



# **MARINE BIOASSAY PROJECT**

## **SEVENTH REPORT**

### **Refinement of Effluent Toxicity Testing Protocols for Four Marine Species**

**94-2WQ**

**January 1994**

**STATE WATER RESOURCES CONTROL BOARD  
CALIFORNIA ENVIRONMENTAL PROTECTION AGENCY**



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## **Acknowledgments**

The authors thank the staff of the Marine Pollution Studies Laboratory for their assistance with all phases of this project: Shirley Tudor, Witold Piekarski, Byrn Phillips, Lisa Weetman, Michelle Hester, Steve Osborn, Kelita Smith, and Matt Englund. We would also like to express our thanks to the members of our Scientific Review Committee: Steven Bay, Southern California Coastal Water Research Project; Tom Dean, Coastal Resources Associates; Jo Ellen Hose, Occidental College; Howard C. Bailey, University of California at Davis; and Phil Oshida, U.S. EPA Region IX. Their technical advice and overall guidance is greatly appreciated. Several laboratories were involved in the interlaboratory comparisons; the cooperation and suggestions of all of the participants contributed to the development of the toxicity tests. The staff of the California State Mussel Watch performed the chemical analyses of reference toxicant test solutions; their cooperation is appreciated. Extra gratitude is given to Lisa Weetman who assisted with the final preparation of this report.

This report was prepared through agreement with the California State Water Resources Control Board Contract No. 0-092-250-1 with the California Department of Fish and Game, and Subcontract FG-0534 between the California Department of Fish and Game (Project Managers: Dr. Michael Martin and Mark D. Stephenson) and the University of California, Santa Cruz (Principal Investigator: Dr. Ron Tjeerdema). This does not signify that the contents necessarily reflect the views and policies of the California State Water Resources Control Board, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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## Executive Summary

The goal of the Marine Bioassay Project (MBP) authorized by the State Water Resources Control Board (State Water Board) in 1984, is to protect California's ocean resources by determining the impacts of toxic waste discharges to marine waters. The Project's primary objective is development of short-term tests to measure the toxicity of these discharges. A second objective is the actual use of these tests for regulatory purposes, specifically for compliance monitoring of chronic toxicity in complex effluents discharged to the ocean.

Many toxicity tests previously in use were relatively insensitive because the adverse effect measured was lethality to adult organisms. In contrast, a newer generation of tests has been developed by the MBP and other groups. These are designed to estimate more subtle long-term adverse effects of waste discharges that ultimately may damage populations of important marine species. The new tests generally use early life stages of sensitive aquatic organisms and measure sub-lethal effects such as abnormal development and decreased growth or reproduction. Because the toxicity tests developed by the MBP are designed to measure adverse effects of discharges to ocean waters, the toxicity test protocols have emphasized the use of marine species native to California.

The Marine Pollution Studies Laboratory (MPSL), operated by the California Department of Fish and Game, serves as the research facility for the MBP. Protocol development has been closely coordinated with parallel work performed nationally by the U.S. Environmental Protection Agency (EPA). While the MBP protocols use test organisms native to California, the EPA work has focused on test species from other parts of the United States.

This report, seventh in a series, describes the work performed during Phase Six, which occurred from January 1, 1991 to June 1993. The seventh report is organized into six sections. Section 1 focuses on topsmelt, *Atherinops affinis*, one of the most abundant fish species in central and southern California estuaries. Section 2 describes work with the mysid shrimp, *Holmesimysis costata*, a crustacean that occurs in the surface canopy of giant kelp. Section 3 describes work with the red abalone, *Haliotis rufescens*, a large gastropod mollusc that is indigenous to California and is distributed throughout the State coastal waters. Section 4 focuses on the giant kelp, *Macrocystis pyrifera*. California's kelp forests harbor a rich diversity of marine life and are an important source of primary production to the nearshore marine ecosystem. Section 5 investigates the contribution of non-point sources to ambient toxicity and evaluates the effectiveness of four MBP protocols for assessing ambient toxicity in estuaries and marine waters. Section 6 summarizes training provided by MBP staff to Regional Board, discharger and private consulting firm staff on all four MBP protocols that have either been adopted into the California Ocean Plan or are scheduled for adoption. This sixth section relates to the second MBP objective: use of these tests to determine compliance with permit requirements for chronic toxicity. Formal use of the red abalone and giant kelp protocols was approved by the State Water Board in 1990. It is anticipated that use of the topsmelt and mysid shrimp will be approved in 1995.

The topsmelt studies examined the feasibility of year-round rearing the fish species for routine effluent toxicity testing and investigated the most appropriate larval age(s) for test use. By manipulating temperature, topsmelt can be spawned in the offseason for year-round availability. However, there was a statistically significant difference in the weight and length of the larvae spawned during the normal spawning period and those spawned out of season. Fish spawned during the normal summer months were significantly larger in terms of weight and length than those spawned during the winter months.

The second phase of the study focused on the susceptibility of topsmelt to a reference toxicant, copper. Although the mechanisms of copper toxicity to developing fish larvae are complex, increased mortality was correlated with increased larval age, gill surface area and cutaneous surface area, possibly indicating increased copper uptake by older fish. The copper NOEC for topsmelt at 20‰ salinity was 32 ug/liter in the test reported here. Recent studies have indicated that as salinity decreases, toxicity of the toxicants copper and sodium azide increase. The larval topsmelt experiments demonstrated that the organism is amenable to toxicity testing at estuarine salinities ranging from 5 to 33 ppt. Control survival at all salinities was 100%. Sensitivity of the topsmelt to azide and copper was salinity dependent.

Mysid experiments expanded on previous Marine Bioassay Project efforts to provide an effluent toxicity test protocol using *Holmesimysis costata*, an indigenous west coast marine crustacean. Studies were conducted to determine various physical factors on test performance, to estimate inter- and intralaboratory precision, to evaluate growth endpoint using representative effluents, and to compare length and weight as measurements of growth.

The experimental results suggest that for many complex mixtures, *H. costata* growth can be an appropriate indicator of chronic toxicity. In three out of four effluent tests and in the interlaboratory testing using bleached kraft mill effluent (BKME), significant growth inhibition occurred at concentrations below those affecting survival. In addition, test precision evidenced by interlaboratory and intralaboratory comparisons of *H. costata* protocol compared favorably with that of toxicological and chemical methods. Interlaboratory coefficients of variation (CVs) were 26% and 14% in two trials when separate laboratories tested split samples of complex effluent.

Research with the giant kelp evaluated test precision using sodium azide as a reference toxicant. Sodium azide is less toxic than copper to giant kelp, and is less affected by chelation. It was initially regarded as a promising alternative to copper as a reference toxicant. However, experimental results indicate that between test variability was not improved by substituting sodium for copper. Also, sodium azide concentrations were difficult to analytically verify. These reasons, in conjunction with the substantial existing database for copper as a reference toxicant, eliminated sodium azide as a possible reference toxicant alternative.

The red abalone experiments evaluated the biological significance of the short-term (48 hr) routine test by comparing results of both long-term (partial life-cycle tests) and exposure recovery tests. The exposure recovery test is essentially a short term procedure in which the

abalone larvae are exposed to a toxicant for 48 hours and then placed in fresh seawater. After exposure, the larvae are monitored to determine if they will successfully settle to the bottom and change from larval to juvenile abalone. The endpoints for the exposure recovery tests are then compared to long-term toxicity tests to verify that the 48-hour larval shell development endpoint is representative of clearly adverse chronic effects on red abalone.

The toxicants used in the study were zinc (the standard reference toxicant) and bleached kraft mill effluent (BKME). Study results confirmed that the 48 hour larval shell development endpoint was representative of clearly adverse chronic effects on red abalone by demonstrating that concentrations of zinc and BKME which affected larval shell development also inhibited metamorphosis. Larvae continuously exposed to toxicants (the partial life cycle test) showed only slightly greater sensitivity than larvae from exposure recovery tests.

The ambient toxicity experiment had two goals: to investigate the ambient toxicity in the Salinas River drainage, Elkhorn Slough, and adjacent waters, and to examine the applicability of short-term chronic toxicity tests (using four marine species) for assessing non-point source toxicity. Findings were inconclusive due to experimental artifacts (e.g., the toxicity of polyester filters used to prepare samples for testing, an interrupted sampling schedule, and difficulty establishing appropriate salinity levels for the test organisms). However, the results did suggest that these four marine species can be used for ambient toxicity testing if further protocol refinement is conducted to eliminate these artifacts. The training workshops familiarized Regional Board, dischargers, and private consulting firm staff with the four MBP protocols either adopted or scheduled for adoption (red abalone, giant kelp, topsmelt, and mysid toxicity test protocols). The workshops also allowed the MBP staff to receive feedback on ways to improve protocols and testing implementation. The workshops were conducted at three sites: the Cabrillo Museum in San Pedro, the California Academy of Sciences in San Francisco, and the Marine Pollution Studies Laboratory in Monterey. As part of the workshop, a questionnaire was included. In general, responses to questionnaires were positive, and indicated that the hands on approach of the workshops provided excellent training.

## Project Overview and Technical Summary

### Toxicity Testing

Tests initially developed with reference toxicants and clean water must be modified to also work with complex effluents, which contain suspended solids and other materials. To develop adequate marine toxicity tests, the Marine Bioassay Project has required over six years of intensive effort by two principal investigators and several laboratory technicians. Having successfully developed several critical life stage test protocols, the MBP is devoting additional effort to examine issues such as test precision and selecting appropriate statistical approaches to analyze test results.

Aquatic toxicology is a relatively new and evolving area of study that includes toxicity tests to measure adverse effects of toxic chemicals on aquatic organisms. Toxicity tests are laboratory experiments in which aquatic organisms are exposed to several concentrations of a toxicant using a formalized testing procedure or protocol. The term "toxicity test" is used in preference to "bioassay" because it more accurately describes the process of estimating the concentration of a chemical in water that produces an adverse response in aquatic organisms.

There are three general categories of toxicity tests: acute, chronic, and critical life stage. The terms "acute" and "chronic" are occasionally confusing because they may refer to either the duration of exposure or to the adverse effect (measured response) produced by exposure to a toxicant. An acute exposure is a short term period, usually 96 hours or less for toxicity tests. An acute effect exposure generally refers to mortality. For example, when an acute test is conducted on larval fish with an endpoint of mortality and a duration of 96 hours, acute describes both duration of exposure and toxic effect.

Chronic refers to a long exposure; a chronic test may involve exposing the test organism for its entire reproductive life cycle. For fish, the duration may exceed twelve months. Chronic toxicity tests are inherently more sensitive to toxicants than acute tests; that is, adverse effects are detected at lower concentrations of a toxicant. While a chronic effect can be either lethal or sub-lethal, chronic is frequently interpreted to mean a sub-lethal effect. For clarification, when referring to duration of exposure, this report uses short-term instead of acute and long-term instead of chronic. The response of an organism determined in a particular toxicity test is given by the endpoint or effect measured (e.g. mortality, germination, growth, or abnormal development).

A third type of toxicity test, the critical life stage or early life stage, is intermediate to acute and chronic tests in duration and sensitivity to toxicants. These tests generally focus on early periods of an organism's life cycle when it is most sensitive to toxicants but can also refer to a sensitive adult life stage, such as during egg production. When properly designed, a critical life stage test serves as a "short-term estimate of chronic toxicity". The tests under development by the Marine Bioassay Project (MBP) are examples of these critical life stage tests.

In addition to measuring the response of an organism to individual toxicants, toxicity tests can be designed to measure the toxicity of wastewater discharges (whole effluents) or complex mixtures of toxicants. Whereas chemical analyses report concentrations of individual chemicals; whole effluent toxicity tests measure the bioavailability of toxicants in a complex mixture, account for synergistic and antagonistic actions, and integrate the adverse effects of the mixture.

Toxicity test development involves conducting repetitive tests over several years in order to refine, simplify, and standardize methods into a formal protocol. Various factors such as temperature, salinity, season, and dilution water chemistry must be examined to determine their effects on test precision. Interlaboratory calibration and confirmation is necessary to demonstrate that other laboratories and their technicians can reliably perform the test.

### Regulatory Background

Development of toxicity test protocols to estimate long-term effects of waste discharges is consistent with both federal and state requirements. In 1984, the United States Environmental Protection Agency (EPA) issued a national "Policy for the Development of Water Quality-Based Limitations for Toxic Pollutants (49 CFR, No. 48, March 9, 1984). This policy outlined a technical approach for controlling discharge of toxic substances through the federal system of discharge permits. In addition to meeting numerical standards for individual chemicals, the policy requires EPA and the States to use biological testing to complement chemical testing. Biological testing also provides information not available for chemical testing. For example, it incorporates bioavailability and interactions in complicated mixtures of toxic materials.

In 1986, AB 3500 added Section 13170.2 to the California Water Code. In addition to mandating triennial review of the California Ocean Plan, Section 13170.2 requires the State Board to develop and adopt toxicity test protocols. Ocean discharges of 100 million gallons per day or more have been required in their permits to use their toxicity test protocols for monitoring complex effluents since January 1, 1991. Section 3 of AB 3500 expressed legislative intent that the organisms used in testing be representative marine species.

"If the State Water Resources Control Board determines through its Marine Bioassay Project that a multispecies toxicity testing program with representative marine species for monitoring complex effluent discharges is appropriate, the state board shall use the multispecies toxicity testing program with representative marine species in adopting the toxicity test protocols specified in Section 13170.2 of the Water Code."

On March 19, 1990 the State Board adopted a series of amendments to the California Ocean Plan. These amendments included the addition of a chronic toxicity objective for protection of marine aquatic life. The State Board also adopted a list of seven toxicity test protocols deemed sufficiently developed for measuring compliance with the chronic toxicity objective. Included on this list of seven are two protocols developed by the MBP, the giant kelp and the red abalone 48-hour toxicity tests. Current efforts by the MBP are focusing on developing two additional protocols and insuring that implementation is achieved in a scientific and technically-sound manner.

In practice, toxicity requirements in a discharge permit are expressed in toxicity units (TU). A TU is defined as 100 divided by the No Observed Effect Level (NOEL):

$$TU = 100/\text{NOEL}$$

The NOEL is defined as the maximum percent concentration of effluent, or any water being tested, that does not result in any observed effect on test organisms. Permits would usually require that no sublethal toxicity be observed at a concentration lower than those present within an outfall's designated mixing zone (the "zone of initial dilution"). For example, if a discharger has an outfall design that provides 99:1 dilution, then no toxicity should be observed in effluent diluted to one percent. The discharge permit would require that the effluent toxicity limit be 100 toxicity units or less.

$$TU = 100/1 = 100$$

### Project History

The Marine Bioassay Project is designed as a multiple phase program to develop and implement short-term tests for toxicity measurement of complex effluents discharged to the ocean. Actual laboratory work is conducted at the California Department of Fish and Game's (DFG) Marine Pollution Studies Laboratory located south of Monterey. To date, six phases of the Marine Bioassay Project have been completed; chapters of this report describe work performed during the sixth phase. This sixth phase focuses on two major areas: (1) completing protocols for a fish (topsmelt) and a crustacean (mysid shrimp) with sub-lethal endpoints, and (2) ensuring that the toxicity testing program for marine discharges is properly implemented. Implementation includes training of technical staff, providing technical support for participating laboratories, developing sound quality assurance/quality control testing procedures, selecting standard statistical procedures, and establishing a uniform system of database management for interpreting results of compliance monitoring toxicity tests.

#### Phase One (November 1984-February 1986)

During the first phase, efforts were made to obtain wide-spread participation in developing the scope of the project. Initially, a draft report was prepared that described a number of potential marine toxicity test species, recommended twelve of these as most suitable, and presented appropriate protocols for each of the twelve. In March 1985, the draft was sent for review to a number of potentially interested agencies (NOAA and DFG), ocean dischargers in southern California, the Southern California Coastal Water Research Project, and a number of individual scientists.

A workshop to discuss the draft report and outline the project's scope was held on April 29, 1985 at the offices of a major ocean discharger, the County Sanitation Districts of Orange County. The purpose was to discuss the proposed toxicity test species and protocols and address questions raised by reviewers of the draft report. Over 50 people attended the workshop and general session and then participated in one of the five sub-committee meetings. The MBP's First Report included a summary of the workshop proceedings in addition to the species descriptions from the pre-workshop draft.

A separate outcome of the workshop was the establishment of a Scientific Review Committee, composed of a small group of outside experts to discuss progress and provide guidance for the Marine Bioassay Project. The first meeting was held in June 1985, and meetings have continued approximately twice a year. The Committee has recommended a number of significant mid-course corrections that have been implemented by the MBP staff. Overall, a major accomplishment in these recommendations has been to refocus daily work on the primary objective: development of short-term protocols for use in performing toxicity tests on complex effluent discharged to the ocean.

Four important laboratory tasks were also completed during Phase One: (1) the Department of Fish and Game laboratory was extensively refurbished for animal culture and rearing of marine species, (2) methods were developed for maintaining and spawning selected marine species, (3) a mobile laboratory was purchased and used to conduct aquatic toxicity tests, and (4) range-finding and definitive tests were developed on two toxicants (pentachlorophenol and endosulfan) of immediate concern to the State Board. This work is described in the MBP's Second Report, (May 1986).

#### Phase Two (March 1986-October 1987):

In Phase Two, three new short-term protocols were developed after repeated testing using zinc as a reference toxicant. In addition, longer term reference toxicant tests were used with each species to calibrate the relative sensitivity of the short-term test protocols. All three short-term test protocols developed were static tests; that is, the test solutions are not changed during testing. Each protocol measured a different effect or endpoint.

These protocols, designed to estimate chronic toxicity of discharge to ocean waters, utilized sensitive life stages of three marine species: the red abalone *Haliotis rufescens*; a mysid shrimp, *Holmesimysis costata*; and the giant kelp, *Macrocystis pyrifera*. After some refinement, preliminary testing with the three protocols was performed on complex effluents from two representative municipal treatment plants.

The short-term larval abalone toxicity test protocol is a 48-hour test in which abnormal shell development is the endpoint used as the measured effect of toxicity. The short-term giant kelp toxicity test is a 48-hour test that measures two different endpoints: zoospore germination and growth of the germination tube. The short-term mysid toxicity test is a 96-hour test with an endpoint of lethality to juvenile mysids.

#### Phase Three (November 1987-December 1988)

During this phase, the abalone, kelp, and mysid shrimp tests developed during Phase Two and described above were further refined using complex effluent from two large municipal ocean dischargers. In addition, preliminary tests were conducted using a fish species, topsmelt *Atherinops affinis*. The project's fourth report provides detailed descriptions of work completed in Phase Three.

#### Phase Four (January 1989-December 1989)

During phase four, the giant kelp, red abalone, and topsmelt toxicity tests developed in previous phases were refined by testing with complex effluents. In addition to performing toxicity tests of complex effluents, a manual entitled "Procedures Manual For Conducting Toxicity Tests Developed By The Marine Bioassay Project" was prepared for the red abalone, giant kelp, mysid shrimp, and topsmelt protocols. This manual is available from the State Board (Report 90-10WQ).

#### Phase Five (January 1990-December 1990)

In Phase Five, complex effluent tests were conducted and toxicity protocols developed using topsmelt, a fish native to the California coast. Also, a sub-lethal endpoint was established for the mysid shrimp protocol, and additional research was conducted in the areas of complex effluent testing and interlaboratory verification. Finally, technical support was provided by the MBP staff to laboratories using MBP protocols. This support included training of laboratory technicians, providing broodstock organisms, detailing specific requirements for test acceptability of individual protocols and performing interlaboratory tests with discharges exceeding 100 million gallons per day of complex effluent.

#### Phase Six (January 1991-June 1993)

Phase Six completed protocol development by the project and continued implementation of the marine toxicity testing program.

Major tasks completed include:

1. Completing the test protocols for the mysid shrimp and the topsmelt.
2. Providing additional technical training and support for dischargers and consulting laboratories.
3. Evaluating the biological significance of the short term (48 hr) red abalone routine test, by comparing it to both long term and exposure-recovery tests.
4. Investigating the use of marine organisms for measuring ambient toxicity from non-point sources.
5. Evaluating the applicability of sodium azide as a replacement toxicant for copper when conducting toxicity studies with the giant kelp *Macrocystis pyrifera*.



**Section 1**

**Topsmelt (*Atherinops affinis*) Experiments**

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## Introduction

Topsmelt, *Atherinops affinis*, occur from the Gulf of California to Vancouver Island, British Columbia (Miller and Lea 1972). It is often the most abundant fish species in central and southern California estuaries (Allen and Horn 1975, Horn 1980), and accounts for a significant portion of the total annual fish productivity in some estuaries (Allen 1982). Topsmelt are opportunistic feeders and have been characterized both as herbivores and detritivores and as low-level carnivores (Fronk 1969, Quast 1968).

Topsmelt population density and size distributions fluctuate seasonally in bays and estuaries (Horn 1980, Allen 1982). Reproduction occurs from February through August, peaking in May (Fronk 1969), and young of the year are present from September through December. They begin spawning in their second and third years, depositing eggs on eelgrass (*Zostera sp*) and benthic algae (e.g., *Gracilaria sp.*, Croaker 1934, Fronk 1969).

Over a four year period we have evaluated the applicability of several early life-stages of this species for routine effluent toxicity monitoring (Anderson et al. 1991, Hunt et al. 1991). Recent research efforts have been devoted to development of a toxicity test procedure for topsmelt larvae. This protocol, patterned after the *Menidia sp.* 7-day growth and survival toxicity test, was tested over a two year period in which several other factors influencing test performance were also investigated. These included: off-season spawning, age-specific sensitivity, and influence of salinity on larval sensitivity. Each of these topics is discussed individually in the following sections. Results of intra- and interlaboratory testing are presented in the section on off-season spawning.

# Topsmelt Section 1: Off-Season Spawning and Initial Evaluation of a Larval Growth and Survival Toxicity Test

## Introduction

In order to use topsmelt for routine toxicity testing, it is necessary to insure year-round production of embryos. Previous research reported success in laboratory spawning of topsmelt (Anderson et al. 1991, Middaugh et al. 1992). However, to date, there have been no reports of spawning beyond the normal summer spawning period. To investigate whether off-season spawning is possible with topsmelt, previously described methods were applied to several groups of adult topsmelt with asynchronous spawning phases. Seasonal variability in test organisms produced by these animals was investigated over a two year period. Embryo viability and initial size of larvae produced during the normal summer spawning period (May -August) were compared to embryos and larvae produced during the off-season (September-April).

Performance of the 7 day growth and survival protocol was assessed using larvae produced over the two year period. Toxicity tests were conducted with copper chloride, a metal found in high concentrations in municipal sewage and power plant effluents (Young et al. 1977, Martin et al. 1977, Harrison 1982). Copper is a commonly used standard reference toxicant (Anderson et al. 1991, Weber et al. 1988) allowing for direct comparison of toxicity test results between species and for comparisons of inter- and intralaboratory variability (Morrison et al. 1989). Performance of the 7d larval protocol was also assessed in an interlaboratory test using reference toxicant and complex effluent. This work is also presented in Anderson et al. (In Press).

## Materials and Methods

### Facilities

All experiments were conducted between November, 1990, and April, 1992 at the California Department of Fish and Game's Marine Pollution Studies Laboratory (MPSL) located at Granite Canyon on the Big Sur coast in Monterey County, California. Background concentrations of total copper in the laboratory seawater were below the detection limit of  $3 \mu\text{g liter}^{-1}$  in this study.

### **Topsmelt spawning**

Detailed methods for culture and spawning of topsmelt are given in Anderson et al. (1991). Briefly, adult topsmelt were caught in Elkhorn Slough in Central California, using seining methods previously described. Fish were transported to laboratory holding tanks where they were induced to spawn using temperature and lighting cues. The temperature was slowly raised to 18 °C from ambient temperature (~13°C) and lighting was held at 16hL:8hD. To induce spawning, the temperature was "spiked" to 21±1 °C over a 24h period then allowed to return to 18 °C. Spawning usually commenced within 2 or 3 days of each spike. Fish were provided with dyeless yarn as spawning substrates. Substrates were removed and replaced daily and all spawned embryos were incubated in flowing seawater culture tubes at 21°C until larvae hatched. Larval cohorts were isolated into separate screen tubes until needed for toxicity testing. Adult fish were fed Tetramin® flake food supplemented with frozen krill twice daily; larvae were fed newly-hatched *Artemia* nauplii in excess.

Three separate groups of adult fish were spawned to provide test organisms for these experiments. Group A was caught in August 1989 and held for approximately 15 months before spawning. When collected all of the 48 fish in this group were sexually immature. Once mature, these fish were spawned out of season in the laboratory from November 1990 through January 1991. These fish were then rested and spawned again in May and June 1991, and again in October through December 1991. During the resting period the fish were held under ambient lighting and water temperature (~ 11-13 °C). Group B was collected in July 1991 and began spawning immediately thereafter. This group of fish consisted of approximately 55 mature individuals. Embryos from Group B were measured for viability. Larvae produced from this group were measured immediately after hatching and later were used in toxicity tests from July through August 1991. Group C was caught in August 1991 and were first spawned in the laboratory in February 1992. This group of fish consisted of approximately 50 mature individuals. Embryos from Group C were measured for viability. Larvae produced from this group were measured immediately after hatching and later were used in toxicity tests from January through April 1992.

### **Embryo viability and larval size**

In order to investigate seasonal differences in the viability of embryos, individual spawns were monitored continually from November 1990 through April 1992. Embryos were isolated and the proportion viability was assessed by counting the number of viable and non-viable embryos in a randomly selected groups of 100. Viability was determined as

presence of a normal blastodisc; non-viable embryos showed no cell division and lacked lipid globules characteristic of healthy eggs; non-viable eggs never developed. A total of 168 individual spawns was monitored for embryo viability (see Table 1). In addition, newly hatched larvae (Day 0) were preserved each month and measured to determine whether there were differences in the size of larvae produced by different groups of parent fish and to determine if larvae spawned out-of-season differed in size from those spawned in-season. Each month, approximately 25 larvae were preserved in formalin then transferred to alcohol. Total length was first measured then the larvae were dried at 55°C for 24h and weighed to give mean dry weight per larva. These fish were collected before the first feeding.

A separate experiment investigated the effect of differences in embryonic incubation time on variability in size of Day 0 larvae. A single cohort of topmelt embryos were isolated at the early-blastula stage from a September, 1992 spawn. These embryos were sorted using a dissecting microscope to verify that all of the embryos were developing synchronously. The embryos were then transferred to culture screens and cultured at 21 °C as above. Daily hatches of larvae were isolated at 10:00 am each morning for consistency. The cohort hatched over a 3 day period; thus, incubation time varied from 10 to 12 days. Twenty larvae were then randomly selected from each group and measured for total length. Ten larvae were then dried and weighed.

### Toxicity tests

Larval topmelt used in toxicity tests were held at 21 °C and 33 ‰ salinity using the procedures described by Anderson et al. (1991). The larval toxicity test protocol used a 7d static renewal exposure and was patterned after the 7d growth and survival test developed for the inland silverside *Menidia beryllina* (Weber et al. 1988). A detailed description of the topmelt protocol is given in Hunt et al. (1991). In brief, five 9 day-old topmelt larvae were randomly placed in test containers with 200 ml of test solution. Test containers were acid-cleaned polyethylene plastic containers. Containers were placed in an incubator at  $20 \pm 1$  °C and covered with plexiglass to prevent evaporation. Total copper concentrations tested were 0, 56, 100, 180, and 320 µg/liter. Each treatment was replicated five times. Topmelt larvae were fed newly-hatched *Artemia* nauplii twice daily (40 nauplii/larva). The photoperiod was 14L:10D at  $12 \mu\text{E m}^{-2} \text{s}^{-1}$ . Survival was monitored and dead larvae were removed daily. All solutions were renewed every 48h. On day 7, total survival was tallied and the surviving larvae were dried as above and weighed. Dry weight per larva was the growth endpoint measured. All tests were conducted with 9 day-old larvae; this age has proven to be the most sensitive to copper

chloride in age-specific sensitivity tests conducted at our laboratory (McNulty et al. In Press). A total of twelve toxicity tests were conducted from November 1990 through April 1992.

One interlaboratory comparison of the topsmelt 7d growth and survival protocol were conducted in August 1991. General methods for interlaboratory comparisons are given in Hunt et al.(1991). The interlaboratory comparison was conducted between the Marine Pollution Studies Laboratory (MPSL) and the Chevron Research and Technology Company Environmental Research Group (Under the direction of Dr. Gary Rausina). Approximately four hundred, 7 day-old topsmelt larvae were cultured at MPSL and divided into two groups. The first group was transported in ice chests to Chevron using standard packaging techniques for transport of larval fish. The second group of fish was held at MPSL under identical conditions. The larvae used in this study were acclimated to 20 ‰ salinity seawater for one week prior to testing. This comparison used a synthetic effluent formulated by Chevron and was tested at 20 ‰ to assess toxicity in a receiving water salinity characteristic of the Richmond area of San Francisco Bay. The synthetic effluent consisted primarily of organic acids (G. Rausina, personal communication). A copper reference test was conducted concurrently at both laboratories during this comparison; the reference test was also conducted at 20 ‰ salinity. Both laboratories used dilution water provided by MPSL.

Analysis of Variance followed by Dunnett's multiple comparison test were used to derive No Observed Effect Concentrations (NOEC's); the trimmed Spearman Karber method (Hamilton et al. 1977) was used to calculate median lethal concentrations (LC50's) and the 95 % Confidence Limits for all tests.

### **Chemical and physical measurements**

Physical parameters in all experiments described in this report were monitored in each test concentration at the beginning and end of each test, and before each renewal. The D.O. and pH was measured using an Orion Model SA 520 pH/millivolt meter. Salinity was measured using an Atago model S-10 refractometer. Temperature was monitored daily with a hand thermometer.

One random sample of each test concentration was taken at the beginning and end of each test for chemical analysis. Total copper concentrations were verified using a Perkin Elmer Model 6003 Atomic Absorption Spectrophotometer. Analytically measured copper concentrations were close to nominal concentrations. Measured concentrations varied  $9.8\% \pm 4.4\%$  (mean variation  $\pm$  standard deviation) from nominal concentrations, which is

within the accuracy of the analytical technique. All copper values are reported as nominal values of total copper.

## Results

### Induced spawning

Using a combination of increased temperature and springtime lighting we were able to induce wild-caught topsmelt to spawn throughout the year. Group A produced embryos in November 1990 through January 1991. From there on, by rotating spawning in Groups A, B, and C we were able to continuously produce topsmelt larvae for toxicity testing from May, 1991 through April 1992 (Table 1). We were not able to obtain topsmelt larvae in September 1991, because the adult fish that were spawning during this month developed a parasitic copepod infestation and the majority of these fish died before an effective treatment method was implemented.

For the following discussion, the characteristics of individual spawns are arbitrarily divided into spawns which occurred during the "normal" spawning period for Elkhorn Slough, California (May-August) and those that occurred during the off-season (October - April). Characteristics for September spawns are not included in this discussion because no spawns occurred in September (as described above). Mean overall embryo viability for the three spawning Groups was 81.5%, and ranged from a high of 93.2 % viable in December 1990 and a low of 64.4 % viable in July 1991. There was no significant difference in viability between larvae spawned in the normal spawning period, May-August and those spawned out of season, October-April (t-test  $p = 0.21$ ). The mean percent viability of embryos spawned in-season was 83.45%, and the mean percent viability for those spawned out-of-season was 76.65%. There was a difference in viability of embryos spawned from the three Groups of fish. The mean viability of embryos was 81.63%, 69.45%, and 87.30% for groups A, B, and C, respectively.

Mean length of Day 0 larvae was 5.95 mm overall for the three spawning groups and ranged from a high of 6.98 mm in June, 1991 to a low of 5.09 mm in December, 1991. Larvae spawned in the May-August spawning period were significantly larger than larvae spawned in October-April (t test  $p = 0.01$ ). The mean length for the May -August spawning period was 6.51 mm; the mean length for the October-April spawning period was 5.73 mm. The total length of Day 0 larvae hatched from embryos spawned from Group C were smaller overall than were the lengths of the larvae hatched from the other 2 groups. The mean lengths of Day 0 larvae were 6.09, 6.32, and 5.48 mm for groups A, B, and C, respectively.

**Table 1.** Size of Day 0 larvae and percent viability of embryonic topsmelt spawned from November 1990 through April 1992. Groups of 25 larvae were weighed and measured each month.

Date	Wt. of Day 0 Larvae (mg)	Length of Day 0 Larvae (mm)	X Embryo Viability(%)	No. Spawns Monitored	Spawning Group
Nov. 90	0.20	5.86	85.8	28	A
Dec. 90	0.18	5.82	93.2	11	A
Jan. 91	0.16	5.94	74.1	11	A
May 91	0.34	6.41	87.4	20	A
Jun. 91	0.35	6.98	80.3	21	A
Jul. 91	0.25	6.55	64.4	5	B
Aug. 91	0.21	6.09	74.5	4	B
Oct. 91	0.26	6.60	75.0	2	A
Nov. 91	0.24	6.04	89.7	11	A
Dec. 91	0.08	5.09	67.5	8	A
Jan. 92	0.13	5.22	87.7	3	C
Feb. 92	0.23	5.80	84.5	23	C
Mar. 92	0.22	5.70	90.0	10	C
Apr. 92	0.19	5.20	87.0	11	C
Mean $\pm$ S.D.	0.22 (0.07)	5.95 (0.56)	81.50 (8.90)		

Summary Means ( $\pm$  S.D.) for 2 Seasons and for the 3 Spawning Groups A, B, and C.

Date	Wt. of Day 0 Larvae (mg)	Length of Day 0 Larvae (mm)	X Embryo Viability(%)
May-Aug.	0.29 (0.07)	6.51 (0.37)	76.65 (9.72)
Sept. - Apr.	0.19 (0.05)	5.73 (0.46)	83.45 (8.35)
Group A	0.23 (0.09)	6.09 (0.57)	81.63 (8.88)
Group B	0.23 (0.03)	6.32 (0.33)	69.45 (7.14)
Group C	0.19 (0.05)	5.48 (0.31)	87.30 (2.26)

Overall mean weight of Day 0 larvae for the three spawning groups was 0.22 mg and ranged between a high of 0.35 mg in June 1991 and a low of 0.08 mg in December 1991. As with length, larvae spawned in the May-August spawning period were significantly larger than those spawned in October-April ( $t$  test  $p = 0.01$ ). The mean weight in May-August was 0.29 mg; the mean weight in October - April was 0.19 mg. The total weight of Day 0 larvae hatched from embryos spawned from Group C were also smaller overall than were the weights of the larvae hatched from the other 2 groups. The mean weights of Day 0 larvae were 0.23, 0.23, and 0.19 mm for groups A, B, and C, respectively.



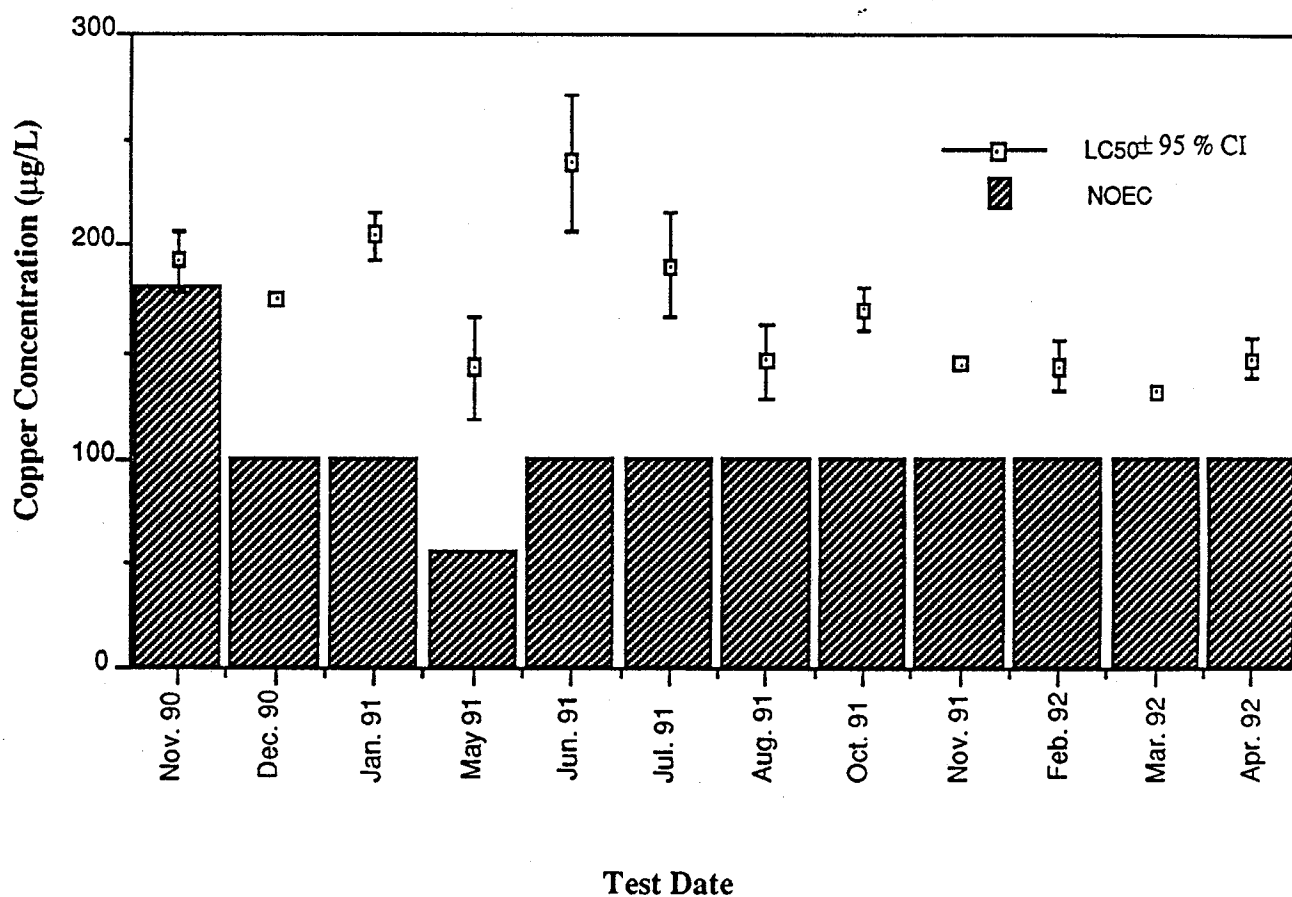
There was a relationship between incubation time and size of Day 0 larvae. Larvae which incubated for 10 days were significantly smaller than those which incubated for 11 and 12 days ( $p=0.0001$ ; ANOVA). The mean length of Day 0 larvae hatched after 10 days incubation was 5.64 mm ( $\pm 0.37$ , sd); the mean lengths for larvae after 11 and 12 days incubation was 6.45 mm ( $\pm 0.24$ ) and 6.24 mm ( $\pm 0.28$ ), respectively. There was no significant difference in the weights of these larvae ( $p>0.05$ ). The mean weight per larva was 0.24, 0.20, and 0.20 for larvae hatched after 10, 11, and 12 days incubation time, respectively.

There was no relationship between the weight of the Day 0 larvae and the weight of control larvae from the toxicity tests conducted in the same months (Table 2; note: the control larval weights were taken at the end of the test with surviving larvae that were 16 days old). Correlation analysis of this relationship revealed no significant correlation ( $r^2 = 0.16$ ). The mean weight of the control larvae from these tests was 1.05 mg ( $\pm 0.26$ , SD;  $n = 12$  tests), the range of weight of these larvae was 0.65 mg in June 1991 to 1.63 mg in August 1991.

Month/Year	Mean Dry Weight/Larva (mg)	
	Day 0 Larvae	Toxicity Test Control Larvae
Nov. 1990	0.20	0.88
Dec. 1990	0.18	0.79
Jan. 1991	0.16	1.27
May 1991	0.34	1.06
June 1991	0.35	0.65
July 1991	0.25	1.02
Aug. 1991	0.21	1.63
Oct. 1991	0.26	0.92
Nov. 1991	0.24	0.94
Feb. 1992	0.23	0.99
March 1992	0.2	1.32
April 1992	0.19	1.12
mean (sd)	0.24 (0.06)	1.05 (0.26)

## Toxicity tests

Results of the toxicity tests showed a consistent response to copper chloride (Fig. 1). The No Observed Effect Concentration (NOEC) for copper in ten of the twelve tests was 100  $\mu\text{g}$  copper/liter; the NOECs in November 1990 and May 1991 were 180 and 56  $\mu\text{g}$  copper/liter, respectively. The LC50's for these tests ranged between 131 and 240  $\mu\text{g}$  copper/liter (mean LC50 = 169.1  $\mu\text{g}$ /liter). Larval growth was generally not inhibited by copper in these experiments; the NOEC for growth was less than that for survival in only one experiment in November 1990 (NOEC = 56  $\mu\text{g}$ /liter; data not shown).



**Fig. 1.** No Observed Effect Concentrations (NOEC's) and Median Lethal Concentrations (LC50's) for 12 larval topsmelt toxicity tests conducted with copper

Results of the interlaboratory test were comparable. The NOEC's for the synthetic effluent was 20% at both laboratories, and the CV of the LC50's was 19%. Only one laboratory detected a significant growth effect during this comparison (NOEC = 10%; Table 3). The NOEC's for the copper reference test conducted concurrently with the effluent test were 32 µg copper/liter at both laboratories, and the CV of the copper LC50's was 3%. The mean coefficient of variation for all interlaboratory comparisons was 11%. Precision of the topsmelt interlaboratory tests compares favorably with coefficients of variation calculated for other interlaboratory tests with marine fish larvae. Morrison et al. (1989) reported a mean CV of 42% in six 96h tests with *Cyprinodon variegatus*, using endosulfan and silver nitrate as reference toxicants.

**Table 3.** Results of an interlaboratory test of the topsmelt 7 day larval growth and survival protocol.†

Test Date	Toxicant	Laboratory	Survival		Growth NOEC
			NOEC*	LC50	
August 1991	Effluent	MPSL	20 %	31.4	ns
August 1991	Effluent	Chevron	20 %	23.9	10
	CV			19%	
August 1991	Copper	MPSL	32 µg/L	55.7	ns
August 1991	Copper	Chevron	32 µg/L	58.4	ns
	CV			3%	

† See text for description of methods.  
 \*The August, 1991 copper test was conducted at 20 ‰ salinity.

Chemical and physical water quality parameters were within acceptable limits in all toxicity tests. The temperatures were  $20 \pm 1$  °C; dissolved oxygen was always greater than 80% of saturation (saturation  $\approx 7.5$  mg/liter at 20°C in seawater); pH =  $8.0 \pm 0.5$ ; salinity =  $33 \pm 1$ ‰ (except the October 1991 and February 1992 tests where salinity was  $33 \pm 2$ ‰).

## Discussion

This study demonstrates that by manipulating temperature and lighting conditions, topmelt may be spawned in the off-season to ensure year-round availability of test organisms. By rotating three spawning groups and resting the fish under ambient conditions between spawning phases, we were able to continuously produce larvae for toxicity testing from May 1991 through April 1992 (except September 1991 due to parasite induced mortality). Although there was some variability, embryo viability remained relatively high during this study (mean viability = 81.5%).

There was a statistically significant difference in larval weight and length between fish spawned in the normal spawning period and those spawned out of season; Day 0 fish spawned during the summer months were significantly larger in terms of length and weight than those spawned during the winter months (Table 1). There was also some variability in size of larvae hatched between spawning cycles. For example, the length and weight of larvae measured from Group A spawns varied between the 3 spawning cycles monitored for this group (Table 1). In addition to these differences, there was also some variability in larval size between larvae produced by the different spawning groups: fish spawned from Groups A and B were generally larger in weight and length than those spawned from Group C (Table 1).

Several factors could have interacted to influence larval size during these investigations. These include incubation time, time of hatch within the spawning cycle, length of time between spawning cycles, and season. Our results indicate that incubation time may influence Day 0 size; longer incubation times produced significantly longer larvae, but there was no statistically significant difference in weight. Alderlice and Forrester (1974) found that size increased with incubation time with the flathead sole (*Hippoglossoides elasodon*). This may result from larvae having more time to develop using yolk reserves. Topmelt larvae typically hatch on day 12 at 20 °C, but hatching of a single cohort of fish may extend over 2 or 3 days even when the embryos start synchronously (Anderson et al. personal observation, Middaugh et al. 1992). In our study, embryo cultures were checked daily to ensure that fish used for Day 0 measurements were no more than 24 hours old; however, incubation time could have varied for the Day 0 larvae collected on different dates. This could have resulted in variability in the size of larvae used in the Day 0 measurements.

We noted differences in the size of larvae produced by individual groups of spawning adults in different spawning cycles. Size of larvae produced from spawning

Group A, in particular, increased from a mean weight of 0.18 mg the first spawning cycle in November 1990 - January 1991 to a mean weight of 0.35 mg in the second spawning cycle in May - June 1991, then declined again to 0.19 mg in the third spawning cycle (Table 1). There is little information on variation in the size of fish produced between spawning cycles within the same year. However, changes in size of fish eggs and larvae have been shown to decline within spawning cycles as the cycle progresses (Bagenal 1971, Cushing 1967). It has been suggested that this is an adaptive strategy evolved so that larval size coincides with seasonal productivity; bigger larvae are produced when planktonic productivity is high and food items are larger and more plentiful and larval size decreases in concert with plankton size and density (Bagenal 1971, Cushing 1967). It is possible the size variability we observed resulted from natural seasonal size fluctuations related to evolutionary adaptations to exogenous factors such as plankton productivity. The duration of the rest period between spawning cycles did not appear to influence larval size because larvae produced during the second spawning cycle for Group A, which followed a 3 month rest, were actually 38% larger than those produced during the third spawning cycle which followed a 4 month rest.

Despite differences in the size of Day 0 larvae over the course of this study, there was no correlation between larval size at hatch during any one spawning period and the size of control larvae in the toxicity tests conducted during these same periods (correlation  $r^2 = 0.16$ ; Table 2). The mean dry weight of the control larvae was  $1.05 \pm 0.26$  mg (mean  $\pm$  sd). Although there appears to be potential for considerable variation in size at hatch of cultured topsmelt larvae, by initiating toxicity tests with older larvae, in this case 9 days-old, initial size differences apparently become insignificant.

Results of twelve copper reference tests were relatively consistent and showed no obvious seasonal trends. The NOEC in all but two of the tests was 100  $\mu$ g copper/liter (mean = 103  $\mu$ g/liter; Fig. 1). The mean intralaboratory LC50 was 169.1  $\mu$ g copper/liter. Precision of the intralaboratory tests compared favorably to precision of intralaboratory comparisons reported for other commonly used 7d larval growth and survival tests for fish. Morrison et al. (1989) reported coefficients of variation of 21.7% and 33.2% for intralaboratory copper tests with *Cyprinodon variegatus* and *Menidia beryllina*, respectively. The coefficient of variation of the LC50's for the topsmelt intralaboratory copper tests reported here was 19%.

Results of the interlaboratory test demonstrated that laboratories having no previous experience with topsmelt could successfully conduct the 7d growth and survival test and obtain comparable results with a reference toxicant and effluent. The coefficients of variation of survival LC50's for three interlaboratory comparisons using synthetic effluent

and copper were 19% and 3%, respectively (Table 3). These results compare favorably with other interlaboratory comparisons using fathead minnows (*Pimephales promelas*) and sheepshead minnows (*Cyprinodon variegatus*). Anderson and Norberg-King (1991) reported a coefficient of variation of 31% for the fathead minnow 7d growth and survival test using chromium as a reference toxicant. Morrison et al. (1989) reported a range of CV's for the sheepshead minnow 96h test from 35 to 50% for interlaboratory comparisons using silver nitrate and endosulfan (LC50).

Topsmelt sensitivity to copper chloride was similar to that reported for larvae of the inland silverside (*Menidia beryllina*). The mean NOEC for survival for twelve topsmelt tests reported here was 103 µg copper/liter (salinity = 34 ‰). The mean NOEC for survival for *M. beryllina* was 113 µg copper/liter for five 7d growth and survival tests conducted at a salinity of 20 ‰ (Weber et al. 1988). It should be noted that the copper NOEC for topsmelt at 20 ‰ salinity was 32 µg/liter in the test reported here. Recent studies have indicated that copper and azide toxicity to topsmelt larvae increases as salinity decreases; this may be due to the interaction of osmotic stress and toxicant bioavailability at lower salinities.

Although Weber et al. (1988) reported that growth was a more sensitive endpoint than survival in two out of five copper tests using *Menidia beryllina*, we found survival to be more sensitive in all but one of the twelve copper tests (growth data not reported). Depending on the nature of the compound being investigated, survival may sometimes be a more sensitive indicator of toxicity than growth in larval fish tests. For example, Goodman et al. (1992) used a 30 day early-life-stage test to evaluate Fenvalerate toxicity to topsmelt and found that survival was a more sensitive endpoint than growth (in weight). These authors noted similar findings in tests with sheepshead and fathead minnow larvae. The relative sensitivity of the two endpoints is dependent upon length of exposure and the mechanism of toxicity of the compound being investigated.

In conclusion, we have demonstrated that topsmelt are amenable to laboratory culture and may be spawned year-round for toxicity testing purposes. Results of both intra- and interlaboratory comparisons of the 7 d larval toxicity test protocol for topsmelt indicate that between-test precision is relatively high. Topsmelt have comparable sensitivity to other atherinids and are easier to culture than *Menidia sp.* because they are able to feed on *Artemia* within 24h after hatching (i.e., they do not require rotifer cultures). Realizing the obvious advantages of using indigenous species for use in regional pollution monitoring programs, we suggest that the topsmelt is an appropriate species to replace *Menidia sp.* for assessment of aquatic pollution within the Northeastern Pacific coastal region.

## Topsmelt Section 2: Age-Specific Sensitivity of Larvae To Copper

### Introduction

Another consideration in the development of the 7d growth and survival toxicity test protocol for larval topsmelt is the determination of the appropriate larval age(s) to use for pollution assessment. In order to develop this protocol for use on a routine basis, it was necessary to compare the relative sensitivity of several ages so that a final protocol could be used for a range of ages having comparable sensitivities. Similar investigations have been conducted with other fish species commonly used in toxicity testing (e.g., *Menidia beryllina*, *L. tenuis*, *M. menidia*, and *M. peninsulae* (Heber et al. 1988, Borthwick et al. 1985).

To investigate age-specific sensitivity of larval topsmelt we compared the relative sensitivity of larvae from 0 to 20d using copper chloride. The goal of this research was to maximize sensitivity of the test and allow for some flexibility on the part of larvae suppliers and testing laboratories. This work is also reported in a recent publication by McNulty et al. (In Press).

### Methods

#### Toxicity Test Procedures

All larvae used in the copper sensitivity tests were hatched on the same day. From that single larval pool, cohorts of ages 0, 1, 3, 5, 7, 9, 11, 15, and 20-d post-hatch were transferred to separate, aerated screen-tubes and provided with flowing seawater until used in toxicity tests. Larvae were fed newly-hatched *Artemia* nauplii, in excess. The different larval age groups were each exposed to a range of copper concentrations to determine their sensitivity. All larvae remained in clean seawater until they were the appropriate age to begin the 7-d exposure to copper.

Age-specific sensitivity was compared using the standardized 7 d larva growth and survival toxicity test described above. The toxicity tests consisted of five replicates, with five larvae in each replicate, exposed to each of six copper concentrations: 0, 56, 100, 180, 320, and 560  $\mu\text{g L}^{-1}$ . The total number of dead larvae per container was counted daily and totaled after 7 days to calculate mortality.

Percent mortality data were normalized using the arcsine/square root transformation (Zar 1984). The no observed effect concentration (NOEC) was calculated using Dunnett's multiple comparison test (Zar 1984). The median lethal concentration (LC50) was

determined using the trimmed Spearman-Kärber method (Hamilton et al. 1977). Age-specific mortality was analyzed using two-way analysis of variance (ANOVA) to determine differences in mortality between larval ages and different copper concentrations. Larval cohorts were divided into statistically similar groups using the Student-Newman-Keuls (SNK) multiple comparison test (Zar 1984). Although the SNK test has been criticized for high type I error rates (Day and Quinn 1989), the division between the two major groups in this study was large (4.15 test statistic vs. 2.77 critical value).

### **Gill Surface Area and Cutaneous Surface Area Estimates**

Lamellar and respiratory surface area were measured on a separate group of larvae which had not been exposed to copper. Larvae ages 0, 1, 3, 5, 7, 9, 11, 15, and 20 d were preserved with formalin then transferred to ethyl alcohol after 48 h. Surface areas were measured on eight larvae from each age group.

Gill surface area (GSA) was quantified by extraction and separation of the gill structure from the left side of the head for microscopic analysis. The most medial gill arch was not dissected because no filaments or lamellae were present. Total GSA of each gill arch was estimated. GSA measurements were accomplished using image analysis software (Image® 1.37) on an Apple® Macintosh™ computer. An image of the gill was captured on the computer screen, "smoothed", and magnified 2x to allow for clear examination of the margin. The gill arch itself was not included in this measurement because it is not a functional respiratory surface area. The lamellar surface areas of the first three gill arches were compared across ages using ANOVA to see if any disproportionate growth occurred within one gill arch that would be masked if only total GSA was examined. All gill measurements were pooled for statistical analyses (total GSA) to minimize variability in the data.

Total length of each larva was measured to the nearest 0.1 mm, and cutaneous surface area (CSA) was estimated as the square of the total length ( $TL^2$ ). Total length squared has been found to be proportional to CSA for oxygen uptake (Blaxter 1988). The possible relationship of GSA and CSA to larval age and toxicity (LC50) was assessed using ANOVA and regression analysis.

Analytical verification of total copper concentration ( $\mu\text{g total Cu L}^{-1}$ ) from selected test solutions showed actual concentrations to be within 13% of nominal values. Endpoint estimates were based on nominal total copper concentrations.



## Results

Results of the 7 d toxicity tests with copper showed higher overall mortality in older larvae, 9- to 20-d-old, than in younger larvae, 0- to 7-d-old (Fig. 2). There was a statistically significant difference in copper sensitivity between the different larval age groups (ANOVA,  $p = 0.001$ ).

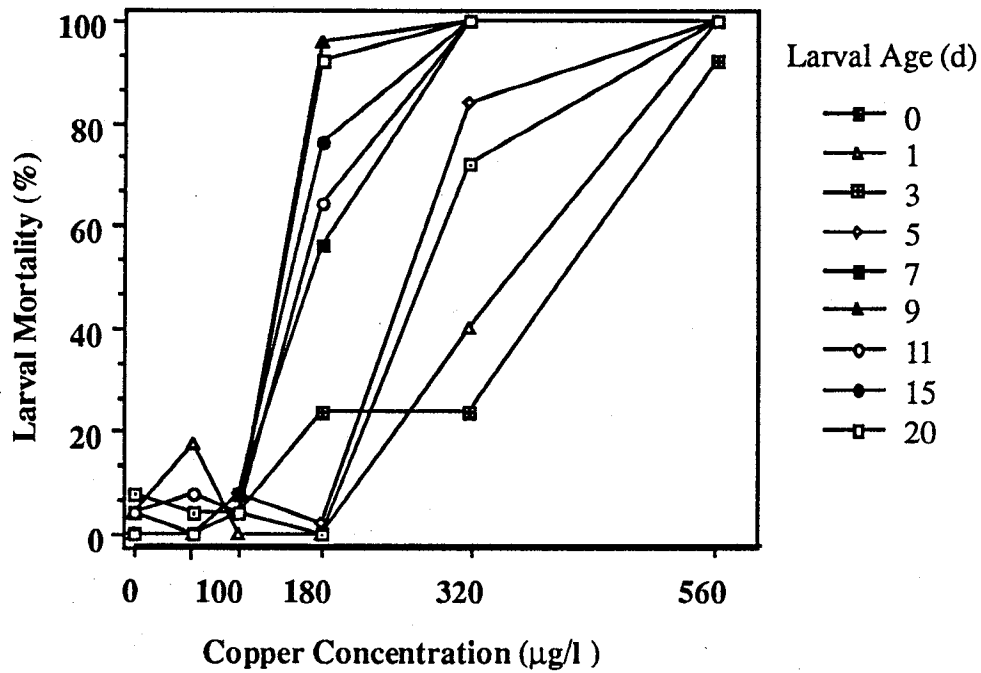


Fig. 2. Dose-response curves for larval topsmelt of various ages exposed to copper at.  $N = 5$  replicates per copper treatment for each age group.

The LC50s decreased steadily from 365  $\mu\text{g L}^{-1}$  Cu for 0 d fish to 134  $\mu\text{g L}^{-1}$  Cu for 9 d fish, nearly a three-fold increase in copper sensitivity with larval age (Fig. 3). The LC50s became asymptotic in 9- to 20-d-old larvae.

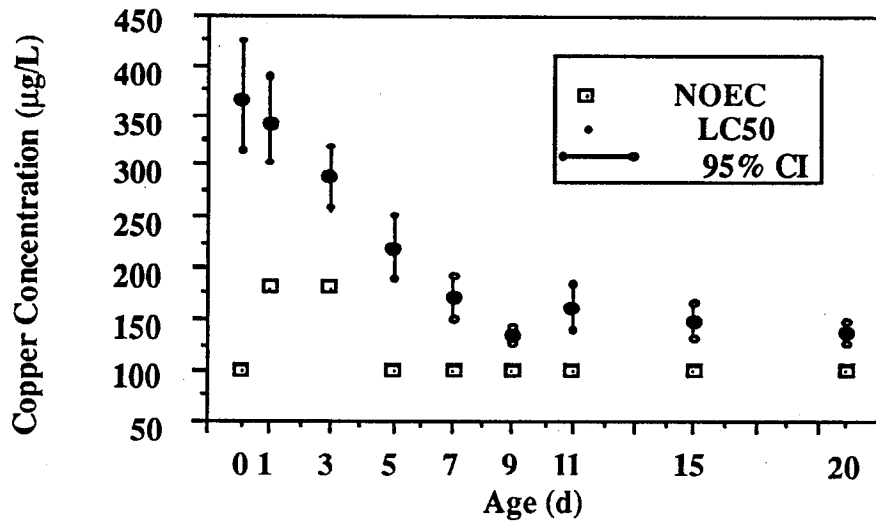


Fig. 3. NOEC and LC50 estimates from toxicity tests with topsmelt larvae of various ages. N = 1 test per age group.

The difference in toxic response between larval age groups was highly significant (ANOVA;  $p = 0.001$ ). Larvae 0- to 5-d-old had significantly higher copper tolerance than larvae 7- to 20-d-old, while there was no significant difference among the older age group (SNK;  $p = 0.05$ ). No observed effect concentrations (NOECs) remained relatively stable ( $100 \mu\text{g L}^{-1}$ ) across all age groups except that at ages 1 and 3 d the NOEC was  $180 \mu\text{g L}^{-1}$  (Fig. 3).

A plot of daily mortality indicated that the mortality rate was higher for older larvae. The graphical depiction of daily mortality indicates that rate could be divided into two groups with 7 to 20 d larvae dying faster than 0 to 5 d larvae (Fig. 4).

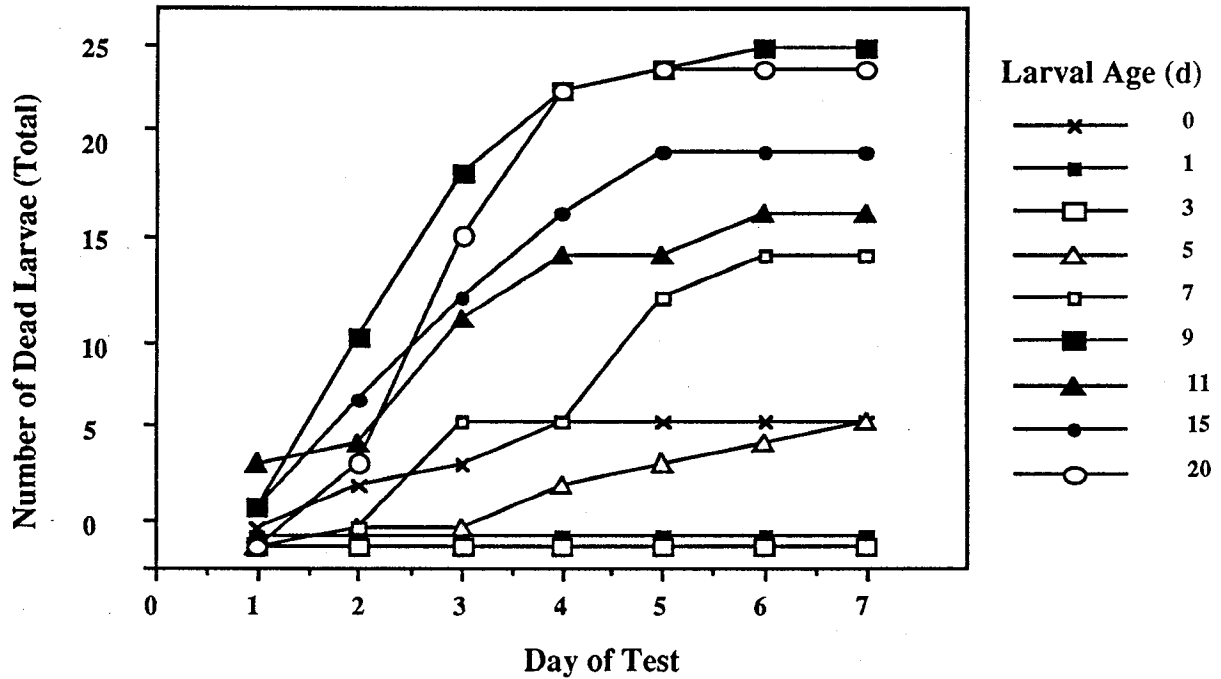
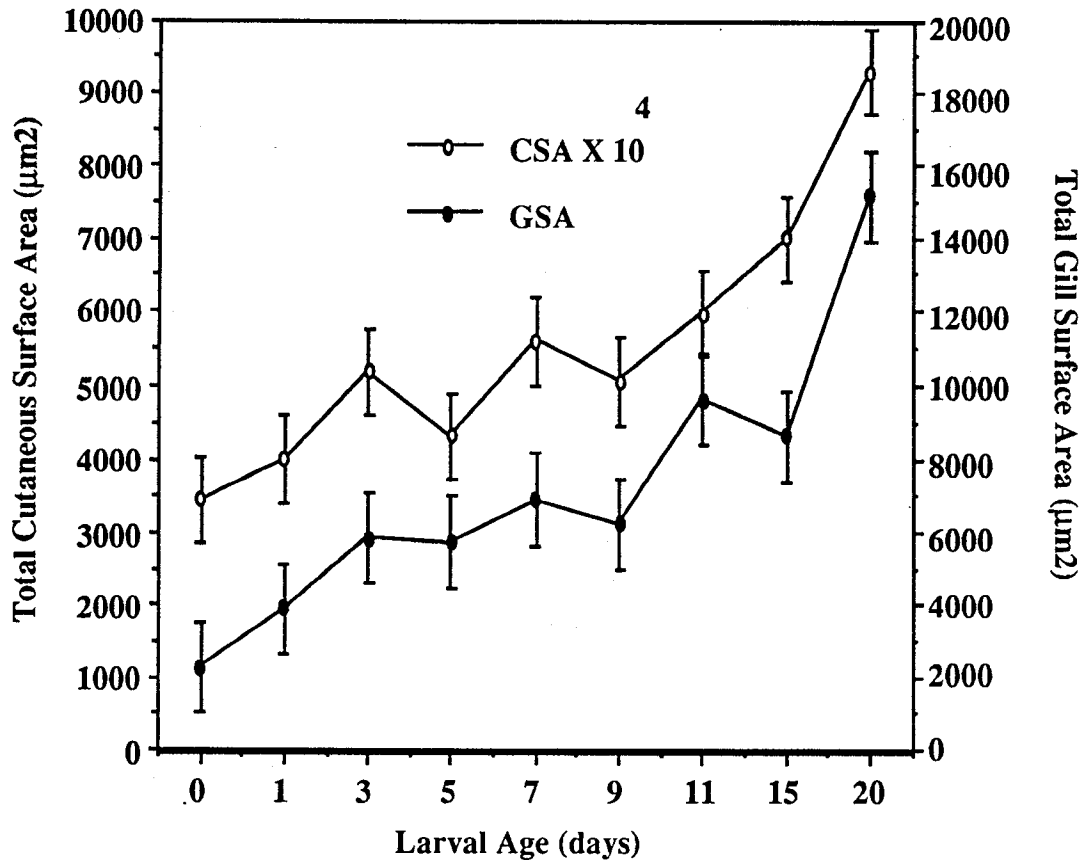


Fig. 4. Cumulative mortalities at 180 µg copper/liter on each day of testing for each age group. Initial number of fish per group was 25. Mortality at 180 µg/liter was compared because this copper concentration elicited intermediate mortality and thus gave an indication of variable sensitivity across the different larval age groups.

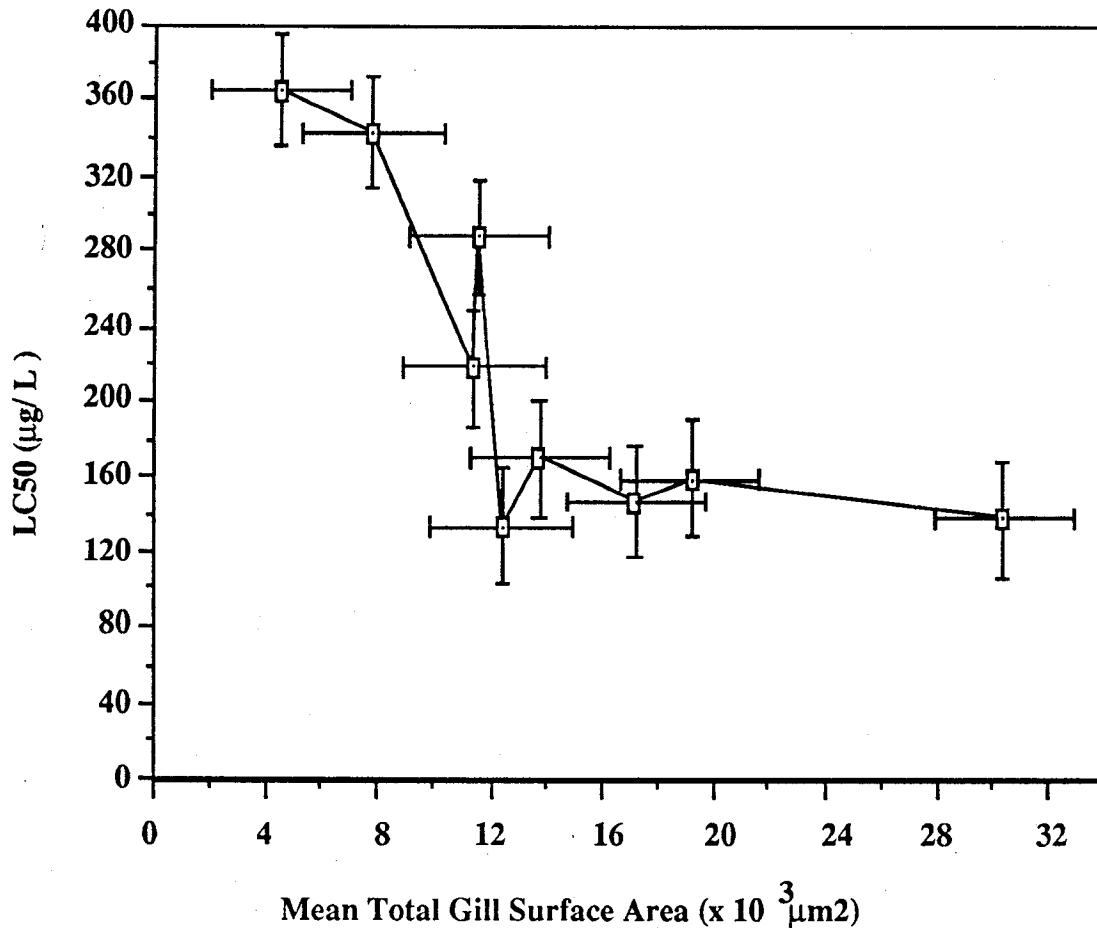
Larvae experienced a seven fold increase in available gill surface area (GSA) between ages 0 d and 20 d (Fig. 5). Mean total GSA was  $4,525 \mu\text{m}^2$  for 0-d-old larvae,  $11,594 \mu\text{m}^2$  for 3-d-old larvae,  $17,250 \mu\text{m}^2$  for 15-d-old larvae, and  $30,391 \mu\text{m}^2$  for 20-d-old larvae. GSA increased more rapidly than cutaneous surface area (CSA; Fig. 5).



**Fig. 5.** Total gill surface area  $\times 10^4$  (GSA) and cutaneous surface area (CSA) compared to larval age in days.  $N = 8$  for both measurements, vertical bars indicate  $\pm$  standard error.

Although CSA was four orders of magnitude larger than GSA, CSA estimates increased only three-fold from 0 d to 20 d (from  $3.5 \times 10^7$  to  $9.5 \times 10^7 \mu\text{m}^2$ ).

Comparison of GSA to LC50 showed an asymptotic relationship (Fig. 6). Sensitivity increases (LC50 decreases) until the larvae reach a mean GSA of 12,000  $\mu\text{m}^2$  or greater.



**Fig. 6.** The relationship between mean total gill surface area and mean LC50 (toxicity). Horizontal bars indicate  $\pm$  standard error. Vertical bars indicate 95% CI around LC50 estimates. Each data point represents an age group.

Beyond this apparent threshold in GSA, sensitivity changes little with increasing GSA. Using exponential regression analysis, LC50 showed a significant inverse relationship with CSA ( $r = -0.760$ ) and GSA ( $r = -0.793$ ), indicating that the relationship of toxicity to both of these measures is similar.

## Discussion

In these experiments, LC50s decreased from 365  $\mu\text{g}$  (copper)  $\text{L}^{-1}$  in 0 d larvae to 134  $\mu\text{g}$   $\text{L}^{-1}$  copper in 9 d larvae (Fig. 3). Copper sensitivity remained relatively constant for larval ages 7 to 20 d with LC50s decreasing from 169  $\mu\text{g}$   $\text{L}^{-1}$  to 137  $\mu\text{g}$   $\text{L}^{-1}$  respectively.

Although the mechanisms of copper toxicity to developing fish larvae are complex, decreasing LC50 values were correlated with increasing larval age, gill surface area and cutaneous surface area, possibly indicating increased copper uptake by older fish. Associated causes of increasing sensitivity include: 1) changes in uptake rate as larvae change from cutaneous to branchial respiration, 2) an increase in gill damage or dysfunction, or 3) development of additional pathways for uptake of toxins.

We hypothesized that if differences in gill surface area (GSA) or cutaneous surface area (CSA) resulted in the increase in copper sensitivity, then statistical comparisons of these morphometrics would result in the same groupings found for the survival data. If this were true, then GSA and CSA for 0 to 5 d larvae would be significantly lower than for 7 to 20 d larvae. However, although there was a statistically significant age-specific difference in CSA and GSA, multiple comparison tests showed no clear distinction between the 0 to 5 d and 7 to 20 d larval groups in terms of respiratory surface area. We found that topsmelt larvae experience an approximately linear increase in both CSA and GSA in their first 20 days. Neither morphometric demonstrates an asymptotic relationship with age like that observed for larval survival (Fig. 4).

One possible explanation for the difference in sensitivity is that copper uptake may be limited by respiratory surface area in larvae 5 days and younger. As larvae grow, respiratory surface area increases and so does the rate of copper toxicity. At approximately 7 days, larvae may achieve a certain threshold level of respiratory surface area that no longer limits copper uptake. Beyond this apparent threshold, larvae have similar rates of mortality even though respiratory surface area continues to increase. The transition from cutaneous respiration to branchial respiration with increasing age indicates a substantial change in the physiological state of the larvae and may affect the toxic response seen between ages 7- and 11-d-old (Fig. 2). Iwai and Hughes (1977) suggest that respiratory distress occurs in larvae during the transition from cutaneous respiration to branchial gas exchange under normal conditions (in the absence of toxic chemicals). As larvae progress from cutaneous to branchial respiration, there is probably a period of time in which the larvae are respiring through both pathways. More developed larvae may be capable of

cutaneous respiration, but at some critical surface-to-volume ratio it becomes inefficient to utilize this avenue as a major respiratory pathway. As larvae make the transition to branchial respiration, a brief increase in copper uptake may occur as both respiratory pathways are utilized concurrently. This may lead to increased sensitivity to copper for a short period of time, as seen in the mortality response of the 9-d-old larvae (Fig. 2).

Physical damage to the gills may also be at least partially responsible for the difference in larval sensitivity (Jagoe et al. 1991). If the primary respiratory pathway for younger larvae is cutaneous, then younger larvae may be less susceptible to the effects of gill damage. As larvae switch to branchial respiration, any physical damage to the gills might cause larval mortality due to hypoxia. Copper has been found to bind to and damage the gills of fishes (Radhakrishnaiah 1988). This response of gills to stress is generally not chemical specific, suggesting that there are generalized physiological reactions to stress that may be a defense response to protect the gills from any kind of irritation (Mallat 1985). The increased mucus and swollen chloride cells make diffusion of gases across the gills difficult. Baker (1969) and Cardeilhac et al. (1979) found increased mucus secretion in the gills of fishes in response to copper exposure, and suggested that the gill damage and dysfunction observed resulted from copper ions binding to the gill tissue. Numerous additional and unmeasured factors could affect the complex process of copper uptake, including ingestion of copper sorbed or accumulated by their brine shrimp food.

These results are similar to other studies investigating age-specific sensitivity of larval fish to copper. Studies with plaice (*Pleuronectes platessa*) showed that older larvae were more sensitive than newly hatched larvae to copper (Blaxter et al. 1977). In addition, 7 to 11 d *Menidia beryllina* larvae were more sensitive to copper and pentachlorophenol than were 0 to 6 d larvae (Heber et al. 1988). Pickering (1992) compared sensitivity of 1, 4, and 7 d fathead minnows exposed to copper and found 7 d larvae to be more sensitive than 4- or 1-d-old larvae.

Age-specific sensitivity varies depending upon the toxic mechanism and uptake routes of different compounds. For example, Hemmer et al. (1992) found no difference in sensitivity between 7 and 28 d topsmelt larvae when exposed to carbophenothion or chlorpyrifos. Borthwick et al. (1985) found *Menidia menidia*, *M. peninsulae*, and *Leuresthes tenuis* larvae 0-, 7-, and 14-d-old were more sensitive to thiobencarb than 28-d-olds, but 0- and 28-d-old larvae were less sensitive than 7- and 14-d-old larvae to chlorpyrifos. *Menidia beryllina* larvae were more sensitive to copper and pentachlorophenol at 7 to 9 d of age than were newly hatched or 4- to 6-d-old larvae (Heber et al. 1988).

Regardless of the mechanisms underlying differences in age specific sensitivity of topsmelt larvae, the results of this study were useful in determining an appropriate age range for a topsmelt larval toxicity test protocol. This information should be supported by similar future studies with additional toxicants to optimize the sensitivity of this toxicity test protocol to unknown or complex toxicants.



## Topsmelt Section 3: Performance of The Larval Protocol at Estuarine Salinities

### Introduction

One desirable characteristic of the topsmelt is its ability to tolerate a wide range of salinities. Carpelan (1955) found that topsmelt may survive and reproduce at salinities up to 72 ‰. Middaugh and Shenker (1988) reported that topsmelt larvae survived salinities from 2 to 60 ‰. Because effluents are discharged into estuarine and coastal receiving waters ranging from brackish to marine salinities, and because changes in salinity may influence toxicity, the use of euryhaline species allows for a more relevant assessment of receiving water toxicity. There is a need for toxicity test protocols using euryhaline species.

A number of studies have investigated the relationship between salinity variation and toxicant sensitivity using heavy metals (e.g., Cd, Zn, Ni, Pb, Cr, Cu, Hg). Most have focused on the interaction between thermal and osmotic stress and exposure to sublethal concentrations of heavy metals. Studies include those with fish embryos and larvae (Lorz and McPherson 1976, Alderlice et al. 1979, Voyer et al. 1979, Voyer et al. 1982) and studies conducted with other phyla, primarily crustaceans (Thurberg et al. 1973, Jones 1975, McClusky and Hagerman 1987, DeLisle and Roberts 1988).

In this paper we evaluate the performance of the 7d growth and survival protocol for larval topsmelt at representative estuarine salinities and describe the relationship between salinity and toxicity of two compounds. The influence of salinity on toxicity of copper chloride was investigated because copper is a commonly used reference toxicant and contaminant (Weber et al. 1988, Anderson et al. 1991), and because copper complexation and cupric ion bioavailability are affected by organic ligand concentrations which may vary with salinity. Salinity dependent toxicity of sodium azide was investigated because the bioavailability of azide is presumably salinity independent (at pH 8.0). There is a general lack of data on azide toxicity to marine species.

Topsmelt larvae were gradually acclimated to salinities from 2 to 34 ‰, and a series of 7d growth and survival tests were first conducted with copper chloride. A similar experiment was then conducted with sodium azide. In a third experiment, topsmelt were acclimated immediately to test solutions with varying salinities as embryos to eliminate acclimation time as a factor, and the hatched larvae were again exposed to sodium azide. The results of these experiments are discussed in terms of the possible interaction of osmotic stress and copper and azide toxicity, and in the context of using topsmelt and other

euryhaline fish species to test complex effluent discharged into receiving waters of varying salinity.

## Methods

### Toxicity tests

All toxicity tests were conducted using methods described above. The copper test was conducted with 9 day-old larvae; the azide tests were conducted with 13 day-old larvae.

To examine the interaction between salinity and toxicity, a single cohort of topsmelt larvae was isolated at hatch at 33‰ salinity. Approximately 200 larvae were isolated and left in 33‰ seawater and the remaining fish were acclimated overnight to 29‰. Salinity was adjusted by slow-dripping distilled water into the culture reservoir at the appropriate volume to give a final target salinity 4‰ less than the original salinity. The culture reservoirs were well aerated to facilitate mixing. Every 24 hours thereafter the salinity was adjusted down 4‰. As salinity was lowered, approximately 200 larvae were isolated and held at the appropriate salinity (e.g., 33, 25, 17, 10, and 5‰) until needed for the experiment. After the lowest salinity was reached all larvae were allowed to acclimate before the toxicity test was initiated. This procedure was used for the experiment assessing copper toxicity and the first experiment assessing azide toxicity. This method of salinity adjustment resulted in different salinity acclimation times for the larvae used in the first two experiments. For example, in the copper experiment, the larvae were acclimated to the target salinities of 10, 17, 25 and 33‰ for 4, 6, 8, and 9 days, respectively. In the first azide experiment, the larvae were acclimated to the target salinities of 5, 10, 17, 25, and 33‰ for 5, 7, 9, 11, and 12 days, respectively.

To eliminate acclimation time as a factor, a third experiment assessed azide toxicity using larvae that were acclimated to the test salinities immediately as embryos. Approximately 500 embryos in the early blastula stage were taken from the spawning substrates ( $S = 33‰$ ) and immediately transferred to culture tubes at salinities of 33, 25, 17, and 10‰. Water in these tubes was held static and renewed daily. The embryos were cultured at 20 °C, as described above, until hatching commenced. As larvae hatched, cohorts were isolated daily into separate screen tubes at the appropriate salinities. When the surviving larvae were 13 days old concurrent toxicity tests were conducted at all salinities as described above.

The relative sensitivity of the fish that were salinity acclimated during larval development vs fish that were acclimated during embryonic development were analyzed by

comparing the LC50s and using ANOVA. The two groups of larvae were grouped by salinity for this comparison (at 10, 17, 25 and 33‰ salinities).

Analysis of Variance followed by Dunnett's multiple comparison test was used to derive No Observed Effect Concentrations (NOECs) for all tests, and trimmed Spearman-Kärber method (Hamilton et al. 1977) was used to calculate LC50's and the 95 % CI for all tests.

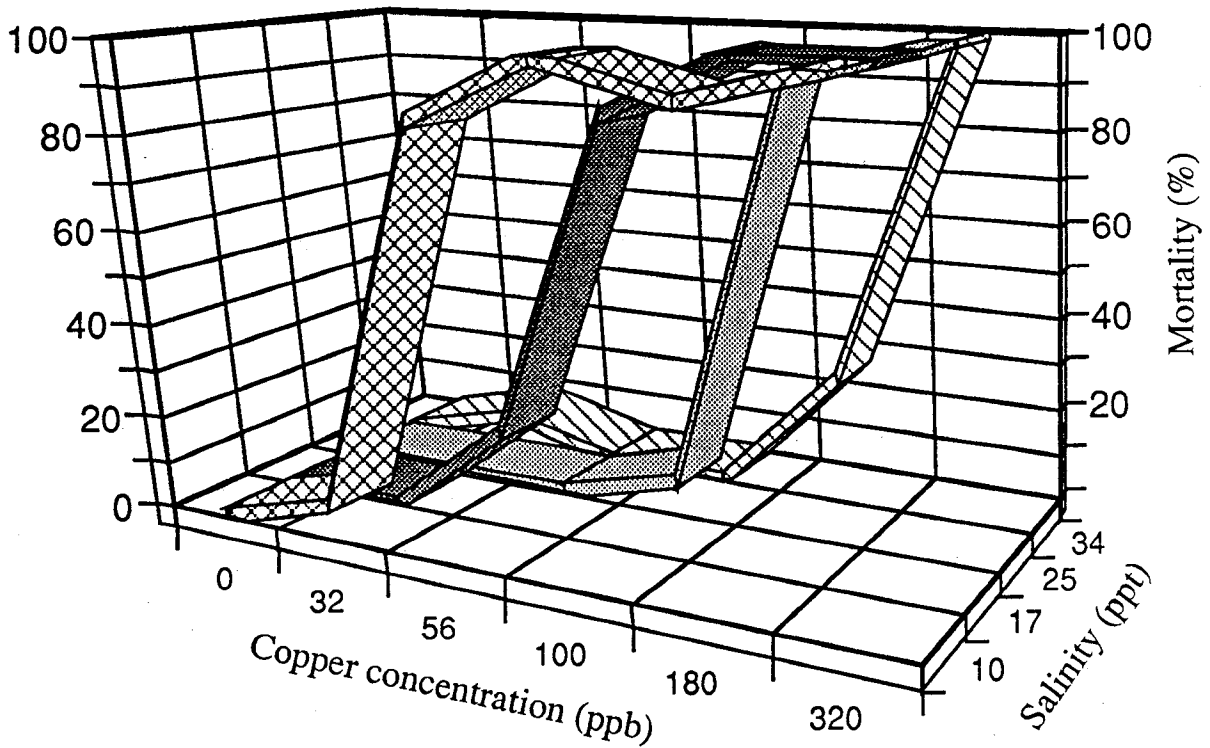
### **Determination of iso-osmotic point**

The isosmotic point (where the culture media osmotic pressure, measured as osmolality, is the same as that of the blood; after Hoar 1975) was determined for both adult and 10 day-old larval topmelt. Adult fish were transferred from culture tanks at 33‰ into 20 L aquaria and slowly acclimated to culture salinities of 5, 10, 17, 25 and 33‰ by adjusting the salinity down 4‰ a day using fresh well water. Three fish were placed into each aquaria. After the target salinity was reached, all fish were held for an additional 5 days and then blood samples were taken from the ventral aorta to determine blood osmolality. Blood and culture media osmolality were measured using a Wescor Vapor Pressure Osmometer (model 5500). For larval measurements, early blastula stage embryos were transferred from the culture tank (33‰) directly to culture tubes at 5 to 33‰ and the hatched larvae were cultured at the respective salinities for 10 days. Larvae had insufficient blood volumes for direct measurement of osmolality, so approximately 100 larvae were removed from the culture tubes and thoroughly blotted dry then placed into a centrifuge tube where they were briefly homogenized. The resulting homogenate was then placed in the osmometer for measurement. Three measurements were taken for all fish at all salinities.

To further ascertain the effects of salinity on larval health, a second group of early blastula stage embryos was transferred from seawater (33‰) to culture tubes at salinities from 5 to 33‰ and the hatched larvae were held for an additional 60 days. After 60 days, the growth of larvae at each salinity was determined. Growth was measured as standard length and dry weight on fifteen larvae for each parameter.

## Results

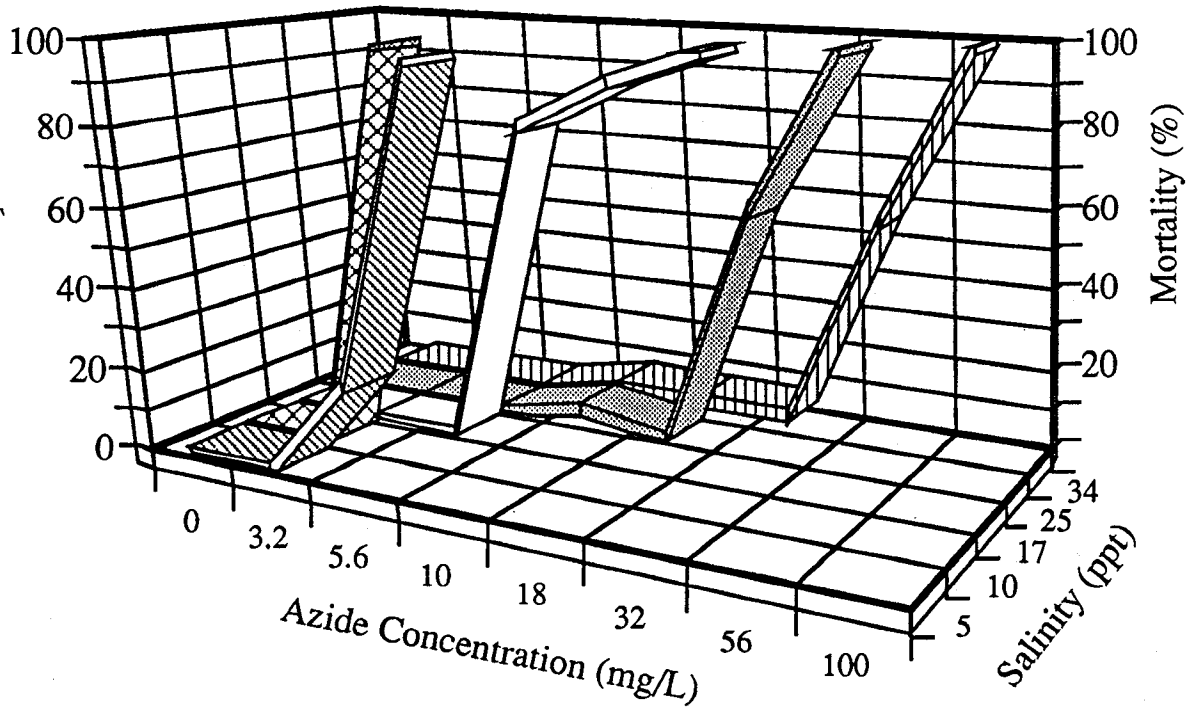
The toxicity of copper and azide to topsmelt larvae increased as salinity decreased in these experiments. This is evidenced by increasing mortality in lower copper concentrations at lower salinities (Fig. 7).



**Fig. 7.** Toxicity of copper chloride to topsmelt larvae exposed to salinities from 10 to 34 ‰.

The LC50's for copper ranged from 205  $\mu\text{g}/\text{liter}$  at 33‰ to 44  $\mu\text{g}$  copper/liter at 10‰ (Table 4). Despite the dramatic increase in larval mortality at lower salinities, control survival at all salinities was 100% in this experiment, indicating that topsmelt are amenable to toxicity testing at estuarine salinities within this range (Fig. 7). However, in the 2‰ salinity treatment, there was 100% mortality at all test concentrations including the control; these fish appeared skittish and all larvae died within the first 24 hours of the experiment.

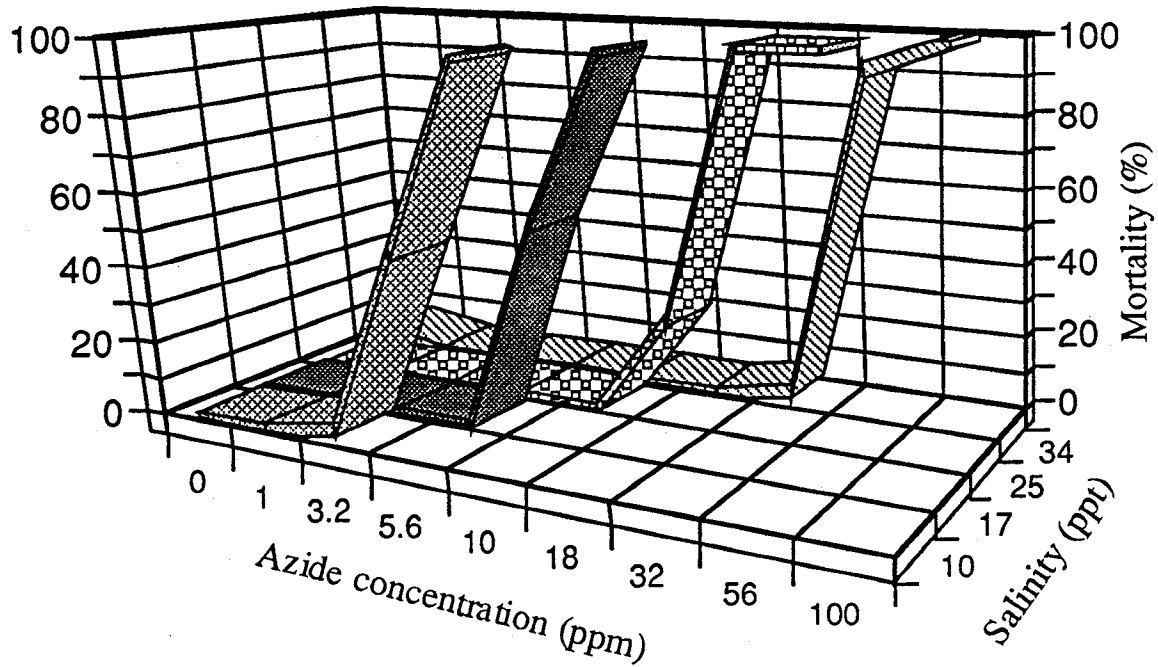
Results of the first azide experiment were similar to the copper experiment; mortality increased at lower azide concentrations as salinity decreased (Fig. 8).



**Fig. 8.** Toxicity of sodium azide to topsmelt larvae exposed to salinities from 5 to 34 ‰.

The LC50's for azide ranged from 54 mg azide/liter at 33‰ to 6.5 mg azide/liter at 5‰, (Table 4). As in the copper test, control survival in this test was 100% at all salinities.

The second azide experiment in which topsmelt embryos were incubated and hatched at the test salinities gave results similar to the first test. Topsmelt mortality also increased as salinity decreased in this experiment, while control survival was 100% (Fig. 9).



**Fig. 9.** Toxicity of sodium azide to topsmelt larvae at salinities from 10 to 34‰. All larvae tested were acclimated to the respective salinities through embryonic and larval development.

The LC50's for azide ranged from 43 mg azide/liter at 33‰ to 5.4 mg azide/liter at 10‰, respectively (Table 4). Control survival in this test was 100% at all salinities. No significant weight effects were detected in any of the experiments reported here; the following discussion refers to larval mortality only .

**Table 4 .** Median lethal concentration ( LC50) for topsmelt larvae used in 3 toxicity tests, conducted at several salinities. Tests used copper chloride, sodium azide with no embryo acclimation, and sodium azide with embryo acclimation. Numbers in parentheses are  $\pm$  95% confidence intervals. NC = not calculated.

Salinity (ppt)	Copper test ( $\mu$ g/L)	Azide test #1 (mg/L)	Azide test #2 W/ Embryo Acclimation (mg/L)
5		6.53 (6.22, 6.85)	
10	43.70 (41.80, 45.7)	5.66 (NC)	5.38 (4.71, 6.14)
17	72.04 (67.83, 79.51)	<10.00 (NC)	9.68 (8.62, 10.86)
25	134.14 (128.62, 139.9)	29.90 (28.18, 31.68)	20.39 (18.37, 22.64)
34	205.07 (194.1, 216.65)	53.6 (50.37, 57.06)	43.31 (40.14, 46.74)

The LC50's from the two azide experiments were similar, particularly at 10 and 17‰ salinity (Table 4). The LC50s from the two experiments diverged somewhat at 25 and 34‰ salinity. There was no significant difference in sensitivity between the two groups at 10 and 17‰ salinity. There was a significant difference between the two groups at 25 and 33‰ (ANOVA  $p \leq .01$ ). At the higher salinity the fish acclimated as larvae were significantly less sensitive than the fish acclimated as embryos.

The isosmotic point calculated for both larval and adult topsmelt was approximately 12‰ (400 mmol/kg), indicating that larval and adult topsmelt have a similar capacity to osmoregulate (Fig. 10).

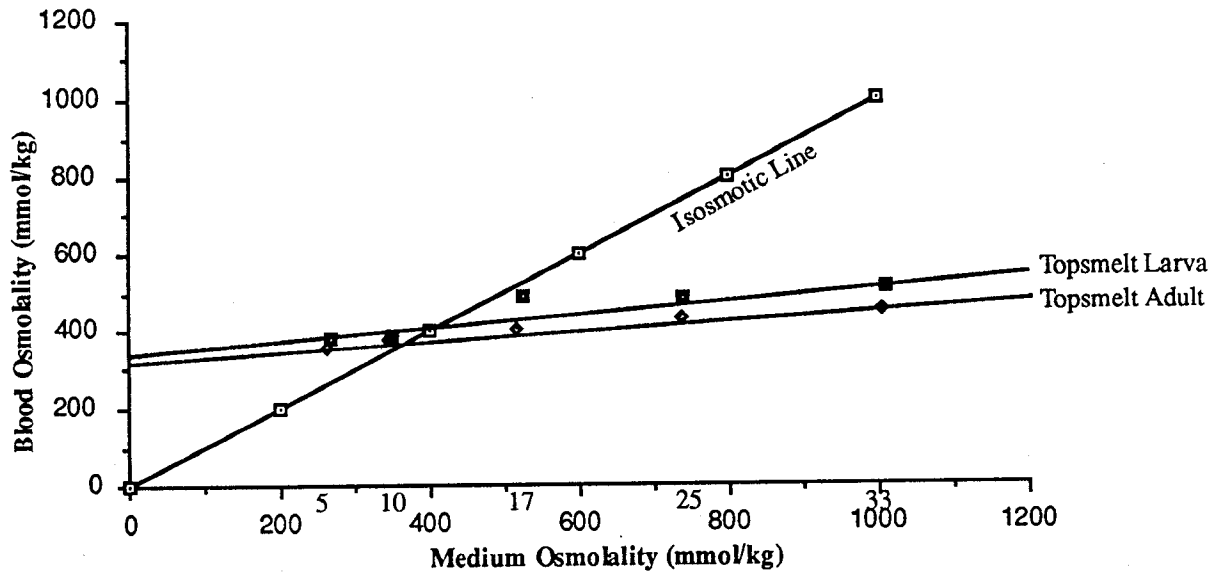
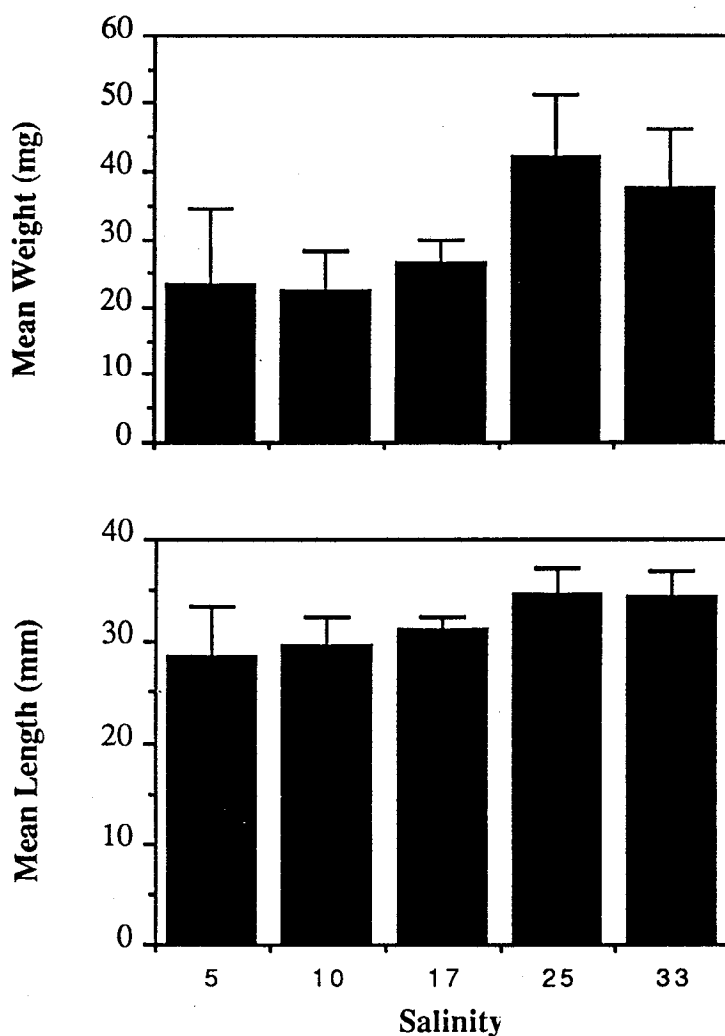


Fig. 10. Isosmotic point for adult and larval topsmelt acclimated to five salinities. The numbers immediately below the X Axis are salinities (‰) corresponding to the medium osmolalities.



To further investigate the effects of salinity on larval viability we cultured topsmelt larvae at salinities from 5 to 33‰ for 60 days then measured the growth of these fish. Results indicate that larvae cultured at lower salinities (5 - 17 ‰) were significantly smaller in length and weight than those cultured higher salinities (25 - 33‰;  $p = .0001$  for length and weight). Thus, although topsmelt are isosmotic at 12‰, growth of fish cultured at lower salinities is significantly less than growth of fish cultured at higher salinities (Fig. 11).



**Fig. 11.** Mean dry weight and standard length of topsmelt larvae acclimated and cultured for 60 days at five salinities.

Water quality parameters were within acceptable limits in all of these experiments. The temperature in all experiments and at all salinities was  $21 \pm 1^\circ\text{C}$ ; dissolved oxygen was always greater than 80% saturation; and measured salinity was within  $\pm 1.5\%$  of nominal

values. In the copper experiment the mean pH was 7.97 and pH ranged between 7.82 and 8.11 in all salinities and copper concentrations. In the first azide experiment mean pH was 8.11 and pH ranged between 7.91 and 8.24 in all salinities and azide concentrations. In the second azide experiment the mean pH was 8.00 and pH ranged between 7.72 and 8.54 at all salinities and azide concentrations.

## Discussion

These experiments demonstrate that larval topsmelt are amenable to toxicity testing at estuarine salinities from 5 to 33‰. Control survival at all salinities in all experiments was 100%. The relative sensitivity of topsmelt larvae to copper and azide was salinity dependent. Decreased salinity resulted in increased mortality in all experiments.

Results with copper suggest that the increase in larval mortality with decreased salinity increased cupric ion bioavailability coupled with increasing physiological challenge of osmoregulation. Coale and Bruland (1988) found that in the upper 200 m of Northeast Pacific waters, greater than 99.7% of copper is bound by "strong" organic complexes. Engel et al. (1981) also reported that copper is bound primarily by organic ligand complexes in fresh and brackish water systems. In our experimental system, seawater was diluted to the lower salinities using distilled water, a method commonly used to adjust salinity in toxicity tests (Weber et al. 1988). This probably diluted the concentration of organic ligands present, thereby increasing the concentration of free (cupric) ions and therefore toxicity (Sunda and Guillard 1976). Variable pH was presumably not a factor in copper speciation in this experiment because the pH remained near 8.0 at all salinities and copper concentrations. Our intention in these experiments was to assess the performance of the toxicity test for larval topsmelt at varying salinities, not to measure the effects of copper ion toxicity *per se*. The conclusion that we inadvertently increased cupric ion concentration through dilution of organic ligands is only speculative, however, because we did not measure free copper in this experiment.

A second possible effect of diluting seawater with distilled water is the dilution of ions necessary for osmoregulation (e.g.  $\text{Na}^+$ ,  $\text{Ca}^{++}$ ,  $\text{K}^+$ ,  $\text{Cl}^-$ ,  $\text{Mg}^{++}$ ). With fewer ions present at lower salinities the larvae might have been forced to expend even more energy to maintain proper osmotic balance.

It is likely that there was an interaction between osmotic stress and copper toxicity in these experiments. Several researchers have found evidence that copper inhibits enzyme systems necessary for osmoregulation (Thurberg et al. 1973, Stagg and Shuttleworth 1982). In a review of mechanisms of copper toxicity, Corbett et al. (1984) suggest that

copper interferes with the pyruvate dehydrogenase system integral to the production of ATP. A reduction in ATP, or inhibition of Na, K-ATPase would impair function of the Na-K pump necessary for osmoregulation (Crespo and Karnaky 1983); see review by Heath (1987). Bouquegneau and Gilles (1979) suggest that the primary physiological mechanism for the interaction of salinity with metal toxicity in invertebrates is the disruption of osmoregulation, with divalent metal ions competing with calcium and magnesium at uptake sites. It is also possible that effects of copper on osmoregulation was a secondary effect. Cardeilhac et al. (1979) found that copper ions caused physical damage to the gills of sheephead (*Pimelometopon pulchrum*). These authors found that copper disrupted membrane integrity in the gill lamellae and this lead to an elevation in the concentration of plasma potassium which disrupted cardiac function.

Our results are consistent with the results of numerous other studies investigating the relationship between salinity and heavy metal toxicity to estuarine and marine organisms. Studies with fish embryos and larvae using primarily cadmium as a representative metal have found increased toxicity at lower salinities (Alderlice et al. 1979, Voyer et al. 1979, Voyer et al. 1982). Studies with crustacea have also found increased sensitivity to heavy metals as salinity decreased (Jones 1975, Bjerregaard and Vislie 1986). Although the primary mechanism depends upon the metal in question, the prevailing conclusion of most of these studies is that metal toxicity results in disruption of osmoregulation. Future experiments will attempt to confirm whether copper disrupts osmoregulation by measuring variations in blood osmolality in fish exposed to copper at different salinities. In addition, we will investigate whether the difference in copper sensitivity can be attributed to variable copper uptake at different salinities.

Results of experiments with  $\text{NaN}_3$  were consistent with the results of the copper experiment and supported our hypothesis that there was a synergistic effect of toxicity and osmotic stress as salinity decreased. The  $\text{LC}_{50}$ 's in the first azide experiment declined with salinity from a high of 54 mg azide/liter at 33‰ to a low of 7 mg azide/liter at 5‰ (Table 4). Sodium azide is an inhibitor of cytochrome oxidase and therefore inhibits respiration and the eventual production of ATP (Kidder and Awayda, 1989). It is possible that the mechanism of azide toxicity is similar to that proposed above for copper where ATP production is reduced thereby limiting the larva's ability to osmoregulate. At lower salinities the combination of osmotic challenge and limitation on the ATP dependent Na-K pump may have resulted in lower tolerance to azide. In seawater  $\text{NaN}_3$  rapidly converts to  $\text{HN}_3$ . The pK of  $\text{HN}_3$  is 4.8, so that at the pH of these experiments (8.0) azide is almost completely disassociated into  $\text{N}_3^-$ , regardless of the salinity. Thus, azide bioavailability should be relatively independent of salinity, and the relative differences in sensitivity

between the larvae tested at different salinities should be related primarily to differences in degree of osmotic stress.

In the second azide experiment, the pattern of increasing sensitivity to azide with decreasing salinity was consistent with previous results, again indicating that the differences in sensitivity with salinity was due to osmotic stress. The larvae used in this experiment were all hatched and acclimated at the various salinities simultaneously so that there was no difference in acclimation time between the different larval groups. A statistical comparison of the azide tests with and without variable acclimation times showed no difference in azide sensitivity between the two experiments at 10 and 17‰. There was, however, a statistically significant difference between the results of the two experiments at 25 and 33‰. For some reason, sensitivity to azide was significantly higher at the high salinities in the second azide exposure even after increased acclimation time for the larvae. In designing this experiment we presumed that, if there was a difference, we would find increased tolerance in the experiment where larvae had a longer acclimation time. The culture and exposure procedures were identical in both experiments at 33‰ so the observed difference between the two tests at 33‰ indicates that the difference may be simply due to between-test variability.

In a review of the effects of salinity on toxicity, Sprague (1985) suggested that euryhaline fish in general may be more effective in dealing with pollutants at their isosmotic point, which may be due to a reduction in uptake rate. Similarly, in separate studies with two different euryhaline mysid species, McClusky and Hagerman (1987) and DeLisle and Roberts (1988) found that maximum tolerance to cadmium occurred at each species isosmotic point, and that mortality increased at both lower and higher salinities. Our results indicate that this generalization is not true for topsmelt. The isosmotic point for topsmelt is approximately 12‰, but our experiments indicate that both copper and azide toxicity increased as test salinity approached the isosmotic point. It may be that although topsmelt are euryhaline and thus able to tolerate lower salinities (as indicated by 100% control survival at all salinities), this species is physiologically better adapted to more marine conditions. In general, topsmelt spend most of their life at marine salinities; they spawn in harbors and estuaries during the drier summer months then migrate offshore in the fall. Although some young fish may remain, adult fish are generally not present in estuaries during the winter months when hypo-osmotic conditions might be expected to occur. Further evidence that topsmelt become stressed at lower salinities is supported by the larval growth data which showed that length and weight of larval fish declined with salinity in the absence of any toxicant (Fig. 11).

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**Section 2**

**Mysid (*Holmesimysis costata*) Experiments**

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## Introduction

The Marine Bioassay Project (MBP) has conducted numerous experiments with the kelp forest mysid *Holmesimysis costata* in order to provide an effluent toxicity test protocol using an indigenous west coast marine crustacean. This work has been guided by criteria established by the California State Water Resources Control Board, their toxicity test protocol review committee, and consensus within the scientific community. These criteria for candidate toxicity test organisms include sensitivity to toxicants, ecological and economic importance, amenability to laboratory culture of early life stages, indigenous distribution along the Pacific coast, dependability of supply, previous use in toxicity testing, and availability of information on life history (Linfield et al. 1985).

Mysids have been shown to be sensitive to numerous toxicants (Nimmo et al. 1977, Benfield and Buikema 1980, Gentile et al. 1982, Breteler et al. 1982, Lussier et al. 1985), and *H. costata* has been found to be more sensitive to some trace metals than other mysids described in the literature (Martin et al. 1989, Hunt et al. 1991). *H. costata* is ecologically important as a food web link between lower trophic levels (including detritus, primary producers, and zooplankters) and higher predators, especially juveniles of economically important kelp forest fishes (Mauchline 1980, Clark 1971, Hobsen and Chess 1976). *H. costata* is easily collected year-round from the surface canopy of giant kelp (*Macrocystis*) forests, and gravid females held in the laboratory will readily release juveniles for use as test organisms. Laboratory populations have produced gravid females that release juvenile test organisms, but additional research is necessary to sustain high fecundity rates in culture (Turpen et al. in review). *H. costata* ranges from La Jolla, California to the Queen Charlotte Islands, British Columbia (Holmquist 1979), and its biology has been described from field and laboratory studies (Clutter 1967, Clutter 1969, Green 1970, Turpen et al. in review). There have been a number of previous toxicity studies with *H. costata* (Tatem and Portzer 1985, Davidson et al. 1986, Machuzak and Mikel 1987, Reish and LeMay 1988, Asato and Reish 1989, Martin et al. 1989, Singer et al. 1990), and various toxicity testing protocols have been published (Reish and LeMay 1988, Anderson et al. 1990, Hunt et al. 1991, ASTM 1993). Many previous studies have referred to this organism as *Acanthomysis sculpta*, though this designation has been revised by Holmquist (1979, 1981) in her comprehensive review of northeast Pacific mysids. We have considered her designation of *Holmesimysis costata* to be definitive (see also Mauchline 1980).

...Although one long-term experiment has been described with this species (Davidson

et al. 1986), most studies with *H. costata* used 96 hour acute mortality tests. This report expands on Marine Bioassay Project efforts to develop a seven-day sublethal test that incorporates growth and survival to estimate chronic toxicity (Hunt et al. 1991). A sublethal endpoint is required of protocols adopted by the State of California for assessing compliance with chronic toxicity objectives in discharge permits. Test organism growth represents an integration of a number of physiological processes and is an easily quantified measure of organism stress.

This section discusses further evaluation of the seven day *H. costata* growth and survival toxicity test protocol. Experiments were conducted to determine the effects of various physical factors on test performance, to estimate inter- and intralaboratory precision, to evaluate the growth endpoint using representative effluents, and to compare length and weight as measurements of growth in these small crustaceans.

## Methods

### Facilities

Experiments were conducted between January 1991 and June 1993, at the California Department of Fish and Game Marine Pollution Studies Laboratory (MPSL) at Granite Canyon. The laboratory is located on the Big Sur Coast in Monterey County, California. The MPSL seawater intake is at least 15 km from any known source of pollution. The site is 0.5 km from a California State Mussel Watch station at Soberanes Point that has consistently shown low concentrations of trace elements, pesticides, and petroleum hydrocarbons (Stephenson et al. 1979, Martin and Castle 1984).

### *Holmesimysis costata* Toxicity Test Protocol

Methods for conducting 7 d growth and survival tests are described in the *H. costata* toxicity test protocol (Hunt et al. 1991). Additional methods used in protocol development are presented below. Performance evaluation of the protocol during this phase of the project consisted of: 1) experiments investigating the effects of various physical factors on test performance, 2) effects of replication on the test's ability to detect a significant difference between treatment and control, 3) interlaboratory tests with complex effluents, 4) repeated testing with a reference toxicant, 5) examination of growth effects in tests with complex effluent, 6) tests assessing sodium azide as a potential reference toxicant, and 7) comparisons of length and weight as measures of mysid growth. All tests began with three-day-old mysids, and only tests with control survival  $\geq 80\%$  are presented. Effluents and reference toxicants are reported as nominal concentrations.

## Effects of Physical Factors

The *H. costata* 7 day protocol requires  $\geq 80\%$  control survival as part of the test acceptability criteria. The following experiments were conducted to examine the effects of various physical factors on control response. Manipulations of some of these factors are being considered in the current protocol revision. Experiments were conducted to investigate the effects of the following factors on control survival: test temperature, number of mysids per test container, number of renewals of test solution during the 7 day exposure, location and shading of test containers, use of various substrates as habitat, and different methods of shipping mysids for interlaboratory tests. In each experiment, mysids released from females in the same aquarium on the same day were randomly assigned to all treatments. Test temperature was initially 15°C, but as evidence accumulated that lower temperatures resulted in better control survival, lower test temperatures were used.

### Test Temperature

In this experiment, 7 replicate test containers containing 5 mysids in clean seawater were assigned to each of four temperature treatments: 11°, 13°, 15°, and 17°C. Mortality was observed daily and tallied at the end of the 7 day exposure. Water was renewed on days two, four, and six, and mysids were fed newly hatched *Artemia* as specified in the protocol (40 *Artemia* per mysid per day). Data was analyzed using ANOVA and Student-Neuman-Kuels multiple comparison test (Zar 1974).

### Loading Density

Two experiments investigated the effects of mysid density on control survival. Both experiments were conducted in clean seawater for seven days, with seawater renewals on days two, four, and six. All mysids were fed 40 *Artemia* per mysid per day. In the first experiment, survival of mysids held individually was compared to that of mysids held together. One treatment had five replicate trays, each containing six mysids held individually in separate 15 ml polystyrene tissue culture wells. Their survival was compared to that of six mysids held together in each of five replicate 200 ml polystyrene flasks. Test temperature was 14°C. Treatments were compared using Analysis of Variance.

In the second experiment, there were four replicates of four treatments: large containers with either five or eight mysids, and small containers with either five or eight mysids. Large containers had about 50% more bottom surface area and the same water volume as the small containers (200 ml). Test temperature was 11°C. Treatments were compared using two factor Analysis of Variance.



### Renewals

Two experiments were conducted to look at the effects of test solution renewal on mysid growth and survival. In one experiment, eight replicate test containers were assigned to each of two treatments: clean seawater renewed once in seven days (on day four), and clean seawater renewed three times in seven days (on days two, four, and six). Test temperature was 13°C, and mysids were fed 40 *Artemia* per mysid per day. Treatments were compared using a t-test.

Another experiment consisted of two concurrent tests with dilution series of 0 (seawater only), 32, 56, and 100 µg/L zinc; one test had one test solution renewal on day four, and the other test had three renewals on days two, four, and six. Each test had five replicates per concentration, test temperature was 14°C, and mysids were fed 40 *Artemia* per mysid per day. Treatments were compared using two factor Analysis of Variance.

### Habitat Substrates

In nature, *H. costata* aggregate in small schools that swim next to surface canopy blades of the giant kelp *Macrocystis pyrifera*. To investigate whether the presence of an artificial substrate mimicking a kelp blade would improve mysid survival, three types of substrates were tested. Eight replicate test containers with five mysids in clean seawater were assigned to each of four treatments: no additional substrate, curved dark glass, thin floating polyethylene sheeting, and a blade of *Macrocystis*. All containers were maintained at the same test temperature, and mysids were fed 40 *Artemia* per mysid per day. Survival was recorded after one and two weeks of exposure, and results were compared using Analysis of Variance.

### Transportation

Two experiments were conducted to determine the most reliable method for transporting mysids to other laboratories. In one experiment, mysids were shipped as gravid females and as two-day-old juveniles. Both sets of mysids were packed in bags containing oxygenated seawater in a cooler with blue ice, driven home overnight and returned to the laboratory the next day to simulate overnight delivery. Juveniles were taken from the bags, randomized into test containers, and exposed to a dilution series of zinc sulfate. Gravid females were set up in an aquarium until they released their young, which were then cultured for three days and exposed to an identical zinc dilution series. Control survival, No Observed Effect Concentrations (NOECs) and LC50s from the two tests were compared.

A second experiment assessed the effects of container type and food (brine shrimp) density on juvenile mysid survival. Two-day-old mysids were transported in plastic bags and in polyethylene bottles, both with and without *Artemia*. These shipping containers were packed in coolers with blue ice, transported by car away from the laboratory, and returned the next day, to simulate overnight delivery. Upon arrival at the laboratory, survival was quantified, and dissolved oxygen and temperature were measured.

### **Effects of Treatment Replication on Test Power**

To estimate the effect of replication on test power, we conducted one experiment with eight replicate test containers per concentration. Data from this test was analyzed sequentially by randomly choosing data sets of two replicates per treatment, then three replicates per treatment, and so on, through eight replicates. Each set of data was then analyzed using analysis of variance to allow determination of the Minimum Significant Difference (MSD). The MSD is the minimum statistically significant difference between a treatment and a control, and indicates test power by estimating how small a difference the test can detect. For example, given a test with a control response of 90% survival and an MSD of 10, any concentration producing survival of  $\leq 80\%$  would be significantly different from the control. The MSD was plotted against the number of replicates to indicate any asymptotic relationship that might indicate an appropriate level of replication that balances logistical effort against statistical power.

### **Interlaboratory Testing with Complex Effluents**

Two interlaboratory comparisons are described here. The first was conducted on June 19, 1992 using Bleached Kraft Mill Effluent (BKME). Effluent was lyophilized (freeze dried), stored frozen, split into samples for each laboratory, shipped overnight on ice, and reconstituted at each laboratory by mixing the powdered sample with dilution water, according to methods described by Higashi et al. (1992). Participating laboratories were the Marine Pollution Studies Laboratory at Granite Canyon (MPSL) and Oregon State University at the Hatfield Marine Science Center in Newport Oregon (OSU). Two-day-old juvenile mysids, released from field-collected females at MPSL, were shipped overnight to OSU in plastic bags containing oxygenated seawater in a cooler with blue ice (approximately 15°C). Juvenile mysids from the same cohort were packaged similarly and held at MPSL for one night prior to the test. The following day, the three-day-old mysids were taken from the shipping bags at each laboratory and randomly assigned to test containers. Test concentrations were 0 (control), 0.5%, 1.0%, 2.0% and 4.0% effluent.

The second interlaboratory test was conducted on February 18, 1993 using whole

effluent from a large publicly owned treatment works (POTW) that discharges to California coastal waters. A composite effluent sample was collected at the treatment plant the day before the test, split into identical one-liter samples, and shipped overnight on ice to the participating laboratories. The two laboratories conducting the test were MPSSL and the Aquatic Testing Laboratory (ATL) in Ventura, California. Two-day-old mysids were shipped the day before testing, as described above. Effluent test concentrations were 0 (control), 0 (brine control), 1.8%, 3.2%, 5.6%, 10% and 18%. The brine control solution was a combination of 18% spring water (18% was the highest effluent concentration), enough hyper-saline brine to adjust the spring water up to 33‰, and dilution water. Hyper-saline brine was made by freezing 1 µm filtered MPSSL seawater. Tests at all laboratories in both trials were conducted according to the seven-day growth and survival protocol (Hunt et al. 1991).

### **Reference Toxicant Tests**

Four reference toxicant tests were conducted at MPSSL to evaluate intralaboratory variability of the protocol. Three tests were conducted within a one month time span, and a fourth test was conducted during the previous year. Zinc sulfate was used as the toxicant in all tests. Zinc concentrations were 0 (control), 10, 18, 32, 56, and 100 µg/L. Test precision was estimated by taking the coefficient of variation of test LC50s. LC50s were generated using the Trimmed Spearman-Kärber Method (Hamilton et al. 1977).

### **Effluent Tests**

In addition to the interlaboratory tests, five more tests were conducted to evaluate test performance using whole effluent samples. One effluent sample was from an advanced primary plant treating waste water from a small agricultural city, and the other four were from a combined primary/secondary plant treating waste water from a large urban area. Two of the tests were conducted side by side using a split effluent sample. Effluent concentrations varied depending on the expected toxicity of the effluent. Hyper-saline brine used for salinity adjustment in all tests was made by freezing 1 µm filtered MPSSL seawater. Brine controls used the same amount of brine as the highest effluent concentration.

### **Sodium Azide Tests**

Sodium azide is a cytochrome oxidase inhibitor that was being evaluated as a candidate reference toxicant. Sodium azide was considered an appropriate reference toxicant because we anticipated that it might inhibit mysid growth at concentrations lower

than those causing significant mortality, therefore providing reference toxicant data useful for evaluating both endpoints. Three tests were conducted, two range finding tests and a definitive test with more closely spaced toxicant concentrations. Sodium azide concentrations in the definitive test were 0 (control), 10, 18, 32, 56, 100, and 320 µg/L.

### **Weight Measurement as an Indication of Mysid Growth**

The protocol used in all of the experiments described above quantified growth effects by measuring mysid length. Weight measurement was not considered to be feasible initially because of the very small mass of the juvenile mysids. Micro-balances are becoming more widely used in toxicity testing laboratories, however, and these are capable of accurately measuring mass in the 1 µg range. Depending on the equipment available, weight measurements may be less labor intensive than measuring length. Weight measurement may also offer more resolution in estimating growth, since weight tends to increase with volume, which should increase as a cube function of length.

In six of the experiments described above, all preserved mysids from each replicate test container were rinsed with de-ionized water and dried together for twenty-four hours on preweighed aluminum microweighboats at 50°C. Mysids were then held in a dessicator to cool to room temperature before weighing. Mysids were weighed in the weigh boats, the weigh boat weight was subtracted, and the remainder was divided by the number of mysids to get the mean individual weight per replicate.

Weight measurements were compared to growth measurements in two ways. Mean mysid length per replicate and mean mysid weight per replicate were paired and correlated using a Spearman Rank Correlation (Statview 512 for the Macintosh). No Observed Effect Concentrations (NOECs) were calculated for both sets of data and compared to determine concordance.

## **Results**

### **Effects of Physical Factors**

Many of the manipulations of physical factors produced differences in control survival, growth or sensitivity to zinc, but not all differences were significant and not all trends were consistent. These data are presented here regardless of statistical significance to illustrate how various factors affect test performance, especially control survival.

### Test Temperature

Mysids held simultaneously in test containers at different temperatures had different rates of survival (Table 5). Survival was 100% at 11°C, and 89% at 15°C. Survival was less than the 80% test acceptability criterion at 13° and 17°C. Survival at 11°C was significantly higher than at 13°C, but there were no other significant differences (ANOVA, SNK  $p = .05$ ;  $n = 7$ ). Although a significant difference was found, the relationship between survival and temperature was not consistent in this experiment. Each treatment was held in a different constant temperature room or water bath, each in a separate laboratory building (due to limited availability of temperature control equipment). Each setting had different levels of light intensity, vibration and visual disturbance, possibly confounding temperature effects. There were no significant differences in mysid growth among treatments (ANOVA,  $p > .05$ ).

**Table 5.** Means and standard deviations for mysid survival in clean seawater at various test temperatures. (N = 7 replicates per treatment).

<u>Test Temperature (°C)</u>	<u>Mysid Survival (%)</u>
11°	100 ± 0
13°	71 ± 16
15°	89 ± 16
17°	74 ± 32

### Loading Density

In the first experiment, mean survival of mysids held individually was  $90 \pm 15\%$ , while mean survival of mysids held together was  $72 \pm 23\%$ . This difference was not statistically significant, but did indicate higher survival when mysids were separated from each other.

In the second experiment, the mean survival was higher with five mysids per test container than with eight, regardless of container size, though this difference was not significant (Table 6).

**Table 6.** Mean percent survival  $\pm$  standard deviation for mysids held at different stocking densities in different sized containers (n = 4).

	<u>5 Mysids</u>	<u>8 Mysids</u>
Small Container	100 $\pm$ 0	94 $\pm$ 13
Large Container	100 $\pm$ 0	97 $\pm$ 6

### Renewals

In the first experiment, mean survival of mysids held in seawater renewed once in seven days was 100%. Mean survival of mysids held in seawater renewed three times in seven days was 73  $\pm$  35%. This difference was significant (t-test, p = .03; n = 8).

In the second experiment, in which mysids were exposed in two concurrent zinc dilution series, the test with one renewal had slightly better control survival, was slightly less sensitive to zinc, and had significantly greater overall mysid growth than did the test with three renewals (2 way ANOVA; p = .004, n = 5; Table 7).

**Table 7.** Mean percent control survival  $\pm$  standard deviation, No Observed Effect Concentrations (NOEC) for survival and growth, and median lethal concentration (LC50) for simultaneous zinc tests with one and three renewals (n = 5).

	<u>Control</u>	<u>Survival NOEC</u>	<u>Growth NOEC</u>	<u>LC50</u>
One Renewal:	88 $\pm$ 18%	56 $\mu$ g/L	> 56 $\mu$ g/L	65 $\mu$ g/L
Three Renewals:	84 $\pm$ 17%	56 $\mu$ g/L	32 $\mu$ g/L	57 $\mu$ g/L

### Habitat Substrates

Mysids in containers with four different types of habitat substrates had different rates of survival, but the differences were not significant (ANOVA, p > .05). In two experiments, one in which survival was measured after both one and two weeks, mean survival tended to be greatest in containers with polyethylene strips or natural blades of *Macrocystis*, and least in containers with curved dark glass or no additional substrate (Table 8).

**Table 8.** Mean percent survival  $\pm$  standard deviation in seawater with different types of substrates added as habitat (n = 8).

<u>Substrate Type</u>	<u>Test 1 (7 d)</u>	<u>Test 2 (7 d)</u>	<u>Test 2 (14 d)</u>
Plastic Sheet:	100 $\pm$ 0	93 $\pm$ 10	87 $\pm$ 15
<i>Macrocystis</i> :	95 $\pm$ 9	95 $\pm$ 9	80 $\pm$ 15
No Added Substrate:	95 $\pm$ 9	88 $\pm$ 21	77 $\pm$ 27
Curved Glass:	93 $\pm$ 10	83 $\pm$ 22	76 $\pm$ 24

### Transportation

Juvenile mysids transported as two-day-olds had better control survival and greater sensitivity to zinc than did juvenile mysids hatched from shipped gravid females (Table 9). The difference in control response was not significant (t-test,  $p > .05$ ,  $n = 5$ ), and both tests produced acceptable results according to protocol criteria.

**Table 9.** Mean percent control survival  $\pm$  standard deviation, No Observed Effect Concentrations (NOEC) for survival, and median lethal concentration (LC50) for 3-d-old juvenile mysids exposed to zinc. Mysids were transported to the testing facility either as 2-d-old juveniles or in the marsupia of gravid females (n = 5).

<u>Life Stage Transported</u>	<u>Control Survival</u>	<u>NOEC</u>	<u>LC50</u>
Two-day-old Juveniles	100 $\pm$ 0	10 $\mu\text{g/L}$	27 $\mu\text{g/L}$
Gravid Females	88 $\pm$ 18	32 $\mu\text{g/L}$	55 $\mu\text{g/L}$

Two-day-old mysids transported with high densities of *Artemia* in polyethylene bottles suffered 100% mortality and arrived in seawater only 40% saturated with dissolved oxygen. Mysids transported with high densities of *Artemia* in plastic bags suffered approximately 10% mortality and arrived in seawater approximately 70% saturated with dissolved oxygen. Mysids transported with very low densities of *Artemia* had almost no mortality and arrived in seawater nearly saturated with dissolved oxygen, regardless of the type of container.

### Effects of Treatment Replication on Test Power

Statistical power increased as the number of replicates increased in an analysis of mysid growth and survival after exposure to a zinc dilution series. The greater the number of replicates, the smaller was the difference between treatment and control that the test was able to detect at an alpha level of .05. The Minimum Significant Difference (MSD) for survival decreased asymptotically from a 41% difference from the control with two replicates to a 16% difference from the control with eight replicates (Fig. 12a). Likewise the MSD for growth decreased from a 0.176 mm difference in length between control and treatment mysids with two replicates to a 0.085 mm difference with eight replicates (Fig. 12b).

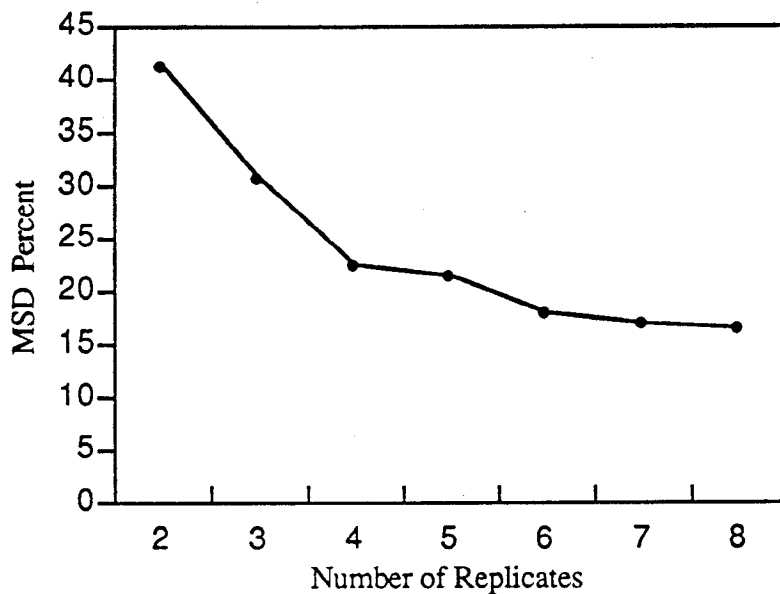
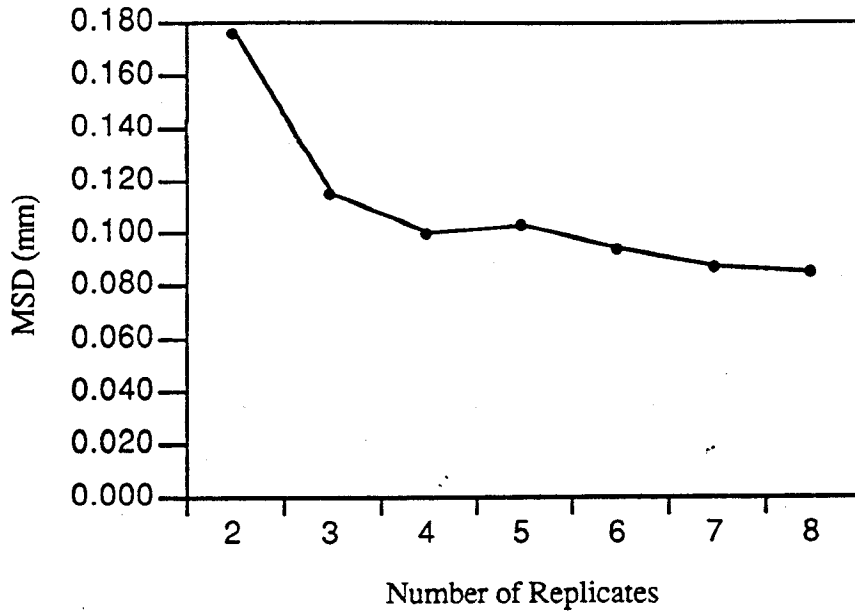


Fig. 12a. Relationship between number of replicates and the Minimum Significant Difference between treatment and control (MSD) for survival data from one mysid zinc test.



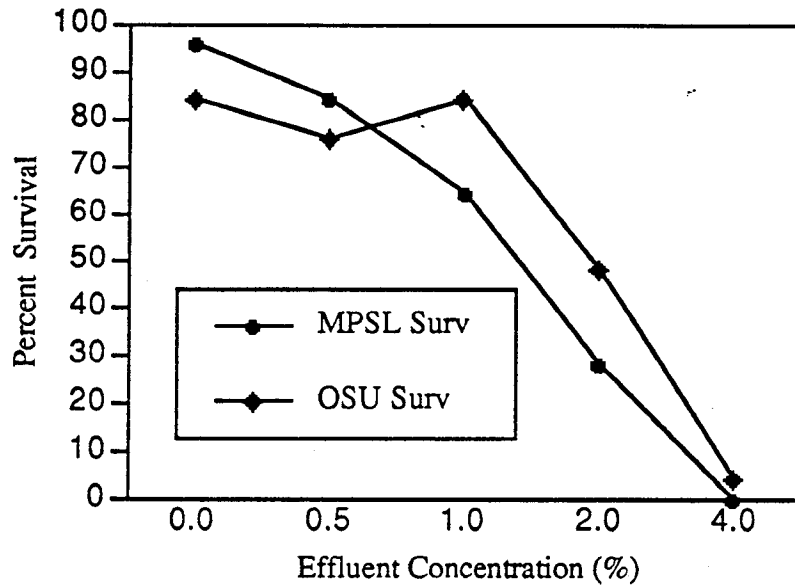


**Fig. 12b.** Relationship between number of replicates and the Minimum Significant Difference between treatment and control (MSD) for growth data from one mysid zinc test.

Five replicates per concentration, as suggested in the protocol, produced MSDs of 22% for survival and 0.103 mm for growth (mysids are generally about 2.5 mm long at the end of the test). The relationship between replication and power, while predicted, was characterized using only one data set. A smoother curve would likely be generated by multiple tests and simulations.

### Interlaboratory Testing with Complex Effluents

In the interlaboratory test using Bleached Kraft Mill Effluent (BKME), there was good agreement between laboratories. The survival NOEC values at both the Marine Pollution Studies Laboratory (MPSL) and Oregon State University (OSU) were the same at 1% effluent (Fig. 13a). The growth NOEC values at both MPSL and OSU were also the same at 0.5% effluent (Fig. 13b). The LC50 values were 1.26% at MPSL and 1.84% at OSU, giving a coefficient of variation of 26%. Both tests met test acceptability criteria.



**Fig. 13a.** Mean percent survival for mysids exposed to split samples of lyophilized Bleached Kraft Mill Effluent (BKME) at the Marine Pollution Studies Laboratory (MPSL) and Oregon State University (OSU).

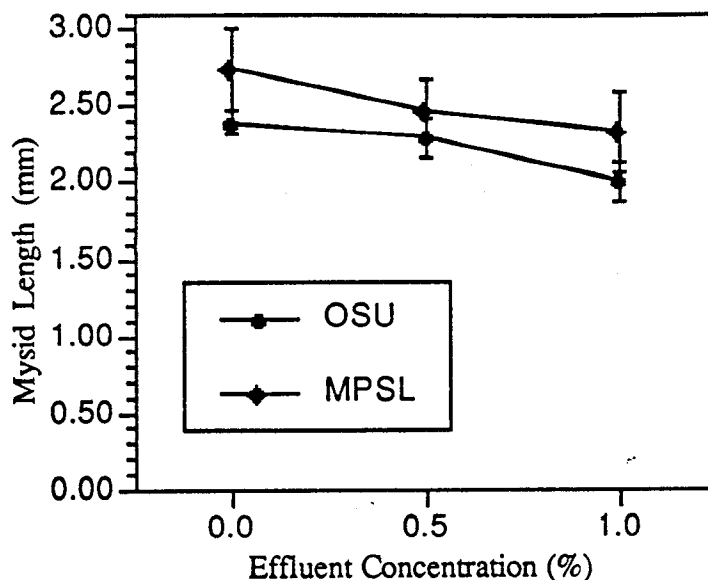
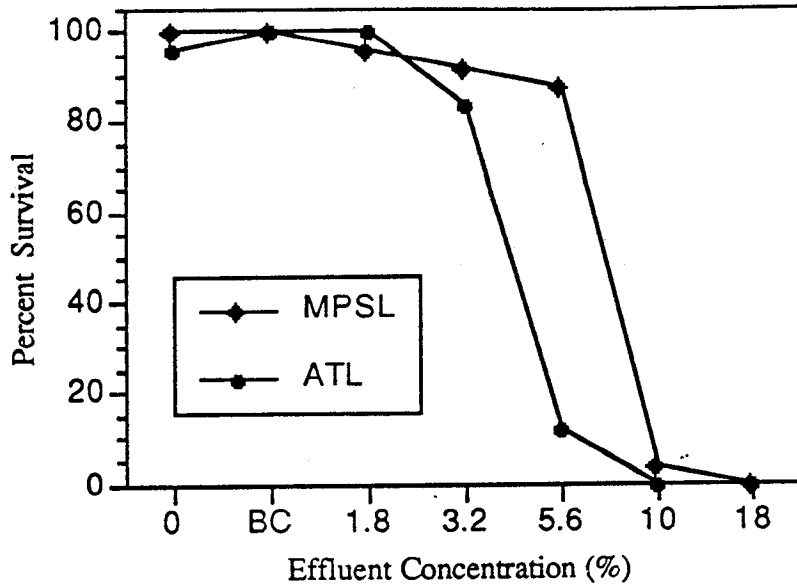


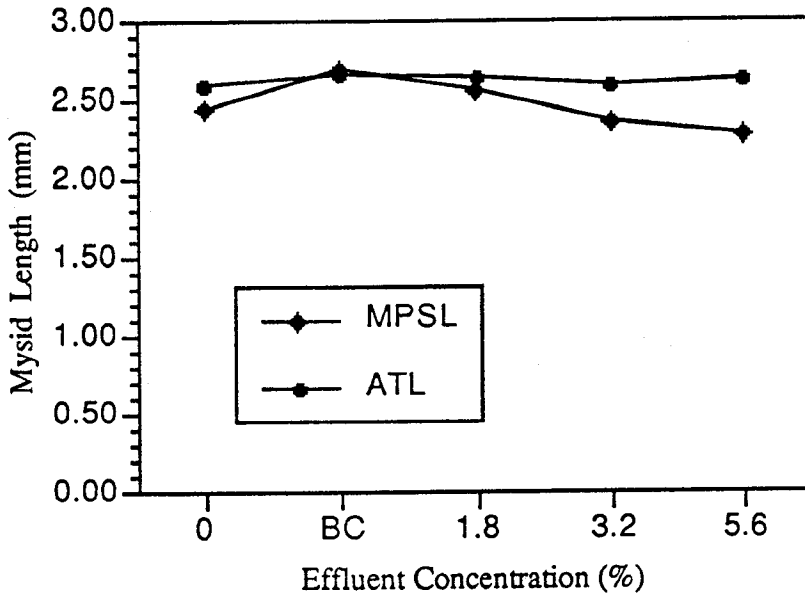
Fig. 13b. Mean length  $\pm$  standard deviation for mysids exposed to split samples of lyophilized Bleached Kraft Mill Effluent (BKME) at the Marine Pollution Studies Laboratory (MPSL) and Oregon State University (OSU).

In the second interlaboratory test, which used fresh effluent from a large publicly owned treatment works (POTW), there was also good agreement between laboratories. The survival NOEC values at both MPSL and the Aquatic Testing Laboratory (ATL) were the same at 3.2% effluent (Fig. 14a). There was no significant growth effect at either MPSL or ATL (NOEC values were  $>5.6$ ; Fig. 14b). The LC50 values were 5.07% at MPSL and 4.14% at ATL, giving a coefficient of variation of 14%. Both tests met test acceptability criteria.

Data from these two interlaboratory tests met test acceptability criteria and produced good agreement between laboratories. Both of these comparisons were preceded by initial unsuccessful tests at the participating laboratories. The unsuccessful tests had lower than acceptable control survival; each was the first test of this protocol at the participating laboratory. The cause of high control mortality was not clear. Of interest is that the investigators at ATL conducted a successful test in 50 ml Petri dishes at the same time and with the same cohort of mysids as the failed test (which used 200 ml beakers according to the *Holmesimysis* protocol). It should be noted that each laboratory had better success during their second trial.



**Fig. 14a.** Mean percent survival for mysids exposed to split samples of POTW effluent at the Marine Pollution Studies Laboratory (MPSL) and the Aquatic Testing Laboratory (ATL).



**Fig. 14b.** Mean length of mysids exposed to split samples of POTW effluent at the Marine Pollution Studies Laboratory (MPSL) and the Aquatic Testing Laboratory (ATL).

### Reference Toxicant Tests

Survival data from four zinc tests produced similar dose-response curves (Fig. 15). Survival was a more sensitive indicator of zinc toxicity than growth in all four tests (Table 10). Three of the tests were conducted within a few weeks of each other; comparison of LC50s from these tests produced a coefficient of variation of 5 %. The fourth test was conducted the previous year with fewer test concentrations (10 and 18  $\mu\text{g/L}$  concentrations were omitted), and this test had a lower LC50. The coefficient of variation for all four tests was 14%.

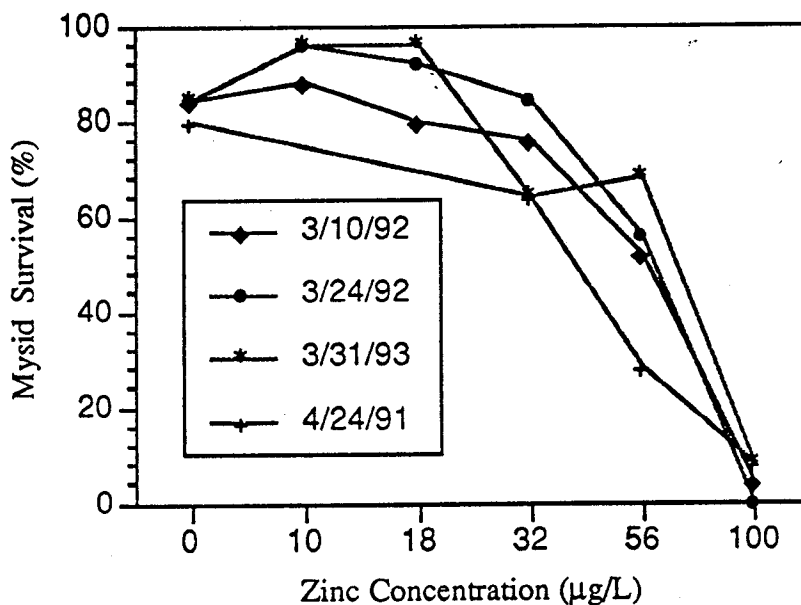


Fig. 15. Dose response curves for four mysid zinc reference toxicant tests.

Table 10. Results of four 7 d mysid growth and survival tests with zinc. No Observed Effect Concentrations (NOECs) and Median Lethal Concentrations (LC50s) are in  $\mu\text{g/L}$  zinc.

Test Date	Control Survival	LC50	Survival NOEC	Growth NOEC
4/24/91	80%	47	32	> 32
3/10/92	80%	59	32	> 32
3/24/92	84%	62	56	> 56
3/31/92	84%	65	56	> 56
Coefficient of Variation of LC50s =			14%	

## Effluent Tests

Five tests conducted with POTW effluent produced typical dose-response relationships, indicating that the test is suitable for determination of effluent toxicity. As expected, NOEC and LC50 values varied with the source, treatment level and time of sampling (Table 11). Growth was a more sensitive indicator of effluent toxicity than survival in three out of four tests in which growth was measured (Table 11). Growth and survival were equally sensitive in the fourth test. An intralaboratory comparison, in which two tests were conducted side by side using a split effluent sample, produced comparable results; comparison of LC50s from these tests produced a coefficient of variation of 6% (Fig. 16). Survival in brine controls was acceptable in all five tests, and was actually higher than in dilution water controls in four of the five tests (Table 11).

**Table 11.** Results of five 7 d mysid growth and survival tests with effluent. No Observed Effect Concentrations (NOECs) and Median Lethal Concentrations (LC50s) are in percent effluent. Effluent tested on 3/14/91 was primary effluent from a small municipality; all others were primary/secondary effluent from the same POTW operating in a large urban area.

<u>Test Date</u>	<u>Control Survival:</u>		<u>LC50</u>	<u>Survival NOEC</u>	<u>Growth NOEC</u>
	<u>Dilution</u>	<u>Brine</u>			
3/14/91	80%	88%	3.1	3.2	1.0
6/14/91 A	100%	96%	7.7	5.6	--
6/14/91 B	96%	100%	8.3	5.6	5.6
3/24/92	92%	100%	7.6	5.6	1.8
3/31/92	96%	100%	5.8	3.2	< 1.8

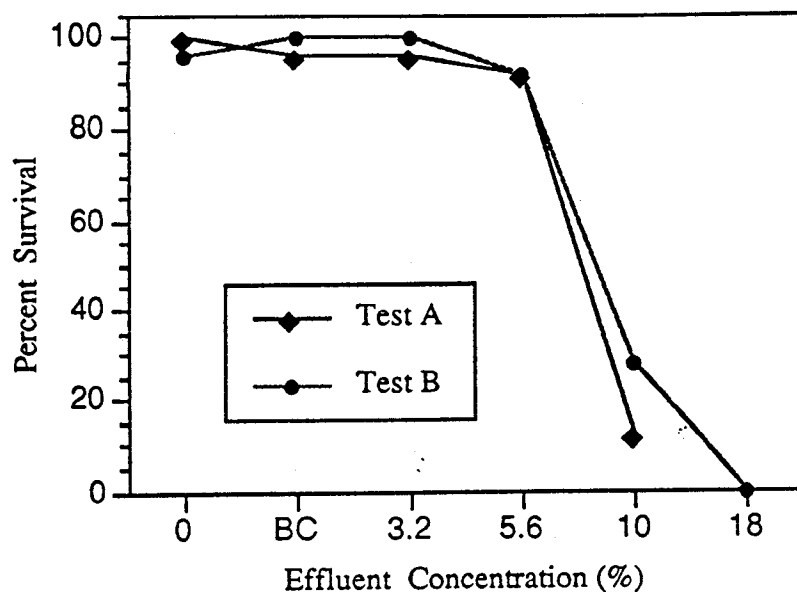


Fig. 16. Dose-response curves for two side-by-side intralaboratory mysid tests conducted at MPSL using split effluent samples.

### Sodium Azide Tests

Mysids were particularly sensitive to sodium azide. However, contrary to our expectations, sodium azide did not inhibit mysid growth at concentrations lower than those inhibiting survival (growth data not shown). A range finding test found 100% mortality at sodium azide concentrations from 1 to 320 mg/L. A second range finding test found 100% mortality at concentrations above 100  $\mu\text{g/L}$ . A definitive test with concentrations closely spaced in the 10 to 320  $\mu\text{g/L}$  range resulted in a NOEC of 100  $\mu\text{g/L}$  and an LC50 of 149  $\mu\text{g/L}$ . Control survival was 88% in the definitive test.

## Weight Measurement as an Indication of Mysid Growth

Of six tests in which both length and weight were measured, the two measures were significantly correlated four times, and the NOEC values for growth were the same four times (Table 12). Three of the tests produced both correlation between the measures and equal NOECs. In the two cases where there was a difference between length and weight NOECs, the weight data produced the lower NOEC, indicating better resolution to detect differences between treatments and controls in those tests.

**Table 12.** Comparisons of length and weight as measures of mysid growth. Calculated Spearman Rank Correlation coefficients for each test are given alongside critical values. Rank coefficients larger than the critical value indicate a significant correlation at the alpha = 0.05 level, and are indicated with an asterisk \*.

<u>Test Date</u>	<u>Toxicant</u>	<u>Length NOEC</u>	<u>Weight NOEC</u>	<u>Corr. Coeff.</u>	<u>Critical</u>
10/20/92	Effluent	≥ 10 %	5.6 %	0.298	0.362
10/20/92	Zinc	≥ 10 µg/L	≥ 10 µg/L	0.784 *	0.521
1/20/93	Ambient	Same site out of seven was toxic		0.454 *	0.362
1/20/93	Zinc	≥ 100 µg/L	≥ 100 µg/L	0.156	0.375
2/18/93	Effluent	5.6 %	5.6 %	0.545 *	0.415
3/10/93	Effluent	5.6 %	3.2 %	0.441 *	0.362

## Test Success Rate

During three years of experimenting with the 7-day growth and survival protocol, test success rate has been determined primarily by ability to meet the control survival test acceptability criterion of 80%. Of 28 tests conducted at MPSL, using both effluents and reference toxicants, 86% met this criterion in dilution water controls and 79% had acceptable survival in both dilution and brine controls. Of 10 effluent tests conducted during this period, 80% had acceptable dilution water controls, and 60% had acceptable survival in both dilution and brine controls. All but two of the test failures occurred during the first year of testing with the protocol, and all of the effluent test failures occurred during that first year. Test temperature during the first year was 15°C, and lower test temperatures were used in subsequent years. These test success rates are based on tests that were completed. A small but unrecorded number of unsuccessful tests were terminated early as a result of high control mortality in the first few days of the test. As mentioned above, test success at other laboratories conducting interlaboratory tests improved after the first attempt.



## Sensitivity to Toxicants

Data from these experiments were compared to values from the literature to evaluate the sensitivity of the *Holmesimysis costata* toxicity test (Table 13). Test sensitivity to zinc was compared to tests with five crustacean species, including the east and Gulf coast mysid *Mysidopsis bahia*, the west coast epibenthic mysid *Mysidopsis intii*, zoea larvae of the market crab *Cancer magister*, the copepod *Tisbe holothuridae*, and adult grass shrimp *Callinassa australiensis*. Data comparing *H. costata* and *Mysidopsis bahia* tests are presented for four additional toxicants. *H. costata* test sensitivity to pesticides was compared against available data for tests with two molluscan species, and sensitivity to sodium azide was compared against other Marine Bioassay Project protocols and tests with the California mussel *Mytilus californianus*. Copper and zinc data for the red abalone test are presented so the mysid test could be compared with a sensitive mollusc protocol. Of the crustacean tests reported, the *H. costata* test was found to be the most sensitive to zinc, copper, and dodecyl sodium sulfate, and it was the most sensitive of all protocols reported to endosulfan and sodium azide (by two to three orders of magnitude). It was less sensitive than the *Mysidopsis bahia* test to mercury and slightly less sensitive to PCBs.

Table 13. Comparison of toxicant sensitivity among various test species. Lower LC50 or EC50 values indicate greater sensitivity to the toxicant. NaPCP is an abbreviation for sodium pentachlorophenate; NaN<sub>3</sub> is sodium azide; DSS is dodecyl sodium sulfate; PCB is polychlorinated biphenyl (Aroclor 1254). All values are in µg/L.

Group	Species	Toxicant	Duration	Endpoint	LC50 or EC50	Source
Crustacean	<i>H. costata</i>	Zinc	7 d	Survival	58	This Report
"	<i>H. costata</i>	Zinc	7 d	Survival	47	Hunt <i>et al.</i> , 1991
"	<i>H. costata</i>	Zinc	96 h	Survival	97	Martin <i>et al.</i> , 1989
"	<i>Mysidopsis intii</i>	Zinc	96 h	Survival	331	Langdon <i>et al.</i> , 1989
"	<i>Mysidopsis bahia</i>	Zinc	96 h	Survival	499	Lussier <i>et al.</i> , 1985
"	<i>Cancer magister</i>	Zinc	96 h	Survival	456	Martin <i>et al.</i> , 1981
"	<i>Tisbe holothuridae</i>	Zinc	48 h	Survival	713	Verriopoulos, 1988
"	<i>Callinassa australiensis</i>	Zinc	96 h	Survival	10,200	Ahsanullah <i>et al.</i> , 1981
Mollusc	<i>Haliotis rufescens</i>	Zinc	48 h	Larval Develop.	68	Hunt and Anderson, 1989
Crustacean	<i>H. costata</i>	Copper	96 h	Survival	17	Martin <i>et al.</i> , 1989
"	<i>Mysidopsis bahia</i>	Copper	96 h	Survival	181	Lussier <i>et al.</i> , 1985
Mollusc	<i>Haliotis rufescens</i>	Copper	48 h	Larval Develop.	9	Hunt and Anderson, 1985
Crustacean	<i>H. costata</i>	Mercury	72 h	Survival	9	Tatem and Portzer, 1985
"	<i>Mysidopsis bahia</i>	Mercury	72 h	Survival	4	Tatem and Portzer, 1985
"	<i>H. costata</i>	DSS	72 h	Survival	960	Tatem and Portzer, 1985
"	<i>Mysidopsis bahia</i>	DSS	72 h	Survival	3,800	Tatem and Portzer, 1985
"	<i>H. costata</i>	PCB	72 h	Survival	12.5	Tatem and Portzer, 1985
"	<i>Mysidopsis bahia</i>	PCB	72 h	Survival	14.2	Tatem and Portzer, 1985
Crustacean	<i>H. costata</i>	Endosulfan	96 h	Survival	0.2	Martin <i>et al.</i> , 1986
Mollusc	<i>Crassostrea gigas</i>	Endosulfan	48 h	Larval Develop.	55	Dinnel, 1991
"	<i>Crassostrea virginica</i>	Endosulfan	48 h	Larval Develop.	460	Mayer, 1987
Crustacean	<i>H. costata</i>	NaPCP	96 h	Survival	84	Martin <i>et al.</i> , 1986
Mollusc	<i>Crassostrea gigas</i>	NaPCP	48 h	Larval Develop.	48	Woelke, 1972
"	<i>Crassostrea virginica</i>	NaPCP	48 h	Larval Develop.	370	Zarogian, 1981
Crustacean	<i>H. costata</i>	NaN <sub>3</sub>	7 d	Survival	149	This Report
Algae	<i>Macrocystis pyrifera</i>	NaN <sub>3</sub>	48 h	Sporo. Prod.	<5,000	This Report
"	<i>Macrocystis pyrifera</i>	NaN <sub>3</sub>	48 h	Germination	79,000	This Report
"	<i>Macrocystis pyrifera</i>	NaN <sub>3</sub>	48 h	Growth	101,000	This Report
Fish	<i>Atherinops affinis</i>	NaN <sub>3</sub>	7 d	Survival	49,000	This Report
Mollusc	<i>Mytilus californianus</i>	NaN <sub>3</sub>	48 h	Larval Dev.	28,000	Cherr <i>et al.</i> , 1990

## Discussion

The *Holmesimysis costata* 7 d growth and survival toxicity test provides regulatory agencies with a short-term test designed to estimate chronic toxicity of aquatic pollutants to a west coast marine crustacean. Successful application of the test depends on its logistical feasibility, test organism supply, rate of test success in routine use, and its evaluation relative to criteria established by the regulatory and scientific community.

The amount of effort required to conduct this test is comparable to other 7 d tests using mysids, the crustacean group used most often in marine toxicity testing. Although commercial cultures do not exist, *H. costata* is readily collected in many coastal areas of California, and a list of potential suppliers is available through the Marine Bioassay Project. The logistics and coordination of supply efforts will likely improve as the test organisms come into greater demand. Our experience has been that mysid juveniles and adults can be shipped to remote locations for testing (see Table 9, and Fig. 13 and 14). Like most sublethal endpoints for crustacea, measurement of mysid growth is time-consuming. Weighing mysids may involve less effort than measuring length, depending on equipment available. However, analysis of length and weight data resulted in different NOEC values for two out of six tests. The lack of a strong correlation between the two measures is most likely the result of error caused by the potentially low signal to noise ratio in weighing the small test mysids, or by variation in length associated with dorsal curvature or contraction. We were unable to determine which was the more accurate measure, although between-replicate variability was low for each (Fig. 13b and unpublished data). Weight provided greater resolution to detect differences from the control in these tests (Table 12). Our intention is to modify the protocol to use weight measurement to determine growth effects. Further analysis of data from intra- and interlaboratory testing is underway to verify the appropriateness of weight measurement and to set acceptability criteria.

Test success rate was determined by control survival. Our estimated success rate over more than 28 tests was less than 80%, but success rate improved with a combination of experience and lower test temperature. We do not know how lower temperatures would affect the control survival of mysids collected in Southern California, however, since we used mysids collected in Monterey Bay. Presumably, southern populations could tolerate cooler than ambient temperatures, although it is unknown whether this would improve control survival in 7 d tests. Temperature affects metabolic rate and toxicant uptake kinetics, possibly affecting test sensitivity. The ASTM acute protocol, developed primarily using Southern California mysids, recommends test temperatures of 17° and 15° ± 2°C for

mysids collected south and north of Point Conception, respectively (ASTM 1993). Test temperatures specified in the *H. costata* 7 d growth and survival protocol are 15° and 13°C for mysids collected south and north of Point Conception, respectively. Although the experiment comparing control survival at different test temperatures showed poor survival at 13°C (Table 5), we question the validity of that result because of the inconsistent relationship between temperature and survival in that experiment. Results were probably confounded by factors related to the different constant temperature equipment used to maintain the various test temperatures. The coolest temperature (11°C) produced the best survival (100%) in that experiment. The test success rates we have experienced while using temperatures of 12° and 13°C (90% and 80%, respectively) are better than for tests conducted at 15°C (56%). We intend to modify the protocol to allow target temperatures to vary by  $\pm 2^\circ\text{C}$  to allow adjustments for higher or lower ambient temperatures at mysid collection sites.

Other factors that may affect success rate include physical and visual disturbance. We conducted experiments comparing shaded and unshaded tests, and comparing tests conducted in mobile laboratories versus tests conducted in buildings with less vibration. No significant differences could be attributed to shading or vibration (unreported data). Control survival was improved slightly by addition of some artificial substrates as habitat (Table 8), but care must be taken to limit introduction of materials that may sorb toxicants or impart additional toxicity to test solutions. Thin teflon sheets may provide a chemically inert substrate, but we have not tried this material. Manipulation of these factors did not result in significant changes in control survival, and no protocol modifications were made based on results of these experiments. In some testing situations, however, physical or visual stimuli may affect test mysids, and controlling these factors may improve test success rate.

A number of compromises have been incorporated in the protocol to reduce effort and optimize success rate; many of them are illustrated by the results of these experiments. The suggested loading density of five mysids per container appeared to provide a reasonable compromise, by reducing density dependent mortality (Table 6) and providing sufficient statistical power (see, for example ASTM 1992). Use of five replicate test containers per concentration balances logistical effort and statistical power (Fig. 12a and 12b), although determining the exact relationship between replication and power would require numerous simulations from multiple tests. The protocol specifies two test solution renewals rather than three, to reduce effort and disturbance (Table 7) while maintaining adequate water quality and sample integrity. However, tests conducted at higher temperatures or with known volatile toxicants may need to include additional renewals to

provide satisfactory estimates of sample toxicity.

Data derived from these experiments allow further evaluation of the 7-day growth and survival protocol relative to established criteria. These include sensitivity, precision, cost and logistical simplicity (Rand and Petrocelli 1985, Stebbing 1980). Logistical and cost considerations have been addressed above. Sensitivity is best evaluated by comparison against other tests using common reference toxicants. The *Holmesimysis costata* test is more sensitive to a number of toxicants than other crustacean tests (Table 13). It is more sensitive to zinc, copper, and DSS, and far more sensitive to endosulfan and sodium azide than tests with species from other phyla. These comparisons underscore the importance of using crustaceans as test organisms in regulatory programs, especially when pesticides may be present in discharged effluents.

The experimental results suggest that, depending on the nature of the toxicant, growth can be an appropriate indicator of chronic toxicity using *H. costata*. In three out of four effluent tests and in the interlaboratory test using BKME, significant growth inhibition occurred at concentrations below those affecting survival (Table 11). Coupled with survival, the growth endpoint should provide analysts with additional information on the chronic toxicity of effluents.

Sodium azide was considered a potentially useful reference toxicant for assessing growth effects because it is an inhibitor of cytochrome oxidase. Inhibition of this enzyme blocks electron transport during mitochondrial respiration. We hypothesized that this might result in an inhibition of mysid growth at concentrations lower than those inhibiting survival. Mysids are apparently quite sensitive to this toxicant, however, and the results demonstrate the complexity of cellular interactions regulating growth and survival.

Precision of test methods is crucial for regulatory programs to verify that detected toxicity is related primarily to the composition of the effluent rather than to variation in the test method. The test precision evidenced by inter- and intralaboratory comparisons of the *H. costata* protocol compares favorably with that of other toxicological and chemical methods. Interlaboratory coefficients of variation (CVs) were 26% and 14% in two trials, each trial having two separate laboratories testing split samples of complex effluent. A split effluent sample tested at the same time in the same laboratory produced a CV of 6% (Fig. 16). A previous interlaboratory test using zinc sulfate produced a CV of 31% (Hunt et al., 1991). These values compare favorably to the range of 14% to 88% (mean of 50%) for nine marine and freshwater test species reviewed in the literature (Hunt and Anderson 1993). Intralaboratory precision was estimated by a CV of 14% for four tests with zinc. Previous intralaboratory testing of the protocol with reference toxicants produced a CV of 20% (Hunt et al. 1991). Acceptable coefficients of variation for chemical analyses of

concentrations near EPA water quality criteria limits range from 18% to 129% for trace metals and 12% to 91% for organics, with CVs tending to increase as concentrations approach detection limits (EPA 1989, APHA 1985).

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**Section 3**

**Red Abalone (*Haliotis rufescens*) Experiments**

**Patrick Conroy  
John Hunt  
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## Introduction

The red abalone (*Haliotis rufescens*) toxicity test has been accepted by the State Water Resources Control Board for NPDES permit monitoring of effluent discharges in California. The test is currently widely used throughout the state, and any protocol modifications will probably be based on comments from laboratories that conduct the test routinely, rather than on further experimentation by the Marine Bioassay Project. Some modifications have already been suggested by the Southern California Toxicity Assessment Group, and those will be considered by State Board staff in Ocean Plan revisions. The purpose of the experiments described here was to further evaluate the biological significance of the short-term (48 h) routine test, by comparing it to both long-term and exposure-recovery tests. These experiments used dilution series of zinc sulfate and complex effluent to determine whether toxicant concentrations causing short-term larval shell abnormalities were similar to those that inhibited metamorphosis in longer-term tests. The use of the metamorphosis endpoint in full larval-stage exposures allowed an evaluation of how well the short-term test estimates chronic effects.

A toxicity test can be categorized as acute, chronic, or critical life stage (CLS). An acute toxicity test is generally defined as a test of 96-hours or less in duration which measures lethality as the endpoint. A chronic toxicity test is defined as a long-term test in which sublethal effects are measured on such processes as reproduction or development, often in addition to lethality. Traditionally, chronic tests have been full life-cycle tests, or shortened tests of about 30 days, known as early life stage tests (Norberg-King 1989). Critical life stage tests focus on the early developmental periods of an organism's life cycle when it is assumed to be most sensitive to toxicants. CLS tests are usually between one hour and seven days in duration, and are intermediate to acute and chronic tests in sensitivity to toxicants. When properly designed, a CLS test serves as a short-term estimate of chronic toxicity (Hunt et al. 1989).

A number of marine mollusc toxicity tests use abnormal larval shell development as an indication of chronic toxicity in short-term (usually 48 hour) exposures (Hunt & Anderson 1993). Although standard short-term molluscan toxicity test protocols are frequently used in regulation of effluent discharges, there is little data indicating the degree to which short-term larval development endpoints estimate long-term deleterious effects. This study expands on previous research (Hunt & Anderson 1989) attempting to correlate the short-term larval shell development of *H. rufescens* with a longer term, clearly adverse endpoint of metamorphosis inhibition. The life history of the red abalone, combined with

techniques to cue larval settlement and metamorphosis (Morse 1979, Seki & Kan-no 1981), allow the opportunity to directly compare the effects of toxicant exposure at different developmental stages. Larval development is temperature dependent; at 15°C, *H. rufescens* larvae develop to the stage necessary for settlement and metamorphosis in approximately six days, although they can spend up to two weeks or more in the plankton (Hahn 1989). Competent larvae selectively settle onto an appropriate substrate and metamorphose into the adult benthic form. Metamorphosis and settlement involve a rapid and dramatic physiological and behavioral change from a free-swimming planktonic veliger larva to a benthic post-larva. Successful metamorphosis into a benthic post-larva is marked by the secretion of the adult shell form and is a significant and necessary process in the abalone life-cycle.

Although there are clear limitations in extrapolating laboratory experiments to field effects (Kimbal and Levin 1985), it is important that the endpoint measured in laboratory toxicity tests can be linked to meaningful effects at the organism or population levels (Underwood and Peterson 1988). By exposing abalone embryos through their complete larval cycle in the presence of two separate toxicants, the effects of the toxicant concentration and duration of exposure were examined to determine the relationship between short-term and longer-term endpoints.

An additional objective of the study was to assess whether toxicant exposure caused an actual inhibition or simply a delay in settlement and metamorphosis. A 15-day experiment was conducted to allow time for measurable post-larval shell growth that could be compared between the different exposure and concentration treatments.

## Methods

The experiments were performed at the Marine Pollution Studies Laboratory (California Department of Fish and Game) located at Granite Canyon, 20 kilometers south of the Monterey Peninsula. The laboratory is continuously supplied with sea water pumped directly from the ocean using stainless steel pump impellers and PVC pipes (Ebert et al. 1974).

Experiments were conducted with two different toxicants: the reference metal compound zinc sulfate ( $ZnSO_4$ ) recommended in the test protocol (Anderson et al. 1990), and lyophilized Bleached Kraft Mill Effluent (BKME). For each toxicant, three experiments utilizing planktonic larvae of the red abalone *Haliotis rufescens* were conducted concurrently: 1) a short-term 48-hour toxicant exposure (ST), 2) a 48-hour toxicant exposure followed by an 8-day recovery period (ER), and 3) a 10-day long-term

exposure (LT). Toxic effect was indicated by abnormal larval shell development in the ST test, and by inhibition of metamorphosis in the Lt and ER tests.

Sexually mature *Haliotis rufescens* broodstock (7-10 cm) were induced to spawn using the hydrogen peroxide method (Morse et al. 1977). Male and female abalone spawned in separate 15L polyethylene buckets, and the gametes were mixed to assure synchronous fertilization prior to introduction into the tests containers. Approximately one thousand fertilized eggs were pipetted into each test container within one hour of fertilization, prior to the first cell division.

### Zinc Experiments

Zinc sulfate is the recommended reference toxicant for toxicity testing with this species (Anderson et al. 1990), and was used in a series of flow-through experiments. Zinc is stable in solution, relatively easy to analyze chemically in sea water, and is often found in high concentrations in municipal and industrial effluents (Schafer 1984, Hunt & Anderson 1989). Three experiments were initiated concurrently using the same cohort of embryos; the short-term 48-hour test (ST), the long term (LT) continuous exposure 10-day test, and the exposure-recovery (ER) 10-day test. 250 mL polypropylene beakers containing 200 mL of test solution were used in all tests. Sea water used in all initial test solutions was UV treated and 0.5 micron-filtered. Zinc concentrations were 0 (control), 5.6, 10, 18, 32, 56, and 100  $\mu\text{g/L}$ , with each toxicant concentration replicated four times. Test solutions were made by volumetric dilutions of a stock solution of 10,000  $\mu\text{g/L}$  zinc sulfate.

All test containers were arranged randomly in a constant temperature water bath maintained at  $15^{\circ}\text{C} \pm 2^{\circ}\text{C}$  for the initial 48 hours of the experiments. During this time period the embryos develop into trochophore larvae, hatch from the egg membrane, and transform into veliger larvae (Crofts 1937). At 48 hours, the ST test was terminated. The entire contents of each test container was poured through a 90 mm Nytex sieve that retained all the larvae. Larvae were then washed from the sieve through a funnel into a 10 mL glass screw-top vial, and buffered formalin was added to the vial to preserve larvae in a 5% formalin solution.

At 48 hours, the LT and ER test containers were placed in separate flow-through systems for the duration of these experiments. The larvae from each beaker were transferred to a 2" diameter PVC screen tube with a 90 mm Nytex screen placed 1 cm from the bottom, then each tube was replaced in the beaker. Test solution was delivered through a silicone hose into the screen tube where the developing larvae were retained, flowing through the Nytex mesh bottom, and then out the beaker pour spout. The LT experiment



utilized a proportional diluter system (Lemke et al. 1978) which regularly introduced 125 mL of fresh toxicant solution at the desired toxicant concentration into each test container. The diluter consisted of water cells of known volume which receive various volumes of clean sea water, and chemical cells of known volume which receive various volumes of a 2000 µg/L zinc test solution. Water from the various dilution water cells siphons down and mixes with the test solution from the various chemical cells to produce the desired test concentrations. Chambers to promote mixing of the test solution and dilution water are located between the diluter and the test chambers. The diluter system contained no metal parts; all components were glass, silicone, or inert plastic.

The cycle time of the diluter was controlled by water-level probes which activated electric solenoid valves located between each cell. The proportional diluter system used in the LT test ran for eight days (192 hours), completing 690 cycles which each delivered 125 mL of the desired test solution to each test container approximately every 17 minutes. At the start of diluter operation and each time the toxicant stock was renewed, samples of each zinc concentration were taken for subsequent chemical verification.

The ER experiment utilized a flush-fill system to deliver clean sea water to the test containers at approximately the same rate as the LT delivery system. The flush-fill system had no cycle counter, and flow was adjusted daily to match that of the proportional diluter system. All sea water used in both the LT and ER experiments was filtered through 5, 3, and 1 m cartridge filters. Test containers remained in the constant temperature water bath for the duration of the experiment. Water bath temperatures were monitored daily during the course of the long-term experiments. Dissolved oxygen, pH, salinity, and temperature of the test solution were measured in one randomly selected replicate from each concentration, at the start and termination of each experiment.

### **BKME Experiments**

Bleached Kraft Mill Effluent (BKME) was used in a series of static-renewal experiments. BKME is the combined aqueous waste of a major chemical pulp-making process. Waste waters from acid, alkaline, chlorine oxidant, and other chemically diverse processes are sewered together to produce the whole mill discharge (Owens 1991). The mixing and ensuing reactions of these streams lead to a final effluent that is highly complex, consisting of simple inorganic salts as well as over 250 identified organic and inorganic compounds (Higashi et al. 1992). Northern California paper and pulp mills discharge over 40 million liters of BKME daily into near shore coastal waters adjacent to the red abalone's habitat.

Three static-renewal experiments (48-hr, LT and ER) using Bleached Kraft Mill Effluent (BKME) were initiated concurrently, as described previously for the zinc experiments. BKME concentrations used in these experiments were 0 (control), 0.32%, 0.56%, 1.0%, 1.8%, and 3.2% BKME in 0.2 mm filtered seawater. Each concentration was replicated four times. All test containers were randomly arranged in trays and remained in a constant temperature room maintained at 15°C. An additional 48-hour reference toxicant test using zinc sulfate was conducted concurrently with the BKME tests to provide information on relative test organism sensitivity. Zinc concentrations used were 0 (control), 18, 32, and 56 mg zinc/L, each replicated four times. Dissolved oxygen, pH, salinity, and temperature of the test solution were measured in one randomly selected replicate from each concentration at the start and termination of all tests, and at each renewal of test solution in the LT and ER tests.

The ST tests were terminated and sampled as described in the zinc experiment. At 48 hours, the developing larvae in the LT and ER containers were transferred to screen tubes as described in the zinc experiments, but were not placed in a flow-through system. Approximately 150 mL of the old test solution was siphoned out of the test container from outside the screen tube, allowing the developing larvae to be retained within the screen tube in 50 mL of test solution. For the LT experiment, freshly reconstituted BKME was then poured into the test containers (but outside the screen tube to prevent excessive turbulence to the larvae), providing a minimum of 75% renewal of test solution. For the ER experiment, the test solution was renewed with clean filtered seawater in the manner described above. Renewals of test solutions were performed on days 3, 5, 7, and 9 of the 10-day experiments.

### Preparation of Settlement Substrates

Larvae were provided a substrate of benthic diatoms and mucous films to induce settlement. Benthic diatoms cultured in white polyethylene buckets (see Ebert and Houk 1984) were wiped from the buckets, then rinsed with 1 mm filtered seawater and poured through a 5 mm filter bag to inoculate a second set of screen tubes to be used as settlement substrates. These diatoms were allowed to adventitiously settle in the PVC screen tubes for 2-3 days under grow- lights. A faint tannish-colored film on the substrate indicated diatom growth. Juvenile 1-2 cm-long red abalone were then allowed to graze this diatom film for 24 hours. The combination of a diatom film and mucous trails left by conspecific grazing juveniles has been shown to be a positive chemotactic inducer for the settlement of red abalone larvae (Seki and Kan-no 1981, Slattery 1987). On day 6 of the LT and ER experiments, larvae from the initial screen tubes were transferred to these settlement screen

tubes, which then were placed into the original test beakers. Zinc containers were maintained in their respective flow-through systems, and BKME test containers were maintained in the static-renewal system. By this time, larvae had developed four lobes of the cephalic tentacles and cilia around the margin of the foot, characteristics that indicate their competence for settlement (Ebert and Houk 1984, Hahn 1989). The larvae were left to settle in these prepared screen tubes for the next 4 days. On day 10, the LT and ER experiments were terminated, and all test containers were sampled. Screen tubes containing test larvae were immersed in a solution of 30% ethanol in heated seawater to remove metamorphosed post-larval abalone from the container walls. Larvae were rinsed from the container with a squirt bottle and collected on a 90 mm sieve, then washed through a funnel and preserved in glass vials as described previously in the 48-hour test.

### Chemical Analyses

For the zinc experiments, zinc concentrations were verified by chemical analysis on a Perkins Elmer 5000 atomic absorption spectrophotometer. For each test, one random replicate from each concentration was sampled at the beginning of the test and after 48 hours. In addition, samples were taken for chemical verification from the proportional diluter each time a new batch of toxicant stock was introduced in the system. These samples were taken as the toxicant flowed from the diluter hoses into the test containers. Prior to analysis, all samples were stored in 30 mL polyethylene vials with 1% by volume additions of 14 N double-quartz distilled nitric acid. Nominal and measured concentrations of zinc test solutions varied by an average of 43% in the short-term test, 42% in the long-term test, and 5% in the short-term reference toxicant test concurrent to the BKME test (Table 14).

**Table 14.** Verified zinc concentrations using atomic absorption spectrometry. 48-hour samples were taken from initial volumetric dilutions, and long-term (LT) samples were delivered from the proportional diluter system. Agreement between the two is indicated by the Relative Percent Difference, which is the difference between the two values divided by the nominal value times 100.

Test Date and Duration	Nominal Concentration ( $\mu\text{g/L}$ zinc)	Verified Concentration ( $\mu\text{g/L}$ zinc)	Relative Concentration (Percent)
Feb. 1991 48-hour	0	1	-
	5.6	9	61
	10	12	20
	18	24	33
	32	46	44
	56	70	25
	100	177	77
Stock Solution	10,000	10,650	6.5
			<u>Mean = 43%</u>
Feb. 1991 10-day LT	0	2	-
	5.6	6	7
	10	12	20
	18	32	78
	32	51	59
	56	88	57
	100	128	28
Stock Solution	2,000	2,090	4.5
			<u>Mean = 42%</u>
Jan. 1992 48-hour	0	6	-
	18	18	0
	32	30	6
	56	58	4
	Stock Solution	10,000	12,300*
			<u>Mean = 5%</u>

\* Stock 10,000  $\mu\text{g/L}$  zinc solution was diluted 4000:1 prior to analysis.

Lyophilized BKME was obtained from Dr. Gary N. Cherr of Bodega Marine Laboratory. The lyophilization procedure is described in detail in Higashi et al. (1992). To obtain representative BKME composition and toxicity, three lyophilized samples were mixed in equal quantities to create a composite sample, which represented an average effluent sample over two months of discharge. For toxicity testing, BKME was reconstituted by adding the appropriate mass (2.5  $\mu\text{g/L}$ ) of lyophilized composite BKME to distilled water and dispersing it for 5 minutes with the aid of an ultrasonic bath. This stock BKME solution was then diluted volumetrically with filtered seawater to the desired test concentrations. No chemical analysis was performed to verify the nominal BKME test concentrations.

## **Endpoint Determination**

To avoid investigator bias, all samples were analyzed without knowledge of toxicant concentration by assigning random numbers to the sample vials. Larvae were pipetted from each vial onto a Sedgewick-Rafter counting slide and were examined at 100x under a compound light microscope. For the 48-hour test, approximately 200 larvae from each replicate were scored either "normal" or "abnormal" as determined by their larval shell characteristics. "Normal" larvae had smooth snail-shaped shells with calcified striations, while shells with indentations or severe deviations from the snail shape were scored as "abnormal" (Hunt and Anderson 1989). For the LT and ER tests, approximately 200 larvae from each replicate were examined microscopically as in the 48-hour test and scored as either metamorphosed or non-metamorphosed. Metamorphosis was indicated by the presence of the juvenile shell with its characteristic radiating lines.

## **Statistical Analysis**

The percentage of normal larvae (48-hr test) or successfully metamorphosed larvae (ER and LT tests) was determined for each replicate of each concentration. This percentage was transformed to the arcsine of its square root to normalize the distribution of the percentage data. The transformed data were then analyzed using an analysis of variance and a Dunnett's multiple comparison test to compare each concentration to the control (Zar 1984). The highest concentration not significantly different from the control was defined as the "no observed effect concentration" (NOEC). The median effective concentrations (EC<sub>50</sub>s) were determined using the non-parametric trimmed Spearman-Kärber method, plotting the percentage of normal or metamorphosed larvae against toxicant concentrations (Hamilton et al. 1977). This method requires at least one concentration with close to 100% effect, one concentration with close to 0% effect (the control) and at least one concentration with partial effect (Rand & Petrocelli 1985).

## **Post-Larval Shell Growth**

Because abalone larvae can spend extended periods in the plankton, a 15 day experiment was conducted to assess the possibility of a delay in metamorphosis induced by toxicant exposure. This experiment consisted of LT and ER treatments exposed to four BKME concentrations: 0, 0.32%, 0.56%, and 1.00% BKME, each replicated four times. After termination of the experiments, larvae from each replicate were pipetted onto a Sedgewick-Rafter counting slide, and post-larval shell length of ten randomly selected metamorphosed larvae from each replicate was measured with an ocular micrometer. A two factor analysis of variance (ANOVA) was performed to determine the effects of

toxicant concentration and duration of exposure on post-larval shell growth. Assuming that the rate of juvenile shell growth was consistent among treatments, delayed metamorphosis would be evidenced by shorter juvenile shell lengths in affected treatments. Inhibition of metamorphosis would be indicated by more uniform shell lengths across treatments, regardless of the percentage of larvae that successfully settled.

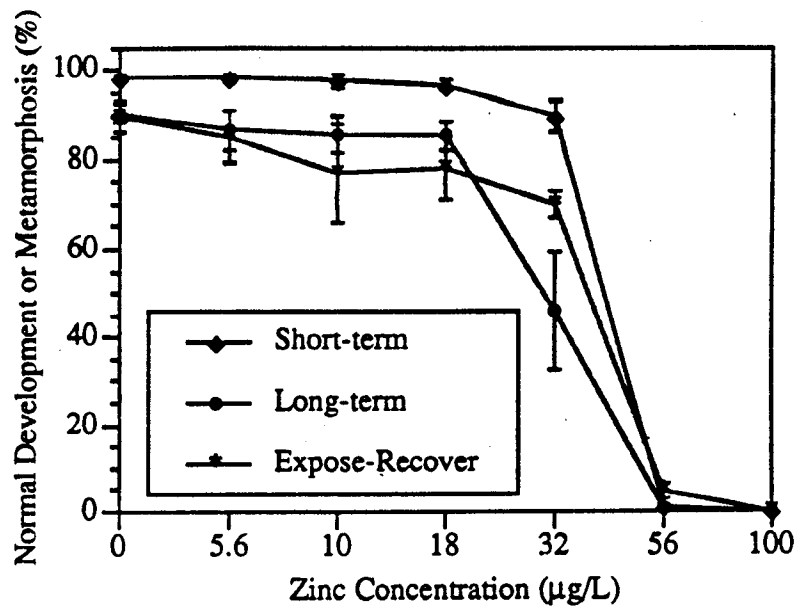
## Results

A comparison of the short-term (ST) and long-term (LT) test results indicate that inhibition of metamorphosis occurs at similar but slightly lower concentrations than larval shell deformity as measured in the 48-hour tests. Zinc concentrations above 18  $\mu\text{g/L}$  and BKME concentrations above 0.56% caused significant increases in the proportion of abnormally developed larvae in the 48-hour tests, and a similar decrease in successful metamorphosis in the 10-day tests. For the zinc experiments, the  $\text{EC}_{50}$  values for the 48-hour and LT tests were 40 and 29  $\mu\text{g/L}$ , respectively. For the BKME experiments, the  $\text{EC}_{50}$  values for the 48-hour and LT tests were 1.00% and 0.67%, respectively (Table 15).  $\text{NOEC}$  and  $\text{EC}_{50}$  values for all tests are reported in Table 14. Because of the low between-replicate variability in the data,  $\text{NOEC}$  values are affected by small differences in treatment means.  $\text{EC}_{50}$  values are affected by differences in control means between the two types of endpoints (shell development and metamorphosis). The dose-response curves for shell development and LT and ER metamorphosis have similar shapes, indicating the similarity of the response between the short-term and long-term tests (Fig. 17 and 18).

**Table 15.** Summary of toxicity test results for *Haliotis rufescens* experiments. NOEC (no observed effect concentration) and EC<sub>50</sub> (median effect concentration) values are µg/L zinc for the zinc sulfate experiments, and % effluent for the BKME experiments. C.I.: Confidence Interval.

<u>Experiment</u>	<u>Toxicant</u>	<u>NOEC</u>	<u>EC<sub>50</sub></u>	<u>95% C.I.</u>
Feb. 1991				
48-hour	Zinc sulfate	18 µg/L	40 µg/L	38.4 - 41.8
Exp.-recov. 10-day	Zinc sulfate	5.6 µg/L	32 µg/L	28.1 - 36.2
Long-term 10-day	Zinc sulfate	18 µg/L	29 µg/L	28.1 - 32.6
Jan. 1992				
48-hour	Zinc sulfate	<18 µg/L	37 µg/L	36.6 - 37.9
48-hour	BKME	0.56%	1.00%	0.95 - 1.04
Exp.-recov. 10-day	BKME	0.56%	0.78%	0.75 - 0.81
Long-term 10-day	BKME	0.32%	0.67%	0.66 - 0.68

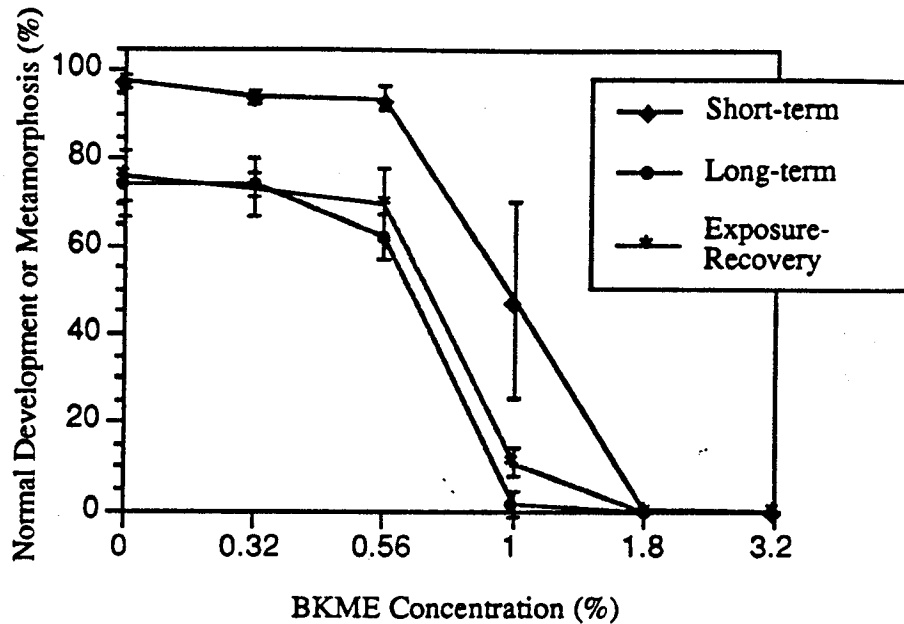
A comparison of the LT and ER test results indicate that abalone larvae exposed to toxicant concentrations which inhibit normal larval shell development do not recover when transferred to clean seawater. Larvae maintained in clean seawater following a 48-hour toxicant exposure did not have an increase in successful metamorphosis compared to larvae exposed to toxicants for the entire larval period. Results of the LT and ER tests indicate an inhibition of metamorphosis at similar toxicant concentrations, regardless of exposure duration. Dose-response curves from Lt and ER exposures were nearly identical (Fig. 17 and 18). In the zinc experiments, the LT and ER EC<sub>50</sub> values were 29 and 32 µg/L zinc respectively. In the BKME experiments the LT and ER EC<sub>50</sub> values were 0.67% and 0.78% respectively (Table 15). There were no metamorphosed abalone observed with damaged larval protoconchs. This indicates that the observed damage done to the larvae occurred during the initial 48 hours.



Concentration (mg zinc/l)	48 Hour Mean (SE)	Long-Term Mean (SE)	Exposure Mean (SE)	Recovery Mean (SE)
0 (control)	98.7 (0.5)	90.4 (2.0)	89.8 (3.6)	
5.6	98.2 (0.8)	86.7 (4.4)	85.1 (5.6)	
10	97.7 (1.6)	85.9 (4.1)	76.8 (11.1)	
18	96.9 (1.1)	85.4 (3.0)	78.0 (7.1)	
32	89.9 (3.5)	45.9 (13.6)	69.8 (3.0)	
56	0.8 (1.0)	0.2 (0.4)	4.4 (1.8)	
100	0 (0)	0 (0)	0 (0)	

Fig. 17. Mean percent normal (48-hour test) or mean percent metamorphosed (long-term and exposure recovery tests) relative to zinc concentration.





Concentration (%BKME)	48 Hour Mean (SE)	Long-Term Mean (SE)	Exposure Recovery Mean (SE)
0 (control)	97.6 (1.3)	74.7 (7.6)	76.1 (5.9)
0.32	94.2 (1.4)	74.3 (2.5)	73.4 (6.8)
0.56	93.9 (2.8)	62.2 (5.3)	69.7 (8.0)
1.0	47.8 (22.5)	1.4 (2.8)	11.0 (3.1)
1.8	0.1 (0.3)	0 (0)	0 (0)
3.2	0 (0)	0 (0)	0 (0)

Fig. 18. Mean percent normal (48-hour test) or mean percent metamorphosed (long-term and exposure recovery tests) relative to BKME concentration.

Length measurements of post-larval shells of metamorphosed abalone from the 15-day experiment indicated that toxicant exposure caused an inhibition rather than a delay of metamorphosis. Cochran's Test for Homogeneity of Variance (Winer 1971) showed no significant differences between sample variances in the ER (Cochran's critical  $C = 0.6841$ , calculated  $C = 0.361$ ), and the LT experiments ( $C = 0.508$ ). Analysis of Variance (ANOVA) indicated there was no significant difference in shell length due to toxicant concentration in the ER ( $p = 0.4243$ ) or LT experiments ( $p = 0.3091$ ). Variation in post-larval shell length was small and consistent both within and between treatments, and variation was small relative to overall shell growth (Fig 19). Assuming a constant growth rate between treatments, or assuming at least that growth was not faster in larvae whose settlement may have been delayed, it appeared that larvae in all treatments settled at about

the same time, both in low concentrations and in the higher concentrations where metamorphosis was inhibited.

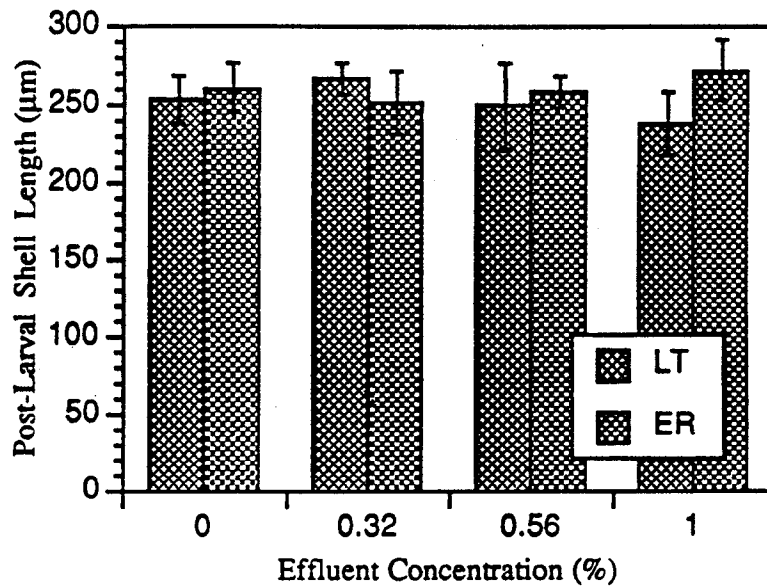


Fig. 19. Post larval shell lengths ( $\mu\text{m}$ ) of metamorphosed abalone from 15-day effluent long-term (LT) and exposure-recovery (ER) experiments.

Temperature, salinity, pH, and dissolved oxygen values were always within acceptable ranges, as indicated in the toxicity test protocol (Anderson et al. 1990; Table 16). It was assumed that variation of these factors within these ranges had negligible effect on organism response to toxicants.

Table 16. Ranges of water quality measurements of test solutions used in toxicity tests with *Haliotis rufescens*. Measurements were recorded at the start and the end of all 48-hour tests, and every 48 hours in the long-term and exposure-recovery tests.

Test Date	Toxicant	D.O. (mg/L)	pH	Salinity (‰)	Temperature °C
Feb. 1991					
48-hour	Zinc	7.09-7.45	7.83-7.92	33	13.0-14.0
Exp-recov. 10-day	Zinc	7.10-7.27	7.81-7.91	33	12.5-14.0
Long-term 10-day	Zinc	7.09-7.30	7.75-7.92	33	12.5-14.0
Jan. 1992					
48-hour	Zinc	5.98-6.29	8.08-8.19	33	14.0-14.5
48-hour	BKME	5.98-6.08	8.06-8.14	32	14.0-14.5
Exp-recov. 10-day	BKME	6.08-7.97	8.06-8.22	33	14.0-15.0
Long-term 10-day	BKME	6.10-7.83	8.06-8.22	33	14.0-15.0

## Discussion

Results of this study demonstrate that zinc and BKME concentrations which effect larval shell development also inhibit metamorphosis (Fig. 17 and 18), indicating that the 48-hour larval shell development endpoint is representative of clearly adverse chronic effects on red abalone. None of the successfully metamorphosed abalone had deformities of their larval shell, indicating that larval shell abnormality precludes survival beyond the planktonic stage.

The comparison of test endpoints allows for an estimation of the biological significance of the effects measured by a short-term chronic toxicity test protocol. Chronic toxicity tests designed for regulatory use generally are not true chronic exposures, but rather are short-term exposures that if designed properly estimate chronic effects on test organisms. There is an emerging paradigm that earlier developmental stages of marine organisms are more sensitive to pollutants than later ones, yet no consistent pattern has emerged from the relatively few studies that have examined different life stages of the same species (Krause et al. 1992). Even when multiple life stages of a species have been considered, it is difficult to extend laboratory-based results to natural situations, in part because of uncertainty as to whether the concentrations and exposure durations used are realistic.

It has been reported that seven-day larval fish tests may accurately predict chronic toxicity within a factor of two (Norberg-King 1989), although that conclusion has been challenged (Suter 1990). Otherwise there are few comparisons of short-term tests to full life-cycle tests reported in the literature. Full life-cycle testing with molluscs is difficult, and may be less important because the larval endpoints are relatively sensitive, but establishing the relationship between short-term and chronic effects is central to understanding the biological relevance of commonly used mollusc protocols (Hunt and Anderson 1993).

Controlled laboratory experiments have limitations in detecting and predicting toxic effects. The strength of laboratory testing lies in the ability to establish cause and effect relationships by controlling all but the variable(s) of interest, although these relationships may be impossible to extrapolate to the natural environment. Because populations of marine invertebrates with planktonic larvae are thought to fluctuate in size with variation in recruitment success, Underwood and Peterson (1988) suggest that measures of reproductive output alone will not predict the future effects on a population. Since this

recruitment pattern may apply to *H. rufescens*, the laboratory tests conducted in this study measure toxicant sensitivity at possibly the most critical life stage of this organism.

The initial 48 hours of larval development appear to be a critical life stage for this species. Abalone larvae displayed an inhibition of metamorphosis at similar concentrations in both the long-term (LT) and exposure-recovery (ER) tests (Fig. 17 and 18), indicating that larvae do not recover to metamorphose in clean seawater after an initial 48-hour toxicant exposure. Continuous exposure of larvae to a toxicant (the LT test) produced only slightly lower EC<sub>50</sub> values (i.e. greater sensitivity) than the exposure-recovery tests.

Planktonically dispersed larvae may differ in the extent to which they are susceptible to point source pollution. Principle attributes affecting susceptibility are the duration of the planktonic phase and the behavior of individuals while in the water column, as these characteristics influence both the likelihood of encountering a waste plume and the duration of exposure once encountered (Raimondi and Schmitt 1992). Prince et al. (1987) suggest that haliotid larvae in their natural environment may be strongly benthic, with limited patterns of dispersal. In a manipulative field study, they calculated dispersal distances for larval *Halotis rubra* of approximately 100 meters. Raimondi and Schmitt (1992), in a field study of *H. rufescens* larval settlement, propose that the relatively short (4-7 days) meroplanktonic stage coupled with transport by coastal currents, may allow for a range of dispersion of ~1-10 kilometers from the point of origin.

It is known that when faced with less than optimal settling conditions invertebrate larvae may postpone settlement (Thorson 1950), and it has also been shown that the discrimination of larvae in selecting a suitable settlement substrate decreases as larval life is prolonged (Knight-Jones 1953). Larvae of the red abalone are functionally incapable of capturing particulate foods, and have been assumed to subsist entirely on endogenous yolk reserves. However, it has been shown that trochophore and veliger larvae can acquire energy by incorporating dissolved organic matter directly from seawater (Jaeckle and Manahan 1989a, Jaeckle and Manahan 1989b). Toxicant effects may be most pronounced at these transition phases in the life history when biochemical pathways are initiated or repressed (Morse 1990).

Because abalone larvae can remain in the plankton for extended periods of time, a 15-day experiment was conducted to allow sufficient time for any and all competent larvae to settle. If toxicant exposure acts to delay the initiation of metamorphosis, a decrease in post-larval shell length of metamorphosed abalone should be observed with increasing toxicant concentration or the duration of exposure. Measurements of metamorphosed abalone from 15-day effluent LT and ER experiments indicate very little variability in post-larval shell length within any test concentration, and no significant differences could be

attributed to either increasing effluent concentration or the duration of exposure (Fig. 19). It appears all the successfully metamorphosed abalone settled at about the same time, or not at all. Although this conclusion assumes a constant growth rate, the observed result could otherwise only be obtained if larvae that settled later grew faster. The mean growth rate of 25 m/day observed in these experiments is greater than the rate of 13 m/day reported by McBride (1990), but less than the 65 m/day growth rate reported by Morse (1981) in experiments using the addition of insulin and mammalian growth hormones.

Biochemical mechanisms responsible for shell deformities and inhibition of metamorphosis have been suggested, including interference of calcification, interruption of shell gland mitosis, and the inhibition of response to known inducers of metamorphosis (Baloun and Morse 1984, Hunt and Anderson 1989). Scanning electron micrographs and polarization microscopy of larvae from this study (Conroy 1993) indicate toxicant interference with calcification of larval shells. Since similar larval shell abnormalities have been observed after exposure to toxicants and effluents of varying compositions (Hunt and Anderson 1989, Hunt et al. 1989, Singer et al. 1991), it is difficult to determine exactly which metabolic processes have been affected by the different chemical constituents. The rapid cellular differentiation preceding shell development is likely to be susceptible to subtle chemical interference.

The results of this study indicate that the 48-hour larval shell development endpoint corresponds with an adverse chronic effect on *Haliotis rufescens*. Toxicant concentrations causing abnormal larval shell development also inhibit metamorphosis, precluding survival past the planktonic stage. The initial 48 hours of larval development appear to be a critical life stage, as larvae maintained in clean seawater following a 48-hour toxicant exposure do not recover to metamorphose. The approximately simultaneous metamorphosis of larvae, even in toxicant concentrations where successful metamorphosis was greatly reduced, indicates that the effluent studied caused an inhibition of metamorphosis rather than a delay.

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**Section 4**

**Giant Kelp (*Macrocystis pyrifera*) Experiments**

**Brian S. Anderson  
John W. Hunt**

## Introduction

In the giant kelp (*Macrocystis pyrifera*) 48-hour toxicity test, germination of kelp spores and initial growth of embryonic gametophytes are effect measures used to determine the toxicity of chemical compounds, complex effluents, and ambient marine waters. In this procedure, motile kelp zoospores settle onto glass slides in test solutions. After a 48-hour exposure, the slides are examined microscopically to determine the percentage of spores which germinate and the length of the embryonic gametophytes (Anderson et al. 1991a).

Development of this protocol has emphasized repeated reference toxicant and complex effluent testing. Results of reference toxicant tests have been used to evaluate both intra- and interlaboratory test variability; copper chloride has been used as the reference toxicant. The primary goal of kelp research during Phase Six of the project has been the evaluation of reference test precision using an alternative reference toxicant, sodium azide. Our interest in an alternative to copper chloride has evolved from our need to define the between-test precision of all of the Marine Bioassay Project (MBP) protocols. Although the intralaboratory precision of kelp copper tests conducted at our laboratory is within acceptable limits, we have been interested in evaluating other reference toxicants to determine whether between-test precision can be better characterized. Although there are several possible sources of variability, we have hypothesized that a certain amount of the variability demonstrated in the copper tests can be attributed to variation in chelation of divalent copper ions (Anderson et al. 1990b).

We considered sodium azide to be a promising alternative reference toxicant because its toxicity is less affected by chelation. In addition, because azide is less toxic to kelp than copper, higher concentrations are necessary to elicit a toxic response, and the signal-to-noise ratio is therefore higher than that of copper. It was hoped that between-test variability associated with temporal and spatial differences in the chelation capacity of dilution waters could be reduced through the use of azide. A series of nine 48h reference tests were conducted over the course of 17 months. The results of these tests are discussed below.

## Results and Discussion

Azide inhibits kelp germination and germination tube growth at similar concentrations (Table 1). The mean NOEC for germ-tube growth was 14.1 mg/liter; the mean NOEC for germination was 17.9 mg/liter. The mean IC<sub>50</sub> for germ-tube growth was 101.4 mg/liter; the mean EC<sub>50</sub> for germination was 78.8 mg/liter. There have been few studies evaluating the toxicity of azide to marine species and, to our knowledge, there have been no published studies assessing azide toxicity to marine algae. Comparison of our data with previous reports on the toxicity of azide to other marine species indicate that kelp has comparable sensitivity to a larval fish and the embryo-larval stages of a mollusc. The NOEC for topsmelt (*A. affinis*) larval survival was 32 mg/liter; the LC<sub>50</sub> for topsmelt was 48.5 mg/liter (see Topsmelt Experiments, Section 1). Cherr et al. (1990) reported a mean EC<sub>50</sub> of 28 mg/liter in azide tests with mussel (*M. californianus*) embryos. It is interesting to note that the mysid *Holmesimysis costata* is considerably more sensitive to azide with a NOEC of 100 µg/liter (see Mysid Experiments, Section 2).

These results indicate that between-test precision using azide is comparable to that of tests using copper chloride. The coefficient of variation of the azide IC<sub>50</sub>'s for germ-tube growth was 28.4%; the coefficient of variation of the azide EC<sub>50</sub>'s for germination was 36.7% (Table 17). A summary of the 1990 copper reference toxicant tests conducted at MPSL was reported in a previous MBP report (Hunt et al. 1991). The coefficient of variation of the germ-tube growth response IC<sub>40</sub>'s for copper was 38.8%. The coefficient of variation of the germination EC<sub>50</sub>'s for copper was 32.9%. Intralaboratory coefficients of variation for both azide and copper are comparable to the coefficient of variation reported for the marine alga *Champia parvula*; Morrison et al. (1989) reported a coefficient of variation for *Champia* of 38.6% (EC<sub>50</sub>'s) using copper.

**Table 17.** Summary of nine 48h kelp toxicity tests with sodium azide. All azide concentrations are nominal (mg/liter).

Test Date	Endpoint				
	Germ-Tube Growth			Germination	
	IC25	IC50	NOEC	EC50	NOEC
2/11/92	39.5	133.7	18.0	52.3	18.0
2/18/92	34.1	96.5	18.0	72.6	32.0
6/29/92	57.5	142.2	32.0	132.1	32.0
7/07/92	33.1	92.5	10.0	79.2	10.0
7/15/92	42.8	138.9	18.0	117.8	18.0
7/16/92	25.0	68.4	5.6	48.3	10.0
7/22/92	30.2	80.6	10.0	62.4	18.0
10/9/92	25.1	80.0	5.6	60.3	5.6
7/2/93	24.8	80.1	10.0	84.0	18.0
Mean (mg/L)	34.7	101.4	14.1	78.8	17.9
S.D.	10.7	28.8	8.4	28.9	9.2
CV (%)	30.8	28.4		36.7	

Although it is still possible that between test variability is partly due to differences in chelation, these results do not indicate that precision is improved appreciably using azide. Azide concentrations are also difficult to analytically verify. We therefore suggest that it would be inappropriate at this time to replace copper with azide as a standard reference toxicant because an extensive database has already been established for copper.

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**Section 5**

**Assessment of Ambient Toxicity Using Four Marine Species**

**Shirley Tudor  
John W. Hunt  
Brian S. Anderson**

## Introduction

The principal application of the short-term chronic toxicity tests developed by the Marine Bioassay Project is monitoring of point-source toxicity. Recently, there has been increasing interest in ambient toxicity which may originate from non-point sources. Federal and state regulatory agencies are now developing monitoring criteria to investigate and eventually regulate non-point source toxicity. As part of this effort, the Central Coast Regional Water Quality Control Board (Region 3) funded a small scale project to investigate ambient toxicity at several sites within the Salinas River drainage, Elkhorn Slough and associated water bodies in Monterey County. Much of the property bordering the Salinas River drainage and Elkhorn Slough is farmed intensively, and the resulting agricultural runoff, along with municipal wastewater, contributes a considerable volume to the system, particularly during the rainy season (ABA Consultants, 1986). This runoff may contain potentially high levels of pesticides, trace metals, organic compounds, and other toxic materials, which when transferred to the nearshore marine environment may have a significant impact on local marine biota. Studies which utilize the newer generation of short-term chronic toxicity tests to investigate the presence of ambient toxicity have been limited (for examples, see Clark 1989, Bailey et al. 1991, Thursby et al. 1992, Khan et al. 1993).

The present study had two goals: The primary goal was to use short-term chronic toxicity tests to investigate ambient toxicity in the Salinas River drainage, Elkhorn Slough, and adjacent waters. Toxicity results from this study will be used as baseline information for future studies on the effects of non-point source toxicity in Region 3. A second goal was to evaluate the effectiveness of the four toxicity tests developed by the Marine Bioassay Project for assessing ambient toxicity in estuarine and marine waters. Four MBP protocols were used in this study, all of which use species indigenous to the Monterey Bay area. The four tests used were the 48-hr abalone (*Haliotis rufescens*) larval development test, the 7-d mysid (*Holmesimysis costata*) growth and survival test, the 48-hr kelp (*Macrocystis pyrifera*) growth and germination test, and the 7-d topsmelt (*Atherinops affinis*) growth and survival test.

## Materials and Methods

Samples were obtained from seven sites in the Elkhorn Slough and the Pajaro and Salinas River areas on the morning of 20 January 1993 (Fig. 20). The water was collected

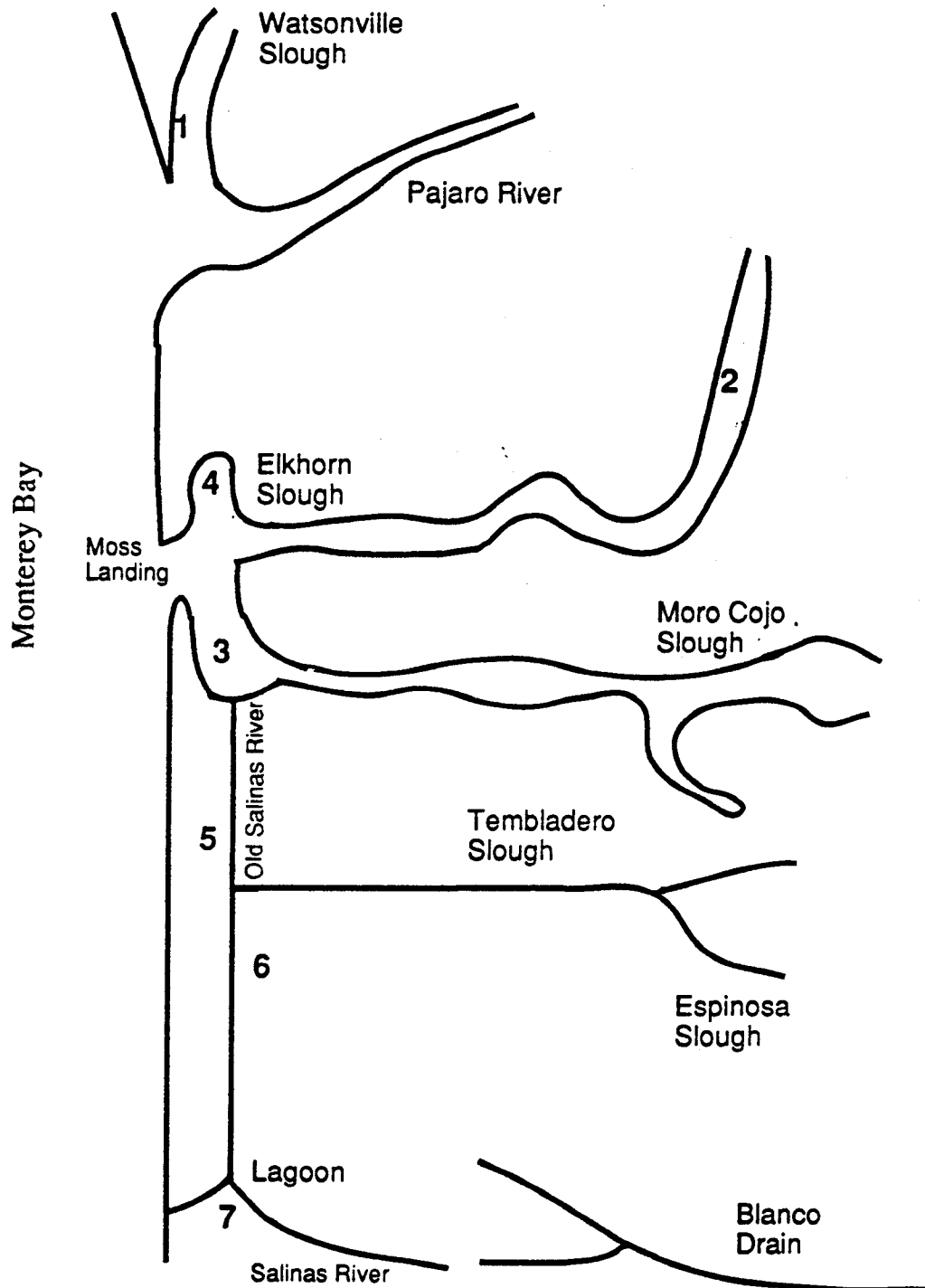


Fig. 20. Sample site locations for ambient toxicity study.

from a depth of approximately 0.5m below the surface in 10L polyethylene Cubitainers™ which had been acid-washed then rinsed and leached with 20 µm-filtered seawater for one week before sampling. The samples were transported on ice, and held at 4°C for 24 hours before the initiation of the tests. After test initiation, sample water for test solution renewals in the mysid and topsmelt tests was held at 15°C for the duration of the tests.

All tests followed the most recent version of the Marine Bioassay Project protocols given in Hunt et al. (1991). In the months prior to sampling, the water bodies received large amounts of fresh-water storm runoff, and as a result, salinity was low at many of the sites sampled (Table 18). All samples were adjusted to marine salinity ( $34 \pm 2$  ‰) using hypersaline brine following procedures described in Hunt et al. (1991). In addition, the samples contained a large load of particulate matter. Due to concerns over interferences which might result from high concentrations of particulates, in particular, smothering of kelp gametophytes and abalone embryos, all samples were filtered to 1µm using a polyester "sock" filter which had been pre-leached in filtered sea water for 1 hour prior to use. A separate filter was used for each sample. Three controls were run with each test: a brine control containing 36‰ brine by volume, a filter control consisting of Granite Canyon seawater filtered through a separate sock filter, and a control consisting of Granite Canyon seawater. Appropriate positive control reference toxicant tests were conducted concurrently with all ambient sample tests. Copper was used as the reference toxicant for the kelp and topsmelt tests; zinc was used for the mysid and abalone reference tests.

**Table 18.** Salinity and site locations of samples.

Site #	Site location	Salinity (‰)
1	Pajaro River	0
2	Kirby Park	11
3	Moro Cojo	7
4	Moss Landing	27
5	Old Salinas River (bridge)	0
6	Old Salinas River	0
7	Lagoon	0

### Statistical analyses

Data were analyzed using Analyses of Variance followed by a Dunnett's multiple comparison test, a test designed specifically to compare each sample individually against the control. The arcsine square root transformation was used to normalize all percentage data.

## Results

Statistical comparisons of the three controls indicated there were no significant differences between controls for either topsmelt or kelp tests. In both the abalone and mysid tests, however, filter and brine control means were significantly lower than the seawater controls (Table 19). Filter controls elicited the greatest toxic response in the abalone test ( $4 \pm 5.0\%$  normal larval development), and in the mysid test (0% survival). In both of these tests, however, percent survival or normal development was as high as 95-100% at some of the sites suggesting that the toxic effect of the filtration process did not impact all samples to the same degree. Note again, that a separate filter was used for each sample. The brine control for the abalone test also exhibited toxicity. However, samples requiring the greatest volume of brine (sites # 1, 5, 6, and 7) all had normal development.

Results of the toxicity tests for all species are summarized in Table 19. All sites were compared to the respective brine control for each protocol to determine statistical significance, except for the abalone test, which used the Granite Canyon seawater control for comparison. Three of the test protocols indicated a significant toxic effect compared to the respective controls. Normal abalone development was significantly inhibited at sites 2, 3, and 4, although all three of these sites had greater than 80%. Given the toxicity found in the brine and filter controls, these samples should be considered non-toxic to abalone. Mysid survival was significantly reduced at sites 4, 5, and 6 with mean survival ranging from 0-32%. Mysid growth was inhibited at site 4, with a mean length of 2.2 mm. Significant inhibition of kelp germ-tube growth was detected at sites 2, 4, and 6; all had a mean germ-tube length of 10.7  $\mu\text{m}$ . Kelp germination was not significantly affected at any site. In the topsmelt tests, survival was at least 96% at all sites, and there was no significant inhibition of either survival or growth at any of the seven sites.

The number of tests that identified significant toxicity at a given site varied. One site (#4) showed toxicity using four of the seven endpoints measured, two sites (#2 and #6) showed toxicity with two endpoints, and two sites (#3 and #5) showed toxicity with one endpoint. Samples from sites 1 and 7 showed no toxicity.

Water quality parameters (D.O., pH, temperature and salinity) were all within ranges specified in the test acceptability criteria for these protocols. All reference test results were within normal ranges demonstrated for these protocols, except for the kelp copper reference test (Table 19). The kelp test showed no effect of copper on germ-tube growth but a significant effect on kelp germination at the lowest copper concentration. The reference test acceptability criteria for this protocol requires that the germ-tube growth NOEC be less than 35  $\mu\text{g/liter}$ ; the NOEC for germination must be less than 110  $\mu\text{g/liter}$ .

**Table 19.** Summary of toxicity test data from Salinas River study of ambient water samples collected from 7 sites. Data are means  $\pm$  standard deviations for each of four toxicity test protocols, 3 of which employ two separate endpoints: mysid and topsmelt tests both use survival and growth endpoints; the kelp test uses germination and growth endpoints. Standard deviations are calculated from 5 laboratory replicates per site. Asterisks indicate sites showing significant toxicity compared with either the control or the site with the least toxic response, whichever value is greater.

Site	Abalone Mean % normal larvae	Mysid Mean % survival	Mysid Mean growth	Topsmelt Mean % survival	Topsmelt Mean growth	Kelp Mean % germination	Kelp Mean growth
1	95 $\pm$ 2.8	96 $\pm$ 8.9	2.6 $\pm$ 0.16	96 $\pm$ 8.9	1.5 $\pm$ 0.2	83 $\pm$ 5.9	11.6 $\pm$ 0.3
2	*82 $\pm$ 2.1	96 $\pm$ 8.9	2.6 $\pm$ 0.23	100 $\pm$ 0.0	1.8 $\pm$ 0.3	86 $\pm$ 1.1	*10.7 $\pm$ 0.9
3	*88 $\pm$ 8.9	100 $\pm$ 0	2.7 $\pm$ 0.20	96 $\pm$ 8.9	1.9 $\pm$ 1.0	81 $\pm$ 5.3	12.1 $\pm$ 1.3
4	*86 $\pm$ 3.4	*32 $\pm$ 17.9	*2.2 $\pm$ 0.38	96 $\pm$ 8.9	2.6 $\pm$ 1.6	83 $\pm$ 18.3	*10.7 $\pm$ 1.0
5	95 $\pm$ 2.8	* 0 $\pm$ 0	----	100 $\pm$ 0.0	1.8 $\pm$ 0.2	83 $\pm$ 12.9	11.7 $\pm$ 0.9
6	96 $\pm$ 2.3	* 0 $\pm$ 0	----	100 $\pm$ 0.0	2.2 $\pm$ 0.7	84 $\pm$ 8.8	*10.7 $\pm$ 0.4
7	95 $\pm$ 2.5	100 $\pm$ 0	2.7 $\pm$ 0.06	100 $\pm$ 0.0	1.6 $\pm$ 0.3	81 $\pm$ 4.4	11.8 $\pm$ 0.9
BC	18 $\pm$ 9.3	88 $\pm$ 17.9	2.7 $\pm$ 0.05	100 $\pm$ 0.0	1.9 $\pm$ 0.9	74 $\pm$ 7.8	12.4 $\pm$ 1.1
FC	4 $\pm$ 5.0	0 $\pm$ 0	----	100 $\pm$ 0.0	1.8 $\pm$ 0.1	74 $\pm$ 4.0	11.7 $\pm$ 0.5
GC	98 $\pm$ 1.0	100 $\pm$ 0.0	2.4 $\pm$ 0.10	100 $\pm$ 0.0	2.2 $\pm$ 1.0	76 $\pm$ 7.2	11.3 $\pm$ 0.5

Therefore, this test met the criterion for germination, but not for germ-tube growth. It is not clear to us why this discrepancy occur. Because the sample had been discarded before the results were analyzed, it was not possible to retest the samples with the kelp protocol.

## Discussion

Although the toxicity results are inconclusive due to toxicity associated with sample handling, this study demonstrates that the short-term chronic toxicity test protocols developed by the Marine Bioassay Project are applicable for assessing ambient toxicity of estuarine and marine waters. The results were confounded by problems with filters, which can be attributed partly to sample manipulations that were necessary to compensate for sampling in an unusually rainy season.

Sampling had been initially scheduled for the fall of 1992, but had to be postponed to January 1993 due to test scheduling conflicts. In the months preceding the sampling date, there was considerable rainfall, and as a result the samples contained a large amount of particulate matter. In general, we try to avoid filtering samples to minimize reductions in toxicity which might result from the elimination of compounds bound to particulates. However, because we were concerned that particulates would interfere with the test organisms, the samples were pre-filtered to ensure that any toxic effects observed would be due to contaminants and not physical effects such as smothering. Some of these filters, notably those used in the abalone and mysid tests, appear to have contaminated the samples. There was 100% mortality in the mysid test filter control and 96% abnormal development in the abalone test filter control, although filter controls for the other tests exhibited no toxic effects. In addition, brine addition was required to raise the salinity of samples that were essentially fresh water (sites #1, 3, 5, 6, &7) to salinities tolerable to marine species. The abalone test brine control exhibited reduced rates of normal development, suggesting that the brine was toxic to the abalone larvae. However, the abalone test results are confounded by the fact that the samples which were adjusted with this brine exhibited normal abalone larval development.

Despite poor survival in the sample manipulation controls, samples from four of the sites exhibited almost 100% survival in the mysid test, and all samples had at least 82% survival in the abalone test. Samples from three sites in the mysid test showed very high toxicity, with mean survival rates of 0-32% at three sites. This suggests that toxicity associated with sample handling affected samples to different degrees. As a result, statistically significant toxicity in the mysid and abalone tests cannot conclusively be

ascribed to ambient toxicity at these sites. We can, however, conclude that low mortality rates characterize sites that were relatively uncontaminated during this sampling period.

In both topsmelt and kelp tests the brine and filter controls performed well. The kelp growth test indicated the presence of significant toxicity at three sites. As discussed above, we cannot determine whether reduced germ-tube growth was due to contaminants present at these sites (2, 4, and 6), or simply due to toxicity associated with the filters. Topsmelt survival and growth, and kelp germination detected no significant toxicity at any of the sites. None of the sites were toxic to topsmelt larvae or inhibited kelp germination, and only one site (4) was toxic to both abalone and mysids. These data indicate that none of the samples were highly toxic.

In conclusion, the results suggest these four species can be used to investigate ambient toxicity. Procedures need to be modified to eliminate artifacts associated with hypersaline brine and sample filtering in any future studies. Non-toxic filters of appropriate material are available commercially, but we had not anticipated the necessity of filtering prior to this study; the sock filters were used only as a last resort.

The issue of salinity adjustment methods is more complicated. For some of the species tested it is impossible to test a sample at 100% strength using frozen seawater brine because the volume of brine necessary to adjust a freshwater sample to 33‰ would result in approximately 40% sample dilution. One alternative is to use dry commercial sea salts to adjust sample salinity, but we have found these to be toxic to some of the MBP protocol species. More time needs to be devoted to investigating alternative sea salt formulae.

The choice of toxicity test species depends on the season and the questions being addressed in the study. We assumed that ambient salinity at most of the sample sites would be estuarine to marine (15 to 33‰) therefore marine species were considered appropriate test animals. However, due to storms, the samples were predominately fresh water. In this case it might have been more appropriate to use freshwater toxicity test species at the freshwater stations so that the samples could be tested at 100% strength without salinity adjustment. An alternative solution would have been to time sampling to occur immediately after the rainy season began when the sampling sites were at higher salinities.



## **Section 6**

### **Training Workshops and Protocol Evaluation**

## Introduction

The Marine Bioassay Project, the U.S. Environmental Protection Agency, and other agencies and universities are continuing to develop short term biological tests to estimate the chronic toxicity of complex effluents. The State of California has begun the process of implementing these protocols by requiring their incorporation into monitoring programs and discharge permits. With implementation, it will be important to maintain communication between groups developing the toxicity tests, regulators incorporating the tests into permits, and dischargers responsible for conducting the tests on a routine basis. In anticipation of using the new tests for effluent toxicity testing, the Marine Bioassay Project (MBP) staff conducted a series of workshops with members of the California Regional Water Quality Control Boards, biologists from the major treatment plants and power plants that discharge into California marine waters, and personnel from private consulting firms. These workshops familiarized Regional Board, discharger, and private consulting firm staff with all of the MBP protocols scheduled for adoption by the state: the red abalone, giant kelp, topsmelt, and mysid toxicity test protocols. The workshops also allowed the MBP staff to receive comments on how to improve the protocols and the implementation process.

### Hands-On Technical Workshops

A series of one-day workshops were held at various locations in the State between August, 1991 and June, 1993. The date, location and protocols presented at each workshop are given in Table 20. The workshop format allowed each participant to conduct every aspect of both protocols to facilitate a technical exchange between the researchers who developed the protocols, regulators who will use the toxicity test data, and biologists who will conduct the tests on a routine basis as implementation proceeds.

<b>Table 20. Date, location, and protocols demonstrated at three separate workshops presented by the Marine Bioassay Project.</b>			
<b>Date</b>	<b>Protocol Presented</b>	<b>Location</b>	<b>No. Attendee</b>
August 27, 1991	Kelp	CMM*	~ 25
August 28, 1991	Abalone	CMM	~ 25
August 29, 1991	Topsmelt	CMM	~ 25
December 4, 1991	Kelp	CAS‡	~30
December 5, 1991	Abalone	CAS	~30
December 6, 1991	Topsmelt	CAS	~30
June 23, 1993	Mysid	MPSL°	~25
June 24, 1993	Mysid	MPSL	~25
* Cabrillo Marine Museum, San Pedro			
‡ California Academy of Sciences, San Francisco			
°Marine Pollution Studies Laboratory, Monterey			

### **Abalone Workshop**

The first two workshops (Cabrillo Marine Museum; California Academy of Sciences) each devoted one day to the red abalone toxicity test. Each workshop began with a slide presentation of the protocol, so that participants would receive an overview and could ask questions on general principles and underlying assumptions of the test procedures. Participants then divided into three small groups that proceeded to a different work-station where each person conducted a segment of the toxicity test. Each group spent approximately one-and-a-half hours at each station, then rotated to the next. The following agenda lists the tasks performed at each of the red abalone protocol stations:

## Red Abalone Toxicity Test Workshop Stations

### STATION 1: TEST SOLUTIONS

#### Reference Toxicants

- Stock solutions

- Standard dilution series

#### Preparation of Hypersaline Brine

- Types of commercial sea salts

- Mixing and decanting brine

- pH adjustment and salinity measurement

#### Effluent Dilution

- Standard dilution series

- Salinity adjustment worksheet

#### Water Quality Measurements

- Temperature, salinity, pH, dissolved oxygen, ammonia

- Buffers and standards

### STATION 2: TEST ORGANISMS

- Handling and inspecting broodstock

- Spawning induction

- Fertilization

- Washing and concentrating embryos

- Density estimation

- Delivery into test containers

### STATION 3: PRESERVATION AND ANALYSIS OF LARVAE

- Sampling test solutions for water quality and chemical analysis

- Collecting larvae on sieve

- Transferring larvae to test tubes

- Preservation in formalin

- Microscopic analysis

- Endpoint interpretation

- Alternative tissue culture flask method

At Station One, participants performed serial dilutions of stock reference toxicants and effluents, prepared hypersaline brines, set up a standard reference toxicant test, and measured water quality parameters. At Station Two, participants learned about maintenance of abalone broodstock, induced male and female abalone to spawn using hydrogen peroxide, fertilized abalone eggs and then counted and delivered embryos to test containers. At Station Three, participants terminated a standard zinc toxicity test using two methods for isolation and preservation of the larval abalone. Participants analyzed representative test containers, and compared their results to previous zinc reference toxicant test results.

At each station, participants discussed equipment requirements and possible variations in techniques that might be needed to adapt the protocol for their individual laboratories. Suggestions were also given for improvements to the test protocols.

## Kelp Workshop

The kelp workshop followed the same format as described above, except that instead of reiterating methods of mixing toxicant solutions, a station was devoted to data analysis and computer formats. This protocol was also demonstrated at the first two workshops: Cabrillo Marine Museum; California Academy of Sciences.

### Giant Kelp Toxicity Test Work Stations

#### STATION 1: SPOROPHYLL PREPARATION and SPORE RELEASE

- Sporophyll collection
- Washing sporophylls
- Preparing sporophylls for storage and transport
- Zoospore release
- Test container set-up
- Determination of spore viability
- Fixing spore samples and estimating density
- Inoculation of test containers

#### Equipment:

sporophylls, 1-liter beaker, 0.2  $\mu\text{m}$ -filtered seawater, paper towels, clean tray, test tubes (each marked for 9 and 10 ml volumes), disposable pipettes, hemacytometers, compound microscope (100x), sporophylls worksheet

#### STATION 2: ANALYSIS OF SPORES AND GAMETOPHYTES.

- Sampling test solutions for water quality and chemical analysis
- Removal of settling slides from test containers
- Microscopic analysis of spores and gametophytes
- Germination endpoint determination
- Growth endpoint determination

#### Equipment:

compound microscope, data sheets.

#### STATION 3: STATISTICAL ANALYSIS OF TOXICITY TEST DATA.

- Data preparation and transformation
- Experimental design
- Statistical tests

- ANOVA (Analysis of Variance)
- Dunnnett's Multiple Comparison
- Probit

- Computer programs for Macintosh
- Computer formats for reporting raw data to central data base

At Station One, participants sorted and prepared *Macrocystis* reproductive blades (sporophylls) for zoospore release, used a hemacytometer to estimate spore density, and inoculated test solutions with zoospores. At Station Two, participants learned how to identify and count germinated and non-germinated spores, and measured gametophyte germ-tube growth. Station Three topics included experimental design, data input and

transformation, statistical analysis (Analysis of Variance, Dunnett's test) and No Observed Effect Concentration (NOEC) calculation, and central data base design.

### **Topsmelt Workshop**

The topsmelt protocol was demonstrated at the first two workshops: Cabrillo Marine Museum; California Academy of Sciences. The topsmelt workshop also followed a similar format with an introductory slide show followed by a laboratory demonstration. The slideshow described spawning and culturing techniques for this species and gave an overview of the toxicity test protocol for larvae. The hands-on workshop included the following stations:

#### **Topsmelt Toxicity Test Work Stations**

##### **STATION 1: TEST INITIATION**

- Larval handling and feeding
- Preparation of test solutions
- Inoculation of test containers
- Test solution renewals

##### **STATION 2: TEST TERMINATION**

- Quantification of mortality endpoint
- Preparation of larvae for weighing
- Quantification of growth endpoint

##### **STATION 3: STATISTICAL ANALYSIS OF TOXICITY TEST DATA**

- Data preparation and transformation
- Experimental design
- Statistical tests
  - ANOVA (Analysis of Variance)
  - Dunnett's Multiple Comparison
  - Spearman Karber
- Computer programs for Macintosh
- Computer formats for reporting raw data to central data base

### **Mysid Workshop**

The 7 day growth and survival toxicity test protocol for juvenile mysids (*Holmesimysis costata*) was presented in two, 1 day workshops at the Marine Pollution Studies Laboratory at Granite Canyon in Monterey. The format for this workshop followed the format described above for the other MBP protocols. The hands-on workshop included the following stations:

## Mysid Toxicity Test Work Stations

### STATION 1: TEST INITIATION

- Isolation of gravid females
- Isolation of juveniles for testing
- Randomization of juveniles
- Loading of test containers
- Feeding and maintenance

### STATION 2: TEST TERMINATION

- Test solution renewals
- Endpoint Analysis :
  - Mortality
  - Growth: Sample preservation and length measurement

### STATION 3: STATISTICAL ANALYSIS OF TOXICITY TEST DATA

- Data preparation and transformation
- Experimental design
- Statistical tests
  - ANOVA (Analysis of Variance)
  - Dunnett's Multiple Comparison
  - Spearman Karber

## Workshop Evaluation

By the conclusion of the workshops, each person had participated in all aspects of the toxicity tests. They were each asked to complete a questionnaire to provide the MBP staff with written comments on technical and general aspects of the test protocols, and on format and utility of the workshops themselves (see below).

### Workshop and protocol evaluation questions:

1. Have you previously conducted this test before?
2. What other toxicity tests has your laboratory used?
3. Please identify any logistical constraints your laboratory might have working with this test species?
4. Are the test methods clearly presented in the protocols?
5. What changes to the methods or protocol would you suggest?
6. Were the methods clearly presented in the workshop?
7. What changes would you suggest to the workshop format?

In general, responses to the questionnaires were positive, and indicated that the hands-on approach of the workshops provided excellent training. The most common modifications to the protocols and suggestions for future workshop formats are given below (by species).

## **Response to Workshop Questionnaires**

### **Kelp Toxicity Test Workshops:**

The percentage of workshop participants who had previous experience with this protocol varied depending upon the region. At the southern California workshop at Cabrillo Marine Museum, 57% of the participants had used this protocol. At the northern California workshop at California Academy of Sciences, only 16% of the participants had used this protocol; most of these people were more familiar with the EPA chronic toxicity procedures. The major logistical constraints listed for this protocol involved the amount of time it takes to terminate the test and read the two endpoints. Based on these comments we have modified the protocol to allow for preservation of the cultures for later analysis. Another suggestion involved modifications to the way we randomly select spores for growth measurements. There were no other significant changes to the protocol suggested. Most of the participants liked the hands-on nature of the workshop, and all felt they learned a lot about the procedures using this format. Some of the participants suggested that more time be spent on data analysis and statistics.

### **Abalone Toxicity Test Workshops:**

The percentage of workshop participants who had previous experience with this protocol also varied depending on the region. At the southern California workshop at Cabrillo Marine Museum, 47% of the participants had used this protocol. At the northern California workshop at California Academy of Sciences, only 14% of the participants had used this protocol; most of these people were also more familiar with the EPA chronic toxicity procedures. The major constraint listed for this protocol involved the use of formalin. Based on these comments we have modified the protocol to allow for the exposure and preservation of the abalone larvae in tissue culture containers. This minimizes exposure to formalin fumes. Another suggested modification was that we rinse the newly fertilized abalone eggs on a Nitex™ screen to remove excess sperm. There were no other significant changes to the protocol suggested. As in the other workshops the participants felt that more time should be spent on data analysis and statistics.



### **Topsmelt Toxicity Test Workshops:**

Few of the workshop participants had previous experience with this protocol; some people however, did have experience with the EPA procedure for *Menidia sp.* a protocol after which the topsmelt test was modeled. At the southern California workshop at Cabrillo Marine Museum, 29% of the participants had used the *Menidia* protocol. At the northern California workshop at California Academy of Sciences, 38% of the participants had used this protocol. The major constraint listed for the topsmelt protocol involved the lack of test organisms, and the amount of time necessary to conduct the 7 day static renewal test. Some of the participants suggested we use a greater number of larvae per test container and fewer replicate test containers.

### **Mysid Toxicity Test Workshops:**

As with the topsmelt test, few of the workshop participants had previous experience with the mysid protocol, but some had experience with the EPA procedure for *Mysidopsis bahia* a protocol similar to the *Holmesimysis* procedure. At the southern California workshop at Cabrillo Marine Museum, 79% of the participants had used the *Mysidopsis bahia* protocol. At the northern California workshop at California Academy of Sciences, 36% of the participants had used this protocol. Overall, about 27% of the people had some experience working with *Holmesimysis*. The major constraint listed for the *Holmesimysis* protocol involved the lack of test organism suppliers, and a lack of equipment for measuring the length endpoint. Almost all of the participants suggested we develop a weight endpoint for quantifying mysid growth as an alternative to length because weight is quicker and easier to quantify. It was pointed out that weight is used in the *Mysidopsis* protocol. We are now investigating the applicability of this endpoint (see Mysid Experiments: Section 2 of this report).

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