult to definitively interpret toxicity results there are several general observations for the three Phase I test events:

- o There is a strong positive linear correlation between total ammonia concentrations and 48-hour mortality in the undiluted effluent (r^2=.98).
- o *Ceriodaphnia* exposed to undiluted (100%), unaltered effluent experienced >50% mortality within 72 hours.
- o Mortality varied between 10 and 70% for *Ceriodaphnia* exposed to 80% effluent.
- o Mortality was not significant for *Ceriodaphnia* exposed to 64 and 50% effluent concentrations.

Table II summarizes the relation of effluent pH to unionized and total ammonia concentrations.

Table II. Unionized Ammonia and pH

Test Date	Initial pH	Total Ammonia[1]	Unionized Ammonia[2] Initial pH	Unionized Ammonia[3] Final pH
3/31	6.9	30.5	0.14[4]	1.27[4]
4/17	6.5	27.4	0.03	1.14[4]
4/30	6.7	33.0	0.07[4]	1.37[4]

1 measured concentrations (mg/L)

2 estimated concentrations (mg/L) calculated from tables compiled by H.P. Skarheim; SERL Report No. 73-5, using measured pH, temperature, and total ammonia values

3 estimated concentrations (mg/L) calculated from tables compiled by H.P. Skarheim; June 1973, SERL Report No. 73-5, using measured total ammonia and temperature values and estimated final pH of 8.1

4 exceeds EPA CMC for freshwater organisms less sensitive than salmonids (EPA 440/5-86-001)

pH adjustment

Inconsistent responses to pH adjustment makes it difficult to draw any definitive conclusions about observed tests responses. A decrease in toxicity was observed after 72 hours when effluent was adjusted to pH 3.0 in the 3/30 and 4/16 tests, but not in the 4/29 test. Adjustment of the effluent to pH 11.0 appeared to decrease toxicity for the 3/30 and 4/16 tests, but had no effect on the 4/29 test.

Aeration

Results from the combination of effluent pH adjustment and aeration were consistent, but inconclusive. Aeration consistently reduced the toxicity of effluent at pH_i below the levels observed in the baseline tests. At pH 11.0 toxicity was reduced in the 3/30 and 4/16 tests, but had no effect in the 4/29 test. Toxicity was increased by aeration in combination with pH adjustment to pH 3.0 in the 3/30 sample, but decreased in the 4/16 and 4/29 samples. During the 3/30 test, the electrode used to measure low pH values leaked AgCl into the test fractions potentially affecting the toxicity tests, and making the pH 3.0 test results suspect.

For the pH-adjusted samples, the results of this fractionation step are confounded by the chemical manipulations required. The addition of significant volumes of acid and base may have reduced *Ceriodaphnia* response by diluting the levels of toxic agents in the effluent sample. For example, in the 3/30 test, aeration at pH 11.0 decreased toxicity, but a total of 3.45 ml of acid and base were added to the original 35 ml volume of sample resulting in a 10% dilution.

Filtration

Responses to the combination of effluent pH adjustment and filtration (1.0 or 0.2um) were not consistent. In the 3/30 and 4/29 tests, filtration in combination with pH adjustment reduced toxicity beyond that achieved solely by adjustment of effluent to pH 3.0. In the 4/16 test there was no reduction beyond that achieved by pH adjustment alone.

The combination of adjusting effluent pH to 11.0 and filtration in the 3/30 and 4/16 tests did not reduce toxicity below levels observed in the baseline tests or beyond that achieved by only adjusting effluent to pH 11.0. Increasing pH to 11.0 increased toxicity beyond the baseline level in the 4/29 test. Filtration subsequently reduced toxicity to a level below baseline. Filtration at pH_i did not reduce toxicity below baseline on any of the three dates, and seemed to slightly increase toxicity in the 3/30 and 4/16 tests. However, no toxicity was observed in filtration blanks.

All filters in the 4/29 test were backwashed with a volume of dilution water equal to the volume of effluent originally filtered. No toxicity was observed in tests of these backwashes.

C18

The ability of C18 to reduce toxicity must be distinguished from the effects of other physical manipulations that were required before effluent samples could be passed through the C18 columns. For example, filtration was shown to be effective in reducing toxicity in fractions that had been adjusted to pH 3 in two of the test events. The reduction of toxicity in such fractions eluted through C18 columns could not be confidently attributed to either the removal of particle-bound toxicants or the adsorption of nonpolar organic toxicants.

Conversely, filtration did not significantly reduce toxicity in effluent fractions whose pH was not altered, i.e. pH_i. In both the 3/30 and 4/16 tests, toxicity was slightly reduced after passing effluent samples through the C18 column.

EDTA chelation

The addition of EDTA over a narrow concentration range (0.4-0.6ml of a 4.1 g/L sodium EDTA stock solution) to a 10 ml effluent test volume reduced toxicity below baseline levels in the 3/30 and 4/16 tests. In the 4/29 test EDTA addition resulted in no toxicity reduction relative to baseline levels. Similarly, in a test performed on 3/9/93 no reduction in toxicity was observed with the addition of EDTA.

Oxidant reduction

The addition of sodium thiosulfate (STS) over a narrow concentration range (0.1-0.6ml of an 8.5 g/L stock solution) to a 10 ml effluent test volume reduced effluent toxicity below

baseline levels in all three test events. The most consistent reduction occurred with the addition of 0.4 ml of stock solution.

Graduated pH

For all test events, 100% mortality occurred in undiluted effluent samples of pH 8.5 within 24 hours. Figure 1 shows the relation between effluent pH and time to mortality. In all cases a greater toxic effect was elicited more quickly at higher pH. To determine the reason for mortality in exposures beyond 24 hours, dissolved oxygen (DO) concentrations were measured in each of the 4/29 test treatments after 100% mortality was observed. Oxygen levels were sufficient (5.4 mg/L) in effluents of pH 8.5 with complete mortality within 24 hours. In contrast, measured final DO levels in effluents of pH 6.5 and 7.5 were below 0.1 mg/L. Therefore, insufficient oxygen probably caused the mortalities in tests conducted with effluents of pH 6.5 and 7.5.

CONFIRMATORY TEST RESULTS

Confirmatory tests, guided by the results of Phase I tests, were begun in late April, 1992. Although initial evaluations were intended to determine if effluent toxicity was caused, in part, by organic chemicals, much of the confirmatory tests effort focussed on assessing the influence of ammonia and other inorganic constituents on observed acute toxicity.

Organic chemicals

There was increased survival in *Ceriodaphnia* exposed to unaltered effluent (pH_i) that had passed through a C18 column compared to baseline tests and beyond that achieved by filtration alone. These results indicated a reduction in toxicity apparently attributable to lower organic chemical concentrations. This result was evaluated further by exposing Ceriodaphnia to the compounds eluted from those C18 columns used in the 3/30 and 4/16 tests.

Theoretically, a 90-fold concentrated fraction resulted from eluting the columns in 2ml of 25, 50, 75, 80, 90, 95 and 100% methanol (180 ml effluent originally passed through column). A 150ul volume of each concentrated fraction was added to 10 ml of control water resulting in a 35% concentration increase over the original effluent sample. Toxicity was not observed in solutions made from C18 eluate in either test event.

Further evidence (based on methods by Walsh and Garnas, 1983) that organic chemicals do not contribute to the toxicity of the District's effluent to *Ceriodaphnia* are presented later in this report.

Ammonia

Graduated pH tests from all three Phase I tests indicated that effluent toxicity was related to ammonia concentrations. Efforts to validate this relation were begun in late May, 1992 using methods described by Mount and Mount (1992).

Initially, the stability of effluent pH over time was evaluated by comparing pH changes under traditional test conditions with effluent pH changes in vials having differing concentrations of CO_2 in a closed headspace. The pH of effluent under closed CO_2 headspace remained unchanged over a 72 hour period. In contrast the pH of effluent exposed to air increased by 0.5 pH units within 24 hours. Subsequent evaluations exposing *Ceriodaphnia* under static renewal test conditions, showed that pH increased in closed vials that did not have CO_2 in the headspace, as well as in open containers. Increases in pH were paralleled by elevated mortalities in these treatments compared to *Ceriodaphnia* exposed under CO_2 headspace which had minimal increases in pH.

During the first (6/9) of these tests total ammonia concentrations in the samples ranged form 31.9-36.8 mg/L. Mortality was highest in the effluent exposed to air, where the pH increased as much as 1.5 units (to pH 8.2) over a 24-hour period. Mortality was lowest in treatments where pH did not increase (pH control was not uniformly successful due to differences in CO_2 introduction technique).

The second (6/23) test included positive ammonia controls. Dilution water was spiked with nominal concentrations of $(NH_4)_2SO_4$ to equal measured total ammonia concentrations in effluent. One treatment was begun at the initial pH of the effluent, and the other was set to approximate the pH increase measured after 24 hours in effluent having the same initial pH as the first treatment. *Ceriodaphnia* were exposed to undiluted effluent under a sealed headspace CO_2 environment and in open cups.

The response to undiluted effluent was similar in the pH-controlled environment and in the open test chambers. At 72 hours there was higher mortality in the open cups (81%) versus the CO_2 environment (47%), but by 96 hours mortality was 100% in both treatments.

The spiked ammonia treatments showed no significant mortality over the 96 hour period. This seems to contradict conclusions that ammonia is an important toxicant in the District's effluent. However, the ammonia concentrations were nominal, not measured, and these results should be weighed accordingly.

Walsh/Garnas fractionation

To resolve some of the ambiguous results from Phase I tests using EPA procedures, an earlier protocol developed by Walsh and Garnas (1983) was used.

Effluent was initially passed through a column packed with XAD-2 resin (8/10/92 and 10/27/92). The effluent collected after passing through the column contained the inorganic

fraction of the sample. The 8/10 column was subsequently eluted with 100ml of acetone to obtain the organic fraction. For the 10/27 test the inorganic fraction was split and treated with anion and cation exchange resins.

Ceriodaphnia were exposed to: 1) unaltered effluent; 2) the inorganic effluent fraction collected after passing through the XAD columns; 3) effluent after treatment with cation and anion exchange resins; and 4) the organic fraction eluted from the column. In the 8/10 test, complete mortality occurred within 48 hours in the undiluted effluent sample and in the inorganic effluent fraction. In contrast, *Ceriodaphnia* exposed to a whole effluent sample in an enclosed headspace under CO_2 experienced only 6.7% mortality in 96 hours. The pH of the whole effluent sample and the inorganic fraction increased from an initial pH of 6.9 to a pH of 8.2-8.4 following exposure to air for 48 hours. The unionized ammonia concentrations (2.26 mg/L and 2.80 mg/L for whole effluent and the inorganic fraction, respectively) estimated from the measured total ammonia concentration (35.1 mg/L) are expected to be acutely toxic.

Ceriodaphnia were exposed to the collected organic fraction which was evaporated to a volume of ~0.5 ml and subsequently increased to 250 ml (the original effluent volume passed through the column) with dilution water. No toxicity was observed in *Ceriodaphnia* exposed to the organic fraction for 96 hours.

The 10/27 test was delayed 24 hours because of an insufficient number of neonates. This delay may have altered the chemical nature of the effluent and the effluent fractions. After 48 hours 53% mortality was observed in the unaltered effluent compared with 22% mortality in effluent under CO_2 sealed headspace. The unionized ammonia concentration of the unaltered effluent was estimated to be 2.39 mg/L (based on measured total ammonia of 37.1 mg/L at pH 8.3). However, the test delay may have resulted in an over-estimation of actual levels of unionized ammonia. Also, at pH 8.3 unionized ammonia is not nearly as toxic as at lower pH values (explained in detail in the Discussion section).

In the 10/27 test, *Ceriodaphnia* exposed to the inorganic fraction experienced 93% mortality after 48 hours. The toxicity of the inorganic fraction mixed with the cation exchange resin increased, although survival in the control treatments was acceptable. The inorganic fraction mixed with the anion exchange resin had reduced mortality at 48 hours (27%).

CERIODAPHNIA DISCUSSION

Determining the causative agent(s) for the acutely toxic response of *Ceriodaphnia* to the District's effluent is difficult, because the response is only consistently elicited at the highest effluent concentrations (100% and 80%).

Ammonia

It is important to note the unique relationship between ammonia toxicity and pH. While a larger fraction of unionized ammonia (the toxic form) is present at higher pH levels, a given level of unionized ammonia in freshwater is more toxic at lower pH levels (Appendix B). EPA (EPA 440/5-85-001) has reported cladoceran 48-hour LC50 values for unionized ammonia ranging from 0.53-4.94 mg/L at various pH levels. Using the EPA's formula for normalizing those LC50's to pH 8.0 (EPA 440/5-85-001) and taking the geometric mean of those values, the calculated mean LC50 at pH 8.0 for cladocerans is 1.82 mg/L unionized ammonia. This value indicates that cladocerans are relatively insensitive to unionized ammonia. Levels of unionized ammonia routinely present in the District's effluent at the end of a 24-hour exposure period (1.0-1.6mg/L, Table I, Appendix A) are certainly high enough to contribute to acute toxicity, but are not high enough to account for all of the observed acute toxicity.

Toxicity results for the effluent samples subjected to Phase I graduated pH adjustments and confirmatory pH control evaluations indicate that toxicity reductions are attributable to decreased unionized ammonia concentrations. This is particularly illustrated by the 8/10 test, in which mortality was 100% after 48 hours in the unaltered effluent (final pH 8.3) and in the inorganic fraction (final pH 8.4) exposed to air. Mortality was only 7% in unaltered effluent under sealed CO_2 headspace (final pH 6.6). Differences between effluent pH and time to mortality indicate a pH-dependent toxicant.

The ion exchange resin procedures, which are part of the Walsh/Garnas fractionation, involve pH alterations (to >10.0 for the anion exchange resin; to <3.0 for the cation exchange resin) followed by stirring for 24 hours. For the anion exchange resin, which reduced toxicity for *Ceriodaphnia* exposed to the District's effluent, this procedure is likely to drive off ammonia which would be in the volatile, unionized (NH_3) form at pH >10.0.

Organics

Toxicity reductions following aeration indicate that the toxicant(s) may be oxidized to less toxic forms or may be removed from the solution by sparging. The District's activated sludge secondary treatment process (using pure oxygen) should thoroughly oxidize any oxidizable constituents in the effluent. Toxicity reductions via sparging might indicate the presence of volatile organic constituents in the effluent, but, as described elsewhere in this report, there is no evidence that organic compounds contribute to the toxicity of the District's effluent. The decrease in toxicity due to aeration could be explained by the removal of surfactants by the bubbling process. However, the presence of such compounds is not indicated by the results of the Walsh/Garnas fractionation or the C18 column eluate.

Fractionation results using the Walsh/Garnas procedure clearly indicate that the toxicity of the District's effluent to *Ceriodaphnia* is not attributable to organic toxicants. Toxicity was not observed in the organic fraction eluted from the XAD-2 column. Phase I test results provide additional confirmation that toxicity was not caused by organic chemicals. Samples collected

after passing effluent through C18 columns at pH_i consistently had lower toxicity than untreated effluent samples, but the toxicity was not recovered in the methanol eluates. This confirms that toxicity in the baseline samples was not caused by organic chemicals.

Metals

The C18 column may have reduced toxicity by removing zinc, nickel, aluminum or copper (Norberg-King, 1992) or by removing constituents via filtration. For zinc, the Criterion Maximum Concentration (CMC) at hardness of 160 mg/L (average for the District's effluent) is 174 μg/L (EPA 440/5-86-001, updated for zinc May 1987). For the cladocerans *Daphnia magna* and *Ceriodaphnia reticulata* LC50 values of 125 μg/L and 136 μg/L, respectively, have been reported (values adjusted to hardness of 160 mg/L $CaCO_3$, as per EPA, 1987). The average zinc concentration in the District's effluent (Table III) was 75 μg/L with a maximum of 90 μg/L and a minimum of 50 μg/L. Based on measured effluent concentrations, zinc is not a likely cause of the acutely toxic response of *Ceriodaphnia dubia* to the District's effluent.

Nickel concentrations in the District's effluent (average = 24 μg/L) are consistently well below the CMC of 2636 μg/L at 160 mg/L $CaCO_3$ hardness (EPA 440/5-86-001). Effluent aluminum concentrations exceeded the CMC of 750 μg/L (EPA 440/5-86-008) on 1 of 7 occasions. Measured aluminum concentrations indicate that the concentration of this constituent in the District's effluent is highly variable and is not likely to be a consistent contributor to acute toxicity. Although after zeolite treatment the aluminum concentration was 8,200 μg/L, only 10% mortality was observed for *Ceriodaphnia*. This is strong evidence that aluminum is not a constituent of concern.

Table III. Metals for District effluent at TIE sample point.

Metal	Average[1] (ug/L)	Maximum (ug/L)	Minimum (ug/L)	s.d. (ug/L)	n
Ag	<3.0	3.0	<3.0	NA	7
Al	314	1100	<10	367	7
Cd	<3.0	<3.0	<2.0	NA	7
Cu	11	16	<6.0	4.4	7
Hg	<10	<10	<10	NA	5
Mn	98	110	87	9.3	7
Ni	24	50	<10	13.9	7
Pb	31	40	<30	NA	7
Se	<70	<70	<30	NA	7
Zn	75	90	50	14	7

1 Averages include values at detection limit.

The average LC50 for copper, based on exposures at ~100-200 mg/L $CaCO_3$ hardness, for cladocerans is 41 μg/L (EPA 440/5-84-031). An LC50 as low as 6.5 μg/L has been reported for *Daphnia magna* (EPA 440/5-84-031). The CMC for copper is 27.6 μg/L at effluent hardness of 160 mg/L $CaCO_3$ (EPA 440/5-84-031). Referring to Table III, the average copper concentration in the District's effluent was 11 μg/L for the seven samples. These data indicate that copper is not contributing substantially to the acute toxicity of the District's effluent to *Ceriodaphnia.*

Effluent sample toxicity was consistently reduced over a narrow concentration range of sodium thiosulfate and EDTA additions. In the 3/30 and 4/16 tests, the toxicity reduction caused by the addition of EDTA combined with the addition of STS suggests that copper, cadmium or mercury may have contributed to toxicity (Norberg-King, 1992). The test in which EDTA had no effect (4/29), but STS reduced toxicity, silver or selenium may have contributed to toxicity (Norberg-King, 1992).

Copper toxicity, as it relates to the District's effluent, has been discussed. Cadmium and mercury would not be expected to contribute to consistent acute toxicity in the District's effluent. The CMC for cadmium (at 160 mg/L $CaCO_3$ hardness) is 6.7 μg/L (EPA 440/5-86-001). The average cadmium concentration in the District's effluent (Table III) was <3.0 μg/L. For mercury the CMC is 2.4 μg/L and an LC50 as low as 2.2 μg/L has been reported for the cladoceran *Daphnia pulex* (EPA 440/5-84-026). Table III shows average mercury results based on only five samples. All levels are below the detection limit of 10 μg/L.

Reviewing data from a different final effluent sampling point for the period from January 1989 to March 1993 revealed that 4 of 195 samples analyzed by cold vapor (detection limit = 0.5 μg/L) had mercury concentrations above 2.4 μg/L and only 1 was above 2.2 μg/L. Based on these data, copper, cadmium and mercury in the District's effluent are not causing consistent acute toxicity to *Ceriodaphnia.*

Silver is highly toxic to freshwater organisms, and the cladoceran *Daphnia magna* is the most sensitive species reported (USEPA 1987). For freshwater species, the CMC is 0.92 μg/L (USEPA 1987). Data in Table III show that this level is below the detection limit except on one occasion. Again, reviewing data from 1/89 - 3/93 reveals that silver levels in the District's effluent, when detectable, consistently exceed this value (average concentration = 3.2 μg/L, maximum = 38 μg/L, minimum = 1 μg/L, n=140). However, these measures represent total concentrations of silver in the District's effluent and may significantly over-estimate the amount of silver that is bioavailable (i.e. toxic).

Data presented in Table III show that selenium was not present above detection limits (usually 70 μg/L) for any sample. The CMC for selenium is 260 μg/L (EPA 440/5-86-001), therefore selenium would not be expected to be a source of acute toxicity in the District's effluent.

Evidence against metals

Despite evidence for metals toxicity, test results following pH manipulation of effluent samples indicate that toxicity is not caused by metals. Reducing the effluent to pH 3 and readjusting to the initial effluent pH would be expected to increase effluent toxicity by dissolving metal complexes and, making metals more bioavailable. Contrary to expectations, toxicity did not increase above levels observed in baseline tests, and in the 3/30 and 4/16 tests toxicity decreased slightly.

Likewise, the results of reducing effluent pH combined with filtration are contrary to metals toxicity. Dissolving metals through pH reduction and then filtering the effluent results in a solution of metals with no solids to bind to. With a greater percentage of metals in a more bioavailable form, effluent toxicity should have increased, but in two instances (3/30 and 4/16) toxicity was reduced by the filtration step. In the 4/29 test toxicity was not affected by filtration.

Finally, it should be noted that national water quality criteria for freshwater are based on toxicity tests performed in relatively clean waters (i.e. low conductivity, low hardness, low organic carbon content). Metals are highly bioavailable in such waters. By comparison, the District's effluent has relatively high conductivity, hardness and organic carbon content. It is expected that only a portion of the total metals measured in the effluent would be bioavailable.

Inorganics other than ammonia and metals

Another source of toxicity may be a variety of changing dissolved constituents that act in combination to consistently supplement the acute toxicity caused by unionized ammonia and metals. This hypothesis is supported by the fact that toxicity was lowered in samples in which the pH was raised to pH 11.0. This treatment reduced toxicity below levels observed in baseline test levels in 2 of the 3 effluent samples.

Toxicity reduction may be explained by the complexing of previously free ions (particularly Cl^- and $K^{+)}$ when the sample pH was elevated to pH 11.0. EPA (EPA/600/4-90/027) has reported LC50 values for *Ceriodaphnia* as low as 256 mg/L KCl. This is confirmed by results from NaCl reference toxicant tests conducted by the District during the Effluent *Toxicity* Characterization Program in which the average LC10 was 610 mg/L Cl^-. District effluent Cl^- levels are routinely above 250 mg/L.

The results of the 6/23/92 test, in which *Ceriodaphnia* were exposed to effluent under sealed CO_2 headspace and in open containers, indicate that a toxicant other than ammonia is probably contributing to acute toxicity. The District's database was searched for unusual chemical spikes that may have occurred during June, 1992. Chloride ion concentration had been measured on 6/4/93 and was found to be 510 mg/L. Monthly measurements taken between 9/91 and 10/92 showed a maximum of 510 mg/L, a minimum of 250 mg/L and an average of 350 mg/L Cl^-. These levels of Cl^- are in excess of LC50 values reported in EPA interlaboratory studies (EPA/600/4-90/027).

The anion exchange resin results provide further evidence that dissolved constituents, particularly Cl^-, are contributing to the acute toxicity of the District's effluent. Toxicity was reduced by the anion exchange resin treatment. The measured Cl^- concentration in untreated effluent for the 10/27 test was 580 mg/L, clearly enough to cause *Ceriodaphnia* mortality.

MENIDIA BERYLLINA RESULTS

Efforts to determine the source of the acute toxic response of *Menidia beryllina* exposed to the District's effluent were begun in October 1992. Because EPA Phase I procedures are not designed for use with saltwater species, methods described by Walsh and Garnas (1983) were used, as well as draft EPA saltwater TIE procedures (Burgess, 1992) and in-house methods were used. Biological responses are summarized in Appendix C.

WALSH/GARNAS FRACTIONATION

Effluent was fractionated by methods described by Walsh and Garnas (1983). Effluent was passed over an XAD column on 10/26/92 (XAD-2) and on 1/4/93 (XAD-4). The XAD resin binds organic constituents so that effluent coming off the column represents the inorganic

fraction. The post-column effluent was further treated with cation and anion exchange resins. Effluent fractions were salted to 29ppt salinity. Toxicity tests were run at 100, 80 and 64% effluent fraction concentrations.

Toxicity was not removed by the XAD column in either test. In the 1/4 test, the toxicity of the post-column fraction increased, although control treatments were acceptable.

Treating the inorganic fraction with an anion exchange resin produced inconsistent results. In the 10/26 test toxicity was reduced below baseline levels. This response was most pronounced in undiluted (100%) effluent fraction. In the 1/4 test the anion exchange resin did not reduce toxicity below baseline levels, but did remove the toxicity observed in the post-XAD effluent for that test. The cation exchange resin reduced toxicity below baseline levels in both the 10/26 and the 1/4 test events.

ZEOLITE

The zeolite mineral clinoptililolite was used to determine whether reductions in toxicity were attributable to ammonia removal. Removing ammonia from the effluent was predicted to reduce effluent toxicity. The total ammonia concentration was reduced from 21.8 mg/L in untreated effluent to 2.8mg/L following treatment. Survival of *Menidia* exposed to undiluted, untreated effluent for 72 hours was 27%. There was no mortality in effluent treated with clinoptililolite for the same time period.

In addition to ammonia, zeolite minerals remove other cations, including metals. Table IV lists concentrations before and after zeolite treatment. The concentrations of copper and zinc were reduced 43% and 75%, respectively, following zeolite treatment. The concentrations of aluminum and iron increased ten- and six-fold, respectively, following zeolite treatment. The concentrations of other metals were unaffected by zeolite treatment.

Table IV. Metals reductions from zeolite treatment.

Metal	µg/L Baseline	µg/L Zeolite	CMC[1] µg/L
Ag	<3	<3	7.2
Al	80	8,200	750
As	<60	<60	69
Cd	<3	<3	43
Cr	<6	6	10,300 (III) 1,100 (VI)
Cu	7	4	2.9
Fe	560	3,200	NA[2]
Hg	<10	<10	2.1
Mn	110	110	NA
Ni	20	20	140
Pb	<30	<30	140
Se	<70	<70	410
Zn	80	20	95

1 References for water quality criteria may be found in References section of text.
2 Not Available

EDTA

The draft EPA Phase I TIE SOP for saltwater species (Burgess, 1992) was utilized to determine the effect of EDTA on effluent toxicity to *Menidia*. There was a slight reduction in the toxicity of undiluted effluent due to EDTA addition at 48 hours, with complete

mortality in both treatments by 72 hours. At lower effluent concentrations EDTA caused a slight reduction in toxicity compared to baseline.

CO_2 SEALED HEADSPACE

Menidia baseline toxicity tests under CO_2 sealed headspace conditions as described by Mount and Mount (1992) were initiated unsuccessfully on two occasions. Results were inconclusive because of excessive control mortality. Final dissolved oxygen measurements in chambers that had been sealed with a CO_2-enriched headspace indicated that control mortality was attributable to oxygen depletion (consistently <4.0 mg/L dissolved oxygen).

MENIDIA DISCUSSION

Dissolved oxygen

The District has conducted this acute TIE to try to determine causes of the occasional toxic response of *Menidia* observed during the 1989-90 Effluent Toxicity Characterization Program. A review of the water quality data from that program reveals that low dissolved oxygen levels may have contributed significantly to mortality of fish exposed to undiluted effluent. Those tests in which dissolved oxygen (DO) was measured at the end of each 24 hour exposure period, 12 of 13 tests had at least one DO measurement below the acceptable level of 4.0 mg/L (EPA/600/4-90/027). In 11 of those 13 tests, mortality was >10%; in 8 of the 13 tests, mortality was greater than 30%. On just one occasion there was significant mortality (93%) with no DO excursions below 4.0 mg/L. On one other occasion there were DO measurements below 4.0 mg/L and only 6.7% mortality.

Ammonia

The anion exchange resin reduced toxicity below baseline levels in the 10/26 test, but not in the 1/4 test. As described previously, the anion exchange procedure involves increasing the pH of the effluent to >10.0 and stirring for 18-24 hours. This treatment would be expected to drive off ammonia, approximately 80% of which is in the volatile, unionized form at pH 10.0 (EPA 440/5-85-001). Initial water quality measurements for the two tests showed total ammonia levels of 37.1mg/L and 19.7mg/L for the 10/26/92 and 1/4/93 tests, respectively. It is presumed that the substantial differences in ammonia concentrations account for the greater toxicity in the baseline test on 10/26 as well as greater toxicity reduction by the anion exchange resin for that same test.

Reduction of toxicity by the cation exchange resin indicates that toxicity is attributable to ammonia. Effluent pH during this treatment is reduced to <3.0 so that all ammonia would be in the ionized, NH_4^+, form and be effectively removed by the resin.

As described above, zeolite reduced effluent total ammonia from 21.8 mg/L to 2.8 mg/L. Survival in undiluted effluent improved dramatically with the reduction in ammonia. This strongly indicates that ammonia is the major source of toxicity to *Menidia* in the District's effluent.

Metals

The zeolite, cation exchange resin and EDTA may have reduced toxicity by removing metals from the effluent. While the zeolite and cation exchange resin results do not identify specific metals, the slight reduction in toxicity resulting from EDTA addition indicates that effluent toxicity may be attributable to cadmium, copper, mercury, zinc, manganese, lead or nickel (Norberg-King, 1992). A discussion of each of these metals is warranted. Copper also needs to be addressed in the context of the zeolite test because that metal was present above the CMC level before treatment.

Cadmium, zinc, manganese and lead were not present in the District's effluent above CMC levels for any of the samples included in Table III. EPA reports (EPA 440/5-86-001) acute values (i.e. LC50's) of cadmium for saltwater fishes ranging from 577 μg/L to 114,000 μg/L. For zinc the LC50 values for saltwater species (based on data for 26 invertebrates and 7 fishes) range from 191.5 μg/L to 320,400 μg/L (EPA 440/5-86-001, 1987 update). For *Menidia beryllina* the EPA reports an LC50 for lead of >3,140 μg/L (EPA 440/5-84-027). There are no water quality criteria for manganese for the protection of aquatic life. Because the measured concentrations of these metals in the District's effluent are far below CMC criteria their contribution to the acute toxic response of *Menidia* is predicted to be negligible.

For copper the CMC for saltwater species is 2.9 μg/L (EPA 440/5-84-031). In at least 6 of the 7 samples presented in Table III (including samples which were subsequently treated with zeolite or EDTA) copper was present in the effluent at concentrations above the CMC level. However there is evidence that *Menidia* are considerably less sensitive to copper than other biota and the CMC value may not be very relevant. First, zeolite treatment did not reduce the copper concentration below the CMC (7 μg/L before treatment; 4 μg/L after), but toxicity was reduced by the zeolite. Second, data from the District's Effluent Toxicity Characterization Study show an average 7-day LC50 for copper of 98.6 μg/L, based on nominal reference toxicant concentrations. Finally, other laboratory test results for the related species, *Menidia menidia*, also demonstrate substantially less sensitive responses, with LC50 values ranging from 66.6 - 216.5 μg/L (EPA 440/5-84-031). Based on these data, the levels of copper in the District's effluent would not be expected to be acutely toxic to *Menidia beryllina*.

The acute criterion for mercury of 2.1 μg/L (EPA 440/5-84-026) is below the detection limit (10 μg/L) achievable by the District's Inductively Coupled Plasma (ICP) method. Acute effects in saltwater fishes have been observed in exposures of mercury ranging from 36 μg/L to 1,678 μg/L (EPA 440/5-84-026). These concentrations are much higher than those measured in the District's effluent (Table III). It is unlikely that observed toxic effects are attributable to mercury.

Nickel levels in untreated effluent for all of the samples presented in Table III were well below the CMC of 140 μg/L (EPA/440/5-86-001). Additionally, the concentration of nickel was not reduced by zeolite treatment (Table IV), but there was a dramatic decrease in toxicity. This indicates that nickel was not the cause of acute toxicity.

Organics

Toxicity was not removed by XAD resins in either the 10/26/92 or the 1/4/93 test events. This indicates that organic constituents are not contributing to acute toxicity of the District's effluent to *Menidia*.

CERIODAPHNIA AND *MENIDIA* SUMMARY

No TIE manipulation removed all baseline toxicity observed in tests with *Ceriodaphnia* (with the exception of one CO_2 headspace treatment on 8/10) and *Menidia*. Presumably some toxicant, or group of toxicants, other than what has been identified in this evaluation is contributing to acute toxicity.

There are two possible explanations for the remaining toxicity. First is simply that TIE methods are not refined enough to discern all possible sources of toxicity.

Second, and most likely, toxicity tests performed in a TIE differ from the way tests were conducted during the Effluent Toxicity Characterization Study (ETCS). For this TIE the *Ceriodaphnia* tests were performed under static conditions (with two exceptions, noted in Appendix A). During the ETCS *Ceriodaphnia* tests were performed under static renewal conditions with fresh effluent samples collected daily. The TIE *Menidia* tests were performed under static-renewal conditions, with renewals made from a single effluent sample collected and manipulated on the first day. Again, this test method differs dramatically from the static-renewal methods used during the Effluent Toxicity Characterization Study in which effluent was collected daily.

While every source of toxicity in the District's effluent cannot be identified, evidence has been presented which supports the following conclusions:

1. Ammonia is a constituent in the District's effluent that regularly contributes to observed acute toxicity for *Ceriodaphnia dubia* and is the major toxicant for *Menidia beryllina*.

2. Silver may contribute to the acute toxic response of *Ceriodaphnia* to the District's effluent; there is no evidence that other metals contribute to this acute toxic response. The toxic response of *Menidia* to the District's effluent is not attributable to metals.

3. Changing, dissolved inorganic constituents (Cl^- in particular) are possibly contributing to the toxicity of the District's effluent to *Ceriodaphnia*. For *Menidia* there may be

inorganic constituents other than ammonia contributing to acute toxicity, but those constituents are unidentified.

4. Acute toxicity of the District's effluent to *Ceriodaphnia* and *Menidia* is not caused by organic constituents.

5. Other factors (e.g. low dissolved oxygen) may have heightened sensitivity of *Menidia* to other toxicants by stressing the organisms.

APPENDIX A.1: BIOLOGICAL RESPONSES OF *CERIODAPHNIA DUBIA* TO EPA PHASE I PROCEDURES TEST START DATE: 3/30/92

BASELINE - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	90	50	30	10
80%	100	100	50	10
64%	90	80	80	40
51%	91	91	91	91
0%	100	100	100	100

PH MANIPULATION, PH 3.0 - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	100	100	100	0
80%	100	100	100	50
64%	100	100	100	100
51%	100	100	100	100

PH MANIPULATION, PH 11.0 - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	100	100	100	60
80%	100	100	100	100
64%	100	100	100	100
51%	100	100	100	100

APPENDIX A.1 (cont.): BIOLOGICAL RESPONSES OF *CERIODAPHNIA DUBIA* TO EPA PHASE I PROCEDURES
TEST START DATE: 3/30/92

FILTRATION, PH_i - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	100	0	0	0
80%	100	100	20	0
64%	100	100	100	100
51%	100	100	100	100
BLANK	100	100	100	100

FILTRATION, PH 3.0 - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	100	100	80	80
80%	100	100	100	100
64%	100	100	100	100
51%	100	100	100	100
BLANK	40	20	20	0

FILTRATION, PH 11.0 - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	100	100	100	60
80%	100	100	100	100
64%	100	100	100	100
51%	100	100	100	100
BLANK	100	100	100	100

APPENDIX A.1: BIOLOGICAL RESPONSES OF *CERIODAPHNIA DUBIA* TO EPA PHASE I PROCEDURES
TEST START DATE: 3/30/92

AERATION, PH_i - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	100	100	80	0
80%	100	100	100	80
64%	100	100	100	100
51%	100	100	100	100
BLANK	100	100	100	80

AERATION, PH 3.0 - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	40	0	0	0
80%	80	0	0	0
64%	0	0	0	0
51%	40	0	0	0
BLANK	0	0	0	0

AERATION, PH 11.0 - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	100	100	100	100
80%	100	100	100	100
64%	100	100	100	100
51%	100	100	100	100
BLANK	100	100	100	80

APPENDIX A.1: BIOLOGICAL RESPONSES OF *CERIODAPHNIA DUBIA* TO EPA PHASE I PROCEDURES
TEST START DATE: 3/30/92

C18, PH_i, 25ML THROUGH COLUMN - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	100	86	71	28
80%	100	100	100	100
64%	100	100	100	100
51%	100	100	100	100
BLANK	100	100	100	100

C18, PH_i, 65ML THROUGH COLUMN - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	100	100	100	100
80%	100	100	100	100
64%	100	100	80	80
51%	100	100	100	100
BLANK	100	100	100	100

C18, PH 3.0, 25ML THROUGH COLUMN - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	100	100	100	100
80%	100	100	100	100
64%	100	100	100	100
51%	100	100	100	100
BLANK	40	0	0	0

APPENDIX A.1: BIOLOGICAL RESPONSES OF *CERIODAPHNIA DUBIA* TO EPA PHASE I PROCEDURES
TEST START DATE: 3/30/92

C18, PH 3.0, 150ML THROUGH COLUMN - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	100	100	100	100
80%	100	100	100	100
64%	100	100	100	100
51%	100	100	100	100
BLANK	40	0	0	0

GRADUATED PH 6.5 - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
6.5	100	80	0	0
7.5	80	0	0	0
8.5	0	0	0	0

EDTA - % SURVIVAL

CONC.	2HR	24HR	48HR	72HR
0.8ML	100	20	0	0
0.6ML	100	100	100	0
0.4ML	100	80	20	0
0.2ML	100	80	20	0

APPENDIX A.1: BIOLOGICAL RESPONSES OF *CERIODAPHNIA DUBIA* TO EPA PHASE I PROCEDURES
TEST START DATE: 3/30/92

THIOSULFATE - % SURVIVAL

CONC.	2HR	24HR	48HR	72HR
0.8ML	100	100	40	0
0.6ML	100	100	60	20
0.4ML	100	100	100	60
0.2ML	100	60	40	0

APPENDIX A.2: BIOLOGICAL RESPONSES OF *CERIODAPHNIA DUBIA* TO EPA PHASE I PROCEDURES
TEST START DATE: 4/16/92

BASELINE - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	90	70	50	50
80%	100	100	90	40
64%	100	100	100	90
51%	100	100	100	90
0%	100	100	100	100

PH MANIPULATION, PH 3.0 - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	100	100	100	80
80%	100	100	100	100
64%	100	100	100	100

PH MANIPULATION, PH 11.0 - % SURVIVAL

CONC.	2HR	24HR	48HR	72HR
100%	100	100	100	100
80%	100	100	100	100
64%	100	100		100

APPENDIX A.2: BIOLOGICAL RESPONSES OF *CERIODAPHNIA DUBIA* TO EPA PHASE I PROCEDURES
TEST START DATE: 4/16/92

FILTRATION, PH_i - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	100	80	0	0
80%	100	100	60	40
64%	100	100	60	60
51%	100	100	100	100
BLANK	100	100	100	100

FILTRATION, PH 3.0 - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	100	100	80	80
80%	100	100	100	100
64%	100	100	100	100
51%	100	100	100	100
BLANK	100	100	100	100

FILTRATION, PH 11.0 - % SURVIVAL

CONC.	2HR	24HR	48HR	72HR
100%	100	100	100	100
80%	100	100	100	100
64%	100	100	100	100
51%	100	100	100	100
BLANK	100	100	100	100

APPENDIX A.2: BIOLOGICAL RESPONSES OF *CERIODAPHNIA DUBIA* TO EPA PHASE I PROCEDURES
TEST START DATE: 4/16/92

AERATION, PH_i - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	100	100	100	100
80%	100	100	100	100
64%	100	100	100	100
51%	100	100	100	100
BLANK	100	100	100	100

AERATION, PH 3.0 - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	100	100	100	100
80%	100	100	100	100
64%	100	100	100	100
51%	100	100	100	100
BLANK	80	60	60	60

AERATION, PH 11.0 - % SURVIVAL

CONC.	2HR	24HR	48HR	72HR
100%	100	100	40	40
80%	100	100	100	100
64%	100	100	80	80
51%	100	100	80	80
BLANK	100	100	100	80

APPENDIX A.2: BIOLOGICAL RESPONSES OF *CERIODAPHNIA DUBIA* TO EPA PHASE I PROCEDURES
TEST START DATE: 4/16/92

AERATION, PH_i - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	100	100	100	100
80%	100	100	100	100
64%	100	100	100	100
51%	100	100	100	100
BLANK	100	100	100	100

AERATION, PH 3.0 - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	100	100	100	100
80%	100	100	100	100
64%	100	100	100	100
51%	100	100	100	100
BLANK	80	60	60	60

AERATION, PH 11.0 - % SURVIVAL

CONC.	2HR	24HR	48HR	72HR
100%	100	100	40	40
80%	100	100	100	100
64%	100	100	80	80
51%	100	100	80	80
BLANK	100	100	100	80

APPENDIX A.2: BIOLOGICAL RESPONSES OF *CERIODAPHNIA DUBIA* TO EPA PHASE I PROCEDURES
TEST START DATE: 4/16/92

C18, PH_i, 25ML THROUGH COLUMN - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	100	100	80	80
80%	100	100	100	80
64%	100	100	100	100
51%	100	100	100	100
BLANK	100	100	80	80

C18, PH_i, 150ML THROUGH COLUMN - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	100	100	100	100
80%	100	100	100	100
64%	100	100	100	100
51%	100	100	100	100
BLANK	100	100	80	80

C18, PH 3.0, 25ML THROUGH COLUMN - % SURVIVAL

CONC.	2HR	24HR	48HR	72HR
100%	100	100	100	100
80%	100	100	100	100
64%	100	100	100	100
51%	100	100	80	80
BLANK	100	100	100	100

APPENDIX A.2: BIOLOGICAL RESPONSES OF *CERIODAPHNIA DUBIA* TO EPA PHASE I PROCEDURES
TEST START DATE: 4/16/92

C18 PH 11.0, 25ML THROUGH COLUMN - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	100	100	100	100
80%	100	100	100	100
64%	100	100	100	100
51%	100	100	100	100
BLANK	100	100	100	100

C18, PH 11.0, 150ML THROUGH COLUMN - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	100	100	100	100
80%	100	100	100	100
64%	100	100	80	80
51%	100	100	100	80
BLANK	100	100	100	100

APPENDIX A.3: BIOLOGICAL RESPONSES OF *CERIODAPHNIA DUBIA* TO EPA PHASE I PROCEDURES
TEST START DATE: 4/29/92

BASELINE - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	85	15	5	5
80%	90	40	15	5
64%	100	95	52	24
0%	95	95	95	95

PH MANIPULATION, PH 3.0 - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	20	7	0	0
80%	93	0	0	0
64%	100	60	40	27

PH MANIPULATION, PH 11.0 - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	47	0	0	0
80%	73	0	0	0
64%	100	0	0	0

FILTRATION, PH_i - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	100	27	13	13
80%	100	93	40	20
64%	100	93	73	60
BLANK	100	100	100	100

GRADUATED PH - % SURVIVAL

CONC.	2HR	24HR	48HR	72HR
6.5	100	100	100	100
7.5	100	100	100	80
8.5	0	0	0	0

APPENDIX A.2: BIOLOGICAL RESPONSES OF *CERIODAPHNIA DUBIA* TO EPA PHASE I PROCEDURES
TEST START DATE: 4/16/92

THIOSULFATE - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
0.6ML	100	100	100	0
0.4ML	100	100	100	100
0.2ML	100	80	80	80
0.1ML	100	100	100	100
0.05	100	100	80	0

EDTA - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
0.6ML	80	80	80	80
0.4ML	100	100	80	60
0.2ML	100	100	60	0

APPENDIX A.3: BIOLOGICAL RESPONSES OF *CERIODAPHNIA DUBIA* TO EPA PHASE I PROCEDURES
TEST START DATE: 4/29/92

FILTRATION, PH 3.0 - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	100	100	100	93
80%	100	100	100	100
64%	93	93	93	93
BLANK	100	100	100	78

FILTRATION, PH 11.0 - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	93	60	27	0
80%	100	80	53	13
64%	100	100	100	100
BLANK	100	100	93	93

AERATION, PH_i - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	100	100	100	100
80%	100	100	100	100
64%	100	100	100	60
BLANK	100	100	80	20

AERATION, PH 3.0 - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	100	100	100	100
80%	100	100	100	100
64%	100	100	100	100
BLANK	80	80	80	60

APPENDIX A.3: BIOLOGICAL RESPONSES OF *CERIODAPHNIA DUBIA* TO EPA PHASE I PROCEDURES
TEST START DATE: 4/29/92

AERRATION, PH 11.0 - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	80	40	10	0
80%	100	100	100	80
64%	100	100	100	100
BLANK	0	0	0	0

GRADUATED PH - % SURVIVAL

CONC.	24HR	48HR	72HR
6.5	100	93	0
7.5	100	27	0
8.5	0	0	0

THIOSULFATE - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
0.6ML	92	62	31	0
0.4ML	100	80	73	20
0.2ML	100	93	0	0

EDTA - % SURVIVAL

CONC.	24HR	48HR	72HR
0.6ML	73	33	0
0.4ML	87	27	0
0.2ML	94	12	0

APPENDIX A.4: BIOLOGICAL RESPONSES OF *CERIODAPHNIA DUBIA*
CONFIRMATORY TESTS
TEST START DATE: 4/22/92

METHANOL ELUATES FROM 3/30/92 C18, PH$_i$ EFFLUENT - % SURVIVAL

MEOH%	24HR	48HR	72HR	96HR
100	100	100	100	100
95	100	100	100	100
90	100	100	100	100
85	100	100	100	100
80	100	100	100	100
75	100	100	100	100
50	100	100	100	100
25	100	100	100	100
MEOH CONT	100	100	100	100
CONT	100	100	73	67

METHANOL ELUATES FROM 4/16/92 C18, PH_i EFFLUENT - % SURVIVAL

MEOH%	24HR	48HR	72HR	96HR
100	100	100	100	100
95	100	100	100	100
90	100	100	100	100
85	100	100	100	100
80	100	100	100	100
75	100	100	100	100
50	100	100	100	100
25	100	100	100	100
MEOH CONT	100	100	100	100
CONT	100	100	73	67

APPENDIX A.5: BIOLOGICAL RESPONSES OF *CERIODAPHNIA DUBIA*
CONFIRMATORY TESTS
TEST START DATE: 6/9/92
STATIC-RENEWAL, DAILY RENEWAL, 100% EFFLUENT

TOTAL AMMONIA RANGE: 31.9 - 33.3 mg/L

CO_2 IN SEALED VIAL vs AIR IN SEALED VIAL vs OPEN CUP - % SURVIVAL

TREAT	24HR	48HR	72HR	UNIONIZED AMM RANGE, MG/L[1]
$C0_2$	80	80	80	0.04-0.14
AIR	93	87	60	0.31-0.42
OPEN	67	53	8.2	1.5-1.6
CO_2 CONT	100	100	100	NA
AIR CONT	100	100	100	NA
CONT	100	100	100	NA

1 estimated from tables compiled by H.P. Skarheim, June 1973, SERL Report No. 73-5, using measured conductivity, temperature, total ammonia and final pH values (pH values for replicates averaged)

APPENDIX A.6: BIOLOGICAL RESPONSES OF *CERIODAPHNIA DUBIA*
CONFIRMATORY TESTS
TEST START DATE: 6/23/92
STATIC-RENEWAL, DAILY RENEWAL, 100% EFFLUENT

TOTAL AMMONIA RANGE: 24.4 - 26.0 mg/L

CO_2 IN SEALED VIAL vs AIR IN SEALED VIAL vs OPEN CUP; AMMONIA SPIKES - % SURVIVAL

TREAT	24HR	48HR	72HR	96HR	UNIONIZED AMM RANGE, MG/L[1]
$C0_2$	93	87	53	0	0.02-0.03
OPEN	93	80	19	0	1.02-1.35

NH3, PH 6	100	100	100	100	0.69-0.73
NH3, PH 8	100	94	94	94	1.07-1.14
CO_2 CONT	100	100	100	100	NA
CONT	100	100	100	100	NA

1 estimated from tables compiled by H.P. Skarheim, June 1973, SERL Report No. 73-5, using measured conductivity, temperature, total ammonia and final pH values (pH values for replicates averaged)

APPENDIX A.7: BIOLOGICAL RESPONSES OF *CERIODAPHNIA DUBIA* CONFIRMATORY TESTS
TEST START DATE: 8/10/92

TOTAL AMMONIA: 35.1 mg/L

XAD2; XAD2 ELUATE - % SURVIVAL

TREAT	24HR	48HR	72HR	96HR	UNIONIZED AMM, MG/L[1]
BASE-LINE	73	0	0	0	2.26
CO_2	100	93	93	93	0.05
XAD2	100	0	0	0	2.80
XAD2 ELU.	93	93	93	93	NA
CONT.	100	100	100	100	NA
CO_2 CONT.	NA	NA	NA	NA	NA
XAD2 CONT.	100	100	100	100	NA
XAD2 ELU. CONT.	60	60	60	60	NA

1 estimated from tables compiled by H.P. Skarheim, June 1973, SERL Report No. 73-5, using measured conductivity, temperature, total ammonia and final pH values (pH values for replicates averaged)

APPENDIX A.8: BIOLOGICAL RESPONSES OF *CERIODAPHNIA DUBIA*
CONFIRMATORY TESTS
TEST START DATE: 10/27/92

TOTAL AMMONIA: 37.1 mg/L

XAD2; ANION EXCHANGE; CATION EXCHANGE - % SURVIVAL

TREATMENT	24HR	48HR	UNIONIZED AMM, MG/L[1]
BASELINE	100	47	2.39
CO_2	100	78	0.04
XAD2	93	7	2.39
ANION	100	73	NA
CATION	13	0	NA
CONTROL	100	100	NA
CO_2 CONTROL	NA	NA	NA
ANION CONTROL	100	100	NA
CATION CONTROL	100	80	NA

1 estimated from tables compiled by H.P. Skarheim, June 1973, SERL Report No. 73-5, using measured conductivity, temperature, total ammonia and final pH values (pH values for replicates averaged)

APPENDIX A.9: BIOLOGICAL RESPONSES OF *CERIODAPHNIA DUBIA* CONFIRMATORY TESTS

TEST START DATE: 2/1/93

TOTAL AMMONIA BEFORE TREATMENT: 21.8 mg/L

TOTAL AMMONIA AFTER TREATMENT: 2.8 mg/L

ZEOLITE - % SURVIVAL

TREATMENT	24HR	48HR	72HR	96HR	UNIONIZED AMM, MG/L[1]
BASELINE	20	0	0	0	1.74
ZEOLITE	100	100	90	90	NA
CONTROL	100	60	0	0	NA
ZEOLITE CONTROL	100	10	0	0	NA

APPENDIX B.2: RELATIONSHIPS BETWEEN PH AND LC50 VALUES FOR NH_3 IN FRESHWATER FOR CLADOCERANS

48-HOUR UNDISSOCIATED AMMONIA LC50'S FOR CLADOCERANS, mg/L[1]

LC50	PH	LC50 NORMALIZED TO PH 8.0	LOG OF NORMALIZED PH
0.77	7.06	1.96	0.293
2.08	8.2	2.08	0.318
2.45	7.95	2.51	0.400
2.69	8.07	2.69	0.430
2.50	8.09	2.50	0.398
2.77	8.15	2.77	0.442
2.38	8.04	2.38	0.376
0.75	7.51	1.07	0.028
0.90	7.53	1.25	0.098
0.53	7.40	0.85	-0.072
0.67	7.50	0.96	-0.017
4.94	8.58	4.94	0.694
1.16	8.1	1.16	0.064
0.613	7.06	1.56	0.194

MEAN OF LOGS = 0.260
ANTILOG OF MEAN = 1.822
GEOMETRIC MEAN = 1.822

1 From data presented in EPA 440/5-85-001

TEST START DATE: 3/9/93

TOTAL AMMONIA: 26.9 mg/L

EDTA STOCK: 4.1 g/L

EDTA - % SURVIVAL

TREATMENT	24HR	48HR	72HR	96HR	UNIONIZED AMM, MG/L[1]
BASELINE	0	0	0	0	2.15
0.4 ML	0	0	0	0	1.74
0.6 ML	0	0	0	0	1.12
CONTROL	100	100	100	100	NA
0.4 ML CONTROL	100	100	100	100	NA
0.6 ML CONTROL	100	100	100	100	NA

1 estimated from tables compiled by H.P. Skarheim, June 1973, SERL Report No. 73-5, using measured conductivity, temperature, total ammonia and final pH values (pH values for replicates averaged)

APPENDIX B.2: RELATIONSHIPS BETWEEN PH AND LC50 VALUES FOR NH_3 IN FRESHWATER FOR CLADOCERANS

TABLE OF LC50's FOR NH_3 AT DIFFERENT PH VALUES BASED ON GEOMETRIC MEAN LC50 OF 1.82mg/L AT PH 8.0

PH	LC50
6.6	0.31
6.7	0.38
6.8	0.46
6.9	0.55
7.0	0.65
7.1	0.76
7.2	0.88
7.3	1.01
7.4	1.14
7.5	1.27
7.6	1.39
7.7	1.52
7.8	1.63
7.9	1.73
$\geq$8.0	1.82

Derived from the following relationships (EPA 440/5-85-001):

$$LC50 = LC50(pH=8) \qquad ; pH \geq 8$$

$$LC50 = \frac{LC50(pH=8) * 1.25}{1 + 10^{7.4-pH}} \qquad ; pH < 8$$

APPENDIX C.1: BIOLOGICAL RESPONSES OF *MENIDIA BERYLLINA* TO TIE PROCEDURES
TEST START DATE: 10/26/92

XAD2; ANION EXCHANGE; CATION EXCHANGE

TOTAL AMMONIA: 37.1 mg/L

BASELINE - % SURVIVAL

CONC.	24HR	48HR	72HR	UNIONIZED AMM RANGE, MG/L[1]
100%	23	8	0	0.05-1.51
80%	83	42	0	NA
64%	77	38	8	NA
CONTROL	100	100	67	NA

1 estimated from tables compoiled by H.P. Skarheim, June 1973, SERL Report No. 73-5, using measured conductivity, temperature, total ammonia and pH values (range derived from lowest initial pH to highest final pH in udiluted effluent)

XAD2 - % SURVIVAL

CONC.	24HR	48HR	72HR
100%	33	8	0
80%	92	17	8
64%	75	50	25
CONTROL	92	83	75

ANION EXCHANGE - % SURVIVAL

CONC.	24HR	48HR	72HR
100%	75	25	17
80%	85	31	15
64%	75	25	25
CONTROL	NA	NA	NA

APPENDIX C.: BIOLOGICAL RESPONSES OF *MENIDIA BERYLLINA* TO TIE PROCEDURES
TEST START DATE: 10/26/92

XAD2; ANION EXCHANGE; CATION EXCHANGE

CATION EXCHANGE - % SURVIVAL

CONC.	24HR	48HR	72HR
100%	90	80	30
80%	92	92	67
64%	73	73	46
CONTROL	100	100	100

APPENDIX C.2: BIOLOGICAL RESPONSES OF *MENIDIA BERYLLINA* TO TIE PROCEDURES
TEST START DATE: 1/4/93

XAD4; ANION EXCHANGE; CATION EXCHANGE

TOTAL AMMONIA: 19.7 mg/L

BASELINE - % SURVIVAL

CONC.	24HR	48HR	72HR	UNIONIZED AMM RANGE, MG/L[1]
100%	93	60	20	0.07-0.51
80%	100	94	62	NA
64%	93	80	40	NA
CONTROL	93	87	87	NA

1 estimated from tables compoiled by H.P. Skarheim, June 1973, SERL Report No. 73-5, using measured conductivity, temperature, total ammonia and pH values (range derived from lowest initial pH to highest final pH in udiluted effluent)

APPENDIX C.4: BIOLOGICAL RESPONSES OF *MENIDIA BERYLLINA* TO TIE PROCEDURES
TEST START DATE: 3/8/93

EDTA

TOTAL AMMONIA: 26.9 mg/L

BASELINE - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR	UNIONIZED AMM RANGE, MG/L[1]
100%	80	7	0	0	0.18-0.88
80%	100	93	20	0	NA
64%	100	100	47	7	NA
CONTROL	100	100	100	100	NA

1 estimated from tables compoiled by H.P. Skarheim, June 1973, SERL Report No. 73-5, using measured conductivity, temperature, total ammonia and pH values (range derived from lowest initial pH to highest final pH in udiluted effluent)

EDTA - % SURVIVAL

CONC.	24HR	48HR	72HR	96HR
100%	100	53	0	0
80%	100	100	60	13
64%	100	100	67	47
CONTROL	100	100	100	80

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IDENTIFICATION AND CONTROL OF ACUTE AMMONIA TOXICITY OF A POTW EFFLUENT USING A CARBON DIOXIDE-ENRICHED ATMOSPHERE

Based on results of the Effluent Variability Test Program, the City and County of San Francisco was required to conduct a Toxicity Identification Evaluation (TIE) to determine the cause(s) of acute toxicity to *Menidia beryllina* larvae. Standard TIE procedures of pH adjustment, aeration, filtration, EDTA additions, as well as fractionation procedures, produced no consistent significant increase in survival relative to baseline tests. Zeolite experiments indicated that effluent ammonia levels, combined with a pH rise under static test conditions, were strongly correlated with test organism survival. Effluent pH ranges from 6.6 to 6.9 when discharged, but drifts upward to $\geq$8.2 within 24 hours under static test conditions, greatly increasing the toxic proportion of unionized ammonia. A CO_2-enriched atmosphere was used to control pH by introducing CO_2 into the airspace above effluent in 300 mL BOD bottles, giving final headspace concentrations of 1, 2.5, and 5 % CO_2. Measurements taken at 96-hr showed little increase in pH, and slight or no increase in total ammonia concentration. Mean survival in CO_2 containers was 78% while concurrent baseline tests had high pH and complete mortality. These results indicated that the acute toxicity observed in static or static-renewal test containers was an artifactual effect of a rise in effluent pH and the associated increase in the proportion of unionized ammonia.

INTRODUCTION

The San Francisco Bay Regional Water Quality Control Board (RWQCB) required the City and County of San Francisco (CCSF) to conduct a Toxicity Identification Evaluation (TIE) on wastewater effluent from the Southeast Water Pollution Control Plant (SEWPCP) to determine the cause of acute toxicity to the inland silversides *Menidia beryllina*. This requirement was based on test results of CCSF's Effluent Variability Test Program (MEC Analytical Systems, May,

1992). Exposure of *Menidia beryllina* to undiluted final effluent during chronic testing resulted in >30% mortality within 96 hours in six of the nine samples tested, exceeding acute toxicity limits specified in the San Francisco Bay Basin Plan.

A TIE Study Plan was submitted and approved, and testing was initiated with *Menidia beryllina* in November 1992. Eight testing events were conducted between November 1992 and March 1993. Tests 1 through 4 were standard 96-hour acute tests to verify that acute toxicity was present. During this period tests were also conducted to establish tolerance levels of *Menidia* to various TIE chemicals and procedures. Tests 5 through 8 were conducted using acute TIE procedures, chemical fractionation, and CO_2 sealed headspace procedures.

The testing period coincided with a significant wet weather season. Because CCSF operates a combined sewer system, final effluent samples had variable water quality. During wet weather events, plant flow is greatly increased, primary treated effluent may be bypassed into the final effluent flow, and measured total ammonia concentrations may be approximately half the average. Precipitation, plant flow, and total ammonia concentrations for each sampling event are reported in the appendix.

TEST DESCRIPTIONS

Menidia larvae (7 to 9 day old) were delivered the morning of each test. All effluent tests were conducted at 25 ppt salinity, using *Artificial Oceans* sea salt. Control water was distilled water salted up to 25 ppt with *Artificial Oceans* sea salt. Reference toxicant tests with copper sulfate ($CuSO_4$) were conducted with each cohort of test organisms. Test solution volume was 500 mL for control and effluent dilutions (acute and baseline tests) and 100 mL for TIE and CO_2 test solutions, reference toxicant test dilutions, and an additional set of 100% effluent replicates. Three replicates for each treatment were used, with ten *Menidia* larvae added to each test container. Baseline tests and all TIE manipulations were conducted simultaneously.

Tests were conducted with 24-hour composite samples of final effluent from the Southeast WPCP. To avoid problems associated with changes in toxicity over time, tests were initiated within 6 hours of collection, except Tests 5 and 8 which were initiated 24 hours after sample collection due to late delivery of test organisms. Sample handling, storage, and preservation conformed to techniques approved by the EPA and the Regional Water Quality Control Board (U.S. EPA 1991b; RWQCB 1991). The following water quality measurements were conducted on effluent samples upon delivery to the laboratory: temperature, pH, dissolved oxygen, hardness, alkalinity, conductivity, total residual chlorine, and total ammonia. Final effluent water quality measurements for each test event are listed in the appendix.

Because there are no approved TIE procedures for use with *Menidia*, initial tests were performed to establish appropriate techniques and to establish tolerance levels to various TIE chemicals. Range finding tests using control water with EDTA, acid/base, and zeolite additions to determine organism sensitivity levels were conducted (U.S. EPA 1991a). Once these tolerance levels were established, these TIE chemicals were added to final effluent samples in an attempt to selectively remove suspected toxicants. A draft copy of U.S. EPA Environmental Research Laboratory

(EPA-ERL)-Narraganset Standard Operating Procedure (SOP) for Marine Phase I TIEs (Burgess 1992) provided limited guidance for these procedures.

RESULTS AND DISCUSSION

96-Hour Acute/Baseline Tests

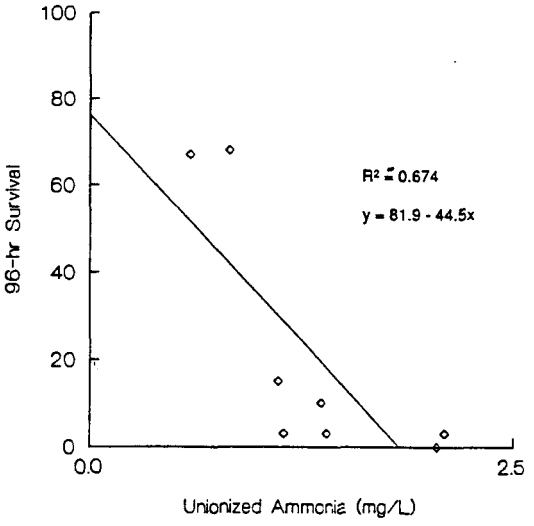
Standard 96-hour acute tests were conducted for all eight testing events. Tests 5 through 8 provided "baseline" data for concurrent TIE manipulations that were conducted. SEWPCP effluent exhibited acute toxicity to *Menidia* in all eight tests, although survival was variable (range of 0% to 68% survival @ 96-hr). Tests 3 and 5 had 68% and 67% survival respectively, and each of these tests was conducted during significant wet weather events when total ammonia concentrations were approximately half of normal values. Test 7 was also conducted during a wet weather event, but ammonia concentrations remained at normal levels (22.2 mg/L), and 96-hour survival in effluent was only 3%. Survival data for all 96-hr acute tests are given in Table 1.

Table 1. 96-hr. acute test survival data, total ammonia amd pH measurements, and unionized ammonia calculations.

Test #	Total Ammonia (mg/L)	Initial pH	Unionized Ammonia @ Initial pH (mg/L)	Final pH	Unionized Ammonia @ Final pH (mg/L)	Mean 96-Hour Survival	
						Control	Effluent
1	31.0	6.99	0.110	7.96	1.108	85%	15%
2	29.7	7.11	0.148	8.08	1.361	70%	10%
3	11.2	6.60	0.018	8.30	0.822	95%	68%
4	28.6	7.24	0.179	8.30	2.099	97%	3%
5	11.9	7.30	0.094	8.15	0.591	97%	67%
6	22.8	6.90	0.072	8.28	1.395	100%	3%
7	22.2	6.99	0.880	8.45	1.410	77%	3%
8	23.7	6.87	0.074	8.51	2.053	80%	0%

Final water quality measurements of test solutions showed that dissolved oxygen, temperature, and salinity were always within acceptable ranges. Total ammonia levels generally remained stable or slightly decreased throughout the course of a test. The initial pH of effluent samples ranged from 6.9 to 7.3, but would drift to 8.0 or higher within 24 hours after test initiation, with an associated increase in the proportion of unionized ammonia (see Table 1). There was a strong negative correlation ($r^2 = {}^{-}0.67$) between 96-hour survival and the concentration of unionized ammonia at 96 hours (Fig. 1).

Figure 1. Linear relationship between calculated unionized ammonia concentrations and 96-hr survival data from 8 acute tests with *Menidia beryllina*.



Toxicant Identification Experiments

In tests 5 through 8, techniques were used to characterize and identify the source(s) of toxicity in the SEWPCP effluent. Mean survival rates of all TIE manipulations (excluding CO_2 experiments) are reported in Table 2.

Table 2. Summary of TIE results.

Test#	Test Type	Mean % Survival 24-hr	48-hr	72-hr	96-hr
5	**BASELINE**	**100**	**90**	**40**	**13**
	Zeolite	100	100	93	37
6	**BASELINE**	**90**	**20**	**0**	**0**
	Zeolite	100	93	27	3
	Aeration pH 3	75	70	45	5
	pH init.	100	5	0	0
	pH 11	95	35	0	0
	Filtration pH 3	90	80	25	0
	pH init.	35	10	0	0
	pH 11	100	55	0	0
	EDTA	93	87	80	60
7	**BASELINE**	**100**	**57**	**20**	**3**
	Zeolite	100	100	65	33
	Aeration pH 3	100	100	94	82
	pH init.	100	100	44	6
	pH 11	94	94	12	0
	Filtration pH 3	100	100	84	38
	pH init.	100	67	25	0
	pH 11	100	92	42	0
	EDTA	100	63	3	0
8	**BASELINE**	**100**	**50**	**3**	**0**
	Aeration pH 3	65	65	50	35
	Filtration pH 3	90	90	80	30
	Cation exchange	90	90	60	15
	Anion exchange	95	20	5	0

Zeolite Column Experiments. Zeolite experiments (U.S. EPA 1989) were conducted in Tests 5, 6, and 7. Zeolite approximates a cation exchange resin and will remove ammonia toxicity, but is not selective and may remove other chemical constituents. Four zeolite treatments were conducted by passing the test solution through a column containing a measured amount of zeolite: 1. Control water passed through a zeolite column to test for artifactual toxicity (negative controls), 2. Control water passed through a zeolite column was then spiked with ammonia to levels approximating final effluent concentrations (positive controls), 3. Effluent passed through a zeolite column, and 4. Effluent passed through a zeolite column and then spiked with ammonia back to original level.

Effluent samples and control water were salted to 25 ppt after zeolite treatment. Total ammonia measurements were taken before and after column treatment. Control water spiked with ammonia to levels equal to that of the effluent showed increased mortality only when the pH drifted to 8.0 or greater. There was no observed artifactual toxicity as measured by the negative controls. Post-column effluent showed consistent results: reduced total ammonia levels produced increased survival, but these results were often overshadowed by the effect of pH on the unionized ammonia levels in the test solutions. Final pH of the effluent test solution was always greater than 8.3. There was always complete mortality in post-column effluent that was spiked back to initial total ammonia concentrations.

EDTA Chelation. EDTA additions to final effluent were conducted in Tests 6 and 7. Survival relative to the baseline test increased from 0% to 60% in Test 6, but measured final total ammonia was also less than 1 mg/L. In Test 7, EDTA addition slightly decreased survival relative to the baseline test. In this test, the final total ammonia measurements in the EDTA test solutions were equal to the initial measurements. Measured metal concentrations in SEWPCP effluent were below levels that would be expected to be toxic to these test organisms. In addition, SEWPCP effluent has a relatively high conductivity and hardness, and it would be expected that only a portion of the total metals measured in the effluent would be bioavailable.

Aeration and Filtration (pH3, pH_i, pH11). Aeration and filtration experiments were conducted in tests 6, 7, and 8 (U.S. EPA 1991a). These procedures are probably the least selective of TIE methods, and as such the results can only be general in nature. Sample pH was adjusted to pH3, pH_i, or pH11 and either aerated in a 1 L column for 1 hour, or 0.45µm filtered, then adjusted back to initial pH and tested. There was no increase in survival in pH_i and pH11 tests, suggesting that volatile organic toxicants were not present in toxic amounts in the effluent samples. Survival relative to baseline tests was increased in all three test events for the pH3 samples. These results may indicate a component of toxicity due to hydrogen sulfide in the effluent, as sulfides would be reduced by aeration or filtration at low pH.

Organic/Inorganic Fractionation. Chemical fractionation manipulations (Walsh and Garnas 1983) were conducted in Test 8. Effluent was fractionated into organic and inorganic portions by passing through a XAD-resin column. The inorganic fraction was then subfractionated into cationic and anionic portions using ion-selective resins. Anion exchange resin selectively removes anionic chemical species from the sample. Effluent was adjusted to pH >10 and mixed for 21-hr in a flask containing a measured amount of resin, then adjusted to initial pH and tested. There

was no increase in test organism survival relative to the baseline test. Cation exchange resin selectively removes cationic chemical species from the sample. Effluent was adjusted to pH < 3 and mixed for 21-hr in a flask containing a measured amount of resin, then adjusted to initial pH and tested. An unintended effect of this procedure was that it also reduced the total ammonia concentration from 23.7 to 7.5 mg/L. There was a slight increase in survival of test organisms relative to the baseline test, but it was unclear whether this was due to removal of cationic metals or due to the removal of ammonia from the sample. The organic fraction was not tested because aeration tests indicated no evidence of toxicity due to organic chemicals.

CARBON DIOXIDE-ENRICHED ATMOSPHERE

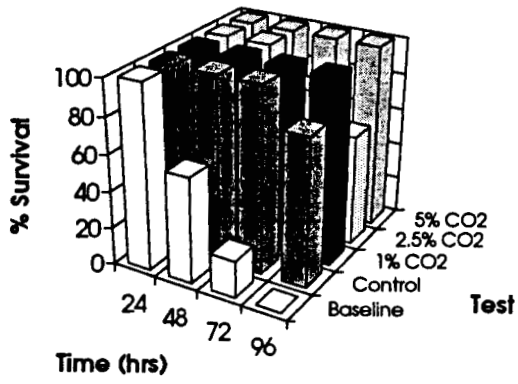
The use of CO_2 for pH control has a number of advantages over other methods such as the addition of acids and bases. Adding a strong acid or base to artificially control pH also adds the counter-ion to the solution, and where large amounts of acid or base are required, toxic artifacts may be introduced due to the toxicity of the counter-ion. Direct adjustment of pH using strong acid or base has the additional disadvantage of further disturbing the sample's existing carbonate system equilibrium. In either case, the rise in test solution pH during toxicity testing may be due to the equilibrium of CO_2 partial pressure in the effluent with that in the atmosphere. Using carbon dioxide to control pH in these tests maintains the natural conditions existing within the effluent at the discharge pH. Perhaps the greatest advantage of using CO_2 is that it uses a natural buffer system, and as such represents an ongoing control of pH rather than a temporary adjustment.

Static tests using CO_2 for control of test solution pH were conducted in tests 7 and 8. To control the pH of test solutions during the course of the experiments, closed headspace test containers with a controlled CO_2 atmosphere were utilized (Mount and Mount 1992). A 10 mL gas-tight syringe was filled with pure (99.9%) CO_2, which was then introduced into the airspace above the test solution in a 300 mL BOD bottle containing 100 mL of effluent. Carbon dioxide volumes of 2.5, 5, and 10 mL were injected into test containers, giving final headspace concentrations of 1%, 2.5%, and 5% CO_2, respectively. Two replicates of each CO_2 concentration, each with eight *Menidia*, were used in Test 7. Three replicates of each CO_2 concentration, each with ten *Menidia*, were used in Test 8. Dissolved oxygen and pH was monitored in "dummy" containers on a daily basis, with CO_2 being reintroduced each day. The headspace of opened test containers was flushed with fresh air prior to reintroducing CO_2 to avoid a build-up of CO_2 in the chamber headspace. Test containers with *Menidia* remained sealed for 96-hr because little or no mortality was observed. Final water quality measurements taken at 96-hr showed little or no increase in pH, and a slight or no increase in total ammonia concentration. Dissolved oxygen concentrations always remained greater than 6.5 mg/L in all sealed test chambers. Concurrent baseline tests were conducted as static 96-hr tests for direct comparison with CO_2 test results.

Survival in baseline effluent tests was 3% and 0% for tests 7 and 8 respectively, while mean survival in effluent CO_2 containers was 83% and 73% respectively (Figs. 2a and 2b). Mean pH measurements (Test 8) were 7.29, 7.09, and 6.85, respectively for the 1%, 2.5%, and 5% CO_2 concentrations, while mean pH of the effluent without a CO_2 atmosphere was 8.51. In a separate set of experiments, control water spiked with ammonia equivalent to effluent concentrations (30

mg/L) was tested with and without a CO_2 atmosphere. After 96 hours, there was complete mortality in the spiked samples and 95% survival in spiked samples maintained under a CO_2 atmosphere (Fig. 2c). Results indicated that the majority of observed toxicity is due to an increase in pH and the associated increase in unionized ammonia; the effluent was not altered in any way other than maintaining the initial pH of the test solution.

Figure 2a. Test 7 results.



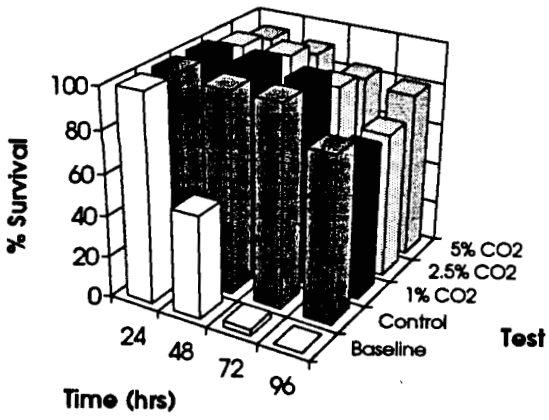


Figure 2b. Test 8 results.

Figure 2c. Control water-ammonia spike results.

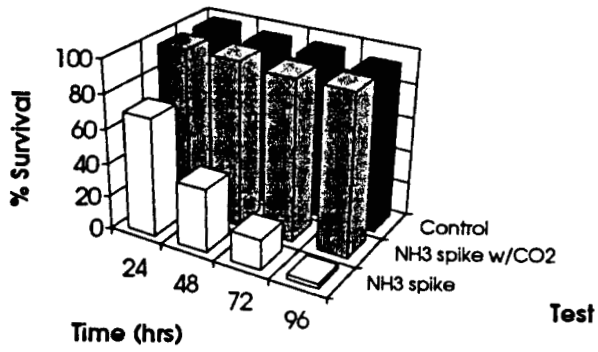


Figure 2. Survival results of carbon dioxide experiments.

CONCLUSIONS

Acute toxicity to *Menidia beryllina* larvae was observed in all eight 96-hour acute tests conducted in this study. Six different procedures were used in an attempt to identify the cause(s) of this observed toxicity. Two of these procedures, zeolite-column treatment and CO_2-enriched atmosphere experiments, indicated that the concentration of ammonia in the SEWPCP effluent, combined with a rise in pH under static test conditions, were the primary cause of the observed toxic response by this test organism. EDTA chelation and inorganic/organic fractionation experiments, combined with metals analysis of effluent samples indicated that the observed toxic response is not attributable to metals or organic compounds. Aeration and filtration procedures suggested that sulfides in the effluent may be a component of the observed toxicity, although these results were inconclusive, and may also be pH-dependent.

Total ammonia concentrations in SEWPCP effluent samples in this study averaged 22 mg/L, although dilution due to wet weather events can reduce ammonia concentrations substantially. The two tests that had the highest survival (Tests 3 and 5) were conducted during wet weather events when the ammonia levels were half the average value. At the discharge pH of 6.6-6.9, the percentage of total ammonia present as NH_3 (toxic form) is approximately an order of magnitude less than that present after 48 hours in test containers of effluent held under static conditions. This is indicated by the correlation between 96-hour survival results and calculated unionized ammonia concentrations of test solutions. Zeolite experiments corroborated these relationships and established that the removal of ammonia increases the survival of test organisms, although survival results were clearly dependent on pH. The CO_2-enriched atmosphere experiments were successful in controlling test solution pH without otherwise manipulating the effluent chemical matrix in any way. Effluent maintained under atmospheres of 1-5% CO_2 remained at or near discharge pH, and the survival of test organisms was always greater than 70% at 96 hours (the acute toxicity limit).

Where acute toxicity limitations are applied to 100% effluent, the discharge pH is the condition of interest, and static or static-renewal test methodologies do not maintain appropriate pH levels for this purpose. The total ammonia concentration of SEWPCP effluent has been shown not to be acutely toxic to *Menidia* when maintained at the discharge pH. Acute toxicity observed in static or static-renewal test containers was an artifactual effect of a rise in pH driven by the carbonate equilibrium system of the effluent, and the associated increase in the proportion of the toxic unionized form of ammonia.

The use of a CO_2-controlled atmosphere has broad applications in toxicity testing, and is particularly effective for the investigation of ammonia toxicity in municipal effluents. In toxicity testing conducted as part of toxicity identification evaluations (TIEs), manipulations of effluent pH are used to reveal the physical and chemical properties of the causative toxicants. The carbon dioxide procedure may be preferable to other methods of pH adjustment, and can be used in conjunction with the various TIE procedures to identify other pH-sensitive toxicants within the chemical matrix of the effluent.

APPENDIX

Summary of rainfall data, plant flow, and effluent water quality measurements.

Test#	Rainfall (in./24 hr.)	Plant Flow (MGD)	pH	Dissolved Oxygen (mg/L)	Alkalinity (mg/L asCaCO3)	Hardness (mg/L asCaCO3)	Conductivity (umhos/cm)	Total Ammonia (mg/L)
1	-	50.4	6.99	8.5	188	254	2646	31.0
2	-	47.8	7.11	10.3	208	282	2985	29.7
3	1.04	134.1 (60)*	6.60	11.4	92	178	1680	11.2
4	-	58.6	7.24	11.0	216	308	2544	28.6
5	1.27	121 (10.7)*	7.30	9.1	140	204	1791	11.9
6	-	58.4	6.90	10.9	230	280	2478	22.8
7	0.82	110.4 (20.6)*	6.99	8.9	192	250	2633	22.2
8	-	55.5	6.87	9.8	226	228	2328	23.7

* = Primary effluent blended into final effluent during wet weather events.

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USE OF TOXICITY TESTING TO EVALUATE PERFORMANCE OF A STORMWATER TREATMENT MARSH.

Ceriodaphnia dubia bioassays were used to quantify toxicity of urban stormwater runoff at Crandall Creek and the downstream Demonstration Urban Stormwater Treatment (DUST) Marsh in Fremont, CA. The Median time to lethality (LT_{50}) was used to compare the relative intensities of toxicity in the system. Measurements taken during or shortly after storm events show horizontal and vertical gradients in LT_{50} and conductivity, with high correlation between the two parameters. Most of the toxic stormwater was contained within the marsh after small and medium-sized (0.2-1.0") storms. The intensity of toxicity was greatly reduced upon mixing of stormwater with preexisting marsh water. A preliminary toxicity dilution model, based on empirically-established mathematical relationship between LT_{50} and sample dilution, was used to predict the reduction in toxicity which is due to dilution only. Comparison of the predicted LT_{50} with the observed values show that substantial reduction in toxicity cannot be explained by dilution only, indicating that removal processes may be involved. Mixing of the water column increased the rate of toxicity diminution in comparison to the rate observed in an unmixed section of the marsh. Filtration of DUST Marsh samples revealed that toxicity was associated with the soluble fraction of the samples and not with particles. Other Phase I TIE manipulations indicated that toxicity was caused by oxidizable, non-polar organics rather than metals. The results of this study suggest that enhancement of mixing may facilitate the removal or attenuation of soluble toxic pollutants, and highlight the importance of integrating engineering design and toxicity monitoring.

A detailed report entitled "Spatial and temporal variations in toxicity in a marsh receiving urban runoff" (1993, LBL # 32837) is available upon request. Call Susan Anderson at (510) 486-4654 or Revital Katznelson at (510) 874-3048.

In order to address these non-point source (NPS) problems, the U.S. EPA published the Final Rule for NPDES Permit Application Regulations for Storm Water Discharges on November 16, 1990. The Rule requires that municipalities establish a storm water management plan which includes the following key components: public information and participation, municipal government activities, new development and construction site controls, illicit discharge identification and elimination, industrial discharges identification and runoff control, assessment of storm water treatment and monitoring. In a pro-active approach, the cities and counties of Santa Clara Valley and Alameda joined together to manage the NPS studies forming the Santa Clara Valley Nonpoint Source Control Program (SCVNPS) in 1987 and Alameda County Urban Runoff Clean Water Program (ACURCWP) in 1988, respectively.

One important aspect of these programs involves implementation of a Toxicity Control Plan. The main objective of the Plan is to characterize and identify which groups of pollutants are causing toxic responses so that they may be controlled though implementation of best management practices (BMP's).

An additional goal of the Plan is to evaluate the potential toxicity of particulate bound metals and other pollutants so that appropriate WQO's may be developed for storm water runoff. This is necessary because the chemical form of pollutants present in storm water runoff is significantly different than traditionally regulated point sources (waste water from industrial and municipal treatment plants) primarily due to the high levels of suspended solids.

This paper presents the results of the Phase I TIE's conducted by SCVNPS and ACURCWP and compares TIE results with exceedances in WQO's.

METHODS

Locations

The location of the study areas and sampling stations are presented in Figure 1. All stations are located in the San Francisco Bay Area. Santa Clara Valley (SC) mixed land-use stations are located in streams that receive storm water runoff from a mixture of commercial, residential, open, transportation and industrial land-uses. The SC industrial station drains a small watershed with heavy industry as the primary land-use. Contained within the catchment are warehouse distribution centers with heavy truck traffic, a print shop, commercial carpet cleaning, and small manufacturing facilities (including metal finishing). Alameda County (AL) mixed land-use (stream) stations drain the south-eastern edge of the San Francisco Bay and receive urban runoff from mixed land-uses (commercial, residential, open, transportation and industrial) within Alameda County. The AL industrial station drains a small watershed with heavy industry as its primary land-use. Contained within the catchment are numerous small businesses including auto wreckers, auto maintenance and repair yard, and a metal galvanizing facility.

Sampling

Samples were collected using automated equipment (ISCO 2700/3700) configured to collect a flow weighted composite sample. Samplers were initiated at the start of the rain event and generally allowed to run until the stream stage reached 1.1 times original base flow levels (total sampling time ranged from approximately 8 to 36 hours). Sample intakes were located 10-20 cm off the bottom of the channels. A complete description of the sampling methods and comparison of automated flow weighted composite method with grab sampling has been presented previously (WCC 1991a, WCC 1991b, WCC 1992).

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APPLICATION OF TIE METHODS TO URBAN STORM WATER RUNOFF FROM INDUSTRIAL AND MIXED LAND-USE STATIONS

Short-term chronic toxicity testing of urban storm water runoff collected in Santa Clara and Alameda Counties (California) indicated significant acute toxicity to Ceriodaphnia dubia. Phase I Toxicity Identification Evaluation (TIE) procedures using C. dubia were used to establish the class of pollutants responsible for the observed toxicity in different land-use areas to aid in selection of toxicity control measures. Results of the phase one TIE manipulations followed by 96-hour acute bioassays indicate that toxicity at two heavy industrial areas was due to cationic metals. Sample LC_{50} values for one industrial site correlated well with measured exceedances in EPA acute water quality objectives (WQO's) for dissolved metals (copper, lead and zinc). In mixed land-use areas (streams), multiple and varied classes of pollutants including neutral non-polar organics, and volatile or oxidizable compounds were indicated as contributing to toxicity. During some storm events acute toxicity criteria for total copper, lead and zinc were exceeded in the mixed land-use areas but no acute toxicity was found for C. dubia. The majority of the metals in exceedance were present in the particulate form, while dissolved metal concentrations were below the acute criteria. Additionally, TIE results from mixed land-use stations showed that moderate concentrations of particulate metals (1-2 times acute WQO) did not contribute to sample toxicity. Comparisons of WQO's with dissolved metal concentrations is suggested as a good indicator of potential toxicity. Future application of toxicity testing will involve focused sampling at upstream locations to isolate various sources of toxicity. Samples will be screened in an improved bioassay design and a subset selected for further detailed characterization using coupled chemical and TIE methods.

INTRODUCTION

The 1972 Clean Water Act (CWA) prohibits the discharge of any pollutant to navigable waters from a point source unless the point source is authorized with a National Pollutant Discharge Elimination System (NPDES) Permit. Efforts to improve water quality under the NPDES program have traditionally focused on reducing pollutants in industrial process wastewater and municipal sewage. In the last few years, it has become evident that more diffuse sources (occurring over a wide area) of water pollution, such as agricultural discharges and urban runoff are also major causes of water quality problems.

Toxicity Testing and TIE Procedures

Short-term chronic toxicity tests were conducted on runoff samples according to the protocol outlined in EPA/600/4-89/001 (US EPA 1989). The bioassay test evaluated mortality and reproduction endpoints. When toxicity testing results indicated acute toxicity within 48 hours of organism exposure, a toxicity identification evaluation was initiated. Toxicity Identification Evaluation (TIE) procedures involve standardized methodology developed to identify chemical constituents responsible for observed acute toxicity to aquatic organisms (EPA, 1988).

The TIE process is comprised of three different phases. Phase I involves toxicity characterization studies designed to establish the four major class of compounds (volatile organics, non-volatile organics, metals and particulates) responsible for the majority of observed toxicity. Phase II utilizes the results from Phase I characterization studies to identify specific compounds that causes toxicity while Phase III involves verification procedures that confirm the suspected toxicants from Phase II studies. To maximize the cost-effectiveness of the assessment, only Phase I TIE's were conducted in the early part of the Toxicity Plan. As additional toxicity information is gathered from Phase I TIE's, we plan to implement Phase II and III TIE's at strategic watersheds.

The procedure incorporates both physical and chemical alterations of samples to eliminate a particular compound or class of compounds, while monitoring the toxicity of the resulting treated effluent. Phase 1 TIE characterization manipulations included toxicity determination at both acidic and basic extremes (pH 3 and 11), filtration, aeration (air), C-18 solid phase extraction chromatography and addition of the EDTA ligand (chelating reagent). The procedures were followed by 96-hour acute bioassays on whole and diluted storm water and chemically treated storm water fractions using C. dubia in a static bioassay system with mortality as the indicating endpoint (EPA 1991). 96 hour exposures were used to enable characterization of samples from mixed land-use (stream) stations which were generally mildly toxic (mean LT_{50} of 46 hours).

RESULTS AND DISCUSSION

Chemical Analysis

Results of the chemical analysis are presented in Figure 2. Presented are the concentrations of total recoverable and dissolved cadmium, copper, lead and zinc and total PAH's for SC and AL stations. Other metal concentrations were generally below WQO's and are not shown. Results are grouped by station land-use type (mixed vs. industrial) for each Program. Data are presented as box percentile plots which provide a graphical representation of the statistical distribution of the data. The top and bottom edges of the box represents the 90th and 10th percentiles, respectively. The middle line represents the median value (50th percentile) while the dashed lines represent the 25th and 75th percentiles.

The metals results indicate a relationship between land-use and metals concentrations, with the industrial sites showing elevated concentrations of total and dissolved cadmium, lead and most notably zinc relative to the mixed (stream) sites. The elevated concentrations are likely due to several factors including lower dilution at the industrial land use sites by clean runoff, and increased usage per unit area. Lower concentrations were observed for copper and lead suggesting industrial sites do not contribute these metals in a greater amount than do other land-use areas.

At the industrial sites a greater fraction of cadmium and zinc were found to be present in the dissolved form as compared to the mixed land-use sites. This suggests that the metals are released in the dissolved form and that chemical composition of the water (e.g. pH, suspended solids, organics) tends to keep the metals in solution. This is supported by total hardness measurements which were much lower at the industrial sites than at the mixed land-use sites.

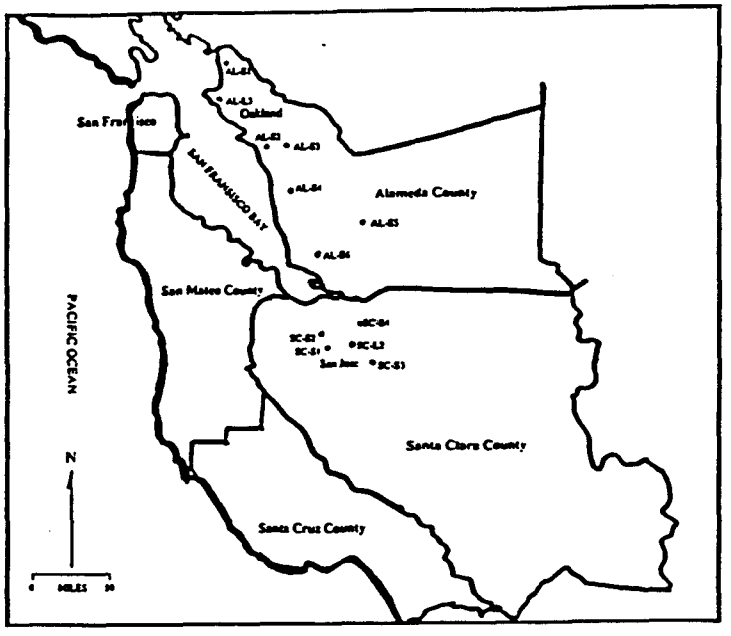


Figure I. Study Area and Sampling Site Locations

Chemical Analysis

Flow weighted composite samples were analyzed for constituents listed in Table 1. The constituents were selected for continued monitoring based on the results of previous characterization using a more extensive suite. Constituents that were consistently undetected or far below water quality objectives and not of concern in the receiving waters or watershed were eliminated from further testing. Analysis methods were based on EPA protocol with modifications to improve method performance (practical quantitation limits) as necessary. QA/QC procedures included the use of field and laboratory duplicates, method and equipment blanks, matrix spike and spike duplicate analysis and external reference standards.

Table 1. Laboratory Analysis Parameters

Parameter	EPA Method	Parameter	EPA Method
Metals -Total Recoverable		**Metals - Dissolved (< 0.45 µm)**	
Arsenic	206.2	Cadmium	213.2
Cadmium	213.2	Copper	220.2
Chromium (Total)	218.2	Lead	239.2
Copper	220.2	Silver	272.2
Lead	239.2	Zinc	289.2
Mercury	245.1		
Nickel	249.2		
Selenium	270.3	**Inorganics**	
Silver	272.2		
Zinc	289.2	pH	150.1
Organics		Hardness	130.2
TOC	9060	Turbidity	180.1
Total Oil and Grease	413.2	TSS	160.2
PAH	Modified 625 (Texas A&M)		

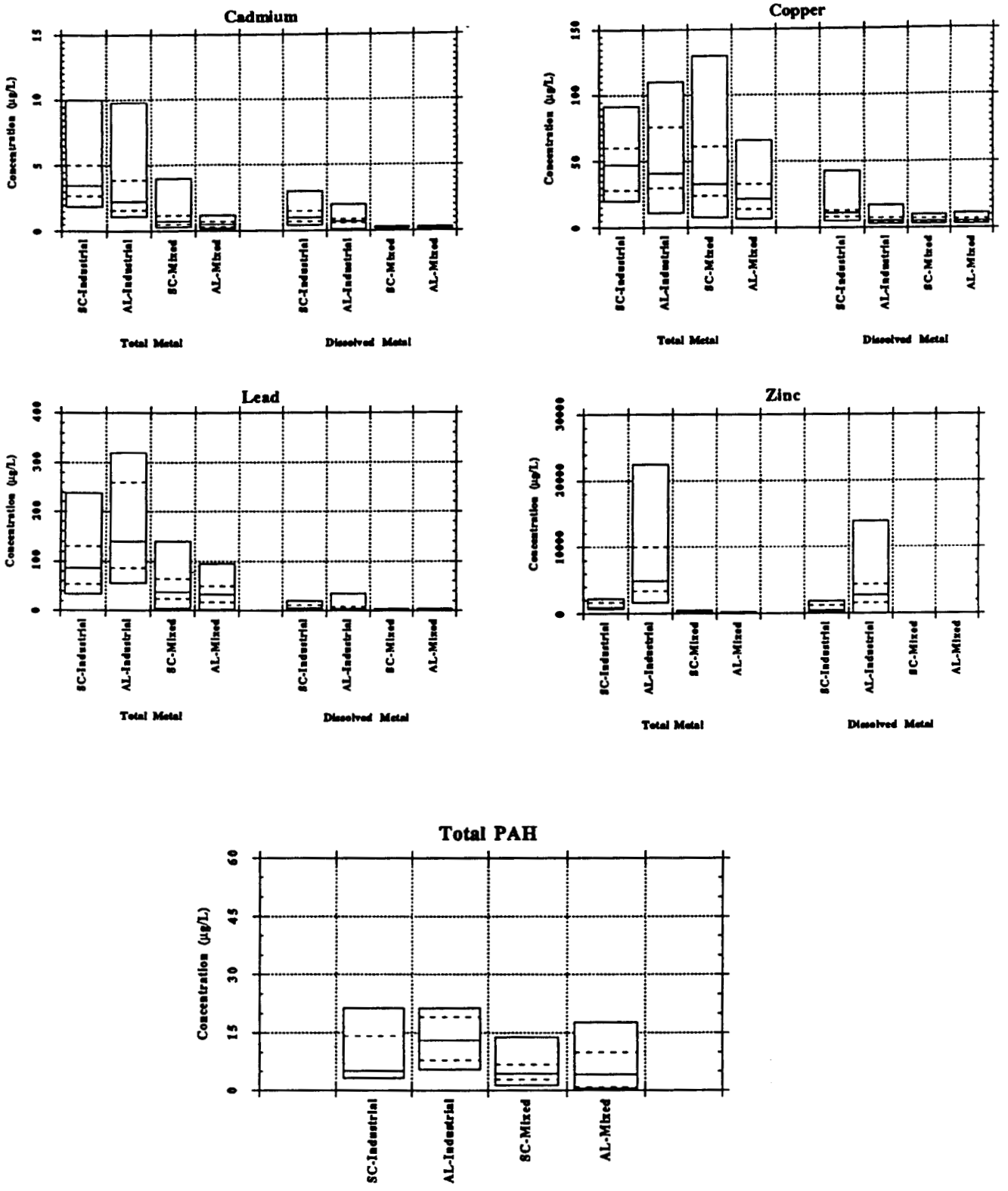


Figure 2. Box Percentile Plots of Trace Metal and PAH Concentrations in Storm Water Runoff at Different Land Use Areas

Comparisons with Water Quality Objectives

Metal concentrations were compared to calculated acute (1-hour) water quality objectives (WQO's) for the protection of aquatic life in freshwater (CSWRCB, 1991). Figure 3 shows the percent of all samples that exceed the objectives for total and dissolved copper, lead and zinc. WQO's are calculated based on sample hardness reflecting the lower bioavailability of cationic metals as ionic strength increases.

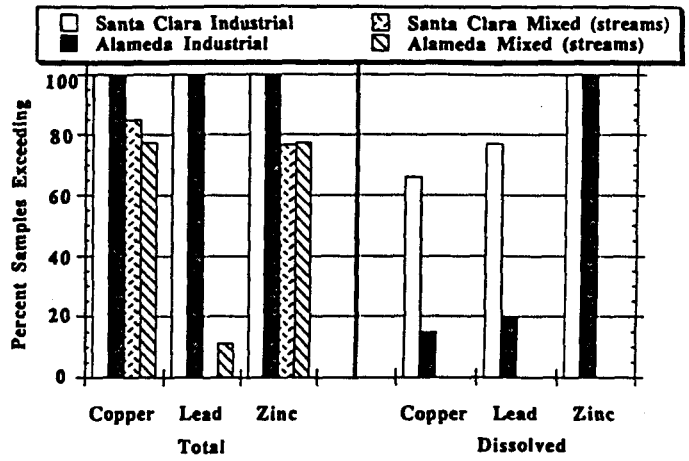


Figure 3. Percentage of Storm Water Runoff Samples Exceeding Water Quality Objectives at Different Land Use Areas

At the industrial sites total copper, lead and zinc consistently exceed WQO's. Consistent exceedances are also observed for dissolved zinc with occasional exceedances of dissolved copper and lead.

At the mixed land-use sites, frequent exceedances are seen for total copper and zinc with occasional exceedances for total lead at the Alameda sites. No exceedances were observed for dissolved metals in any of the mixed land-use sites, indicating the total metals exceedances are due to particulate metal forms. The lower frequency of total metal exceedances and the lack of dissolved metal exceedances are primarily due to lower metal concentrations and higher sample hardness at the mixed land-use sites as compared to the industrial land-use sites.

Toxicity

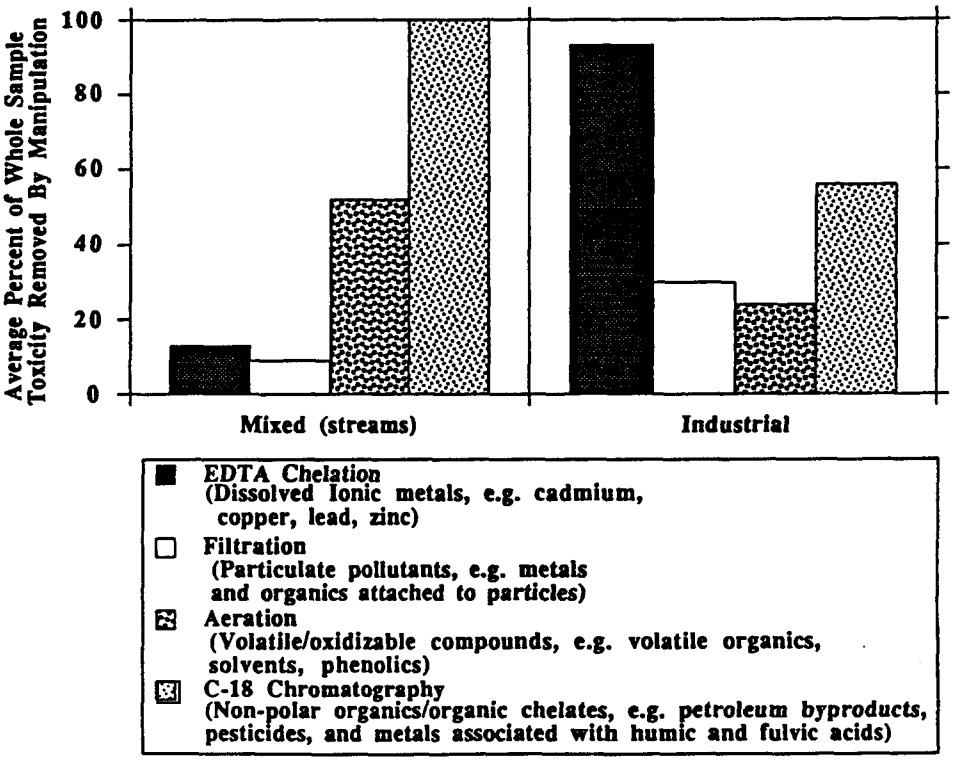
Whole sample toxicity (initial and day 2) and TIE results are presented in Table 2. TIE manipulation test results are presented as the percent of the whole sample toxicity (LC_{50} for Day 2 of sample storage) reduced following the manipulations. The table also presents acute water quality exceedance factors (WQEF's) for total, dissolved, and particulate metals. WQEF's are calculated as the ratio of the metal concentration to the acute WQO and provide a measure of the severity of the water quality exceedance.

The LC_{50} data indicate that runoff samples tested for the industrial sites are more toxic than runoff tested from the mixed land-use sites (average Initial LC_{50} values: Industrial = 23 %; Mixed = 46%). However, after two days of sample storage, whole sample toxicity at the two sites are comparable, suggesting a portion of the toxicity is degraded.

The average percent removal were grouped for all TIE's by land-use type (mixed and industrial) and are presented in Figure 4. Not presented are results of thiosulfate or graduated pH tests as no effect was observed, indicating oxidants and ammonia did not contributed to sample toxicity.

Table 2. TIE Results and Comparisons with Water Quality Objective Exceedance Factors

Date	Station	LC50 Day 1	LC50 Day 2	% Removal pH 3	% Removal pH 11	% Removal Aeration	% Removal Filtration	% Removal C-18 25ml	% Removal C-18 150ml	EDTA	Total WQEF Cd	Total WQEF Cu	Total WQEF Pb	Total WQEF Zn	Sum	Dissolved WQEF Cd	Dissolved WQEF Cu	Dissolved WQEF Pb	Dissolved WQEF Zn	Sum	Particulate WQEF Cd	Particulate WQEF Cu	Particulate WQEF Pb	Particulate WQEF Zn	Sum
Industrial Land-use																									
10/26/91	AL-I3	17	18	0	0	0	0	40	0	74	7.0	7.2	9.0	170	193	0.43	0.94	0.39	29	31	6.6	6.3	8.7	141	163
12/16/90	SC-I2	36	57	7	7	43	43	100	43	100	0.81	2.3	1.4	8.8	13	0.27	0.42	0.12	5.9	6.7	0.54	1.9	1.3	2.9	6.7
2/28/91	SC-I2	10	53	0	100	53	74	5	3	100	4.9	13	10	24	52	0.67	2.4	1.2	5.4	10	4.2	11	8.8	19	43
3/4/91	SC-I2	29	53	0	0	0	4	79	15	100	2.4	5.7	4.3	16	28	1.4	2.5	0.49	13	18	1.0	3.2	3.8	2.8	11
	AVG	23	46	2	27	24	30	56	15	93	3.8	7.1	6.2	55	72	0.69	1.6	0.55	13.4	16	3.1	5.5	8.6	41.4	56
	STDEV	12	19	3	49	28	35	42	20	13	2.7	4.5	4.1	77	83	0.50	1.0	0.46	11.0	11	2.9	3.9	3.7	66.8	73
Mixed Land-Use																									
4/11/92	AL-S3	NA	45	70	70	0	0	100	100	22	0.30	1.8	6.4	1.1	9.6	0.05	0.76	0.28	0.34	1.4	0.25	1.0	6.1	0.79	8.2
10/26/91	SC-S2	35	20	82	21	100	18	100	100	1	0.33	3.9	0.73	2.6	7.6	0.03	0.33	0.02	0.17	0.55	0.30	3.6	0.71	2.5	7.1
10/26/91	SC-S1	38	35	100	94	100	34	100	100	0	0.36	4.2	0.61	3.2	8.3	0.04	0.04	0.02	0.19	0.29	0.32	4.2	0.59	3.0	8.0
12/27/91	SC-S2	50	43	0	0	14	0	100	100	22	0.24	2.7	0.78	2.0	5.8	0.09	0.93	0.06	0.43	1.5	0.15	1.8	0.72	1.6	4.3
12/16/90	SC-S3	36	62	28	43	0	0	15	100	21	0.22	1.8	0.49	1.4	3.9	0.05	0.24	0.07	0.18	0.54	0.16	1.5	0.43	1.3	3.4
12/27/91	SC-S3	71	60	79	0	100	0	100	100	16	0.16	1.8	0.42	1.3	3.7	0.05	0.26	0.01	0.06	0.38	0.11	1.5	0.41	1.3	3.3
	AVG	46	44	60	38	52	9	86	100	13	0.27	2.7	1.6	2.0	6.5	0.05	0.43	0.08	0.23	0.78	0.22	2.3	1.5	1.7	5.7
	STDEV	15	16	38	38	52	14	35	0	10	0.06	1.1	2.4	0.8	2.4	0.02	0.34	0.10	0.13	0.54	0.09	1.3	2.3	0.8	2.3



Note: Percent of Toxicity Removed Using 96-hour LC_{50} Results with C. dubia, Not Shown are Graduated pH and Thiosulfate Manipulation Results Which Had No Effect on Sample Toxicity.

Figure 4. Average Percent of Whole Sample Toxicity Removed by TIE Manipulations in Different Land Use Areas

Classes of Compounds Affected By TIE Manipulations

To aid in the interpretation of the TIE test results, chemical manipulations and the classes of compounds they affect are described below.

pH adjustments to 3 and 11 affect toxicants by shifting equilibrium to their unionized forms making them susceptible to removal by aeration and extraction methods. Increases in sample toxicity upon addition of acid (lowering the pH) may be the result of acid solubilization of metals associated with particulates or organics as metals are known to be more soluble at lower pH. Similarly, decreases in toxicity upon addition of base (raising the pH) may result from removal of metals from solution either by adsorption onto the sample container walls or particles in the sample, as the tendency for some metals to be soluble decreases with increasing pH.

Aeration tests assess the contribution of volatile or oxidizable compounds to sample toxicity. Solid phase extraction using C18 extraction columns extract hydrophobic compounds (non-polar organics and metal chelates) from the effluent as it passes through the column. EDTA Chelation test utilizes the chelating ability of the organic compound EDTA to form strong complexes with metallic ions thereby masking them from test organisms.

Pollutants Responsible for Toxicity At Industrial Sites

At the industrial sites TIE results indicate the majority of the observed sample toxicity was due to dissolved metal ions. In three of four samples tested complete removal of toxicity was achieved by the addition of the EDTA. The results of the EDTA test are supported by the removal of sample toxicity upon increasing the pH and filtering the sample (results not shown), as dissolved metals will adsorb onto particles at high pH and be removed via filtration.

The role of metal ions in causing toxicity is further supported by significant WQEF's for dissolved copper and zinc (Table II). Figure V shows that for the Santa Clara site, dissolved copper and lead concentrations are strongly correlated with initial sample toxicity.

C-18 manipulations removed some sample toxicity in some samples. However, interpretation of the C-18 results is difficult due to the ability of the manipulation to remove both organic molecules and ionic, organically complexed, or colloidal metals depending on the presence of other sample constituents and the particular form of metal present. Nevertheless the results do not exclude organics as contributing to sample toxicity.

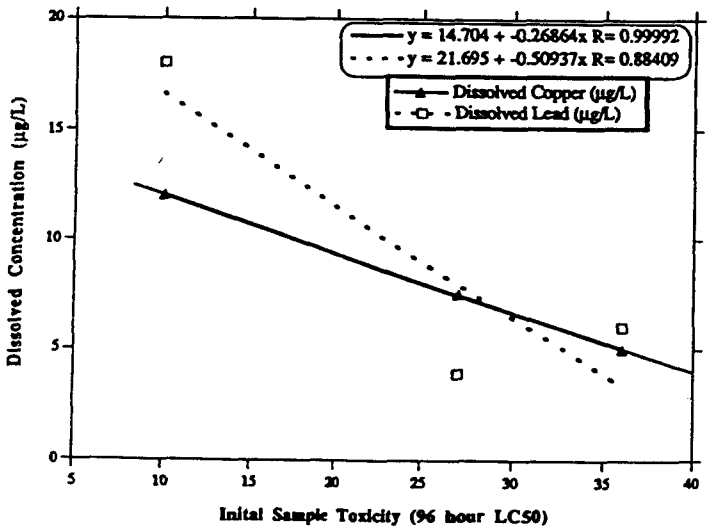


Figure 5. Correlation of Initial Sample Toxicity (96-hour LC_{50}, C. dubia) with Dissolved Metal Concentrations at Santa Clara Industrial Site

Pollutants Responsible for Toxicity at Mixed Land-use (stream) Sites

Organics. At the mixed land-use sites TIE results indicate the majority of the observed sample toxicity was due to dissolved organics and/or volatile/oxidizable compounds. In all the samples tested complete removal of toxicity was found following C-18 treatment. Some examples of this class of organics which may be removed by C-18 treatment include polynuclear aromatic hydrocarbons (PAH), pesticides, surfactants, and also metals associated with natural organics (humic and fulvic acids). In order to evaluate what specific types of organics are causing the toxicity, results of PAH analyses are presented. A total of 35 individual PAH compounds encompassing 2 through 6-ring structures were measured in storm water samples at the ng/L level by using a sophisticated GC-MS method. Figure 2 shows that the median total PAH is highest (13 µg/L) for industrial runoff collected from a station, L3, in Alameda County. A similar industrial station, L2, in Santa Clara County showed a lower total PAH median concentration (5 µg/L), which is only slightly higher than the total PAH concentrations (about 4.3 µg/L) from the mixed land use stations in Alameda and Santa Clara Counties.

In a review of the scientific literature on the toxicity of PAH to aquatic organisms (Eisler, 1987), Table 3 presents acute toxicity data showing that the LC_{50} values (lethal concentration of a specific PAH to kill 50% of the test organism) of most PAHs range from 50 to 3,800 µg/L. These LC_{50} values are substantially higher than the measured PAH concentrations found in the storm water runoff. This comparison indicates therefore that the PAH found in storm water runoff may likely not be the hydrophobic non-polar organics that are responsible for causing acute toxicity. It must, however, be emphasized that PAHs can rapidly bioaccumulate in animal tissues, and at least 20 PAHs are known to be carcinogenic.

Although pesticides were not measured in runoff samples that were submitted for toxicity tests, we have results of stream sediments to show that organochlorine (OC) pesticides (such as DDT and chlordane) are commonly found, and some chlorinated herbicides (e.g. 2,4-D and 2,4,5-T) are occasionally detected. Scouring of stream sediments during storm runoff events will readily introduce these pollutants, as particulate and dissolved forms, into the water column. Another group of pesticides that is commonly used for insect control is organophosphorus (OP) pesticides. Table 4 shows three key environmental properties that distinguish OP from the OC pesticides.

Table 3. Toxicity of Selected PAHs and Pesticides to Aquatic Organisms

Pollutant	Organism	LC-50 (µg/L)	Pollutant	Organism	LC-50 (µg/L)
1. Polynuclear Aromatic Hydrocarbon			**2. Organochlorine Pesticides**		
Fluoranthene	Sandworm	250	2,4-D	Rainbow trout	4,000
Fluorene	Rainbow trout	820			
	Sandworm	1,000	Chlordane	Rainbow trout	0.09
	Amphipod	600			
	Sheepshead minnow	1,680			
Napthalene	Copepod	50	**3. Organophosphorus Pesticides**		
	Sandworm	3,800			
	Sheepshead minnow	2,400	Diazinon	Rainbow trout	2.6-3.2
2-methylnapthalene	Sheepshead minnow	2,000			
Phenanthrene	Sandworm	600	Fenamiphos	Rainbow trout	0.072
1-methylphenanthrene	Sandworm	300			

Ref: Eisler, 1987 and The Agrochemicals Handbook

Table 4. Environmental Fate Properties of DDT and Diazinon

Property	DDT	Diazinon
Persistence (months)	>12	< 1
Sorption (Koc)	243,000	227
Water Solubility (mg/L)	0.0017	40

Ref: Lyman, et al. 1991 and The Agrochemicals Handbook.

In general, the OP pesticides have: higher toxicity; higher water solubility; faster degradation rates; and lower sorption (or affinity) to solids.

Based on these properties, we can predict that the OC pesticides are highly sorbed to particulates and therefore very little is in the dissolved form. In contrast, the OP pesticides are more soluble, and correspondingly a larger amount is in the dissolved form. When coupled with higher toxicity, it is clear that the OP pesticides have higher potential to cause acute toxicity than the OC pesticides.

In fact, recent results of monitoring storm water runoff toxicity at an adjacent watershed showed that an OP pesticide, diazinon, was responsible for causing acute toxicity to Ceriodaphnia (Foe et al., 1993). The source was likely runoff (and possibly aerial deposition) from diazinon application in orchards prior to the storm events. In addition to the previous evidence, preliminary Phase II and II TIE results in stream runoff samples from Alameda County also indicate that diazinon is the main organic pollutant causing acute toxicity to Ceriodaphnia.

Metals. Dissolved metals are not indicated as causing significant amount of toxicity in mixed land-use sites as low reductions in toxicity were observed after addition of EDTA. These results are further supported by a lack of significant dissolved WQEF at the stream sites.

Two factors may contribute to low dissolved metal toxicity in the mixed land-use sites (many of which receive drainage from industrial sites). Concentrations of dissolved zinc are lower than at industrial sites (probably due to dilution) and other constituents are present in the stream samples (such as suspended solids, dissolved organics, salts) which may serve to lessen the bioavailability of the dissolved metals upon discharge to the stream channels.

Particulate bound pollutants (either organics or metals) were indicated as small contributors to toxicity in two of the six samples. It should be noted these samples had among the highest particulate metal WQEF's (Table 2). In four of the six samples, significant particulate metal WQEF's were measured but no evidence of particulate toxicity was observed. This result is consistent with lower bioavailability of particulate as compared to dissolved metals and suggests the need for WQO's based on tests with particulate metal species.

The need to develop particulate based WQO's is further supported by water quality and bioassay results from one stream site located in Alameda County (AL-S1). At this site, particulate metal concentrations consistently exceed acute WQO's, however, no acute toxicity was observed after seven days of organism exposure (C. dubia 7-day static renewal protocol).

A recent publication (EPA 1992) has recognized that current WQO's may be overprotective when applied to total metals concentrations due to different bioavailability of particulate and dissolved metals. Current options which are suggested include comparing WQO's to dissolved metals and development of site specific criteria.

CONCLUSIONS

Toxicity at industrial land-use sites was primarily due to ionic metals. The TIE results are supported by measured exceedances of acute water quality objectives for dissolved metals. Toxicity at mixed land-use stream sites was primarily due to hydrophobic organic compounds, possibly organophosphorus pesticides. Particulate bound pollutants only contributed a small fraction of the total sample toxicity, despite large exceedances of WQO's by particulate metals. These results indicate WQO should be developed that address the different bioavailability of specific metal forms in order to prevent misdirection of resources.

When hydrophobic organics are implicated as causing acute toxicity, chemical monitoring of storm water runoff should prioritize OP pesticides. This is not only applicable in land use areas that include agriculture, but also urbanized areas where OP products are used for insect control in lawn care and rights-of-way.

ACKNOWLEDGEMENTS

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PROGRESS ON THE DEVELOPMENT OF PROCEDURES FOR IDENTIFYING PESTICIDE TOXICITY IN EFFLUENTS AND AMBIENT WATERS

Pesticides have been implicated as sources of toxicity in ambient waters tributary to the Delta, as well as in effluents discharged directly into the Delta. Chemical scans for such chemicals are costly, may not address the chemical of interest, and may lack sensitivity at concentrations that cause toxicity. Similarly, standard Toxicity Identification Evaluation (TIE) procedures provide only general information on the characteristics of a suspected toxicant. By the time the confirmatory stage of the investigation is reached, the material of interest may have degraded to the point where it is no longer quantifiable. Consequently, TIE procedures are being developed that focus on the chemical properties of specific pesticides.

This investigation evaluated three chemicals of interest: two organophosphate pesticides, diazinon and chlorpyrifos; and one carbamate pesticide, carbofuran. The effect of different TIE procedures on each of the pesticides was characterized. The procedures included solid phase extraction (SPE), recovery in methanol eluates, hydrolysis under acid and base conditions, and retention in specific methanol:water fractions. To date, the results suggest that diazinon is labile at low pH and

carbofuran is labile at high pH. Conversely, chlorpyrifos was not affected by pH. Both carbofuran and diazinon were retained ≥ 90 percent on C-8 and C-18 SPE columns but chlorpyrifos exhibited approximately 50 percent retention on the SPE columns. All three pesticides eluted from the columns in specific methanol:water fractions. Carbofuran was the most polar of the three and eluted in the 50 and 70 percent methanol fractions, followed by diazinon in the 75 and 80 percent fractions and chlorpyrifos in the 85 and 90 percent methanol fractions. In addition, the effect of the metabolic inhibitor, piperonyl butoxide, on toxicity of each of the three pesticides is currently being investigated. Further details on this work may be obtained by contacting the authors.

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RESEARCH AND DEVELOPMENT OF PHASE I TIE METHODS FOR WEST-COAST MARINE SPECIES.

TIE methods with west-coast marine species were developed for clients whose effluents exhibited toxicity to echinoderms, giant kelp, and red abalone. Test methods were developed, validated with laboratory waters, and then tested on effluent samples to determine whether characterization of sample toxicity was possible. Laboratory tests included pH adjustment, aeration, filtration, solid phase extraction, and the addition of zeolite, sodium thiosulfate, EDTA, and other chelators. Some aspects of testing that were evaluated were: various means of metals removal, radical pH adjustment, the role of ammonia in these tests, and when to manipulate the salinity of the samples. Results so far have indicated that toxicity can successfully be characterized using these species. For one discharger, it was determined that ammonia was causing a reduction in echinoderm fertilization. For another discharger, it was determined that ammonia was not causing low germination rates in the giant kelp test, but it was affecting red abalone development. Non-polar organics were found to be the source of toxicity to the giant kelp.

BACKGROUND: PHASE I TIE METHODS WITH WEST COAST SPECIES

In 1984, the State Water Resources Control Board commissioned the Marine Bioassay Project to develop bioassay test methods for species native to California waters. Two of these tests, the Giant Kelp Survival and Growth Test and Red Abalone Development Test, have been used throughout California as a means of determining effects of whole effluent toxicity on a representative plant and invertebrate species. Additionally, the echinoderm fertilization test developed by Dinnel is used throughout the west coast using native species. Although the testing requirements for these tests are well established, if effluent toxicity were to exceed compliance limits, there would be no means of determining which classes of compounds were contributing to the toxicity, because no Toxicity Identification Evaluation (TIE) methods have been developed for these species.

Agency Development of Marine TIE Methods

EPA has published acute and chronic methods for TIEs using *Ceriodaphnia dubia* and *Pimephales promelas*. In 1992, EPA began developing Phase I marine TIE methods using species native to the east coast. Through combined state and private efforts, Phase I TIE methods are beginning to be developed with west coast species of echinoderms, kelp and abalone. Presently, EPA guidelines suggest that if a response with a marine species triggers a TIE, that the response be correlated to a freshwater species and TIE procedures be performed with the freshwater species. The difficulty inherent in this process is that species respond differently to different toxicants.

Impetus for Internal Development of TIE Methods

Several MEC clients have exceeded compliance limits with marine species over the past several years. In the Effluent Toxicity Characterization Program (ETCP) for the Regional Water Quality Control Board, San Francisco Bay Region and the California Ocean Plan, three species are tested concurrently. Often, freshwater and marine species are tested concurrently to compare sensitivity. If a marine species responds while freshwater species do not, it would be difficult to determine the causes of toxicity without directly using the marine responder.

Echinoderm Testing. Eleven out of twenty dischargers in the first round of the ETCP found echinoderms to be sensitive in the screening phase and two dischargers triggered TIEs with echinoderms due to their sensitivity. In 1992, TIE methods were developed, validated, and performed on a municipal effluent (POTW 1).

Kelp and Abalone Testing. In 1992, after a series of monthly toxicity tests with one acute and three chronic species, an ocean discharging POTW (POTW 2) was intermittently outside toxicity compliance limits with both acute and chronic species. Acute TIE procedures were performed, which indicated ammonia was the primary toxicant in the effluent along with secondary toxicants. This raised the question of how ammonia and other toxicants were affecting chronic toxicity results. Test methods were developed to enable toxicity characterization.

VALIDATION OF PHASE I METHODS

Objectives

The first step in developing TIE methods was to determine the most appropriate methodology. Methods were based on EPA developed acute and chronic guidelines for freshwater species. Acute methods include more extreme procedures that may introduce artifactual toxicity into the more sensitive chronic tests. Both the kelp and abalone tests are considered short-term chronic tests, so it had to be determined where they fell into the range of testing capabilities. As well, specific questions to be answered were:

> Is radical pH adjustment to be attempted initially as in acute procedures or secondarily as in chronic procedures?
>
> What pH extremes should be attempted?

Should TIE characterizations be performed on marine solutions or on freshwater solutions which are raised in salinity later?

What concentrations of additives and reagents should be added to be effective but not toxic?

How effective is chelation with EDTA in marine solutions?

What is the effect of ammonia on these species?

What is the best means of maintaining rigid pH control in marine solutions?

Results

Echinoderm Tests. We began a case study with POTW1 effluent using the sperm cell fertilization test. Although the effluent had a consistent effect on this endpoint, it was moderate in severity. When the test endpoint was changed from fertilization to development (to the pluteus larva stage), a much greater degree of toxicity was observed (Table 1). We decided to carry out TIE validation procedures with both endpoints in order to quantify and qualify toxic responses between the two endpoints.

Table 1. Results of echinoderm fertilization tests with POTW1 effluent.

% Effluent	% Fertilization	% Normal Development
Control	91.0	95.8
Brine	73.0	96.0
50	87.0	0.0
60	81.5	0.0
70	62.5	0.0

The validation studies indicated that the developed methods should not introduce artifactual toxicity into the test procedures (Table 2). Both the fertilization and the development endpoints were evaluated on freshwater and marine solutions. The freshwater manipulations were performed on deionized water; after manipulations were performed hypersaline brine was added to raise the salinity of the solutions to 30 ppt. The marine solutions were filtered seawater from San Francisco Bay at 30 ppt. There was little difference in response, therefore further manipulations were performed on freshwater solutions which were subsequently raised to test salinity. Radical pH adjustment to pH3 and to pH10 was successful. Threshold levels were established for EDTA, sodium thiosulfate and methanol to both the fertilization and development endpoints.

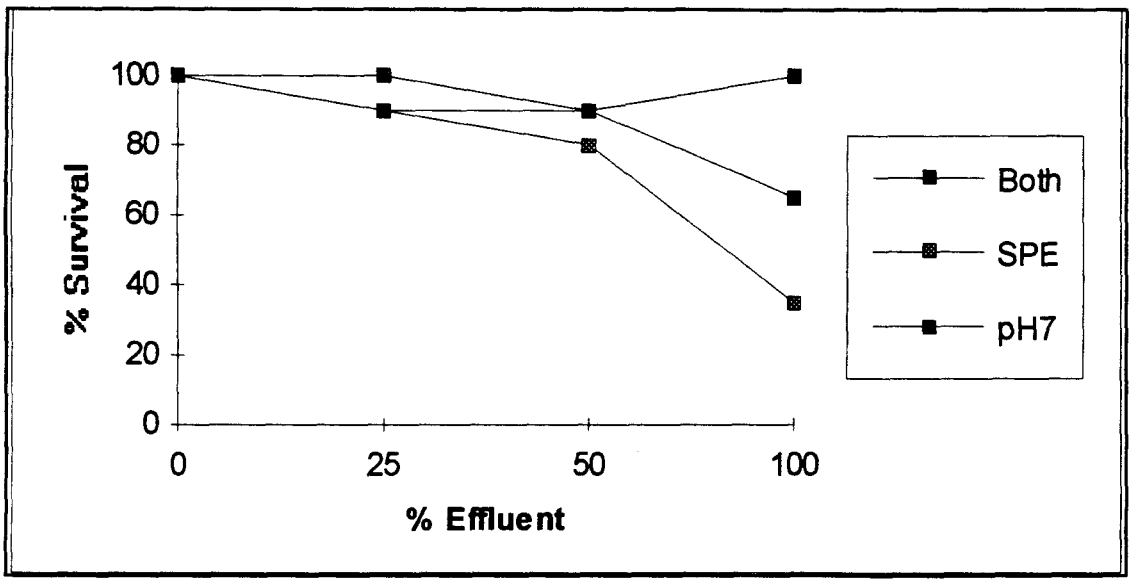
Table 2. Results of TIE validation procedures with echinoderms.

Procedure	% Fertilization (Freshwater)	% Fertilization (Marine)	% Normal Development (Freshwater)	% Normal Development (Marine)
pH3	88	87.9	93.5	84.2
pH10	81.5	86.7	91.5	87.1
Aeration	86	88.6	90.5	93.8
Filtration	72	88.1	76.5	95.1
SP Extraction	63.5	85.4	77.5	80.9

Giant Kelp and Red Abalone Test

We were able to quantify toxicity of POTW2 with acute procedures using the fathead minnow (Figure 1). The results indicated that although ammonia was the primary toxicant, non-polar organics were contributing to overall effluent toxicity.

Figure 1. Mean results of Phase I Acute Tests (n=5)



POTW2 effluent was outside compliance limits for marine chronic tests as well as freshwater acute tests. Since the acute test implicated ammonia and non-polar organics as potential toxicants, the role of these classes of compounds with the giant kelp test and the red abalone tests was evaluated.

Initial studies performed using kelp and abalone in seawater indicated that the kelp NOEC to total ammonia was 20-40 mg/L, while abalone were sensitive to 3 mg/L. We decided to pursue TIE procedures with giant kelp to determine what classes of compounds in the effluent besides ammonia were potentially toxic. Before testing proceeded, the TIE was designed. Validation studies indicated that the design would not introduce artifactual toxicity into test procedures.

Table 3. Red abalone and giant kelp reagent response

Reagent	Abalone NOEC	Kelp NOEC
Sodium thiosulfate	< 50 mg/L	>500 mg/L
EDTA	10-100 mg/L	>10 mg/L
Methanol	0.5 mg/L	1%
BIS-TRIS	< 10 g/L	20 g/L
MOPSO	<10 g/L	20 g/L
POPSO	<10 g/L	20 g/L

CASE STUDIES

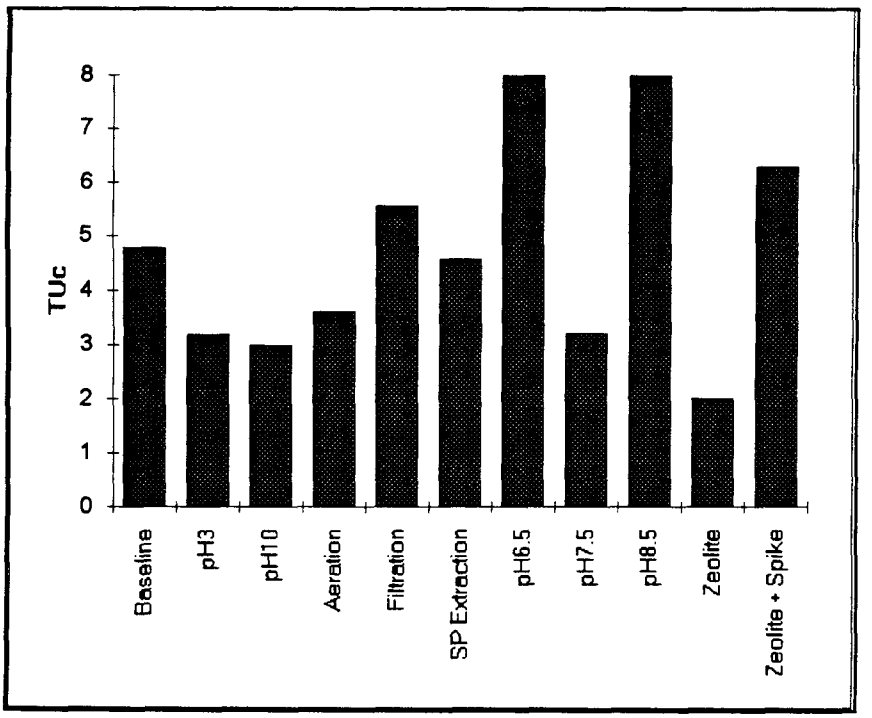
POTW1. The development endpoint was pursued for the case study due to the lack of consistent toxicity in the effluent. Phase I results indicated that the majority of toxicity was due to ammonia. However, the graduated pH test was not successful at pH6.5; this pH is apparently outside the biological range of the test. Zeolite was added to test solutions, which lowered total ammonia from 20 mg/L to 5 mg/L, significantly reducing the toxicity. When ammonia was spiked back into zeolite-effluent, the toxicity was recovered (Figure 2). A slight reduction in toxicity was observed with the addition of EDTA and with the addition of sodium thiosulfate, implicating metals in the toxicity. These reductions have been confirmed, but Phase II and III procedures have not been completed.

POTW2. TIE procedures with giant kelp indicated that toxicity was reduced by

- Adjustment to pH10
- Filtration at pH3,pHi,pH10
- SPE at pH3,pHi,pH10
- EDTA additions (very slight)
- Sodium thiosulfate additions (very slight)

Toxicity was recovered in C-18 columns if elution with 75% methanol was performed immediately. The specific organic toxicant(s) have not yet been identified. The fine powder released from zeolite addition interfered with abalone development, but kelp spores were found to have developed normally underneath the zeolite resin if the settled powder was rinsed away. Reduced toxicity due to filtration in the kelp test may be related to monitoring studies which indicated a correlation between kelp forestation surrounding discharges versus levels of solids in the effluent.

Figure 2. Case study: Echinoderm development - POTW1.



CONCLUSIONS

TIE efforts with west-coast marine species have been successful at identifying classes of compounds causing toxicity. The echinoderm 48-72 hour development test can be used in conjunction with the fertilization test to characterize toxicity. The graduated pH test below pH7 was not successful with the echinoderm development test because below this pH, development is impaired. Red abalone are sensitive to total ammonia levels of 3 mg/L, while kelp are sensitive to 30 mg/L. High solids levels in effluents may be a factor in the abalone and the giant kelp response. The mechanisms behind the reaction to solids is unknown, but is under investigation.

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IDENTIFICATION OF CHRONIC TOXICITY TO ECHINODERMS DUE TO COPPER IN A MUNICIPAL EFFLUENT

ABSTRACT

A municipal effluent produced chronic toxicity to echinoderms. TIE procedures were adapted to the echinoderm bioassay. Phase I TIE procedures implicated cationic metals as the cause of toxicity. Comparison of toxicity values for Ag, Cd, Cu, and Hg with effluent concentrations further implicated Cu. A novel study design involving Cu spiking and serial dilution of effluent samples showed that the toxicity was related to the Cu concentration instead of the effluent concentration. Retrospective analysis of effluent Cu values agreed with echinoderm toxicity data obtained over three years.

INTRODUCTION

As part of the Effluent Toxicity Characterization Program, a total of 18 toxicity testing events were completed on a municipal effluent with three aquatic species. The species included a cladoceran (*Ceriodaphnia dubia*), a fish (*Menidia beryllina*), and echinoderms (*Strongylocentrotus purpuratus, Dendraster sp., and Lytechinus pictus*). Over the course of the program, several effluent samples produced NOECs with echinoderms that were less than 10 percent effluent. Consequently, a Toxicity Identification Evaluation (TIE) was initiated to identify the component(s)

of the effluent that were responsible for the toxicity. The TIE was conducted in three parts: (1) baseline studies were conducted to verify that the Phase 1 TIE procedures could be adapted to the echinoderm fertilization test since procedures have not been published for TIEs with echinoderms; (2) once the test parameters were defined, effluent samples were screened for toxicity and samples that resulted in appreciable toxicity were then subjected to TIE procedures to identify the toxic agent(s) and, (3) confirmation studies were conducted to determine if levels of the suspected toxicant could quantitatively account for the effluents toxicity.

METHODS

Effluent toxicity screening studies

Weekly effluent samples were assayed for toxicity using the echinoderm fertilization test. The level of toxicity was judged not only on the basis of the no-observable effect concentration (NOEC) but also on the magnitude of the effect at the lowest observable effect concentration (LOEC). Thus, not only did the effects have to be statistically significant, they also had to be appreciably large. This approach was justified on the basis of maximizing the usefulness of the TIE approach; since components of the effluent can be lost through handling and degradation of the sample, samples having greater toxicity assure that sufficient material will remain throughout the different steps of the procedure to produce toxicity and facilitate interpreting the results.

Species sensitivity studies

Because of seasonal variations in spawning patterns of different echinoderm species, three different species were used over the course of the variability study. To determine the extent to which the species might exhibit different sensitivity to the effluent, two of the three species were tested simultaneously on the same effluent. *Dendraster* and *S. purpuratus* obtained from wild stocks were held under laboratory conditions to extend their spawning season. All bioassays used a sperm exposure of 20 min. The sperm to egg ratio was determined on the basis of a pre-test that also incorporated a 20 min exposure.

TIE reagent toxicity studies

Two major components of the TIE procedure include chelation of metal ions and removal of non-polar organic materials with C-18 solid phase extraction columns (SPE) columns. Both processes are designed to selectively reduce or remove identifiable components of the effluent. Consequently, a reduction in toxicity after a specific treatment implicates certain materials as the cause of toxicity. The C-18 extraction is carried a step further in that the SPE column is eluted with methanol and the extract is re-tested for toxicity. A positive result confirms that the toxic material was trapped on the column.

These procedures require that the test organism be exposed to chemicals involved in the TIE reagents; EDTA in the case of the chelation step and methanol in the case of the organic extraction add-back step. Sodium thiosulfate may also be used to selectively chelate certain metals. Consequently, it is important to know what concentrations of these chemicals can be tolerated by the test organism before the TIE can be conducted. While some baseline levels of these materials have been published for *Ceriodaphnia* and larval fathead minnows, the effect of these chemicals on the echinoderm fertilization test has not been studied. Therefore, studies were conducted to determine the acceptable concentrations of methanol and EDTA to both *Dendraster* sp. and *S. purpuratus*. No baseline work was conducted with sodium thiosulfate; the response of echinoderms to this material was determined by evaluating fertilization success across a range of concentrations during the actual TIEs.

Ammonia toxicity studies

Because the pH adjustments required for the evaluation of ammonia toxicity affect fertilization success in echinoderms, pH adjustments were not included in the planned TIE. However, because ammonia was potentially an important toxic component of the effluent, an additional bioassay was conducted to determine if ammonia contributed to effluent toxicity, particularly at concentrations of less than 10 percent effluent. The bioassay used ammonium chloride and evaluated fertilization success within a concentration range of 6.2 to 100 mg/L total ammonia.

RESULTS

Effluent toxicity screening studies

The echinoderm toxicity data from the entire variability study are presented in Table 1. In addition to overall toxicity, the results are also shown for the different species used.

Table 1. NOECs obtained for the different echinoderm species used during the variability study.

	NOEC (%)		
Test Date	Lytechinus	Dendraster	S. purpuratus
7/89	4.2	N/T	N/T
8/89	4.2	4.2	N/T
	N/T	4.2	N/T
	N/T	1.0	N/T
9/89	N/T	1.0	N/T
	N/T	16.8	N/T
	N/T	16.8	N/T
10/89	N/T	N/T	33.5
	N/T	N/T	4.2
11/89	N/T	N/T	8.4
	N/T	N/T	33.5
12/89	N/T	N/T	67.0
1/90	N/T	N/T	67.0
2/90	N/T	N/T	67.0
3/90	N/T	N/T	4.2
4/90	N/T	N/T	8.4
5/90	N/T	33.5	N/T
6/90	N/T	N/T	16.8
8/90	N/T	N/T	4.2
Average	4.2	11.1	28.6
Std. Dev.	0.0	12.0	26.8
n	2	7	11

N/T = not tested.

While the *Lytechinus* data set is too small (n=2) to derive conclusions, it is clear that the *Dendraster* and *S. purpuratus* NOEC values overlapped considerably. Even though the average NOEC values differed by a factor of about 2.5, the large variability precluded finding a statistically significant difference ($p<0.05$) between the two data sets. The results may have been further obscured by coincidences between seasonal variability in the effluent's toxicity and seasonal availability of the test organisms. However, overall, approximately 50 percent of the tests conducted with *Dendraster* (4 of 7) and *S. purpuratus* (5 of 11) resulted in NOECs of < 10 percent effluent.

Comparison of species sensitivity to the effluent

The data in Table 1 suggested that *Dendraster* and *S. purpuratus* did not respond similarly to the effluent samples. To characterize this apparent difference in species sensitivity, a simultaneous fertilization test was conducted with both species. The NOEC for *S. purpuratus* was 67 percent effluent while the NOEC for *Dendraster* was < 16.8 percent effluent (the lowest concentration tested). This result suggested an appreciable difference in sensitivity which may have been attributable to the testing procedures, the organisms themselves, or a combination of these factors.

TIE reagent tolerance studies

The appropriate test range for methanol and EDTA which can be used in TIE studies without effecting the fertilization success of *Dendraster* and *S. purpuratus* was determined for each species in three separate tests. The results indicated that fertilization success was not impaired within the following concentration ranges of MeOH (*Dendraster*-0.25 to 2.00 %; *S. purpuratus*-0.06 to 1.50 %). Methanol concentrations in excess of 0.25 % consistently produced granularities in the cytoplasmic membrane of the fertilized egg but this did not interfere with scoring for fertilization on the basis of membrane lift-off.

The sensitivity of fertilization for both species in the presence of different concentrations of EDTA was also determined for *Dendraster* and *S. purpuratus*. Two tests were conducted with *Dendraster* sp. One incorporated a concentration range of 16 to 135 µL EDTA (from a stock concentration of 0.1 M EDTA), while the other incorporated a range of 8 to 60 µL EDTA. In the first test, fertilization success was reduced at concentrations of 23 to 135 µL while concentrations of 33 to 60 uL inhibited fertilization in the second test. In the one test conducted with *S. purpuratus* over an EDTA range of 8 to 66 µL, concentrations of 33 and 66 µL inhibited fertilization success.

Contribution of ammonia to toxicity

The effect of ammonia on fertilization success was evaluated over a concentration range of 6.2-100 mg/L. The controls showed 100 percent fertilization success, compared with 95 percent in the two highest concentrations tested--50 and 100 mg/L. While the reduction in fertilization success was not substantial in these concentrations, it was statistically significant. Since effluent generally contained less than 30 mg/L total ammonia, the results suggested that ammonia was not a significant contributor to toxicity in the effluent toxicity tests.

Phase 1 TIE studies

A total of six effluent samples were screened for toxicity to identify a sample with appropriate toxicity for a TIE investigation. TIEs were pursued with two of these samples (Table 2).

Table 2. Results of TIE evaluations with *Dendraster*.

	Fertilization Success (%)	
Treatment	Sample 1	Sample 2
Control	86.0	92.0
Untreated	64.0	75.0
pH 3	65.0	48.0
pH 11	88.0	78.0
Filtration pH 3	69.0	33.0
Filtration pH i	64.0	56.0
Filtration pH 11	55.0	57.0
Aeration pH 3	45.0	15.0
Aeration pH i	67.0	64.0
Aeration pH 11	68.0	66.0
EDTA (µL)		
8	83.0	88.0
16	73.0	95.0
23	77.0	92.0
33	57.0	93.0
66	25.0	93.0

Table 2, cont'd. Results of TIE evaluations with *Dendraster*.

	Fertilization Success (%)	
Treatment	Sample 1	Sample 2
Sodium thiosulfate (µL)		
12	91.0	72.0
25	74.0	84.0
50	59.0	82.0
100	57.0	80.0
200	39.0	71.0
400	24.0	56.0
800	11.0	63.0
C-18 column pH 3	53.0	*
elution	85.0	*
C-18 column pH i	67.0	*
elution	88.0	*
C-18 column pH 9	69.0	*
elution	88.0	*

*Column blanks exhibited toxicity.

The data in the table suggest that EDTA and sodium thiosulfate were the most effective treatments in reducing toxicity in both samples. In the one sample for which the C-18 extraction data are valid, treatment of the sample with SPE columns did not appear to reduce toxicity. Thus, non-polar organics and weak organic acids and bases were not implicated in toxicity. This is supported by the fact that MeOH did not elute toxicity from the columns. The effectiveness of EDTA suggests that a divalent cation(s) was responsible for toxicity in the samples tested. The concurrent effectiveness of sodium thiosulfate suggested that the potential suite of cations was limited to Ag, Cd, Cu, and Hg.

SUMMARY OF PHASE ONE RESULTS

Tolerances of the two test species (*Dendraster and S. purpuratus*) to EDTA and methanol were determined. These results indicated that the two test species can tolerate these reagents at a level useful in TIE studies. Data from the actual TIEs also suggests that sodium thiosulfate can be used effectively in TIEs with echinoderms. In addition, the sensitivity to ammonia was determined. The relative insensitivity to ammonia is favorable because ammonia is a comparably large component of this and many other municipal effluents. If samples can be tested at higher

concentrations without interference from ammonia, the chances for a successful TIE increase because the difference between the control and treatment response will be greater and thus, there will be less opportunity for random variation to obscure the overall results.

Overall, the Phase 1 results implicated chelatable cations, specifically Ag, Cd, Cu, and/or Hg, as causing the observed toxicity. This conclusion was based on the elimination of toxicity by treatment with the chelating agent EDTA. Moreover, toxicity was also reduced by treatment with sodium thiosulfate. Collectively, these results limited the selection of potential divalent cations to the above four.

CONFIRMATION OF METALS TOXICITY

Sensitivity of echinoderms to cationic metals

To elucidate the role of Ag, Cd, Cu, and Hg in the effluent's toxicity, NOECs were determined for both *Dendraster* and *S. purpuratus*. Metals solutions were prepared in moderately hard freshwater, then hypersaline brine added (1/3 brine:2/3 metal solution) to attain the salinity required for the echinoderm fertilization test. This procedure is analogous to the preparation of effluent (which is freshwater) prior to testing. The concentrations the stock solutions were confirmed analytically prior to use. The results of bioassays with different concentrations of metals on echinoderm fertilization success are summarized below.

Table 3. NOECs (µg/L metal) obtained for *Dendraster* and *S. purpuratus* exposed to different metals.

Metal	*Dendraster*	*S. purpuratus*
Ag	> 13.4	> 13.4
Cd (Test 1)	> 9.4	N/T
(Test 2)	> 67.0	> 67.0
Cu (Test 1)	5.4	N/T
(Test 2)	10.0	20.0
(Test 3)	3.8	N/T

Table 3, cont'd. NOECs (µg/L metal) obtained for *Dendraster* and *S. purpuratus* exposed to different metals.

Metal	*Dendraster*	*S. purpuratus*
Cu (Test 4)	8.0	N/T
(Test 5)	13.1	19.7
Hg (Test 1)	0.1	N/T
(Test 2)	> 0.7	> 0.7
(Test 3)	> 2.2	N/T

N/T = not tested

One test was conducted with Ag. The NOECs for both species were > 13.4 µg/L. Two tests were conducted with Cd. In both cases, the highest concentrations tested (9.4 and 67.0 µg/L) failed to produce any measurable effects on fertilization success. A total of 5 tests were performed with Cu. The NOECs for *Dendraster* ranged between 3.8 and 19.6 µg/L, with an average of 9.4 µg/L. In the two trials in which concurrent tests with *S. purpuratus* were performed, the NOECs were 1.5 to 2 times greater than those obtained with *Dendraster*. A total of three tests were performed with Hg, with mixed results. The first test appeared to give an NOEC, but no-effects on fertilization success were found in the next two studies at concentrations up to 0.7 and 2.2 µg/L, respectively.

Comparison of Toxic Concentrations of Metals with Concentrations Found in the Effluent

Data on the concentrations of metals in the effluent were obtained from the discharger and NOECs for each of the metals were compared to the range of concentrations found in the effluent. Toxicity ratios were calculated for each metal [NOEC (µg/L)/metal concentration (µg/L)]. A ratio substantially greater than one implied that the metal(s) was not present in the effluent at concentrations high enough to account for toxicity. Conversely, a ratio of one, or less, indicated that the concentration of metal approached or exceeded the NOEC and, consequently, could cause toxicity. Any metals that fell into this latter category were subjected to further analysis. These data are summarized below:

Table 4. Comparison of effluent concentrations of selected metals with NOECs derived from laboratory studies with *Dendraster* (all values are as µg/L).

Metal	Effluent Concentration	NOEC	Ratio*
Ag	< 0.2 - 4.0	> 13.4	3.4 to > 67
Cd	< 0.2	> 9.4; > 67	> 47 to > 335
Cu	4.0 to 9.0; sometimes to 19	3.8 to 19.6	0.2 to 4.9
Hg	< 0.2 - 0.4	0.1, > 0.7, > 2.2	Uncertain

* If the ratio (NOEC/effluent concentration) is close to 1, or less, then the metal is a candidate for further study.

Clearly, the relatively large ratios associated with Ag and Cd suggest that the concentrations of these metals were not high enough to produce intermittent toxicity associated with the effluent. The effect of Hg is uncertain because of the discrepancies in the NOECs. However, the two tests aimed at validating the first result (0.1 µg/L) both failed to produce effects in the highest concentrations tested, up to 2.2 µg/L. This suggests that the first result may have been an artefact. Moreover, the range of concentrations of Hg did not appear to be great enough to account for the range of variation in effluent toxicity. Cu was the most promising of the metals to be identified in this analysis since effluent concentrations frequently overlapped the range of NOECs obtained from tests with *Dendraster*. Moreover, *S. purpuratus* exhibited less sensitivity to this metal than *Dendraster*, which agreed with other results that suggested that *Dendraster* were consistently more sensitive to the effluent than *S. purpuratus*.

Confirmation of Suspected Toxic Component

The toxicity ratios for the candidate cationic metals suggested that Cu was the most likely toxic component of the effluent. However, the data presented in previous sections point out potentially problematic aspects associated with identifying toxicity in the effluent and confirming the identity of the causative agent. First, *Dendraster* appeared to exhibit greater sensitivity to Cu than did *S. purpuratus*. In addition, the more sensitive species exhibited a fairly wide range of sensitivity to Cu. Finally, the effluent itself exhibited considerable temporal variations in Cu concentrations; according to the discharger's monitoring data during the period of interest, Cu concentrations averaged 8.6 µg/L , with a range of 3 to 19 µg/L. Consequently, a combination of sensitive organisms, high statistical resolution, and sufficient Cu present in the effluent would be necessary to enable us to assess the contribution of Cu to effluent toxicity. With this in mind, studies were designed to confirm Cu as the primary factor in effluent toxicity. The first involved parallel

bioassays of effluent and seawater spiked with Cu at concentrations found in the effluent. By comparing the response directly across corresponding concentrations of Cu, it would be possible to determine if Cu could account for effects seen in the effluent. The data are summarized in Table 5, below.

Table 5. Comparison of NOECs and LOECs (as µg/L Cu) and Percent Fertilization Obtained from Bioassays on Effluent (sampled 12/10/91) and Seawater Spiked with Cu Using *Dendraster.*

Treatment	NOEC*	LOEC*
Effluent	3.8 (89.3)	7.5 (73.3)
Seawater + Cu	3.8 (88.7)	7.5 (80.0)

* As µg/L Cu; percent fertilization given in parentheses.

Based on Cu concentration, the NOECs and LOECs were consistent between the effluent sample and the concurrent bioassay with Cu. Furthermore, the percent fertilization was very similar in corresponding concentrations of both bioassays. These data suggested that Cu could account for the reduction in fertilization success associated with this effluent sample.

To further test the hypothesis that Cu was a likely cause of effluent toxicity, another study was conducted with a more rigorous experimental design. The approach entailed spiking a subsample of effluent with Cu (as $CuSO_4$) such that the subsample contained twice (2X) the amount of copper as the original sample. Serial dilutions, which incorporated a 50 percent dilution factor, were then prepared with the spiked and unspiked samples. Additional subsamples were also spiked at 1.5 and 3X copper. Depending on the results, it could be determined whether copper was the controlling factor in toxicity in the presence of the effluent matrix. Our reasoning follows:

- If copper controlled toxicity, then the LOEC obtained for the 2X series of dilutions should be one concentration level below that obtained with the unspiked sample. Moreover, the dose-response associated with the samples spiked with increasingly higher copper concentrations should be continuous with the response in dilutions of the unspiked sample.

- Alternatively, if copper did not control toxicity, the LOECs obtained from dilutions of the spiked (2X) and unspiked samples should be identical since, with the exception of copper, the effluent parameters were the same at each dilution. Furthermore, the dose-response associated with the higher copper concentrations (1.5-3X) should be discontinuous with the response in the unspiked sample.

The results of the simultaneous exposures to the dilution series of effluent and effluent spikedwith Cu are shown below.

Table 6. Percent Fertilization Obtained from Bioassays with *Dendraster* on Effluent (sampled 1/7/92) and Effluent Spiked with Cu.

Effluent Concentration (unspiked)			Effluent Concentration (spiked at 2X Cu)		
(% Effluent)	(µg/L Cu)	% Fert	(%) Effluent	(µg/L Cu)	% Fert
0.0	0.0	96	0.0	0.0	96
8.4	0.8	97	8.4	1.6	91
16.8	1.6	97	16.8	3.3	90
33.5	3.3	91	33.5	6.6	83*
67.0 (1X)	6.6	82*	67.0	13.2	76*
67.0 (1.5X)	9.9	74*			
67.0 (2X)	13.2	74*			
67.0 (3X)	19.8	72*			

* Significantly less than controls; $p<0.05$.

For this effluent sample, fertilization success was reduced in 100 percent effluent (1X Cu), but not in 50 percent effluent. In the effluent spiked to 2X Cu, the effect level dropped to 50 percent dilution (1X Cu). Not only were the NOECs were the same, based on Cu concentration, the percent fertilization success was very similar (82 vs 83 percent) in the corresponding Cu concentrations at 1X. These results support the hypothesis that Cu was responsible for toxicity in this effluent sample. Moreover, increasingly higher Cu concentrations (up to 3X) produced further reductions in fertilization success, compared with 100 percent effluent. Note that a concurrent assay conducted with *S. purpuratus*, showed no response to the effluent, even when spiked up to 3X with Cu.

DISCUSSION

Results from the Phase 1 TIE suggested that the metal ions Ag, Cd, Cu, and Hg might be responsible for the effect of CCCSD's effluent on fertilization success in echinoderms. This conclusion was based on reduced toxicity following treatment with EDTA and sodium thiosulfate. Furthermore, it was demonstrated that *Dendraster* was more sensitive to the effluent than *S. purpuratus*.

Follow-up studies were aimed at establishing the sensitivity of the echinoderms to the different metals, comparing their sensitivities to concentrations found in the effluent, and confirming suspect metals as causing effluent toxicity. The results of these studies showed that concentrations of Ag and Cd in the effluent were well below their respective NOECs. Two of the three tests conducted with Hg indicated that its toxicity was limited to concentrations in excess of those found in the effluent. Conversely, the range of Cu concentrations found in the effluent overlapped the range of NOECs obtained for *Dendraster*. As Table 3 suggests, *S. purpuratus* was also less sensitive to Cu than was *Dendraster*.

Confirmatory studies also implicated Cu. Toxicity was demonstrated at Cu concentrations identical to those found in the effluent. Furthermore, fertilization success in a spiked effluent sample was related to Cu concentration, instead of effluent concentration. Finally, a dose-response was demonstrated in effluent spiked with increasingly higher concentrations of Cu.

These results support the hypothesis that Cu was responsible for the effluent's intermittent toxicity demonstrated by the echinoderm fertilization test during 1991 and 1992. Of the four materials identified in the Phase 1 TIE, Cu was the only one that occurred in the effluent at concentrations that overlapped the toxic range. Cu also exhibited greater toxicity to *Dendraster* than to *S. purpuratus*. This is important because extensive screening in 1991 with *S. purpuratus* failed to reveal any toxicity associated with the effluent. However, when *Dendraster* became seasonally available, intermittent toxicity with effluent samples occurred, even though these same samples failed to cause any effect on *S. purpuratus*.

While the results of this study appear to implicate Cu as the primary cause of reduced fertilization success in echinoderms exposed to this effluent, the effects were largely limited to effluent concentrations in excess of 20 percent. Thus, they may not address the low NOECs (< 10 percent effluent) found during the Variability Phase of the Effluent Characterization Study conducted in 1989. To put those results in perspective, analytical data on copper concentrations obtained from the treatment plant for 1989, 1990, and 1991 are shown in Figure 1. Copper concentrations ranged between 7 and 20, 2 and 16, and 1 and 19 µg/L for the three years, respectively. Note that the analyses were performed only quarterly for most of 1989, while the more recent data are based on weekly measurements.

Frequency distributions of the analytical data, as well as LOECs obtained from reference toxicant tests with *Dendraster* and *S. purpuratus* over the three year period, are summarized in Figure 2.

The box plots, which show the 50^{th} and 90^{th} percentiles for each of the data sets, suggest that Cu concentrations in 1990 and 1991 were both lower than concentrations in 1989. In addition, while there was overlap between LOECs obtained for both of the echinoderm species, *Dendraster* was consistently more sensitive to Cu concentrations less than 10 µg/L. As the figure also suggests, *S. purpuratus* would have responded only very occasionally to 1990 and 1991 effluent concentrations of Cu, but would have responded frequently to effluent copper concentrations in 1989. This observation agrees with the experimental data obtained from effluent samples tested with both species over the three year period.

The only question remaining is whether the measured copper concentrations could have accounted for the NOECs that were less than 10 percent effluent. As Figure 2 shows, ten percent of the Cu concentrations during the period of interest exceeded 18 µg/L. As the same figure shows, approximately 10 percent of the LOECs obtained for *Dendraster* were less than 3 µg/L Cu. In order for the effluent NOEC to be less than 10 percent, the LOEC would have to be 20 percent, or less. At an effluent Cu concentration of 18 µg/L, 20 percent effluent would correspond to a Cu concentration of 3.6 µg/L. Clearly, Cu concentrations were high enough in 1989 to account for effluent NOECs of 10 percent, or less, with *Dendraster*. Using a similar argument for the 1990 data, a 20 percent effluent concentration would correspond to a Cu concentration of approximately 2.7 µg/L. This value is lower than the 1989 value and also lower than the 10th percentile LOEC value of 3 µg/L Cu for *Dendraster*. These data suggest that Cu could have produced effluent NOECs of < 10 percent in 1990 but the frequency would have been less than in 1989. Finally, the 1991 data suggest a maximum of only 2.4 µg/L Cu at 20 percent effluent, further reducing the possibility that Cu could account for NOECs of 10 percent effluent, or less. Note that this discussion emphasizes *Dendraster*; with a lower 10th percentile LOEC value of 9.5 µg/L, exceptionally high Cu concentrations ($\geq$ 47.5 µg/L) in the effluent would have been necessary to consistently produce NOECs of less than 10 percent effluent with *S. purpuratus*.

CONCLUSIONS

❒ Phase I TIEs implicated Ag, Cd, Cu, and Hg as potentially causing toxicity in samples of the effluent.

❒ Follow-up comparisons of laboratory derived NOECs for each of these metals with their concentrations in the effluent suggested that Cu was the only metal present at concentrations high enough to cause toxicity.

❒ Additional studies confirmed the presence of Cu toxicity in samples of the effluent. However, effluent samples tested in this phase produced responses in *Dendraster* but not in *S. purpuratus*. This observation agrees with the species sensitivity studies which showed that *Dendraster* were more sensitive to Cu than *S. purpuratus*.

❒ An analysis of analytical data obtained from the period of interest suggests that Cu concentrations could have accounted for the low NOECs associated with effluent samples tested with *Dendraster* in the initial part of the effluent toxicity variability program. Much higher effluent levels of Cu would be necessary to produce NOECs of < 10 percent with *S. purpuratus*.

Figure 1: Effluent Copper Concentrations During 1989 -1991.

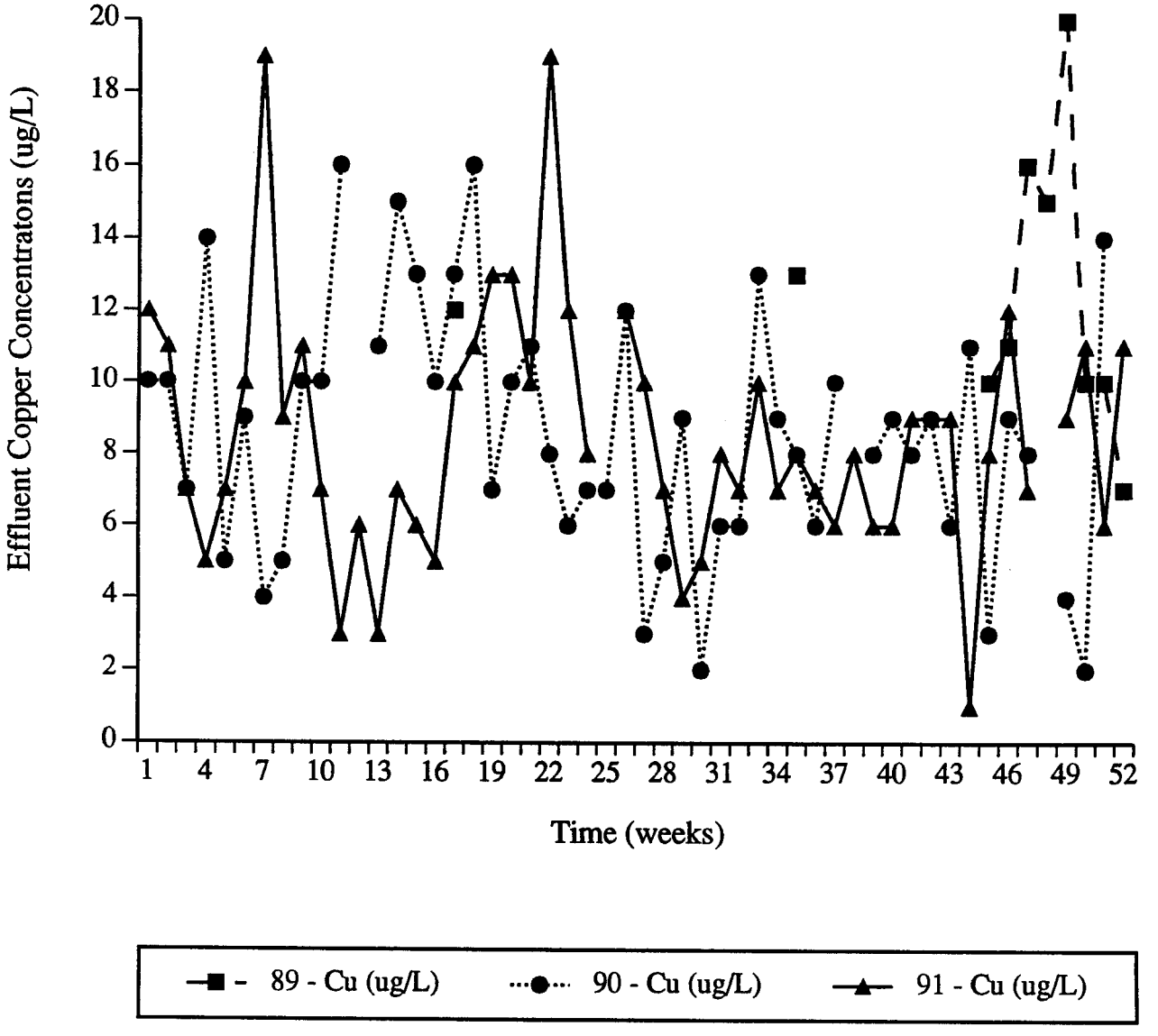
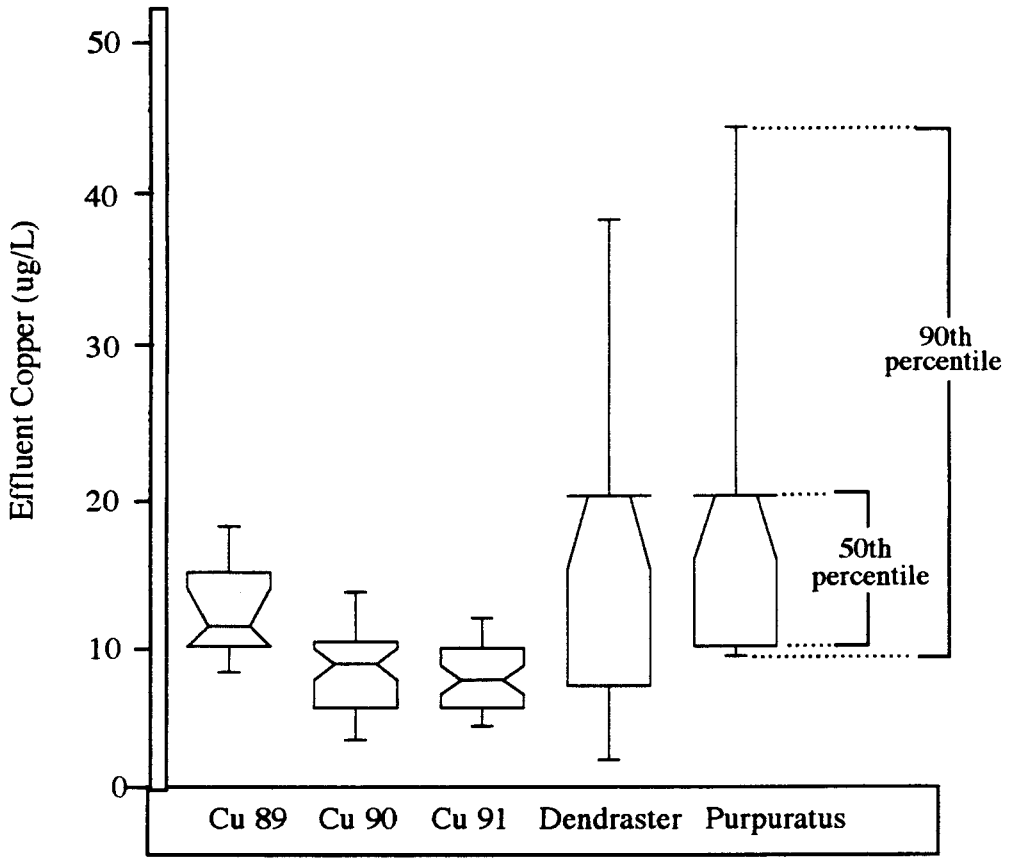


Figure 2: Effluent Copper Concentrations and Echinoderm LOEC's



	Cu 89	Cu 90	Cu 91	Dendraster	Purpuratus
Mean	12.4	8.6	8.3	17.2	23.2
Std Dev	3.2	3.5	3.1	11.4	19.9
10th percentile	-	-	-	2.8	9.5
90th percentile	18.0	13.2	12.0	-	-
N	10	4	5	1	1

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TECHNIQUES FOR DERIVING ADDITIONAL INFORMATION FROM CHRONIC TOXICITY TESTS WITH *CERIODAPHNIA DUBIA*

The protocol for estimating the chronic toxicity of effluents to *Ceriodaphnia dubia* (EPA-600/4-89-001) was extensively used for monitoring of toxicity in storm water samples collected in Santa Clara and Alameda Counties. Calculation of the median time to lethality (LT_{50}) using the daily observation data, yielded a range of values which measured the relative intensity of toxicity in each sample. For processing of reproduction data, a raw data spreadsheet with formula has been developed. The spreadsheet calculates the number of offspring per female per reproductive day (OFRD) for the last three days of the test. Comparison of the OFRD value of a sample with the control OFRD value for the same day enabled us to detect reproductive effects even in samples in which all of the adults died before the end of the test. Moreover, it allowed us to confirm the lack of reproductive effect in some lethal samples. Application of this technique to raw data obtained in the Santa Clara and Alameda NPS Programs indicate that storm water toxicity may be manifested in two distinct effects, one related to lethality and the other to reproduction.

INTRODUCTION

Processing of toxicity test data as instructed in the EPA chronic test protocol (USEPA 1989) is designed for tests using dilution series and for expression of test results in end-points stating the No Observed Effect Concentration (NOEC) or Lowest Observed Effect Concentration (LOEC) for compliance purposes. The data can also be used to calculate point-estimates such as LC_{50} or EC_{50}. In either case, the key variable is concentration. However, when this protocol is applied to screening tests of 100% samples without dilution, end points based on dilution are not available.

Chronic toxicity tests have been widely used in the screening mode (without dilution) in storm water monitoring in the San Francisco Bay Region. We have used additional data-processing steps to derive expressions of toxicity which may allow more rigorous comparisons among various samples and comparisons of samples with controls. These

techniques, as were applied to raw toxicity data obtained for numerous storm water samples collected in the region, are described in the present report.

METHODS

Short term chronic toxicity bioassays were conducted with runoff samples according to the EPA protocol outlined in EPA/600/4-89/001 (USEPA 1989). Ten test animals per treatment were exposed to runoff samples or control water for 7 days, with daily feeding and renewal of test solutions. Survival observations were performed daily and reproduction was monitored for each animal on day 3 through day 7 of the test. The chronic toxicity bioassays for both Alameda and Santa Clara toxicity monitoring programs were performed by ToxScan Laboratory. The reports included statistical analysis of survival data (% survival) using the Fisher Exact test to detect significant differences from the control, and Student's T value or Mann Whitney U-value to analyze reproduction data for samples which were not lethal to *C. dubia.*

Samples collected at the DUST Marsh (Alameda County) were analyzed for toxicity using a modified bioassay design, referred to as the "4x5 design". This test design followed the acute bioassay protocol (USEPA 1991), using five test animals in each of four laboratory replicates, and incorporated modifications calling for daily renewals, seven-day test duration, and increased number of observations. The protocol for the 4x5 design was developed at Lawrence Berkeley Laboratory (Katznelson *et al* 1993).

RESULTS AND DISCUSSION

Methods for calculation of LT_{50} values

The median time to lethality (LT_{50}) is the exposure duration (number of hours) at which 50% of the test organisms had died, and is inversely related to the intensity of toxicity: The shorter the median time to lethality, the higher the toxicity. To determine the LT_{50} values, several methods devised to calculate the median lethal concentration (LC_{50}) values were investigated using a variety of data sets. Examples of values obtained for one data set are shown in Table 1. The Trimmed Spearman-Karber method (Montana State University program) yielded underestimated results, sometimes in contradiction to actual observations. The Moving Average method (manual calculation, USEPA 1985) produced values which were very close to those obtained by the graphical method (manual calculation, USEPA 1991). There was a good agreement between the graphical method and the old version of the Probit method (EMSL Cincinnati, Revision 1.0, 1987). There was a discrepancy in the values obtained by the different versions of the Probit method, and the new program (Version 1.4) was limited in its ability to compute confidence intervals for "problematic" data sets. Eventually, the Probit method (Version 1.0) was chosen for its ability to compute LT_{50} based on probability and to supply 95% confidence interval. Since it is important to use only one method to allow comparison of an array of data sets within one test, the requirement for at least two partial mortalities may need to be overridden in several cases and the results should be interpreted with caution.

Table 1. Comparison of median time to lethality (LT_{50}) values obtained by different calculation methods

Raw data

Time (h)	1	24	54	74	93	116	140	166
No. Exposed	25	25	25	25	25	25	25	25
Mortalities	0	1	1	3	8	16	24	25

Results:

Method	LT_{50}	95% Lower C.I.	95% Upper C.I.
Trimmed Spearman Karber (Montana State University)	91.6	76.8	109.2
Probit Version 1.4 (EMSL Cincinnati)	96.6		
Moving Average Angle (EPA 600/4-85-013)	102.6	95.3	110.2
Probit Version 1.0 (EMSL Cincinnati 1987)	102.5	68.3	153.7
Graphical Method (EPA 600/4-90/027)	105		

Correlation of LT_{50} values with other storm water quality parameters

Figure 1 demonstrates the use of LT_{50} values to correlate toxicity with water quality parameters and with concentrations of other pollutants found in storm water. Data collected at the DUST System during a storm event are plotted for four sampling stations situated along the horizontal axis of storm water flow in the Crandall Creek and the DUST Marsh. Survival endpoints, as expressed by the percentage of survivors at the end of seven-day exposure (Figure 1A), did not provide meaningful comparisons with electrical conductivity (EC) (Figure 1B) or with total copper concentrations (Figure 1C). However, derivation of LT_{50} values from the mortality records (Figure 1D) shows a trend in the relative intensity of toxicity which correlates well with EC and with copper concentrations observed in the same samples. The correlation with EC is to be expected, since both EC values and toxicity intensity reflect the dilution of low-conductivity, toxic storm water with the high-conductivity, non toxic marsh water. Other pollutants such as copper are probably diluted in similar proportions. Measurements of polynuclear aromatic hydrocarbons (PAH) during another storm event monitored in the DUST System yielded a horizontal gradient as well (not shown). Although these correlations do not suggest that copper or PAHs are the cause of toxicity, they

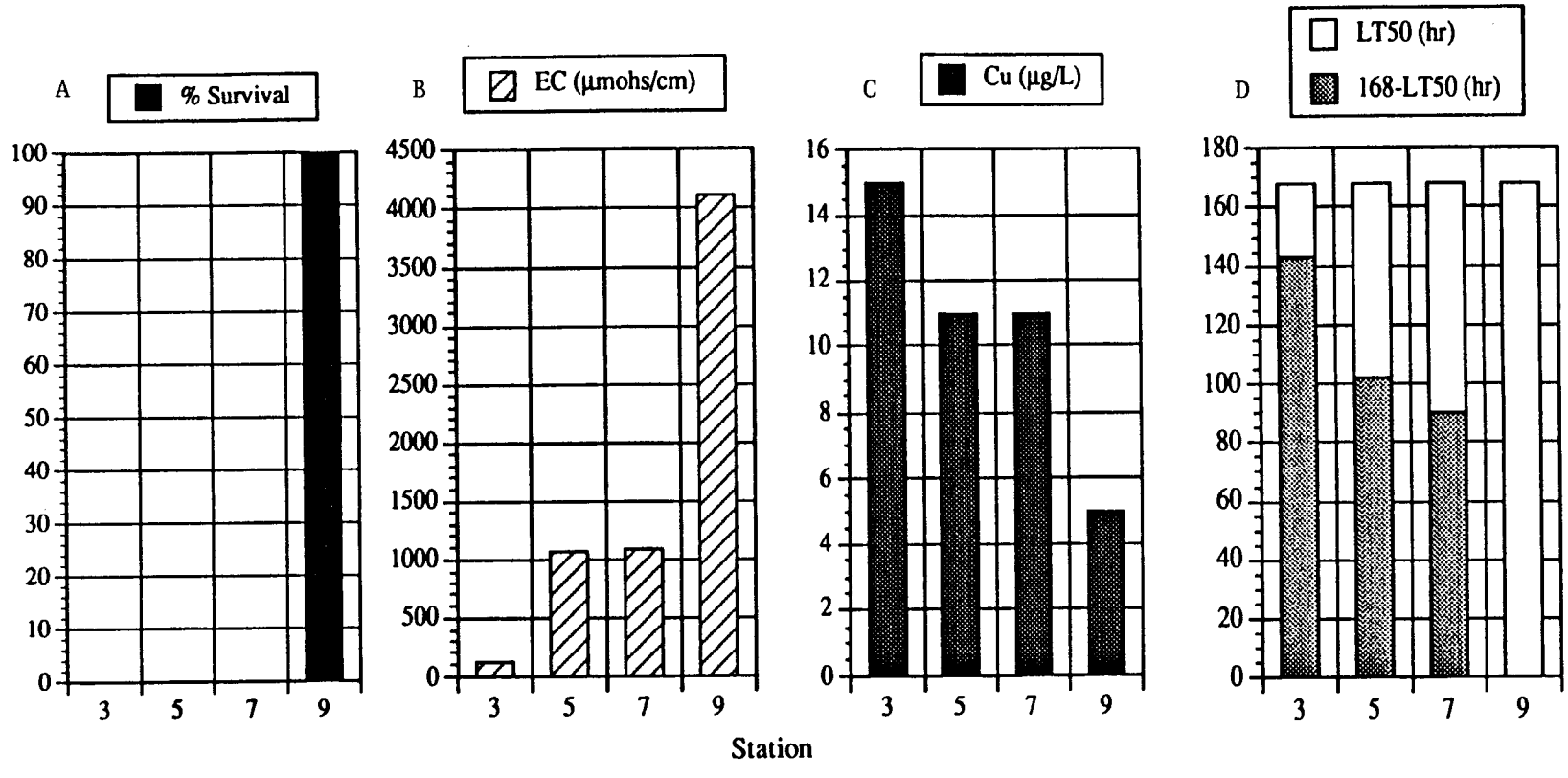

Figure 1. Comparison of toxicity, electrical conductivity, and total copper concentration in samples collected at Crandell Creek and DUST Marsh during a storm event

indicate that the relative intensities of toxicity are good indications for the severity of pollution in storm water in this hydrological system.

Measuring the relative intensity of toxicity

LT_{50} values were used to compare toxicity of runoff samples collected in various land-use stations, which were monitored routinely to provide a long term assessment of toxicity. Samples collected in Santa Clara and Alameda counties during the five winters of 1988-1993 were generally toxic to *C. dubia* (WCC 1989, 1991a, 1991b, 1992a, 1992b, 1993). The results of chronic, 7-day bioassays performed during 1991-1993 were arranged by categories of toxicity intensities (Table 2). The table lists the categories in ascending order of intensity and specifies the useful toxicity expressions (e.g., LT_{50} or LC_{50}) for each category. Categories B, C and D are usually referred to as "chronic toxicity", while samples in categories E and F are considered "acutely toxic". Categories C, D and E share a common scale of LT_{50}. All samples included in these categories are, therefore, comparable to each other. Categories E and F share a common scale of LC_{50} values. Since the chronic toxicity test design (without dilution) which has been used in the monitoring efforts does not yield LC_{50} values, comparisons of intensity of toxicity within category F cannot be made.

Table 2. Categories of toxicity intensities observed in storm water samples subject to chronic toxicity bioassys with *Ceriodaphnia dubia*[1]

Category		Effect		LC_{50}[2] Range	LT_{50}[3] Range	Stream (mixed)	Indus-trial	Trans-portation
		Lethality	Reproduction Inhibition[4]	(% sample)	(hours)	% of Samples		
A	Non-toxic (No effect)	No	No	N/A	N/A	14	6	8
B	Non-lethal (Repro effect)	No	Yes	N/A	N/A	8	-	46
C	Moderately toxic (No Repro effect)	Yes	No	N/A	96-168 h (4-7 days)	19	-	8
D	Moderately toxic (Repro effect)	Yes	Yes			5	-	23
E	Highly toxic	Yes	N/A	50-99%	20-96 h (1-4 days)	46	11	15
F	Extremely toxic	Yes	N/A	2-50%	< 20 h	8	83	-

[1] Flow-weighed composite storm water samples collected at Santa Clara and Alameda Counties during 1991-1993 storm events are included.
[2] 96h LC_{50}: Median lethal concentration: The dilution (percent sample) which causes mortality of 50% of the test organisms within 96 hours of exposure.
[3] LT_{50}: Median time to lethality: The exposure duration at which 50% of the test organisms had died.
[4] Inhibition of reproduction: Number of offspring per female per reproductive day (OFRD) in the sample is significantly lower than control OFRD.
N/A: Not Applicable

The mortality data (Table 2) for samples from various land use stations reveal distinctly different distribution among toxicity categories. The majority (70%) of the stream samples were in categories C, D, and E (moderate to high toxicity), most (83%) of the industrial samples were extremely toxic (category F), while more than 50% of samples collected at transportation stations were not lethal to *C. dubia.*

Reproduction effects in *C. dubia*

In the chronic toxicity test setup, neonate *C. dubia* are placed and maintained in individual test chambers to monitor reproduction. Under favorable conditions, reproduction of cladocerans is parthenogenetic (does not require the presence of males) and the culture is composed of females only. The young animals usually produce the first brood of offspring by day 3 or day 4, depending on the quality of food and on other factors (Winner 1989). Subsequent broods are released on days 5,6 and 7 of the test. Reproduction end points are normally reported only for samples in which most of the test animals survived exposure for 7 days, and are based on the total number of offspring per female for the test duration (USEPA 1989). We have developed a spreadsheet consisting of an input area, where the daily observations are entered, and an output area with formulae which calculate the number of offspring per female per reproductive day (OFRD), for each female separately, based on the number of reproductive days and the number of offspring produced until its death or until day 7, whichever came first. Figure 2 presents a graphical comparison of average OFRD values calculated for days 5, 6, and 7 of a test. It is obvious that reproduction in the control was higher than in the sample (T1). Average OFRD values increase as the test proceeds. This pattern of increasing OFRD values is observed very often in test results, indicating that the fecundity of the young cladocerans increases as they mature. Therefore, when OFRD values obtained in a lethal sample for day 5 or day 6 are compared to control OFRD, it is very important to make the comparison with control OFRD of the corresponding day (5 or 6). Thus, in lethal samples, the OFRD values for the last day in which >50% of the females were present were compared to the control OFRD values obtained for the same day. The choice of a statistical procedure was dictated by each set of test data: If only one sample was to be compared to control for a given day, a t-test was used. For tests in which two or more samples were compared to control (usually using day 7 data), ANOVAs (Dunnett's, Bonferroni or Steel's) were used.

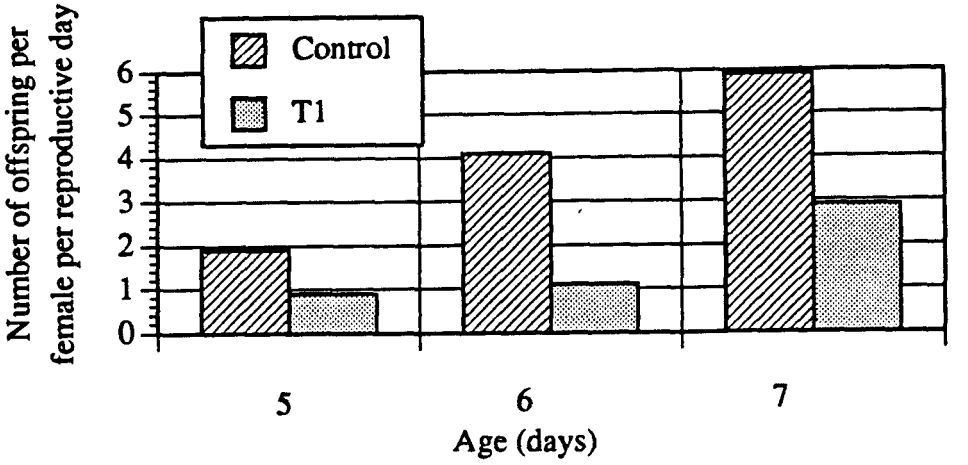

Figure 2. Fecundity of Ceriodaphnia dubia as a function of age in a storm water sample and in control medium.

The new approach for processing of reproduction data allows detection of reproductive effects, or confirmation of lack thereof, in moderately toxic samples (categories C and D in Table 2). By calculating the number of offspring per female per reproductive day (OFRD), we were able to detect significant reproductive effects in the majority of the moderately toxic transportation station samples. When these figures are added to the incidence of reproductive effects in non-lethal transportation samples, another striking difference between land-use stations emerges: 69% of the transportation station samples inhibited reproduction in *C. dubia*, whereas in stream station samples very low incidence (13%) of reproductive effects was observed while the incidence of lethality was extremely high (Table 2). Thus, storm water toxicity may be manifested in two distinct effects, one related to lethality and the other to reproduction.

Effluents and storm water are complex mixtures which may contain at least three major types of toxic substances. Type 1 includes toxicants which are lethal to *C. dubia* but do not affect the organism's capability to reproduce, Type 2 are substances which specifically inhibit reproduction but do not cause death, and Type 3 are elements or chemicals which inhibit reproduction at low doses and cause mortality at higher concentrations. It is reasonable to assume that runoff samples exhibiting toxicity in category C (Table 2) contain only type 1 toxicants. Category B samples are presumed to contain primarily Type 2 substances, but this cannot be verified unless the substance has been shown to be non-lethal at high concentration. A dose response curve, using the appropriate dilution series, may be useful in discerning the various toxicant types in category D. Dilution series in conjunction with chronic test setup (USEPA 1989) may also be used to unmask reproductive effects in samples which cause early mortality in *C. dubia* (categories E and F).

Toxicity bioassay design and data processing procedures may be tailored to answer specific questions. Examples for the use of dilution series to gain information on types of toxicants have been described above. In situations where fine resolution of toxicity intensity is required, denser observation regime will enhance the probability of obtaining partial mortalities and produce data sets which allow calculation of LT_{50} values with reliable confidence intervals. If the difference between samples or treatments is important, a test design with replication will enable analysis of variance.

For samples of unknown intensity of toxicity a flexible test design is recommended: When a sample arrives in the toxicity laboratory, a chronic bioassay will be set up with ten animals in individual test chambers and the test will proceed according to the protocol (USEPA 1989), but if mortality is observed in all ten chambers of a sample on the morning after test setup (after 16-20 hours of exposure), a new test with a dilution series will be set up for that sample to derive LC_{50} values; this option will enable comparisons of toxicity intensities within category F.

CONCLUSIONS

Determination of LT_{50} for stormwater samples taken at stream and transportation land use stations is useful as it allows comparisons of relative toxicity using ambient samples without dilution ("screening" protocols). In toxic samples collected at the DUST Marsh correlations between relative toxicity and environmental parameters were obtained. These correlations were not apparent when endpoints of % survival after 7 days were used as a measure of toxicity.

Calculation of the number of offspring per female per reproductive day (OFRD) in moderately toxic samples allows determination of reproductive effects even in samples that cause mortality and adds valuable information as to the nature of the toxicant and its mode of action.

Toxicity testing is a powerful and cost effective tool in water quality assessment of urban runoff. Furthermore, the ability to quantify toxicity using LT_{50} values and to distinguish between lethality and reproductive effects may be very useful in a number of applications. These may include decision making in order to select stations and drainage areas for further monitoring and to evaluate long term trends, effectiveness assessments of pollution control measures, source identification programs, and toxicity identification evaluations (TIEs) which focus on chronic toxicity.

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