MARINE BIOASSAY PROJECT
SIXTH REPORT

INTERLABORATORY COMPARISONS AND PROTOCOL DEVELOPMENT WITH FOUR MARINE SPECIES

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STATE OF CALIFORNIA

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EXECUTIVE SUMMARY

The goal of the Marine Bioassay Project (MBP), authorized by the State Water Resources Control Board (State Board) in 1984, is to protect California's ocean resources by determining the impacts of toxic waste discharges on marine waters. The Project's primary objective is development of short-term tests to measure the toxicity of these discharges. 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, the sixth in a series, describes the work performed during Phase Five, which occurred from January 1, 1990 to December 31, 1990. The sixth report is organized into four 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 the giant kelp. Section 3 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 4 describes work with the red abalone, *Haliotis rufescens*, a large gastropod mollusc that is indigenous to California and is distributed throughout the State's coastal waters. In addition to the above sections, the report contains five appendices. Four appendices describe the MBP toxicity test protocols (giant kelp, red abalone, topsmelt, and mysid shrimp) and the fifth appendix is a chapter on Quality Assurance/Quality Control. Two of these protocols, the giant kelp and red abalone toxicity tests, have undergone sufficient development and have been approved by the State Board for inclusion into statewide water quality control plans. These and other sensitive test

protocols are now being incorporated by Regional Boards into waste discharge permit requirements for compliance monitoring of chronic toxicity. The mysid shrimp protocol has been developed with both an acute endpoint and is still under development with a sublethal endpoint. The fish protocol should be considered tentative until further testing has been completed. A summary of the test results from this phase of the Marine Bioassay Project is presented in the discussion below.

The topsmelt experiments evaluated three different life stages: fertilization of embryos, embryonic development, and larval growth and survival. Toxicity tests were conducted with reference toxicants and complex effluent. Preliminary experiments on the 48-hour topsmelt fertilization test indicated that this test would be impractical for routine testing. Several factors present problems for the fertilization test: limited sperm viability time, lack of control over fertilization, and limitations on test organism supply. The 12 day embryo development test was evaluated for routine testing. Experiments were conducted to investigate feasibility of supplying early-blastula embryos to other testing laboratories. Additional research is needed to explore this technique so that early-blastula embryos can be shipped to testing laboratories and arrive at or near the early-blastula stage. A 7-day larval growth and survival test was developed as an alternative protocol to the embryo and fertilization tests and is the protocol currently being refined by the MBP. This protocol was patterned after the *Menidia berylina* 7-day growth and survival test developed by EPA. Three experiments were conducted using copper chloride as a reference toxicant; the intralaboratory test precision expressed as percent coefficient of variation was 21% (CV). Test precision is a measure of the ability to provide reproducible results when a series of tests are conducted under the same conditions. Interlaboratory test precision was 37% (CV) with copper chloride.

The mysid *Holmesimysis costata* 7-day survival and growth test was further evaluated with a reference toxicant (zinc sulfate). Reference toxicant tests were conducted during this phase to estimate sensitivity to a common toxicant, success rate, and logistical feasibility, as well as to determine intralaboratory test precision. The intralaboratory precision at MPSL for six zinc reference toxicant tests was 20% (CV). Tests were also conducted to compare sensitivity to zinc between *H. costata* and the epibenthic mysid, *Mysidopsis intii*. In seven day growth and survival tests, *H. costata* was found to be more sensitive to zinc than *M. intii* . However, *M. intii* has a shorter life cycle than *H. costata*, and the EPA is sponsoring work at Oregon State University to develop an *M. intii* test with a reproductive endpoint.

Research with the giant kelp consisted primarily of interlaboratory testing of the 48-hour protocol with complex effluent from four municipal dischargers. Copper reference toxicant tests were conducted concurrently with all interlaboratory tests. The mean interlaboratory precision with copper was 27% (CV) for germination and 36% (CV) for germ-tube length. The interlaboratory precision with effluent tests was 67% (CV) for germ-tube length and not calculated for germination. The intralaboratory precision with copper reference tests conducted at MPSL was 33% (CV) for germination and 38% (CV) for germ-tube length. In addition, two preliminary tests were conducted using sodium azide as a reference toxicant. Sodium azide is a promising alternative reference toxicant to copper chloride because the toxicity of this compound is less affected by binding to constituents of sea water. During Phase Six, additional tests using sodium azide will be examined to measure intralaboratory test precision.

The focus of work with the red abalone protocol was to determine if the protocol was sufficiently detailed to allow different investigators to produce acceptable test results under varying laboratory conditions. Four interlaboratory tests with the red abalone 48-hour protocol were conducted using split effluent samples and reference toxicants. The interlaboratory precision for four effluent tests was 10% (CV); with zinc test precision was 19% (CV). Intralaboratory precision with zinc reference tests conducted at MPSL was 10% (CV).

Recommended future work for the project involves three areas of focus:

(1) Completing development of the topsmelt and mysid shrimp protocols;
(2) Continuing interlaboratory testing with other laboratories; and
(3) Providing workshops and training for technicians who will perform the tests.

TOXICITY TESTING

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 of 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 test, 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 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 example 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 bioavailabilty of toxicants in a complex mixture, account for synergistic and antagonist 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. 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 now devoting additional effort to examine issues such as test precision, and selecting appropriate statistical approaches to analyze test results.

There are two statistical approaches to analyze test results: point estimate techniques and hypothesis testing. Point estimate techniques measure toxicity by plotting toxicant concentration against organism response to identify the concentration that produces a given level of response, such as 50% mortality in an LC 50 analysis. Point estimate techniques used in toxicity testing include probit analysis, moving average angle, Spearman-Karber, and linear interpolation methods. Probit analysis, moving average angle, and Spearman-Karber are used to calculate an Effective Concentration (EC 50) by analyzing percentage (quantal) data from concentration-response tests. Linear interpolation method is a procedure to calculate a point estimate of the toxicant concentration (Inhibition Concentration, IC) that causes a given percent reduction (e.g., 25%, 50%, etc) in the reproduction or growth of the test organisms (EPA /600/4-89/001). Test precision can be quantitatively assessed by calculating a mean and standard deviation and consequently a percent coefficient of variation (CV = standard derviation/mean x 100%) using point estimate techniques.

When hypothesis testing techniques are used to analyze toxicity test data, it is not possible to express test precision. The results of the test are given in terms of two endpoints, the No-Observed-Effect Concentration (NOEC) and the Lowest-Observed-Effect Concentration (LOEC). The NOEC and LOEC are limited to the concentrations selected for the test; the width of the

NOEC/LOEC interval is a function of the dilution series. As a rule of thumb, the precision of tests is considered acceptable when NOECs vary by no more than one concentration interval above or below a central tendency (EPA/600/4-89/001).

Statistical methods for analyzing toxicity test data are continuing to evolve. Standard operating procedures for deriving point estimates and NOEC's for discreet and continuous data have not been finalized. The Marine Bioassay Project is working with a statistician to develop standard operating procedures for all MBP protocols. These procedures will be included in the next iteration of the MBP protocol manual.

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 is especially useful for assessing complex discharges where it may be virtually impossible to characterize toxicity solely by chemical analysis. Biological testing also provides information not available from 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 these toxicity test protocols for monitoring complex effluents since January 1, 1991. The State Board must adopt a schedule by January 1, 1992 requiring the use of these protocols by dischargers of less than 100 million gallons per day. 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 ocean 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, 1987 the State Board adopted a work plan for triennial review of the California Ocean Plan, based on public hearings held in October 1986. The work plan listed 26 issues raised during the hearings and identified seven as being high priority for Ocean Plan review. Refinement of toxicity test protocols and implementation of their use was one of the high priority issues.

In March 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 MBP tests, using giant kelp and the red abalone 48-hour toxicity tests. These marine toxicity tests will be implemented in regulatory programs of the State Board and six coastal Regional Water Quality Control Boards. Part of future efforts by the MBP will focus on 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):

$$TU = 100/NOEL$$

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 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, five phases of the Marine Bioassay Project have been completed; chapters of this report describe work performed during the

fifth phase. The sixth and final phase has been planned for the period from January 1991 to June 1993. The final 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 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 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, thepsmelt *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 to December 1990)

Work conducted during Phase Five is the focus of this report. A description of the phase five research appears in the Executive Summary.

Phase Six (January 1991 to June 1993)

Phase Six will complete both protocol development by the project and implementation of the marine toxicity testing program. Major objectives 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. Insuring that implementation of the Ocean Plan's chronic objective in discharge requirements is achieved through a sound toxicity testing program using proper quality assurance/quality control and testing procedures. To achieve this latter objective, extensive use of MBP staff expertise and knowledge will be important.

Section 1
Topsmelt Experiments

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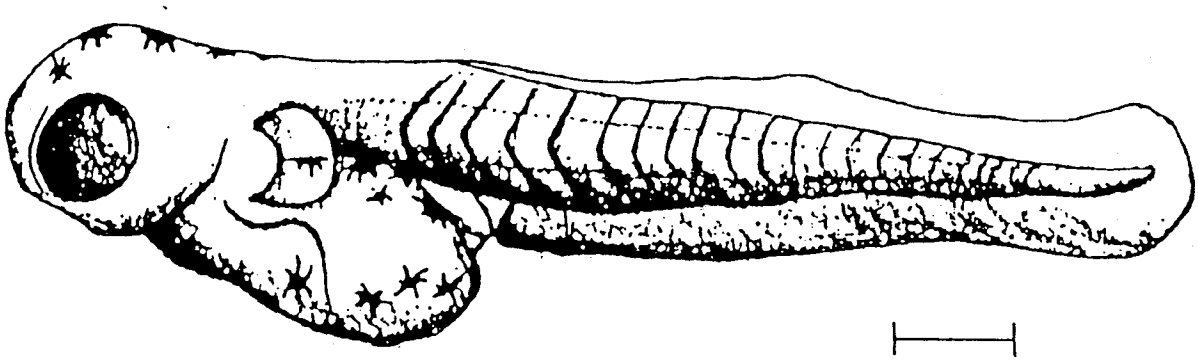


Atherinops affinis - larva

1 mm

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, Allen 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 (Allen 1980) 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 (Allen, 1980). They begin spawning in their second and third years, depositing eggs on eelgrass (*Zostera sp*) and benthic algae (eg., *Gracilaria sp.*, Croaker, 1934; Fronk, 1969).

There is increasing interest in the use of topsmelt in toxicity testing because of their ecological importance, potential exposure to coastal pollution, and amenability to laboratory culture. Because these fish are limited to nearshore and estuarine waters, it is likely they are exposed to domestic or industrial effluents (eg., Hose et al., 1983) and pesticide residues drained from coastal agricultural lands. Topsmelt embryonic development is similar to that of other atherinids used widely in toxicity testing (eg., *Menidia* species, Borthwick et al., 1985; Middaugh et al., 1987; Middaugh et al., 1988), and methods developed to assess sublethal effects with *Menidia* have proven to be easily adapted for topsmelt (Anderson et al., 1991a).

During Phase 3 of the Marine Bioassay Project, a series of preliminary zinc sulfate toxicity tests were completed using 9 day-old topsmelt larvae hatched in the laboratory (Hunt et al., 1989).

Phase 4 topsmelt research focused on two areas: (1) developing methods for spawning adult topsmelt and (2) measuring the response of several life-stages to a reference toxicant. Our research determined the relative sensitivity of gametes, embryos and larvae to copper chloride (Anderson et al., 1991). Methods were developed to induce spawning of laboratory cultured fish, and routine spawnings were successful from June through August.

The Phase 5 topsmelt research reported here continues the evaluation of different topsmelt life stages for toxicity testing using reference toxicants and sewage effluent. Our previous work indicated that sperm and embryos were more sensitive to copper than larvae, so initial work for Phase 5 emphasized experiments with these stages. To further evaluate the 48-h topsmelt fertilization test, experiments were conducted to investigate sperm viability over time; one fertilization experiment was conducted with sewage effluent.

Factors affecting performance of a 12-day embryo development and hatching test were also investigated. A preliminary experiment tested the potential for temperature control of the development rate

of embryos. The affect of embryo density was studied in tests with copper and sewage effluent. Several additional embryo development tests were conducted using sewage effluent.

A preliminary test protocol for topsmelt larvae was also developed and evaluated. This procedure, patterned after the *Menidia sp.* 7-day growth and survival toxicity test, was developed as an alternative to the 12-day embryo test. Three 7-day larval growth and survival tests were conducted with copper. One interlaboratory test of this protocol was completed using copper.

Methods

Facilities

All experiments were conducted from April through August 1990, at the California Department of Fish and Game's Marine Pollution Studies Laboratory (MPSL) located on the Big Sur coast in Monterey County. The facilities are described in detail in Martin et al. (1981). Methods for topsmelt culture and toxicity tests were similar to those described in Anderson et al. (1991a).

Laboratory Spawning

Adult topsmelt were seined from Elkhorn Slough, Monterey County, California, in April, 1990 (water temperature = 16 °C; salinity = 33 ‰). Fish were transported to MPSL in 100-liter aerated holding tanks. Once at MPSL, the fish were treated for 2 days with a general antibiotic (Prefuran®), then divided among two 1000-liter holding tanks. A re-circulating system similar to that described by Middaugh and Hemmer (1984) was used. The system uses a pump to recirculate water (10 liters/minute) from the tanks through vertical filter elements in a separate reservoir and then back into the tanks. Dissolved oxygen levels were maintained at greater than 6.0 mg/liter using aeration. A 600 watt immersion heater maintained constant temperature and provided temperature "spikes" to initiate spawning.

The photoperiod was 16 hours light followed by 8 hours darkness (14L:10D) with lights commencing at 0600. Four 'cool white' 40 watt fluorescent lamps, suspended 1.25 m above the surface of each tank, provided illumination.

A 'tidal signal' of reduced current velocity in each tank was produced once daily, from 2400 to 0200 hrs, by turning off the circulating pump (Middaugh & Hemmer 1984). Temperature was monitored daily and at 1 to 4 hour intervals on days when water temperature was raised to induce spawning ("temperature spikes"). Salinity was measured with a refractometer (to the nearest 1.0 ‰).

Polyester fiber spawning substrates attached to the surface of plastic grids (7 cm x 10 cm x 1 cm) were weighted to the bottom of each tank. These were observed daily for the presence of eggs.

Adult topsmelt in each tank were fed approximately four grams of Tetramin ™ flake food per 30 fish at 1300-1500 hours daily. Tanks were siphoned clean twice weekly.

Temperature Spikes

Temperature "spikes" were employed to induce spawning (Anderson et al., 1991a). Adult topsmelt were held at approximately 18 °C, then the water temperature was increased to 21-22 °C over a 14-hour period. This elevated temperature stressed the fish as evidenced by occasional mortality. Temperature was then reduced to 18 °C overnight. Thereafter, spawning substrates were checked daily for egg production. All eggs were removed, assessed for developmental stage (after Lagler *et al.*, 1962), enumerated, and classified as viable or non-viable at 36x using a dissecting microscope. Additional temperature spikes were provided at 7 to 9 day intervals with water temperature between spikes maintained at 18 °C.

Toxicity Tests

Fertilization Tests

The fertilization test consists of exposing sperm to a toxicant, introducing eggs, and then measuring percent fertilization after 48 hours incubation. Results of multiple copper reference toxicant tests conducted during Phase 4 of the project indicated that there was considerable between-test variability. It was suggested that variability might be reduced by tighter control over sperm-to-egg ratios in the test. In order to determine optimal sperm-to-egg ratios it was first neccessary to know how long sperm would remain viable once they were stripped from male fish.

Preliminary experiments during Phase 5 indicated that sperm observed through a microscope remained motile for approximately one hour, post-stripping. An experiment was conducted to determine whether motile sperm held for different time intervals were competent to fertilize eggs. Sperm were stripped from several male fish into a 100 ml beaker and held at 6 ±1 °C, on ice, for various time intervals from zero to one hour. The sperm were held "dry"; no seawater was added to the sperm solution. By holding the sperm dry we hoped to delay the sperm activation time, and prolong post-stripping viability. The holding times were 0, 10, 15, 30, 45, and 60 minutes. After the appropriate holding time elapsed, two drops of sperm were pipetted into test containers with 10 mls of seawater at 18 °C, and allowed to incubate for 15 minutes, simulating the test exposure period. The test containers were 15-ml capacity polystyrene "wells" set in multiwell tissue culture plates (Falcon ™, 6-well plates). Each isolated well served as one replicate. After the 15 minute exposure to seawater, freshly-stripped eggs were introduced to the test containers.

The sperm and eggs were left to incubate at 21 ± 1 ° C for 48 hours, with a 14L:10D photoperiod (30 $\mu E\ m^{-2}\ sec^{-1}$). At 48-h, the eggs were observed with a Bausch and Lomb Photozoom ™ inverted microscope (40 - 100x) and the percentage of fertilized eggs were determined for each replicate.

Further experiments to determine the optimum sperm-to-egg ratio were unsuccessful because sperm stripped from male fish were only viable for a short time (see results).

One fertilization test was conducted with sewage effluent using methods described above (see also Anderson et al., 1991a). The effluent tested was an advanced primary treated sewage effluent collected as a

grab sample from a local municipal sewage treatment plant. Effluent concentrations tested were 0, 0.56, 1.0, 1.8, 3.2, and 5.6%.

Embryo Tests

Control of Embryo Development Rate

The topsmelt embryo test protocol is a 12-day experiment that assays embyo development and larval hatching success. To maximize sensitivity, it is designed to start with early-blastula-stage embryos (approximately 8 hours post-fertilization). One aspect of the protocol that required further investigation was the feasibility of supplying early-blastula embryos to other testing laboratories. In order for laboratories to conduct the 12-day embryo protocol, embryos would need to be shipped to arrive in the early-blastula stage. Transport times may be as long as 24 hours. Cold temperature treatment was investigated as one possibile method of retarding development during transport so that the embryos could arrive at the testing laboratories at the appropriate stage. The holding temperatures tested were 12, 10, and 7 °C.

Approximately 450 early-blastula embryos were isolated from spawning substrates and placed in 300 mls of 18 °C seawater in a stacking dish. The dish was placed in a water bath and allowed to cool to 14 °C over 2.0 hours. The dish was then placed in a refrigerator and further cooled to 12 °C over 0.75 hours. The embryos were then divided into three equal groups in three separate stacking dishes. The Group One embryos were immediately transferred to a thermos holding 1 liter of 12 °C seawater and sealed for overnight storage. The Group Two embryos were left in the refrigerator and cooled further to 10 °C (over approximately 0.75 hours) then tranferred to a thermos for storage. The Goup Three embryos were cooled to 7 °C (over approximately 1.25 hours) then transferred to a thermos, as above. All thermos bottles were then placed in an ice chest with blue-ice for storage. After 24 hours, the water temperatures in the bottles were recorded, and the embryos were removed and their developmental stages noted. They were then left to warm to 18 °C (~3 to 5 hours) and transferred to three separate screen tubes at 18 °C and allowed to incubate. After 13 days all of the embryos either hatched or died; the proportion hatching from each group was quantified.

Experimental Design Evaluation

The 12-day embryo development and hatching protocol is currently designed to use 20 replicates per test concentration (treatment) with each replicate containing one embryo. The assay has two possible endpoint outcomes: the embryo from each replicate either hatches normally, or it does not. Nominal scale data is generated from this kind of experimental design. The statistical method used to analyze this kind of data is a row by column test of independence. We wanted to investigate whether an embryo test employing the row x column experimental design (ie., one embryo per replicate) resulted in the same NOEC as an embryo test employing an Analysis of Variance (ANOVA) experimental design (ie., multiple embryos per replicate). All of the other Marine Bioassay Project toxicity tests protocols use the mulitple test organism per replicate (ANOVA) design.

Two experiments were conducted using eight embryos per replicate, and six replicates. The test containers were 10 ml capacity polystyrene tissue culture containers. This design was tested once with copper, and was evaluated in terms of test performance (control hatching rates and copper sensitivity). The NOEC and EC_{50} from this experiment was compared to those of previous experiments employing the original test design of one embryo per replicate. This design was also also tested once with the sewage effluent used in the sperm test described above. Effluent concentrations tested were: 0, 0.5, 1.0, 2.0, 3.0, and 4.0%.

Embryo Tests With Complex Effluent

Three 12-day embryo development and hatching experiments were conducted to assess test performance with complex sewage effluent. All effluent samples were grab samples from a municipal treatment plant. The procedures were identical to those described in Anderson et al. (1991a). Single early blastula embryos (stage 8-9, after Lagler *et al.*, 1962) were placed in glass tissue culture tubes (94mm x 16mm) containing dilutions of sewage effluent. Control and dilution water was 0.2 µm-filtered seawater (33 ‰ at 21 ± 1 °C). Each tube contained 9 mm of solution and 7 mm of airspace and was capped with a teflon-lined cap for the duration of the test. Each treatment was replicated 20 times. Tubes were stored horizontally in stainless steel racks to increase the volume of test media exposed to the airspace. Test solutions were not renewed.

In the first test, embryos were checked daily using an inverted microscope (40-100x). Effluent concentrations tested in test #1 were: 0, 0.32, 0.56, 1.0, and 1.8% effluent. Effluent concentrations in test #2 were: 0, 0.56, 1.0, 1.8, 3.2, and 5.6% effluent. Effluent concentrations in test #3 were: 0, 0 (brine control), 3.2, 5.6, 10.0, and 18.0% effluent. Hypersaline brine was used to adjust effluent concentrations of 5.6% and greater. In the second and third tests, the embryos were checked 24 hours after introduction to the test tubes to determine embryo viability. Thereafter, test tubes were inverted four times daily to mix and aerate the solutions. On days 6 through 12, embryos were examined microscopically for viability, developmental abnormalities, mortality, and hatching success. Post-hatch larvae were also examined for terata at the end of the tests. Teratogenic expressions were quantified using the procedure described in Anderson et al (1991; after Weis and Weis,1982). Three categories of developmental abnormalities were quantified: craniofacial, cardiovascular, and skeletal defects.

Upon completion of each experiment, one endpoint was quantified: the number of normally hatched larvae. This includes all hatched live larvae without visible abnormalities. A row by column test was employed to compare all data. This test of independence uses the G statistic and is based on the chi-squared distribution. NOEC's for each test were calculated by pairwise comparisons using an adjusted alpha (alpha' = 0.05) to compare test concentrations to controls (Sokal and Rohlf 1969).

Larval Tests

Our previous research indicated that topsmelt larvae are less sensitive than embryos to copper (Anderson et al., 1991). However, these experiments compared larval survival in 96-hour toxicity tests to embryo development and hatching success in 12-day toxicity tests. To further evaluate the use of larvae in toxicity tests, we adapted a longer-term 7-day growth and survival test with larvae; this protocol was patterned after the *Menidia sp.* 7-day growth and survival test (Weber et al., 1988). The sensitivity of the 7-day topsmelt larval test was compared to previous topsmelt life-stages using copper.

Larval topsmelt were cultured at 18-21 °C and 33 ‰ using our previously described procedures (Anderson et al., 1991).

The 7-day larval protocol is a static-renewal toxicity test that assays growth (dry weight) and survival. Three experiments were conducted using copper chloride as a reference toxicant. Nine day-old larval topsmelt (five larvae per replicate) were exposed to copper solutions in 250-ml, acid-washed, polyethylene plastic food containers. The first and second tests used five replicates each of 0, 56, 100, 180, 320, and 560 µg copper/liter. The third test used 0, 32, 56, 100, 180, and 320 µg copper/liter. Each container held 200 ml of solution (salinity 33 ‰). Solutions were renewed every 48-hours.

Containers were placed in an incubator or water bath at 21 ± 1 °C and covered with plexiglass to prevent evaporation. The photoperiod was 14L:10D at 12 µE m^{-2} s^{-1}. Dead larvae were counted daily and removed. Larvae were fed approximately 60 newly-hatched *Artemia* nauplii per larvae daily (Argentemia® silver label). At the end of the experiment all surviving larvae were dried for twenty four hours at 55 °C. All fish from each replicate were dried together and the weight per larva was calculated for each replicate. Analysis of Variance followed by Dunnett's multiple comparison test were used to derive NOEC's for growth and survival; LC_{50}'s and 95 % confidence limits for the survival endpoint were calculated using the trimmed Spearman-Karber method. The growth endpoint was not compared statistically because there were no growth effects (see results).

Interlaboratory Comparison of Larval Protocol

One interlaboratory comparison of the 7-day larval growth and survival protocol was conducted between MPSL and the Vantuna Research Group (VRG) at Occidental College (under the direction of Dr. JoEllen Hose). Methods for this experiment were similar to the interlaboratory comparisons described in previous Marine Bioassay Project reports (Anderson et al., 1990).

Approximately four hundred, 7 day-old topsmelt larvae were cultured at MPSL and divided into two groups. The first group was shipped via overnight courier in double-bagged, one-liter polyethylene ziplock bags filled with oxygen-saturated, 1 µm-filtered seawater. Transport temperature was approximately 12 °C. The second group was held at MPSL under indentical conditions. After a 24-hour acclimation period, 7-day growth and survival tests were conducted by both laboratories using 9-day old larvae. Copper concentrations used at both laboratories were 0, 32, 56, 100, 180, and 320 µg /liter. Both laboratories used

their own dilution waters; powdered copper chloride was provided by MPSL. Methods for the experiments and interpretation of results were the same as those described above.

Chemical and Physical Measurements

Physical parameters for all toxicity tests were measured in each test concentration at the beginning and end of each test. Dissolved oxygen and pH was measured using a Orion 940® dissolved oxygen/pH/millivolt meter. Salinity was measured using an Atago® model S-10 hand refractometer. Temperature was monitored with hand and digital thermometers daily; pH and dissolved oxygen levels (mg/liter) were recorded every 48 hours in the larval tests.

One random sample of each test concentration was taken at the beginning and end of each test, and with every 48-hour renewal in the 7-day larval tests, for measurement of total copper. Total copper concentrations were verified using a Perkin Elmer Model 6003 Atomic Absorption Spectrophotometer.

Results and Discussion

Fertilization Experiments

Preliminary microscopic observations of sperm held "dry" indicated that topsmelt sperm remained motile for up to 1.5 hours at 6 °C. Results of the experiment to verify whether these sperm were viable indicate that topsmelt sperm stripped from male fish remain viable for fertilization for approximately fifteen minutes when held dry at 6 °C. The proportion of normally developing embryos observed at 48-hours post-fertilization decreased significantly (Chi Square $p = 0.001$) when sperm were held dry for longer than 15 minutes (Table 1). It is not clear why the fertilization rate was zero in the treatment where eggs were added to seawater immediately after sperm was introduced, while the fertilization rate was 92% in treatments where the sperm was incubated for 15 minutes prior to introduction of eggs.

There is a limited time period for sperm viability once the sperm have been stripped from the adult fish. This presents a problem for further development of the topsmelt fertilization test because it limits the time available for estimating sperm densities and for adjusting sperm-to-egg ratios. A lack of standardization of sperm-to-egg ratios limits our ability to control a factor that probably affects both sensitivity and between-test variability (Anderson et al., 1991a; Dinnel et al., 1987).

Table 1. Sperm viability over time. Sperm was held "dry" at 6 ± 1 °C for various time intervals from time zero to one hour (= dry holding time). The sperm was then pipetted into 18 °C seawater and incubated for 15 minutes (= activation time). After a 15 minute sperm exposure, eggs were introduced and left to incubate for 48 hours. The proportion of normal embryos was then observed. Total time = total time from sperm stripping to introduction of eggs to test containers. (N = 5)

Dry Holding Time (min.)	Activation Time (min.)	Total Time (min.)	Prop. Normal Embryos (% ± sd)
0	0	0	0
0	15	15	92.1 (5.7)
10	15	25	76.9 (17.2)
15	15	30	85.7 (6.0)
30	15	45	61.9 (19.3)
45	15	60	57.3 (10.1)
60	15	75	23.2 (13.4)

Problems associated with a limited sperm viability time appeared to confound the results of the effluent fertilization experiment. Effluent concentrations and controls were each replicated three times in this experiment, and it took approximately 25 minutes to introduce the eggs to all of the test containers. This resulted in some of the test containers receiving eggs while sperm were still viable, while other containers received eggs after the optimum viability period had passed. Although eggs were added to the test containers in random order, the results suggest that the lack of adequate time affected the experiment. As a result, there was considerable between-replicate variability. Another problem was a lack of control over fertilization, as indicated by significant fertilization in the no-sperm control. These problems confounded the results and lead to an apparent increase in fertilization rates as effluent concentrations increased.

Table 2. Effect of primary-treated effluent on topsmelt fertilization.

Percent Effluent	Mean Percent Fertilization (± sd)
0	42.0 (41.0)
0 (ns)	26.0 (14.0)
0.56	57.0 (33.0)
1.00	55.0 (25.0)
1.80	69.0 (31.0)
3.20	93.0 (0.60)
5.60	70.0 (25.0)

ns = no sperm control

Another potential problem associated with this toxicity test is test organism supply. Because the fertilization protocol requires that gametes be stripped from gravid topsmelt, it is necessary to have a considerable number of broodstock available to conduct toxicity tests. Test organism supply to laboratories involved in toxicity testing for NPDES monitoring would require suppliers to ship adult fish to testing laboratories; these fish would presumably not be available for future use. This presents a significant supply problem for effluent testing programs if this protocol is implemented on a wide spread basis. Besides the obvious problems associated with transporting adult fish so that they arrive at their destination in spawning condition, it would not be cost effective for suppliers to ship broodstock because broodstock supplies would be constantly depleted. These considerations, and the limited window of viability of stripped sperm, place

considerable constraints on the feasibility of using this protocol in routine testing programs. Although this test is potentially sensitive, we propose that this protocol not be considered for statewide effluent testing programs at this time. Other topsmelt early life stages are more promising and lack the technical and logistical constraints involved in working with gametes.

Embryo Tests

Control of Embryo Development

The attempt to retard development of early-blastula embryos by cooling were partially successful. All embryos remained in the early-blastula stage after 24 hours, but because of over-cooling in the ice chest, embryos placed in a thermos at 10 °C dropped to 5 °C overnight. This apparently killed a signifiacnt proportion of the embryos and resulted in reduced hatching rates in this group (Table 3). The drop in temperature resulted when the 10 °C thermos was placed directly on blue-ice in the ice chest. The temperature in the 12 °C group also declined significantly, but the drop evidently was not enough to result in significant mortality. Hatching rates of the other two groups were similar, and averaged about 71.5%.

We consider the results of this experiment preliminary, however, because no attempt was made to presort the embryos prior to cooling to ensure that all groups started with 100% viable embryos. Viability percentages are generally greater than 90% for embryos harvested from spawning substrates in the culture tanks (Middaugh et al., in press), but occasionally the proportions of viable embryos are lower for unknown reasons. The apparent reduction of hatching rates in this experiment could have resulted from poor initial viability, or from temperature shock resulting from cooling the embryos over a relatively short time period. This technique needs further study, and we intend to repeat this experiment with presorted, 100% viable embryos. A separate treatment to control for negative effects due to rapid cooling will also be included in the next experiment.

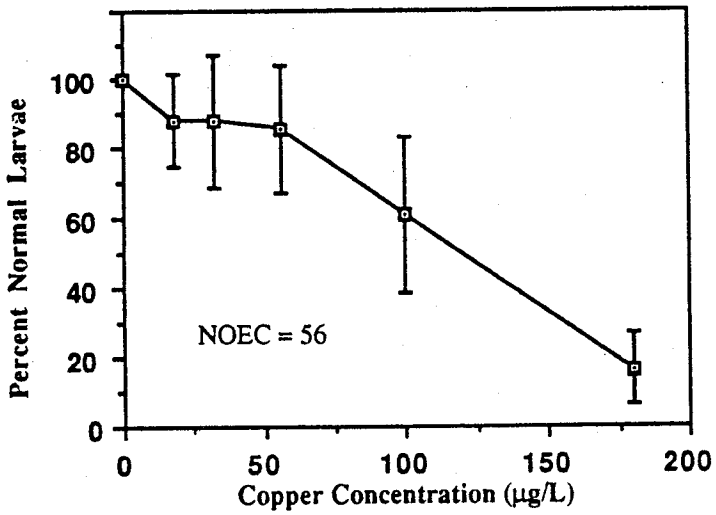
Table 3. Effects of cold storage on topsmelt embryonic development. Equal numbers of early blastula embryos were placed in each of three thermoses at 7.0, 10.0, and 12.0 °C. After 24 hours, their developmental stage was noted and the embryos were gradually transferred to 20 °C seawater and allowed to develop and hatch.

Temperature @ Start (°C)	Temperature @ 24 Hours (°C)	Developmental Stage @ 24-h	Proportion Normal Hatching @ 15-d
7.0	8.0	Early Blastula	73.0
10.0	5.0	Early Blastula	30.0
12.0	6.5	Early Blastula	70.0

Experimental Design Evaluation

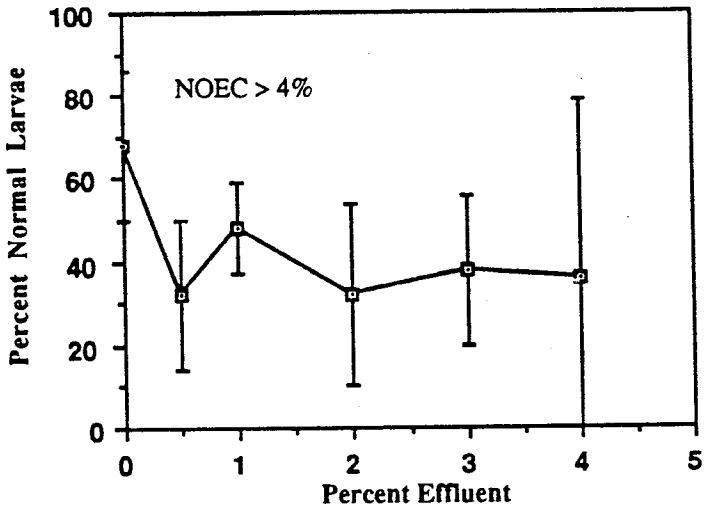
Topsmelt embryos developed normally in the test design with eight embryos per replicate. The NOEC for copper (56 µg/liter) was the same as previous NOEC's using one embryo per replicate, indicating that the multiple embryo and single embryo designs have similar statistical power. Hatching rates in the controls were 100% (Figure 1). The EC_{50} (and 95% Confidence Interval) for this test was 112 (102, 123). The pooled EC_{50} for tests using the single embryo design was 142 (131,157).

Figure 1. Effects of copper chloride on topsmelt embryonic development: ANOVA experimental design (n = 6; eight embryos per replicate).



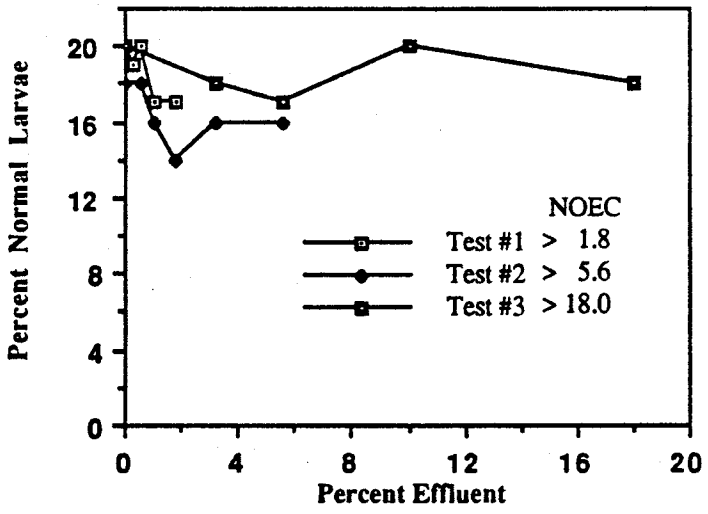
The multiple embryo per replicate design did not appear to work as well with sewage effluent (Figure 2). Control hatching rates were lower in this experiment because some of the embryos became infected with fungus, and the fungus spread to other embryos before they were removed from the test containers. Fungal infection problems compelled EPA researchers to resort to the single embryo design in embryo development and teratogenisis toxicity tests using *Menidia sp.* (Dr. Douglas Middaugh, U.S. EPA Gulf Breeze Environmental Research Laboratory, personal communication). Our evaluation did not indicate any advantage in changing the original experimental design for the 12-day embryo test; further protocol development will continue using one embryo per replicate.

Figure 2. Effects of advanced primary treated sewage effluent on topsmelt embryonic development (n = 6 ; eight embryos per replicate).



Because the results of the multi-embryo per replicate test indicated that there was some toxicity at the lowest effluent concentration tested (0.56%; Figure 2), initial experiments with this effluent using the single embryo test design started with relatively low effluent concentrations (Figure 3). The test concentrations were increased in subsequent tests. All experiments had excellent control hatching rates (mean = 97%), however no significant toxicity was detected in any. Experiment #1 showed limited toxicity at 2.0% effluent, with hatching success declining from 100% in the controls to 85% at 2% effluent. Because this was not a statistically significant reduction, experiment #2 tested higher effluent concentrations, up to 5.6%. Again, there appeared to be some toxicity, although not a statistically significant reduction. The final experiment (#3) tested effluent concentrations up to 18%; but, again, there was no significant response. Thus, while the experimental protocol performed well in all experiments, limited toxicity was detected. A preliminary experiment with 7-day-old topsmelt larvae also failed to detect significant toxicity in this effluent (data not shown). These results suggest that the effluent was relatively non-toxic topsmelt embryos during the period these tests were conducted. This effluent comes from a treatment plant serving a relatively small municipality and there may be considerable temporal variability in toxicity. The effluent used in these experiments was used for protocol development because of logistical considerations. Because the effluent came from a local discharger, we were able to collect grab samples on the morning of renewal days, and thereby avoid shipping constraints. Future evaluations of this protocol will assess toxicity using composited effluents from more highly industrialized areas.

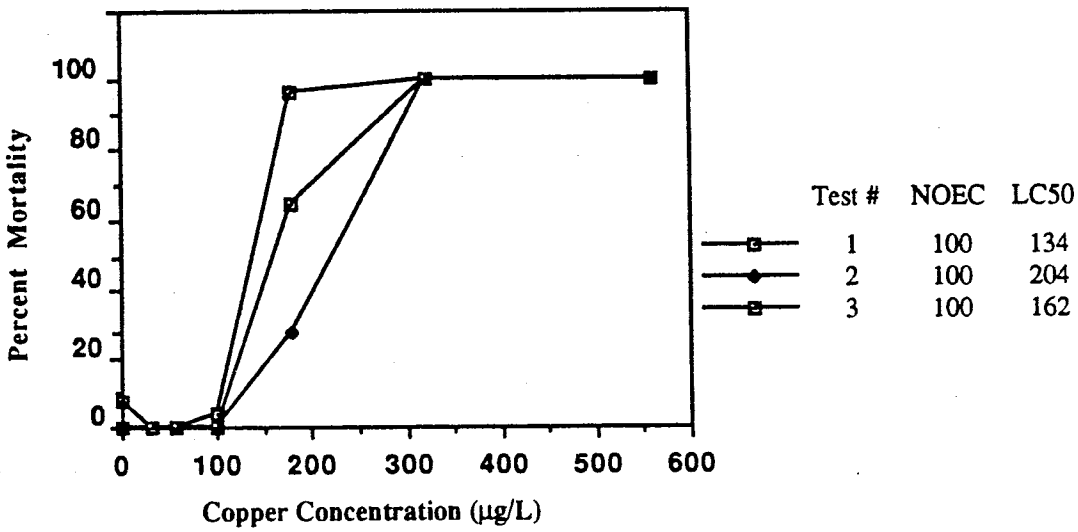
Figure 3. Effects of advanced primary treated sewage effluent on topsmelt embryonic development (n = 20 ; one embryo per replicate).



Larval Experiments

The three 7-day larval growth and survival toxicity tests showed similar responses to copper chloride. Control survival was excellent in all tests (mean = 97%), and the NOEC's for survival were the same for all three tests, 100 µg copper/liter. The LC_{50}'s for survival (95% confidence limits) were 134 (126, 143), 204 (184, 226) and 162 (145, 180) for tests #1 -3, respectively (Fig. 4). Between-test precision with copper was acceptable; the coeffecient of variation between the LC_{50}'s was 21%.

Figure 4. Survival of 9-day old topsmelt larvae in three 7-day copper tests (n = 5 for each test).



Larval survival was a more sensitive indicator of copper toxicity than was larval growth in the three tests; there was no significant reduction in topsmelt larval weight relative to the controls at any of the test concentrations in any of the tests (Table 4).

Table 4. Effect of copper on topsmelt larval growth in three copper tests. No Observed Effect Concentration (NOEC) ≥180 µg copper/liter for all tests (Growth data corresponds to tests #1-3 given in Figure 4; n=5; ±1 standard deviation).

Copper Concentration (µg copper /liter)	Mean Weight/Fish (mg dry weight) (sd)		
	Test #1	Test #2	Test #3
0.0	1.59 (.19)	1.02 (.06)	1.01 (.11)
32.0	*	*	1.03 (.13)
56.0	1.80 (.16)	1.08 (.06)	1.14 (.05)
100.0	1.88 (.23)	1.02 (.05)	1.04 (.08)
180.0	1.30 (†)	0.99 (.09)	1.22 (.39)

* This concentration not included in tests #1 and 2.
† N = 1 for this treatment in test #1.

The apparent lack of a growth inhibition in these experiments may be an artifact of using copper as a reference toxicant. Other researchers have found that copper concentrations in the same range used in our experiments resulted in gross changes in gill architecture in other marine fish (eg., Baker, 1979). Cardeilhac et al. (1979) found that copper damaged gill structure in sheephead, *Archosargus probatocephalus*, and that the primary result was a disruption in blood serum ion concentrations and osmoregulation resulting in death. It is also possible that copper uptake occurs cutaneously in larvae, because young topsmelt larvae may not have sufficiently developed gills to accomodate respiratory needs. Disruption of osmoregulation leading to death might also result from cutaneous uptake of copper. Thus, copper may be lethal to topsmelt larvae at the concentrations we tested, but have no obvious effect on growth over a seven day exposure period.

Survival is sometimes a more sensitive indicator of toxicity than growth in tests with larval fish. For example, Goodman et al. (in review) used a 30-day early-life-stage (ELS) test to evaluate Fenvalerate toxicity to topsmelt and found that survival was a more sensitive endpoint than growth (in weight). These authors also noted that in other tests using Fenvalerate, growth of sheepshead and fathead minnow larvae

was more sensitive than survival in one of six tests, survival was more sensitive than growth in four of six tests, and the two endpoints had equal sensitivity in the last test.

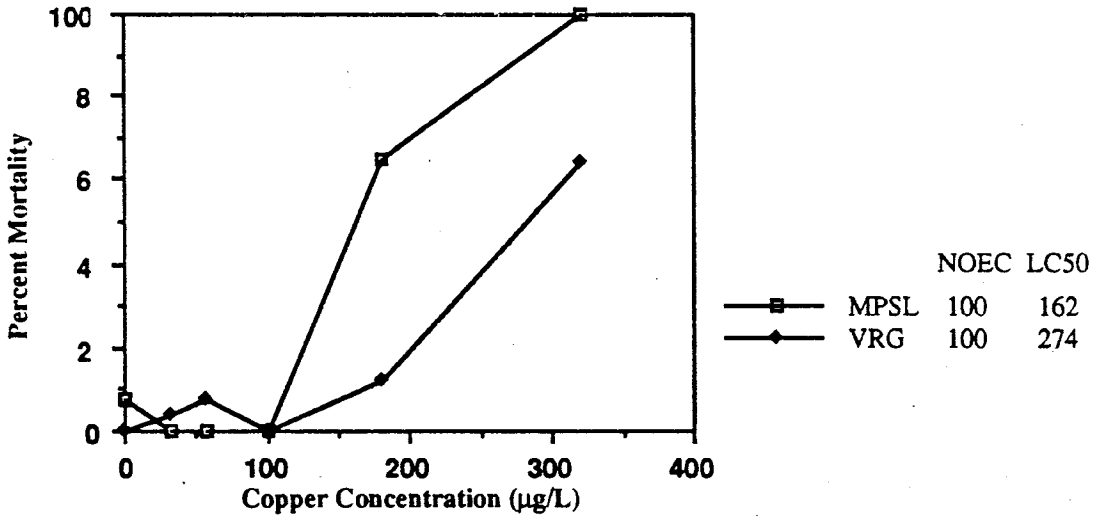
It is also possible that the feeding regimes provided in these tests was too low to allow growth effects to be manifested (see following discussion).

Interlaboratory Comparison of Larval Test

Results of the interlaboratory comparison of the topsmelt 7-day test were comparable. Both MPSL and VRG had the same NOEC for survival (Figure 5); The LC_{50}'s (95% confidence limits) were 162 (145, 180) and 274 (234, 322) at MPSL and VRG, respectively. The coefficient of variation of the LC_{50}'s was 37%.

Neither laboratory found a significant inhibition of growth (data not shown). However, the test at VRG included duplicate control replicates in which the larvae were fed to excess. These fish were significantly larger (in weight) than control fish fed a strict diet of 40 *Artemia* nauplii/larva/day (t-test p = 0.006; Dr. JoEllen Hose, Occidental College - Vantuna Research Group, unpublished data). Future tests will evaluate higher ratios of *Artemia* per larva, and use more than one daily feeding to investigate this factor on growth sensitivity.

Figure 5. Results of interlaboratory comparison of topsmelt 7-day larval growth and survival tests between MPSL and VANTUNA Research Group using copper chloride (n = 5).



The 7-day larval topsmelt growth and survival toxicity test protocol is comparable to the *Menidia sp.* growth and survival test. Recent research at the United States EPA suggests that topsmelt larvae are equally or more sensitive than *Menidia* larvae to a variety of toxicants (Hemmer et al., 1991; Goodman et al., 1991).

The topsmelt 7-day test is an appropriate west coast surrogate to the *Menidia* larval growth and survival test. Future development of the MPSL larval topsmelt test will focus on further investigation of factors that may increase test sensitivity (eg. feeding rates, age-dependent sensitivity, and an optimal salinity regime), and validation of test procedures through continued intra- and interlaboratory testing with complex effluent and reference toxicants.

In addition, recent work at MPSL has demonstrated that off-season spawning of topsmelt is possible through control of environmental factors and feeding, and that LC50's for copper are consistent between seasons (Anderson et al., 1991b). Future research will emphasize year-round spawning techniques and test organism supply.

Reference Chemical Verification and Water Quality

Measured copper concentrations were comparable to nominal concentrations for the Phase 5 topsmelt reference toxicant tests. The mean percent difference (± S.D.) of measured concentrations from nominal concentrations for all tests conducted was 8.5 ± 2.4% (range = 5.6% to 11.1% for all concentrations tested). Water chemistry parameters (DO, pH, salinity, and temperature) were all within acceptable limits prescribed for MPSL toxicity test protocols for all topsmelt tests conducted.

Summary

1. Results of investigations into the feasibilty of using a 48-hour topsmelt fertilization test for routine complex effluent testing indicated that this protocol is not practical for this application for several reasons.

2. An investigation into the feasibility of retarding topsmelt embryonic development through temperature control was partially successful. Future research will futher explore this technique so that early-blastula embryos can be shipped to testing labs and arrive at or near the early-blastula stage.

3. An evaluation of an alternative experimental design using multiple embryos per replicate for the topsmelt 12-day embryo test indicated that the current design of one embryo per replicate was the most appropriate.

4. A series of three embryo tests using complex effluent were successful but failed to detect significant toxicitiy with an advanced primary effluent from a small municipal waste treatment facility.

5. A 7-day larval growth and survival test was developed as an alternative protocol to the embryo and fertilization tests. Three copper reference tests and one interlaboratory comparison were successfully completed.

6. Future research will emphasize the development of a 7-day larval growth and survival test. In addition, methods for year-round spawning and test organism supply are currently being pursued.

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Section 2
Mysid Experiments

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Introduction

Mysid crustaceans are important components of estuarine and marine ecosystems (Nimmo *et al.*, 1977; Mauchline, 1980). Their ecological importance, amenability to laboratory culture and sensitivity to toxicants make them logical candidates for toxicity testing (Nimmo *et al.*, 1977; Benfield and Buikema, 1980; Gentile *et al.*, 1982; Breteler *et al.*, 1982; Lussier *et al.* 1985; Martin *et al.*, 1989). As part of an effort to develop new toxicity tests with indigenous Pacific coast marine organisms, the Marine Bioassay Project has conducted research on the kelp forest mysid *Holmesimysis costata* for six years (Linfield *et al.*, 1985).

Holmesimysis costata (=*Acanthomysis sculpta**, Holmes 1900) is a common near-shore mysid that ranges from La Jolla, California to the Queen Charlotte Islands, British Columbia (Holmquist, 1979). *H. costata* occurs in the surface canopy of the giant kelp, *Macrocystis pyrifera*, where it is ecologically important as a food source for fishes, particularly juveniles of kelp canopy species (Mauchline, 1980; Clark, 1971; Hobsen and Chess, 1976). The biology of this species has been described in relatively few publications (Clutter, 1967; Clutter, 1969; Green, 1970). This species is present in kelp forests year-round and there are no seasonal limitations on availability for collection (S. Turpen, unpublished data). Little is known about pollution effects on *H. costata* populations, but because the species occurs near shore, populations are likely to come into contact with discharged effluents. Laboratory tests have shown *H. costata* to be sensitive to municipal effluents (Hunt et al., 1989), and it is more sensitive to zinc and copper than other crustaceans described in the literature; these two trace metals are found in high concentrations in municipal effluents (Martin et al., 1981; Ahsanullah et al., 1981; Lussier et al., 1985; Tatem and Portzer, 1985; Reish and LeMay, 1988; Verriopoulos and Hardouvelis, 1988; Martin et al., 1989; SCCWRP, 1989). Other previous toxicity investigations with *H. costata** include short- and long-term tests with tributyltin (Davidson *et al.*, 1986) and short-term mortality tests with field-collected adults exposed to drilling fluids (Machuzak and Mikel, 1987). *H. costata* has also been recommended for dredged sediment elutriate tests (Reish and LeMay, 1988).

A 96-hour mortality test protocol has been completed for *H. costata* (Anderson et al., 1990; ASTM, in review) and the test is sensitive to a variety of toxicants (Martin et al., 1989; Singer et al., 1990). The objective of experiments described here is to refine the *H. costata* protocol to include a sublethal endpoint. A sublethal endpoint is necessary to satisfy criteria established for marine toxicity testing in the State of California, and makes possible the detection of toxicity at contaminant levels lower than those causing immediate mortality. Reproductive endpoints are impractical with *H. costata* because of the 70-day length of the mysid's life cycle; as an alternative, we have focused on growth inhibition as a sublethal indication of adverse effect. Growth represents the integration of a number of physiological processes and is relatively easy to measure.

* Note: Holmquist (1979, 1981) in a comprehensive review of northeast Pacific mysids associated with the genus *Acanthomysis*, has determined that the previous designation of this mysid as *Acanthomysis sculpta* was in error. She renamed it *Holmesimysis costata*, the type species for the new genus *Holmesimysis*. We consider this interpretation to be definitive (see also Mauchline, 1980).

Research was initiated on growth inhibition in juvenile *H. costata* in a previous phase of the Marine Bioassay Project (Hunt et *al.*, 1989). At that time, carapace lengths were measured on daily samples as mysids were cultured throughout their entire life cycle. Rapid growth was observed in nine-day-old mysids, but control survival of nine-day-olds was unacceptibly low in seven-day growth tests. Three-day-old mysids have been used in subsequent tests. Results from effluent tests, interlaboratory tests, and repetitive reference toxicant tests are presented here to demonstrate the current status of a preliminary seven-day protocol measuring the growth and survival of three-day-old mysids. The mysid 7 d growth and survival protocol is given in appendix II.

Methods

Facilities

Experiments were conducted between January and December 1990, 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). Detailed methods are given in the mysid toxicity test protocol (Appendix II).

Holmesimysis costata Toxicity Test Protocol

Methods for conducting 7 d growth and survival tests are described in the *H. costata* toxicity test protocol given in Appendix II. Additional methods used in protocol development are presented below. Performance evaluation of the protocol during this phase of the project consisted of: 1) repeated testing with a reference toxicant, 2) testing with a sample of complex effluent, and 3) interlaboratory testing with a reference toxicant.

Reference Toxicant Tests

Zinc (zinc sulfate, $ZnSO_4 \cdot 7H_2O$) was used as a reference toxicant in six repetitive tests and three interlaboratory tests. Zinc was selected as a reference toxicant because it is stable in solution, easy to analyze chemically, relatively non-hazardous to laboratory personnel, and present in high concentrations in municipal effluents (Anderson *et al.*, 1988).

Six 7 d zinc tests were conducted throughout the year to investigate test precision. Test concentrations were 0 (control), 10, 18, 32, 56, and 100 µg/liter zinc. Each treatment was replicated five times. Median lethal concentrations (LC50s) were generated for each test using the Trimmed Spearman-Karber method (Hamilton et al., 1977 and 1978). The coefficient of variation (standard deviation÷mean) among the LC_{50}s was calculated to evaluate

toxicity test precision. No Observed Effect Concentrations (NOECs) were calculated for growth and survival data using Analysis of Variance (ANOVA) followed by Dunnett's Multiple Comparison test. To generate NOEC values for growth data, only those concentrations less than or equal to the mortality NOEC were analyzed. Growth inhibition concentrations (ICp's) were not calculated because there was less than a 10% mean reduction in mysid total length in affected treatments relative to controls. Detailed methods for statistical analysis are given in Appendix 2.

Effluent Test

One test was conducted with bleached Kraft mill effluent to provide a preliminary indication of the suitability of the 7 d protocol for testing complex effluents. Effluent was prepared from a lyophilized (freeze dried) sample that was reconstituted by mixing with 1-µm- filtered seawater. Effluent test concentrations were 0 (control), 0.5, 1.0, 2.0, and 4.0%. No salinity adjustment was necessary. As above, NOEC's were calculated for growth and survival endpoints, and an LC_{50} was calculated for the survival data.

Interlaboratory Testing

Three interlaboratory tests were conducted using zinc sulfate. One test was conducted with Dr. Chris Langdon of Oregon State University (OSU). Two tests were conducted with Dr. Tom Dean of Coastal Resources Associates (CRA) in Carlsbad, California.

In early October, 1990, juvenile mysids were sent by overnight air delivery to the Oregon State University laboratory at the Hatfield Marine Science Center in Newport, Oregon. Mysids from the same cohort were kept in coolers at MPSL to simulate transport conditions. Tests were initiated on the same day at both laboratories when the mysids were three days old. Tests at both laboratories used the same test containers (tissue culture flasks), the same seawater (MPSL seawater held in polyethylene containers for two days prior to testing), the same toxicant (zinc sulfate supplied by MPSL) and the same food for test mysids (newly hatched RAC-II *Artemia* supplied by OSU). Mysids at MPSL were measured after the test using a projecting compound microscope, mysids at OSU were measured on a grid viewed at 50x on a dissecting microscope.

Later in October, 1990, another cohort was randomly divided, and half the juveniles were sent to CRA by overnight delivery. The other half were kept in coolers at MPSL. Both laboratories used seawater and zinc from MPSL. Unfortunately, the temperature control unit at CRA failed during the test, resulting in increased temperature and significant mortality. The results of this comparison are not included in this report.

The CRA interlaboratory test was repeated in November, 1990, this time using the offspring from transported gravid female mysids. Gravid females were held in coolers at MPSL to simulate transport conditions. Juveniles were released from the females at CRA and MPSL, and the test was begun when juveniles were three days old. This test was also unsuccessful due to unexplained excessive mortality in all treatments at CRA. The mortality data from the MPSL portion of this test are presented in the Results and Discussion section.

Species Comparison

Dr. Chris Langdon and his staff at OSU, in conjunction with the US EPA, have been developing toxicity testing techniques with another mysid, *Mysidopsis intii*. *M. intii* has a shorter life-cycle than *H. costata* and may be amenable to toxicity testing using reproductive endpoints. As part of an initial comparison of species sensitivity, we conducted a 7 d growth and survival test with *M. intii* concurrently with the *H. costata* interlaboratory test. The *M. intii* test used zinc concentrations of 0 (control), 10, 18, 32, 56, 100, and 180 µg/liter, with five replicates per treatment. Tissue culture flasks were used as test containers for both species, and both species were tested at the same density (eight mysids per 200 ml of test solution), with the same food and feeding rate (50 RAC-II *Artemia* nauplii per mysid per day). *M. intii* test temperature was 20° ± 2°C, *H. costata* test temperature was 13° ± 1°C. Total length of all mysids was measured with a compound projecting microscope. All data were analyzed as described in the *H. costata* protocol (Appendix 2).

Physical/Chemical Measurements of Test Solutions

Physical/chemical parameters were measured in test solutions from one random replicate of each toxicant concentration and control at the beginning and end of each test and before each renewal. Dissolved oxygen and pH were measured with an Orion EA 940 Ion Analyzer accurate to ± 0.01 mg/liter or pH unit. Salinity was measured with an Atago refractometer acurate to ± 1 ‰, and temperature was measured using a mercury thermometer accurate to ±0.5°C and factory calibrated to NIST standards. Dissolved oxygen probes were calibrated to water-saturated air, pH probes were calibrated to buffer solutions of pH 7.00 and 10.00, and the refractometer was calibrated to a 33.99 ‰ seawater standard originally measured on a Beckman salinometer calibrated to Wormley water. The ranges for physical/chemical measurements from each test are given below (Table 5). All measurements were within normal ranges, except for temperature measurements which varied from the target temperature by 1.5° to 2.5°C, rather than by ±1°C as specified in the protocol. Temperature variation was caused by an inaccurate incubator thermistat.

Table 5. Ranges of physical/chemical data for mysid experiments. Each reported range is for all measurements from a given test. No physical/chemical measurements were taken during the 4/9/90 zinc test or the 4/30/90 effluent test. Test conditions for these two tests were similar to those for other tests reported.

Toxicant	Date	Dissolved O_2 (mg/l)	pH	Salinity (ppt)	Temperature (°C)
Zinc	1/31/90	7.60 - 8.06	7.57 - 7.80	32 - 33	11.5
Zinc	2/2/90	7.61 - 7.79	7.84 - 7.88	33	11.5
Zinc	4/16/90	6.89 - 7.28	7.77 - 7.86	32 - 33	13.0 - 14.5
Zinc *H. costata*	10/9/90	6.49 - 7.40	8.00 - 8.29	31 - 33	10.5 - 13.0
Zinc *M. intii*	10/9/90	7.16 - 7.76	7.71 - 7.88	34	18.0 - 21.0
Zinc	11/13/90	6.38 - 7.31	7.77 - 7.99	34 - 35	11.0 - 13.0

Reference Toxicant Chemical Verification

As in our previous work, reference toxicant concentrations were sampled for chemical analysis at the beginning and end of all tests. Chemical analyses based on the methods of Bruland *et al.* (1979) were performed on Perkin Elmer model 603 or 5000 atomic absorption spectrometers at the DFG State Mussel Watch Laboratory at Moss Landing, California. Data from chemical analyses of test solutions are given below (Table 6). Measured concentrations closely matched nominal concentrations in only two of the tests. In the other six tests, measured values were higher than nominal values by an average of about 40%. This may have been caused by inaccurate weighing or dilution of the toxicant at MPSL, by inaccurate measurement at the chemistry laboratory, or by contamination of test containers, sample vials, or analytical equipment. Because all discrepancies involve higher measured concentrations than nominal concentrations, and because zinc is so ubiquitous in the environment, we suspect that contamination of test solutions and samples was responsible for the observed differences. The actual concentrations to which the animals were exposed probably increased from the nominal level toward the measured level during the course of the test. Because of uncertainty regarding the timing and extent of possible contamination, all results are presented using nominal concentration values.

Table 6. Nominal and chemically measured zinc concentrations for all mysid tests. Measured concentrations for each test are given in columns below their respective nominal concentrations. Measured concentration values are means from samples taken at the beginning, end, and at each water change from one randomly chosen replicate of each test concentration. Dashed spaces indicate concentrations not included in a particular test. All values are in µg/L. ND = not detected, concentration was below the detection limit.

Test Date	**Test Solution Zinc Concentrations**						
Nominal Concentrations:	**0**	**10**	**18**	**32**	**56**	**100**	**180**
1/31/90	5	14.5	19	40.5	70	118	-
2/2/90	ND	9	31.5	48	77.5	113.5	-
4/9/90	ND	10	21	32	58	104	-
4/16/90	2	-	22	35	62	109	-
10/9/90 MPSL *H. costata*	9.5	18	31	49	64	124	-
10/9/90 OSU *H. costata*	16	17	24	77	144	116	-
10/9/90 MPSL *M. intii*	8.5	18	31.5	44	78.5	115.5	224
11/13/90	3	48	23	42	57	101	-

Results and Discussion

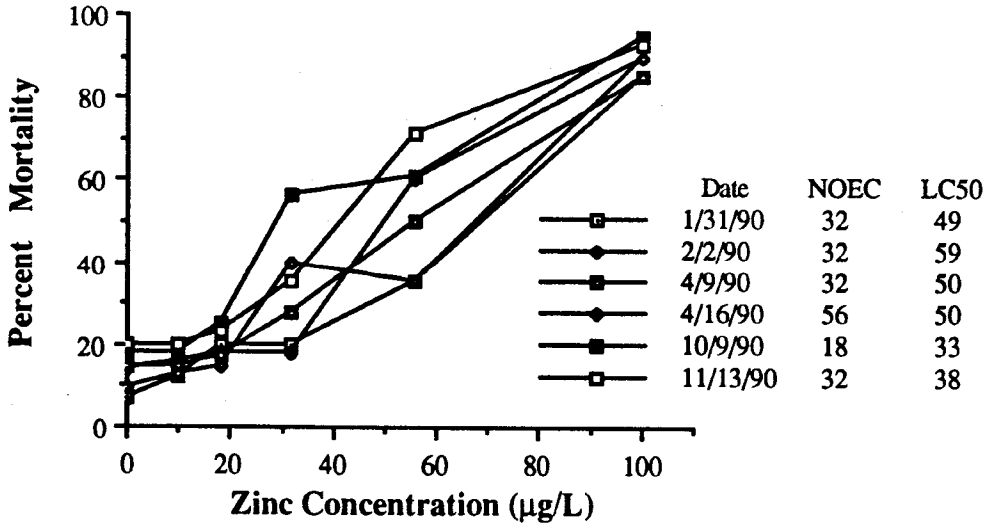
Reference Toxicant Tests

The *H. costata* toxicity test is being developed for routine use in a regulatory setting, where sensitivity, precision, success rate, and logistical feasibility are the main objectives. The reference toxicant tests conducted during Phase 5 of the Marine Bioassay Project were designed to estimate sensitivity to a common toxicant and precision between repeated trials of the *H. costata* 7 d growth and survival protocol.

The sensitivity of the protocol is indicated by six tests with a reference toxicant (zinc sulfate) that produced mean NOECs of 33.7 (± 12.3) µg/liter for survival and 19.2 (±7.9)* µg/liter for growth (Table 7). These compare with NOECs of 112 and 22 µg/liter zinc for *Mysidopsis intii* 25 d survival and 7 d growth, respectively (Langdon, 1989), and 120 µg/liter for *Mysidopsis bahia* 29 d survival and reproduction (Lussier et al., 1985). The mean *H. costata* LC50 was 46.6 µg/liter, compared to 499 µg/liter for *Mysidopsis bahia* and 456 µg/liter for *Cancer magister* (Martin et al., 1981). The lower values for *H. costata* indicate that it is more sensitive to zinc than other crustaceans reported in the literature. Previous research indicates that *H. costata* is sensitive to a number of other toxicants, including mercury, PCBs, DDS (Tatem and Portzer, 1985), copper (Martin et al., 1989), and tributyltin (Davidson *et al.*, 1986).

* The mean growth NOEC is for comparison only, because values with ≤ and ≥ signs were included in the calculation. See Table 7.

Figure 6. Dose response curves for six 7 d zinc toxicity tests with *H. costata*. All points are means of five replicates per treatment.



Precision of the 7 d *H. costata* protocol is indicated by intra- and interlaboratory variability among reference toxicant tests (Table 7, Figure 6)). The coefficient of variation (CV) among six zinc toxicity tests conducted at MPSL was 20%, with LC50 values ranging from 33 to 59 µg/liter. This indicates relatively good intralaboratory precision as compared to CV values available from the literature. A mean CV of 25.0% (range: 0 to 135%) was derived from 16 intralaboratory trials of acute tests using *Mysidopsis bahia, Daphnia pulex, Daphnia magna, Ceriodaphnia dubia*, or *Pimephales promelas* (Rue et al., 1988). A mean CV of 24.5% (range: 1.8 to 46.4%) was derived from 12 intralaboratory trials of chronic tests using *Mysidopsis bahia, Champia parvula, Arbacia punctulata, Cyprinodon variegatus*, and *Menidia beryllina* (Morrison et al., 1989).

Because growth was measured as total length, rather than as change in length during the test period, differences in length between treatments were small compared to the total length of the mysids. This did not affect determination of NOEC values, but meaningful ICp values could not be calculated because the difference between affected concentrations and controls was generally less than 10% of the mean total length. Therefore, the CV for the growth endpoint could not be calculated. The range of growth NOECs was ≤ 10 µg/liter to ≥ 32 µg/liter (Table #). Interlaboratory precision is discussed below.

Table 7. Summary statistics from 7-day growth and survival toxicity tests using the mysid *Holmesimysis costata*. Zinc values are in µg/L; effluent values are in percent. LC_{50} = median lethal concentration; NOEC = No Observed Effect Concentration; CL = confidence limits; CV = coefficient of variation (s.d. ÷ mean); MPSL = Marine Pollution Studies Laboratory; OSU = Oregon State University.

Test Date	Toxicant	Growth NOEC	Survival NOEC	Survival LC_{50} (95% CL)	
1/31/90	Zinc	≤ 10	32	49 (43, 56)	
2/2/90	Zinc	≥ 32	32	59 (52, 67)	For six zinc tests:
4/9/90	Zinc	18	32	50 (44, 57)	Mean LC_{50} = 46.6
4/16/90	Zinc	18	56	50 (44, 57)	CV = 20.1%
10/9/90	Zinc	≥ 18	18	33 (29, 39)	
11/13/90	Zinc	-	32	38 (33, 44)	
4/30/90	Effluent	≥ 2	2	3.4 (2.7, 4.4)	
10/9/90	Zinc	≥ 18	18	33 (29, 39)	Interlab Test: MPSL
10/9/90	Zinc	≥ 32	32	22 (17, 27)	Interlab Test: OSU
					Interlab Mean LC50 = 27.4
					Interlab CV = 30.5%

Sublethal effects are included in the protocol only to determine if organisms are affected at lower concentrations than those causing death. Growth NOECs for *H. costata* were calculated using only test concentrations that showed no significant mortality. This is consistent with the statistical approach used on other multi-endpoint tests recommended by the US EPA (Weber et al., 1988). Calculating NOECs using concentrations with high mortality can mask toxicant effects on growth; our observations indicate that in containers with high mortality, surviving mysids tend to be large. Perhaps larger individuals are more tolerant, or perhaps survivors benefit from the death of their cohorts, either through cannabalism or because of decreased interference during feeding. Feeding rates were adjusted daily to account for mortality, so food per mysid remained constant throughout the tests. In the January and April tests, growth was depressed in medium concentrations, then increased in higher concentrations where significantly fewer mysids were measured (Table 8).

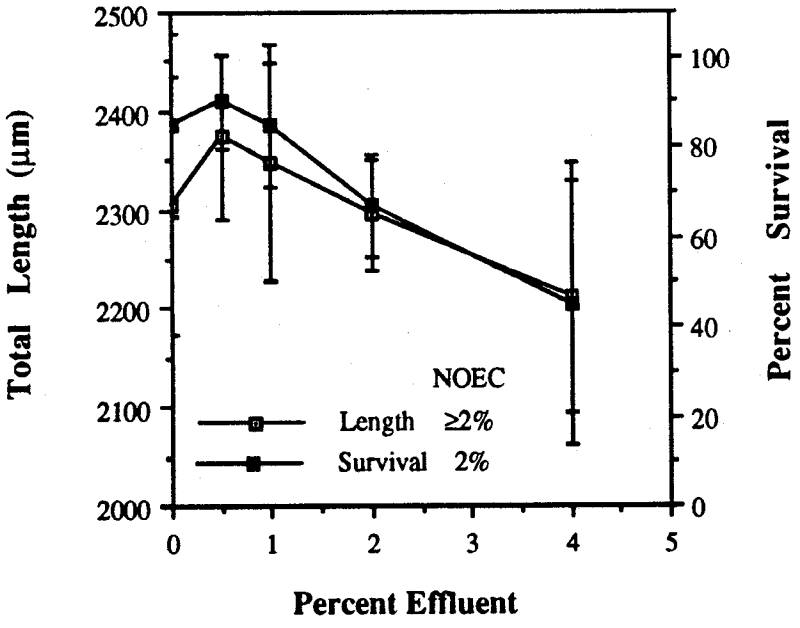
Table 8. Mysid total length data from five zinc tests. Values are mean total length (in microns) of mysids from five replicate containers in each of five toxicity tests using zinc sulfate as the toxicant. Mean length of mysids from the No Observed Effect Concentrations (NOECs) are in bold script. NOEC values are in µg/liter. There was no significant effect on growth in the February or October tests. Mean length values from concentrations having significant mortality are in italics; these were not used in calculation of the growth NOEC (see text). All test dates are 1990. Data from test #1 are carapace length, all others are total length.

			Zinc Concentration (µg/L)					
Test	**Date**	**NOEC**	**0**	**10**	**18**	**32**	**56**	**100**
# 1*	2/2 - 9	≥32	717	714	700	710	*697*	*699*
# 2	1/31 - 2/7	≤10	2696	**2472**	2398	2478	*2538*	*2275*
# 3	4/9 - 16	18	2511	2398	**2436**	2402	*2559*	*2533*
# 4	4/16 - 23	18	2461	2367	**2491**	2256	2265	*2300*
# 5	10/9 - 16	≥18	2179	--	2167	*2238*	*2263*	*2200*

Effluent Test

In the effluent test, response curves for growth and survival were very similar, but the growth NOEC was higher because of greater variability in the growth data (Figure 7). The *H. costata* 7 d growth and survival protocol performed well in testing complex effluent. The effluent was lyophilized bleach Kraft mill effluent, and therefore contained a lower particle load than would be characteristic of many municipal sewage effluents. Past 96 h *H. costata* tests were conducted successfully in municipal effluents, and we do not anticipate problems in conducting the 7 d protocol in other complex effluents. Future evaluation of this protocol will emphasize additional complex effluent testing with municipal sewage effluents.

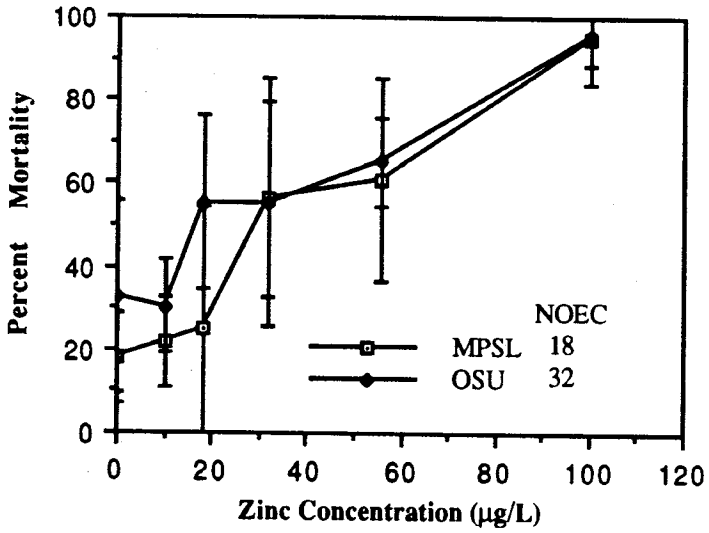
Figure 7. Response curves for measurements of length and mortality in one 7 d effluent toxicity test. Points are means (± 1 s.d.) of five replicates per treatment.



Interlaboratory Tests

MPSL conducted two interlaboratory trials of the *H. costata* protocol with Coastal Resources Associates (CRA). The MPSL data for the November test is included with the reference toxicant data in Table 7 and Figure 6. The two tests at CRA were unsuccessful; one due to temperature control equipment failure, and the other due to unexplained wide-spread mortality early in the test. Shipping and handling stress did not appear to be the primary cause of mortality in the latter test, because excess mysids not used in the test survived well at the CRA laboratory (Dave Guthoff, personal communication).

Figure 8. Response curves for mortality data from two 7 d zinc toxicity tests conducted concurrently at two separate laboratories. Points are means (± 1 s.d.) of five replicates per treatment. OSU = Oregon State University, MPSL = Marine Pollution Studies Laboratory.

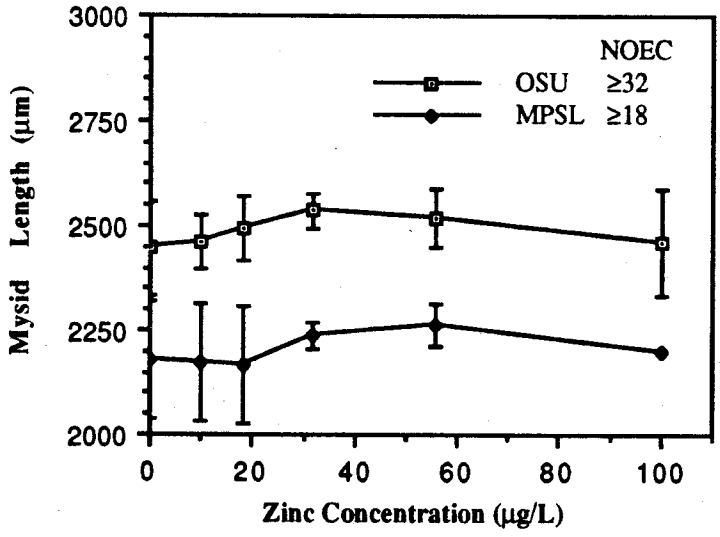


The tests conducted concurrently at MPSL and Oregon State University produced an interlaboratory coefficient of variation (CV) of 30.5% (Table 7); the LC50s were 33 and 22 µg/liter, respectively. This CV for one interlaboratory test compares with a mean interlaboratory CV of 31.1% (range: 0 to 110%) for 13 separate trials of acute tests with *Daphnia magna, D. pulex, Mysidopsis bahia*, and *Pimephales promelas* (Rue et al., 1988). A mean CV of 46.9% (range: 22 to 104%) was derived from 12 interlaboratory trials of chronic tests using *Mysidopsis bahia, Champia parvula, Arbacia punctulata, Cyprinodon variegatus*, and *Menidia beryllina* (Morrison et al., 1989).

The response curves are similar between the MPSL and OSU tests, except that control mortality was higher (33%) in the OSU test data (Figure 9). Because control mortality was high, elevated mortality in low toxicant concentrations was not found to be significantly different from the control. The resulting OSU NOEC (32 µg/liter) was higher than the MPSL NOEC (18 µg/liter). Test acceptability criteria in the *H. costata* protocol require that control mortality not exceed 20%. Mysid handling and transport will be further investigated in attempts to decrease control mortality at laboratories receiving mysid shipments.

Growth data from the two laboratories followed similar response curves, but the two curves were offset, with OSU mysids consistently measuring about 250 µm longer. This consistency indicates that the difference between laboratories was probably more a result of measurement technique than of actual growth differences. Different types of microscopes and measuring equipment were employed at each laboratory. Emphasis will be placed on measurement intercalibration in future interlaboratory tests. There was no significant toxicant effect on growth in either test.

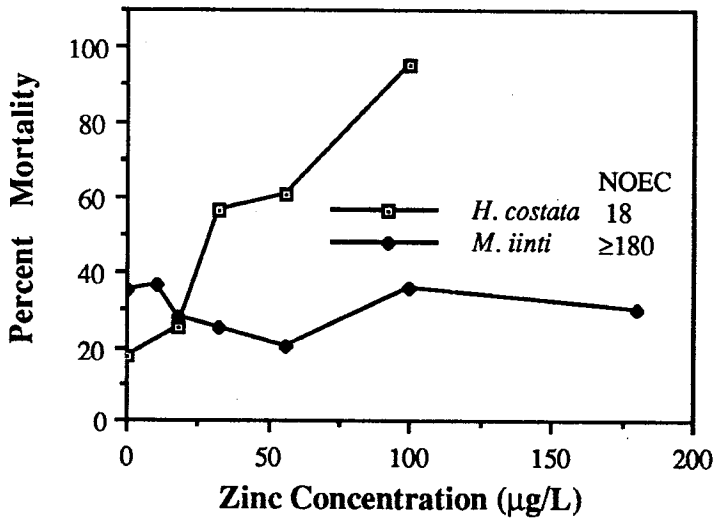
Figure 9. Response curves for length data from two 7 d zinc toxicity tests conducted concurrently at two separate laboratories. Points are mean total length (± 1 s.d.) of five replicates per treatment. OSU = Oregon State University, MPSL = Marine Pollution Studies Laboratory.



Species Sensitivity Comparison

In tests conducted simultaneously at MPSL, there was more than one order of magnitude difference in sensitivity to zinc between *H. costata* and the epibenthic mysid *Mysidopsis intii*; the NOECs for mortality were 18 and ≥180 µg/liter, respectively (Figure 10). *M. intii* were tested at 20°C, 7° warmer than *H. costata*. The warmer temperature should have theoretically increased the toxicant uptake rate and sensitivity for *M. intii* relative to *H. costata*. No comparison of LC50s was possible because there was no 50% response by *M. intii*. There was no significant effect on growth in *M. intii* at concentrations up to 180 µg/liter, nor on *H. costata* in concentrations up to 100 µg/liter in this test. A previous test at OSU found significant effects on growth at 50 µg/liter (NOEC = 22 µg/liter; Langdon et al., 1989). This apparent difference in sensitivity between the two *M. intii* tests may be due to differences in handling (including shipment), dilution water, test container size, or stocking density. *Artemia* for the MPSL *M. intii* test were supplied by OSU, and newly hatched nauplii were used. Nutrition, especially the inclusion of the harpacticoid copepod *Tigriopus californicus*, has been found to be an important factor in the growth rate of this epibenthic mysid (Langdon et al., 1989); *Tigriopus* were not included when feeding *M. intii* in the MPSL test. Although *M. intii* was less sensitive to zinc than *H. costata*, this may not be the case for all toxicants. *M. intii* has a shorter life cycle than *H. costata*, making reproductive endpoints more feasible. EPA sponsored research on *M. intii* is continuing in conjunction with OSU at the Hatfield Marine Science Center, Newport Oregon.

Figure 10. Dose response curves for mortality data from 7 d zinc toxicity tests with *Holmesimysis costata* and *Mysidopsis intii*. Tests were conducted concurrently at MPSL. All points are means of five replicates per treatment.



Summary

1. The purpose of this research was to further develop the toxicity test protocol for *Holmesimysis costata* to include growth as a sublethal endpoint. The seven day growth and survival protocol was tested repeatedly with a reference toxicant (zinc sulfate) and once with a representative effluent (BKME).

2. *Holmesimysis* was found to be more sensitive to zinc than other crustaceans described in the literature, including other mysids currently used or proposed for use in routine toxicity assessment.

3. Intralaboratory precision (given as the CV of test LC50s) was 20% for six zinc tests conducted at MPSL during the year. Interlaboratory precision was 31% in one test conducted concurrently at two laboratories. Both estimates of precision compare favorably with estimates cited in the literature.

4. Further protocol development should focus on continued effluent testing to verify the test's suitability for effluent toxicity assessment, and continued interlaboratory testing to verify that transported mysids can be used successfully under a variety of laboratory conditions.

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Section 3

Giant Kelp Experiments

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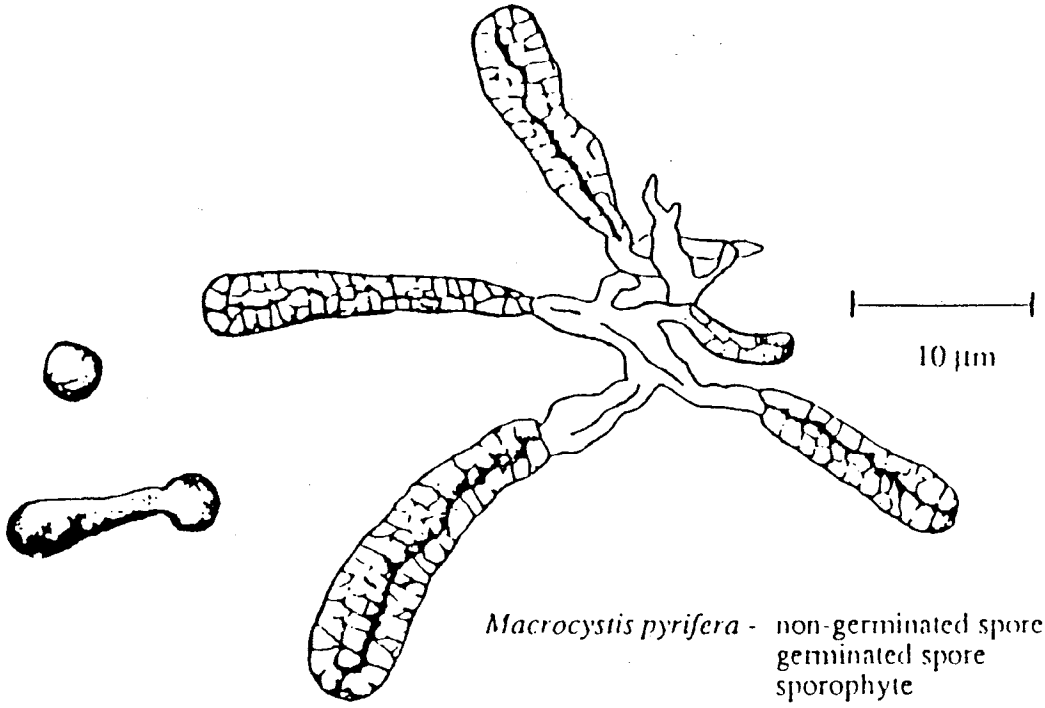


Macrocystis pyrifera - non-germinated spore
germinated spore
sporophyte

Introduction

Forests of giant kelp, *Macrocystis pyrifera,* extend from Baja California to central California. Kelp forests harbor a rich diversity of marine life and are an important source of primary production to the nearshore marine ecosystem. *Macrocystis* was chosen as a macroalgal toxicity test species for the Marine Bioassay Project because of its economic and ecological importance, and amenability to laboratory culture (Linfield *et al.*, 1985). Giant kelp has been cultured extensively in laboratory studies and its life history has been well described (North, 1971, 1976; Luning and Neushul, 1978; Luning, 1980; Deysher and Dean, 1984; see review by Foster and Schiel, 1985). Its use in toxicity studies has been limited. Early studies by Clendenning (1958, 1959, 1960) focused on the effects of toxicants on photosynthesis in adult blades. Smith and Harrison (1978) investigated the effects of copper chloride on growth of kelp gametophytes. Studies on the trace metal requirements of microscopic stages of kelp by Kuwabara and North (1980) and Kuwabara (1980, 1981) are applicable to the interpretation of toxicity test results. James *et al.* (1987) used the microscopic stages of several laminarian species, including *Macrocystis*, to assess the toxicity of PCB's and hydrazine.

Two kelp toxicity tests were developed during Phases 1 and 2 of the Marine Bioassay Project: a short-term 48-hour test, and a longer term 15 to 20-day test. The 48-hour test has two endpoints: germination of the haploid kelp zoospores, and initial growth of the "germ-tube" of the developing gametophyte. The longer term test focuses on sporophyte "production" and is used for comparison with the short-term test. Sporophytes are the product of sexual reproduction between male and female gametophytes.

Results of initial experiments comparing the short- and long-term tests indicated that the 48-hour toxicity test was more appropriate for use in routine effluent testing. The long-term test was time consuming, and cultures were susceptible to microalgal contamination during the 15 to 20-day test. Emphasis was placed on the continued development of the 48-hour test using reference toxicants and complex effluents. These studies demonstrated that this test was suitable for assessing effluent toxicity (Anderson and Hunt, 1988).

Research with *Macrocystis* during Phase 3 of the project focused on testing of complex effluents and repeated replicate reference toxicant tests (Hunt *et al.*, 1989). Results of the effluent tests showed some variability between tests for the same effluent source. Comparisons of quarterly copper reference toxicant tests indicated temporal variability in the response of kelp to copper. Proposed sources of variability included seasonal variability in the sensitivity of kelp to toxicants and variability in the chelation capacity of dilution water used in toxicity tests (Anderson *et al.*, 1990a). An interlaboratory comparison produced consistent results between laboratories using copper chloride as a reference toxicant.

Kelp research during Phase 4 emphasized continued refinement of the *Macrocystis* 48-hour protocol. Interlaboratory experiments demonstrated that laboratories having no previous experience with the 48-hour kelp protocol could successfully conduct toxicity tests using complex effluent. Two separate interlaboratory tests were completed: one with a sewage effluent and one with bleached kraft mill effluent.

Copper reference tests were conducted concurrently with the complex effluent interlaboratory tests. Other experiments investigated different photoperiods, test containers, and handling procedures to determine effects of these variables on test performance.

Kelp research during Phase 5 of the project consisted primarily of interlaboratory testing of the 48-hour kelp protocol with sewage effluents from four municipal waste dischargers. Copper reference tests were conducted concurrently with all interlab tests, and a fifth interlaboratory test was conducted with copper only. In addition, two toxicity tests were conducted with sodium azide, an alternative reference toxicant to copper chloride.

Methods

All experiments were conducted between January and December 1990 at the Marine Pollution Studies Laboratory (MPSL). Detailed methods for the kelp experiments discussed in this section are given in the *Macrocystis* protocol (Appendix III).

Interlaboratory Testing

Four interlaboratory comparisons were conducted using split effluent samples and reference toxicants. A separate interlaboratory test was also conducted using copper only. General methods for all tests followed the procedures for interlaboratory tests given in Anderson *et al.* (1990b). The first interlaboratory test was conducted in March, 1990, using effluent from the County of Orange waste treatment facility. Participating laboratories were: MPSL, ENSECO Chemical Research Laboratory (ENSECO), Aquatic Bioassay and Consulting Laboratories (ABC), and Coastal Resources Associates (CRA). The second and third comparisons were conducted in May, 1990, first using effluent from the City of Los Angeles' Hyperion waste treatment facility, and then using effluent from the County of Los Angeles JWPCP waste treatment facility. Participating laboratories were: MPSL, Environmental and Energy Services Company Inc. (ERCE), and the Hyperion Waste Treatment Plant biology laboratory, The fourth interlaboratory comparison was conducted in December, 1990 between MPSL and ERCE using effluent from the City of San Diego waste treatment facility.

For three of the interlaboratory effluent comparisons, *Macrocystis* sporophylls were collected from adult plants at Granite Canyon the day before the experiments began. The sporophylls were retained in coolers overnight for experiments the next day. For the San Diego effluent comparison, the sporophylls used by both laboratories were collected from adult plants at Granite Canyon, Monterey. Half of the sporophylls were shipped in coolers (12 °C) to ERCE via overnight carrier, and half were held in coolers for use at MPSL.

For all interlaboratory experiments, 24-hour composite effluent samples were collected the day before the experiment. Split samples were shipped in coolers via overnight courier to the participating

laboratories (temperature = approximately 5 °C). All laboratories used their own dilution waters and followed the methods described in the giant kelp 48-hour toxicity test protocol (Anderson et al. 1990b).

The effluent concentrations tested in the Orange County effluent comparison were 0 (dilution water), 0 (brine control), 0.32, 0.56, 1.0, 1.8, 3.2, and 5.6% effluent. A separate copper chloride reference toxicant test was conducted at both laboratories concurrently with the effluent tests. The nominal copper concentrations tested were: 0, 5.6, 10, 18, 32, 56, 100, and 180 µg/liter.

The effluent concentrations tested in the Los Angeles City (Hyperion) effluent comparison were 0 (dilution water), 0 (brine control), 1.8, 3.2, and 5.6, 10, and 18% effluent. The nominal copper reference concentrations were as above.

The effluent concentrations tested in the Los Angeles City effluent comparison were 0 (dilution water), 0 (brine control), 0.56, 1.0, 1.8, 3.2, 5.6, and 10% effluent. The nominal copper reference concentrations were as above.

The effluent concentrations tested in the San Diego effluent comparison were 0 (dilution water), 0 (brine control), 1.0, 1.8, 3.2, 5.6, 10, and 18% effluent. The nominal copper reference concentrations were as above.

A separate interlaboratory comparison was conducted between MPSL and Coastal Resources Associates (CRA) in September, 1990, using only copper reference toxicant. The sporophylls used for these experiments were collected from the kelp population at Granite Canyon and stored and shipped as above. Both laboratories used their own dilution waters and tested the same concentrations listed above.

Sodium Azide Tests

Two tests were conducted using sodium azide (NaN_3), an alternative reference toxicant. Both tests used azide concentrations of 0, 5, 50 and 100 mg NaN_3/liter.

Statistics

No Observed Effect Concentrations (NOEC's) in these tests were calculated using ANOVA followed by Dunnett's multiple comparison. In order to assess between-test precision, EC_{50}'s were calculated for germination data using the Trimmed Spearman-Karber method (Hamilton et al., 1977). Point estimates for the germ-tube growth data were calculated using the Inhibition Concentration Percentage (ICp) approach developed for the U.S. EPA (Norberg-King, 1988). Different point estimates were calculated for effluent, copper and sodium azide because of variation in the toxicity of these substances. Because the effluents were generally less toxic than the copper, concentrations resulting in 10% growth inhibition (IC_{10}) were calculated for germ-tube growth data in the effluent tests. Concentrations resulting in 40% growth inhibition (IC_{40}'s) were calculated for the germ-tube growth data in the copper tests. Concentrations resulting in 10 % (IC10s) or 25 % (IC_{25}'s) were calculated for the germination and growth data, respectively in the sodium azide tests. In each case, we attempted to derive the highest point

estimate possible to allow for comparison of the response curves. Because the different effluents and reference toxicants had variable toxicity to kelp, it was necessary to calculate different point estimates. Between-test precision was assessed by calculating a Coefficient of Variation (CV) of the EC_{50}'s, IC_{10}'s, IC_{40}'s, and IC_{25}'s, where appropriate. **Note:** Statistical methods for analyzing toxicity test data are continuing to evolve. Standard operating procedures for deriving point estimates and NOEC's for discrete and continuous data have not been finalized. The Marine Bioassay Project has recently consulted with a statistician in order to develop a standard operating procedure for all MBP protocols. These procedures will be included in the next iteration of the MBP protocol manual.

Analysis of Copper and Azide Test Concentrations

Copper test concentrations were measured at the beginning and end of all tests using a Perkin Elmer model 5000 atomic absorption spectrophotometer, following methods described by Bruland et al. (1979). Mean measured copper concentrations were within 12% of nominal concentrations (range of mean differences of measured from nominal concentrations = 6 - 29 %).

Sodium azide stock concentrations were measured with an ultraviolet spectrophotometer using methods developed at MPSL by Debra Smalheer. The accuracy of this method improved at concentrations greater than 5 mg/liter. Measured concentrations overall were within 24% of nominal concentrations (range of mean differences of measured from nominal concentrations = 0 - 62%). However, at 1 and 5 mg/liter the mean measured concentrations were within 55% of nominal, while at concentrations greater than 5 mg/liter the mean difference of measured from nominal values declined to 8%.

Results and Discussion

Interlaboratory Tests

Results of all of the Phase 5 interlaboratory tests are summarized in Tables 1 and 2. Table 9 summarizes the germination data; Table 10 summarizes the germ-tube growth data for the same tests. These tables give the results of all of the interlaboratory tests in terms of No Observed Effect Concentrations and point estimates. As described above, the point estimates are either EC_{50}s, IC_{10}s, IC_{25}s or IC_{40}s depending on the endpoint and toxicant being compared. For the purpose of comparing the precision of the interlaboratory tests, the results of individual interlaboratory comparisons with specific effluents (and reference toxicants) are discussed in terms of point estimates only. The point estimates derived by each participating laboratory for the two endpoints are compared using a coefficient of variation.

Table 9. Summary of Phase 5 kelp interlaboratory test results (**germination data**). All effluent concentrations are given as percent effluent; all copper concentrations are given as µg/liter.

Test Date	Interlaboratory Comparison	Laboratory	NOEC	EC_{50} (95% CI)	Coefficient of Variation
March 90	Orange County	MPSL	>5.60	*	
	Effluent (%)	CRA	>5.60	*	
		ENSECO	>5.60	*	nc
	Orange County	MPSL	10.0	46.9 (39.3, 56.0)	
	Copper (µg/L)	CRA	32.0	46.2 (36.7, 58.1)	
		ENSECO	32.0	*	>1.1%
May 90	Hyperion	ERCE	3.2	*	
	Effluent (%)	MPSL	10.0	17.2 (15.1, 19.7)	
		HYPERION	>18.0	*	nc
	Hyperion	ERCE	32.0	164.2 (140.5, 191.9)	
	Copper (µg/L)	MPSL	18.0	60.6 (52.0, 70.7)	
		HYPERION	18.0	67.9 (51.1, 90.2)	59.3%
May 90	JWPCP	MPSL	>10.0	*	
	Effluent (%)	ERCE	1.0	3.7 (3.3, 4.2)	
		HYPERION	>10.0	*	nc
	JWPCP	MPSL	18.0	112.0 (98.8, 127.1)	
	Copper (µg/L)	ERCE	56.0	64.5 (43.2, 96.3)	
		HYPERION	32.0	158.0 (132.8, 188.0)	41.9%
December 1990	San Diego	MPSL	10.0	13.8 (12.8, 14.9)	
	Effluent (%)	ERCE	5.6	8.4 (7.2, 9.8)	34.5%
	San Diego	MPSL	56.0	77.7 (65.2, 92.6)	
	Copper (µg/L)	ERCE	18.0	*	nc
September 1990	CRA	MPSL	56.0	127.4 (106.7, 161.2)	
	Copper (µg/L)	CRA	56.0	114.8 (101.8, 129.4)	7.4%

* No EC_{50} calculated because response was less than 50%
nc = not calculated (insufficient numbers to calculate a coefficient of variation)

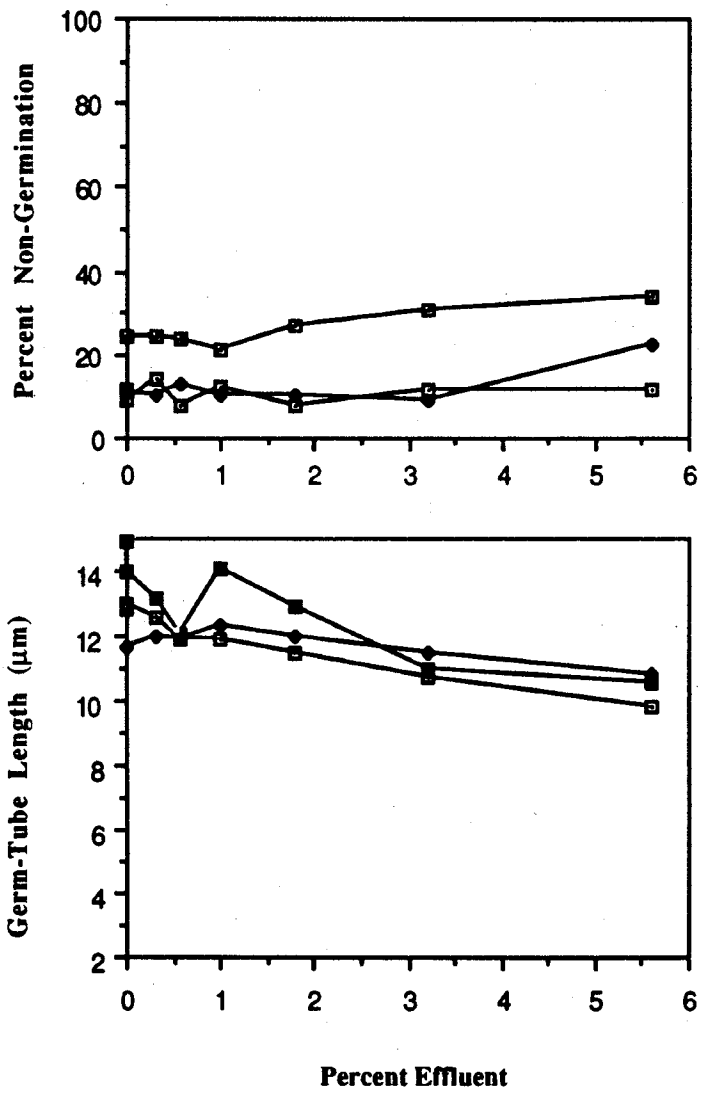
Table 10. Summary of Phase 5 kelp interlaboratory test results (**germ-tube growth data**). All effluent concentrations are given as percent effluent; the IC10 (= the concentration resulting in 10% growth inhibition) was calculated for effluent tests. All copper concentrations are given as µg/liter; the IC40 (= 40% growth inhibition) was calculated for copper tests.

Test Date	Interlaboratory Comparison	Laboratory	NOEC	IC_{10} (eff.) IC_{40} (Cu) (95% CI)	Coefficient of Variation
March 90	Orange County	MPSL	1.0	1.6 (0.5, 2.6)	
	Effluent (%)	CRA	>5.6	3.7 (1.7, 5.4)	
		ENSECO	1.8	0.5 (0, 1.2)	87.9%
	Orange County	MPSL	5.6	122.7 (83.1, 173.2)	
	Copper (µg/L)	CRA	32.0	117.8 (98.1, 144.5)	
		ENSECO	18.0	104.1 (92.4, 121.1)	8.4%
May 90	Hyperion	ERCE	1.8	0.85 (0.3, 1.2)	
	Effluent (%)	MPSL	5.6	0.66 (0, 2.2)	
		HYPERION	>18.0	*	17.9%
	Hyperion	ERCE	<5.6	99.1 (85.2, 116.5)	
	Copper (µg/L)	MPSL	10.0	43.1 (28.2, 64.1)	
		HYPERION	18.0	68.7 (47.8, 94.3)	39.9%
May 90	JWPCP	MPSL	5.6	3.9 (0, 7.7)	
	Effluent (%)	ERCE	<0.56	†	
		HYPERION	>10.0	1.3 (0, 6.9)	70.9%
	JWPCP	MPSL	18.0	70.7 (52.3, 111.3)	
	Copper (µg/L)	ERCE	18.0	91.3 (53.3, 175.2)	
		HYPERION	32.0	134.2 (95.6, 175.1)	32.8%
December 1990	San Diego	MPSL	<1.0	0.5 (0, 1.3)	
	Effluent (%)	ERCE	1.8	2.4 (0.9, 4.1)	89.7%
	San Diego	MPSL	5.6	88.0 (66.1, 99.7)	
	Copper (µg/L)	ERCE	5.6	45.3 (33.6, 54.1)	45.3%
September 1990	CRA	MPSL	32.0	124.7 (95.8, 149.2)	
	Copper (µg/L)	CRA	18.0	54.4 (49.2, 74.1)	55.5%

* No IC40 calculated because response was less than 40%
† No IC_{10} calculated because the brine control was significantly different from the reference control

Interlaboratory experiments between MPSL, ENSECO, and CRA showed comparable germination results with the split Orange County effluent sample (Figure 11 A; the results from ABC have been excluded from this analysis because this test was conducted after the other tests using effluent stored for one week). Although germination rates were lower overall at ENSECO, there was no significant inhibition of germination by the effluent at any of the laboratories. Germination tube growth was inhibited at a lower effluent concentration in the MPSL test (Figure 11 B); the Coefficient of Variation for the germ-tube growth $IC_{10's}$ was 87.9% (Table 10).

Figure 11. Results of interlaboratory comparison between MPSL, ENSECO, and CRA laboratories using effluent from Orange County waste treatment facility. A = Non-Germination; B = Germ- Tube Growth. nc = not calculated.



(A) Non-Germination

	NOEC	EC50
MPSL	>5.6	nc
CRA	>5.6	nc
ENSECO	>5.6	nc

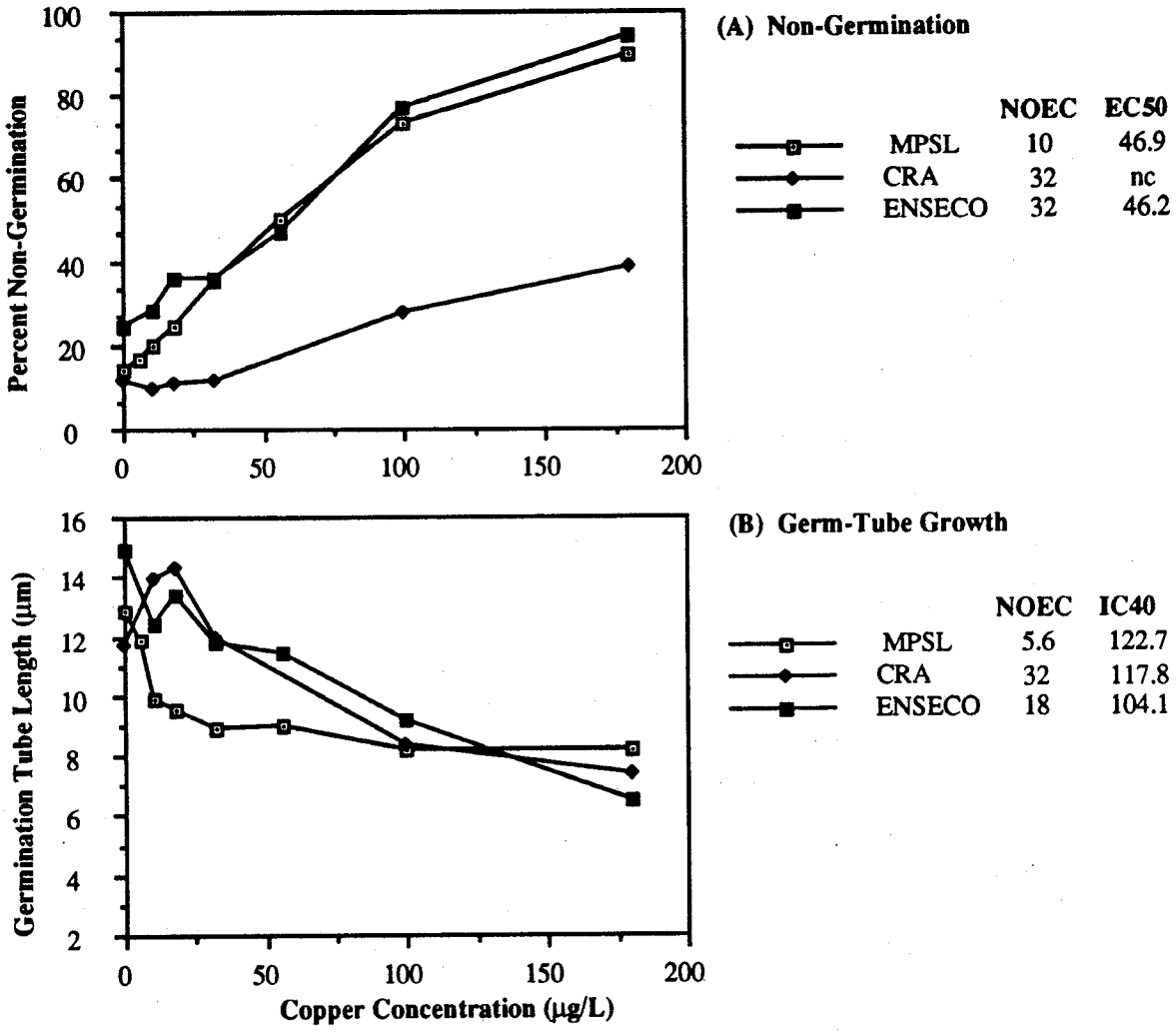
(B) Germ-Tube Growth

	NOEC	IC10
MPSL	1.0	1.6
CRA	>5.6	3.7
ENSECO	1.8	0.5

The reference toxicant tests concurrent with the Orange County effluent tests met test acceptability criteria established for this protocol. The copper response curves for germination were similar between MPSL and ENSECO, although the NOEC at MPSL was lower (Figure 12 A). The coefficient of variation of the EC_{50}'s at MPSL and ENSECO were 1.1%; no EC_{50} could be calculated for the CRA data (Table 9; note: the 56 µg /liter concentration at CRA was excluded for this analysis because insufficient copper was added to this treatment; personal communication, T. Dean, CRA)).

The interlaboratory precision of the IC_{40}s was relatively high (Figure 12 B). The coefficient of variation of the IC_{40}'s was 8.4% (Table 10).

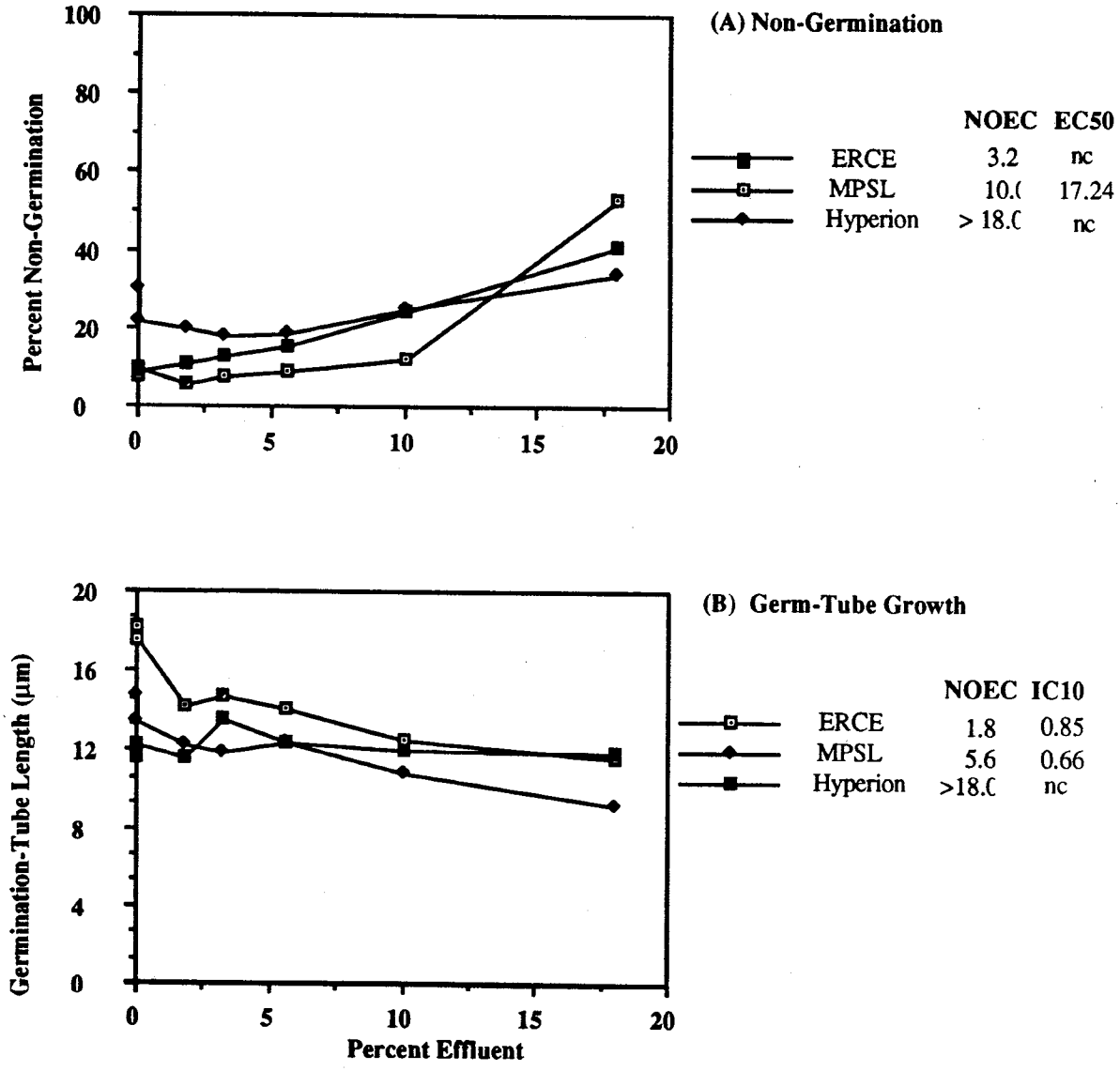
Figure 12. Results of interlaboratory comparison between MPSL, ENSECO, and ABC laboratories using copper reference toxicant (concurrent with Orange County effluent test). A = Non-Germination; B = Germ- Tube Growth. nc = not calculated.



Although the response curves were similar, interlaboratory tests with the Hyperion treatment plant effluent produced different NOEC's between MPSL, Hyperion, and ERCE (Figure 13 A). Control germination rates at Hyperion were 66% and did not meet the minimum criterion of 70% set for the test. It was not possible to calculate a coefficient of variation from these data because a point estimate could only be calculated for the MPSL test.

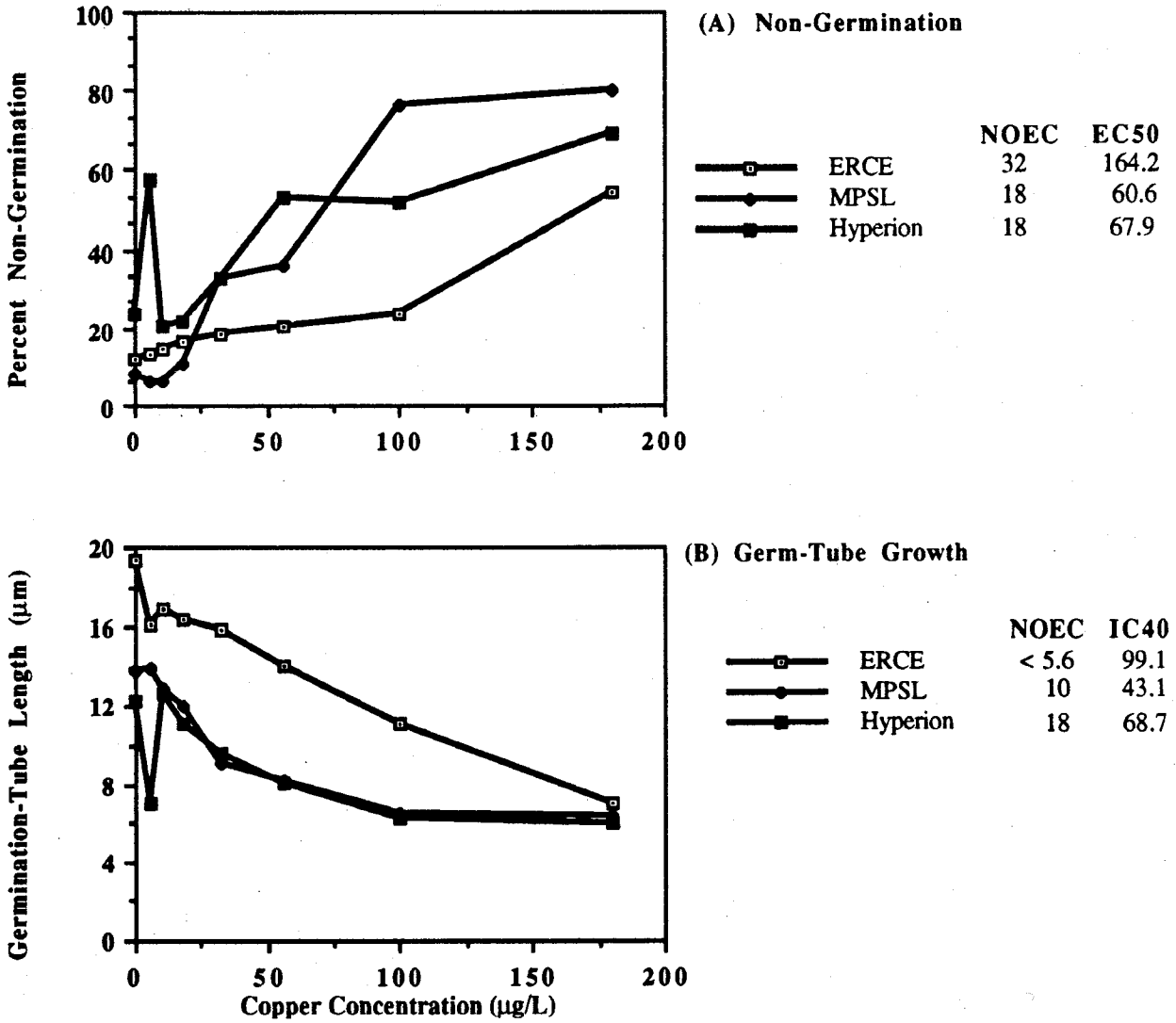
Germ-tube growth also produced different NOEC's between the three laboratories (Figure 13 B). The coefficient of variation of the IC_{10}'s for the MPSL and ERCE tests was 17.9%; no IC_{10} could be calculated for the Hyperion test (Table 10). The longer germination tubes at ERCE were probably due to lower light levels (B. Snyder, personal communication).

Figure 13. Results of interlaboratory comparison between MPSL, ERCE, and Hyperion laboratories using effluent from Los Angeles City (Hyperion) waste treatment facility. A = Non-Germination; B = Germ- Tube Growth. nc = not calculated.



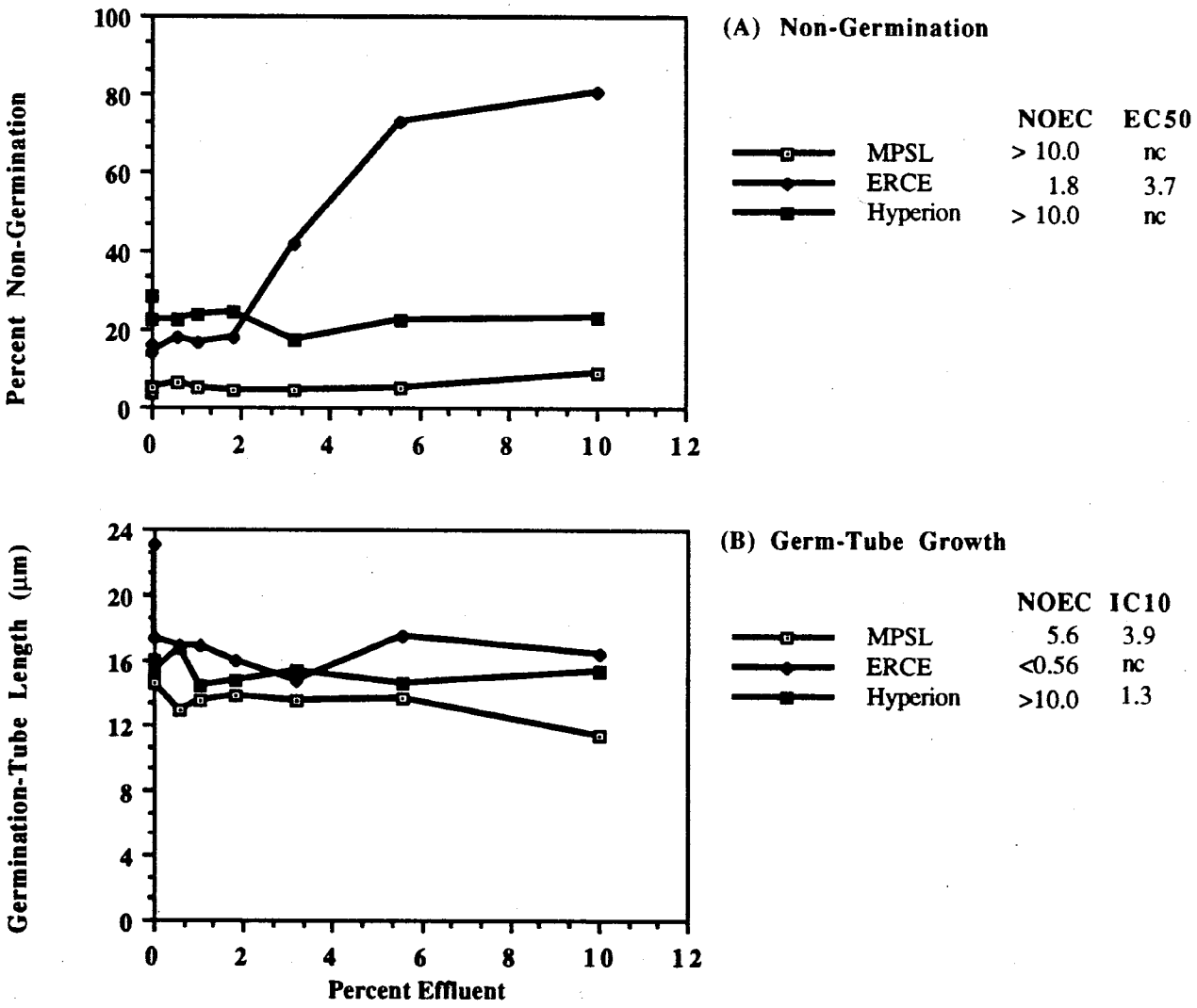
Interlaboratory reference toxicant tests conducted concurrently with the Hyperion effluent test showed similar trends, and all met reference test acceptability criteria. The germination NOEC's were the same for MPSL and Hyperion despite a significant decrease in germination in the 5.6 µg/liter treatment at Hyperion (Figure 14 A). Germination rates at ERCE were generally higher, and are refleceid by a higher NOEC. The CV of the germination EC_{50}'s was 59.3% (Table 9). The decrease in germ-tube growth and germination rates at the 5.6 µg/liter treatment in the Hyperion copper test was apparently due to a significant deviation from the nominal copper concentration (personal communication, P. Chang - Hyperion Biology Laboratory).

Figure 14. Results of interlaboratory comparison between MPSL, ERCE, and Hyperion laboratories using copper reference toxicant (concurrent with Hyperion effluent test).
A = Non-Germination; B = Germ- Tube Growth; nc = not calculated.



As in the effluent test, germ-tube lengths were generally higher at ERCE than the other two laboratories, probably due to lower light levels, and the NOEC was lower. Except for the sharp drop in germ-tube growth at 5.6 µg/liter in the Hyperion test, the response curves at MPSL and Hyperion were nearly identical (Figure 14 B). The NOEC's for these two tests differed by one concentration, apparently due to higher between-replicate variability in the Hyperion test. The Mean Square Error (MSE) value from the ANOVA table gives a measure of between-replicate variability: the MSE value for the copper length data at MPSL was 1.815; the MSE value for the copper length data at Hyperion was 2.677. The coefficient of variation of the germ-tube growth IC_{40}'s was 39.9% (Table 10).

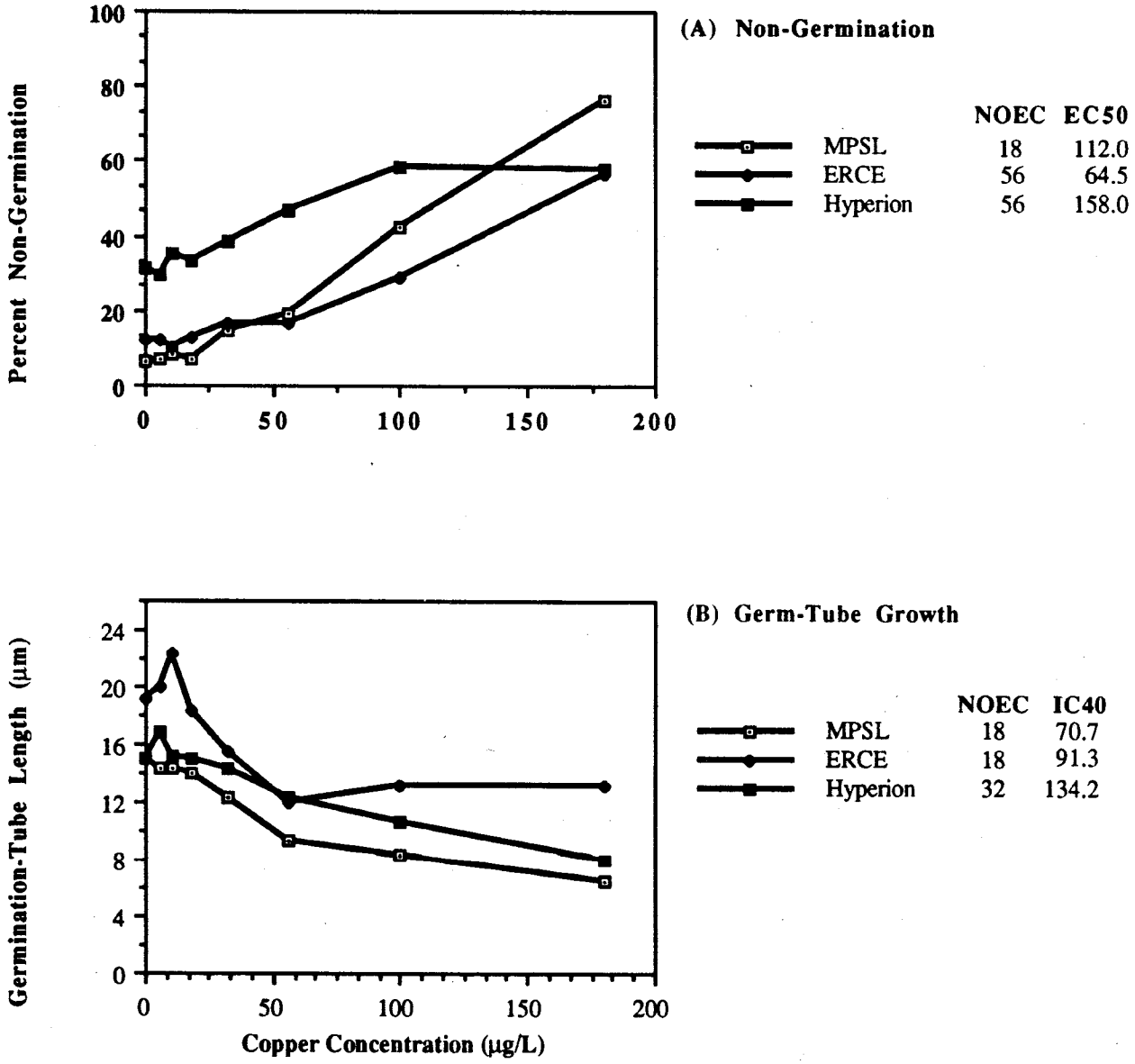
Figure 15. Results of interlaboratory comparison between MPSL, ERCE, and Hyperion laboratories using Los Angeles City effluent (JWPCP). A = Non-Germination; B = Germ- Tube Growth; nc = not calculated.



Germ-tube growth results showed a similar trend; ERCE had significantly longer germ-tubes (for reasons discussed earlier) and a lower NOEC; MPSL found significant toxicity at 10% (NOEC = 5.6%); Hyperion found no significant difference (Figure 15 B). There was a significant difference between the reference and brine controls in the ERCE test, which violates the test acceptability criteria. The coefficient of variation of the germ-tube growth IC10's for MPSL and Hyperion was 70.9% (Table 10); no IC_{10} was calculated for the ERCE test.

Results of the interlaboratory reference toxicant tests were less variable than results from the concurrent JWPCP effluent tests. Although the control germination rate in the Hyperion test was below the acceptable level (70%) the NOEC for this test was the same as ERCE's (56 µg/liter; Figure 16 A). The response curves for the ERCE and MPSL tests were similar but MPSL had a higher germination rate in the control and a lower NOEC. The coefficient of variation of the germination EC_{50}'s was 41.9% (Table 9). The coefficient of variation of the germ-tube growth IC_{40}'s was 32.8% (Table 10).

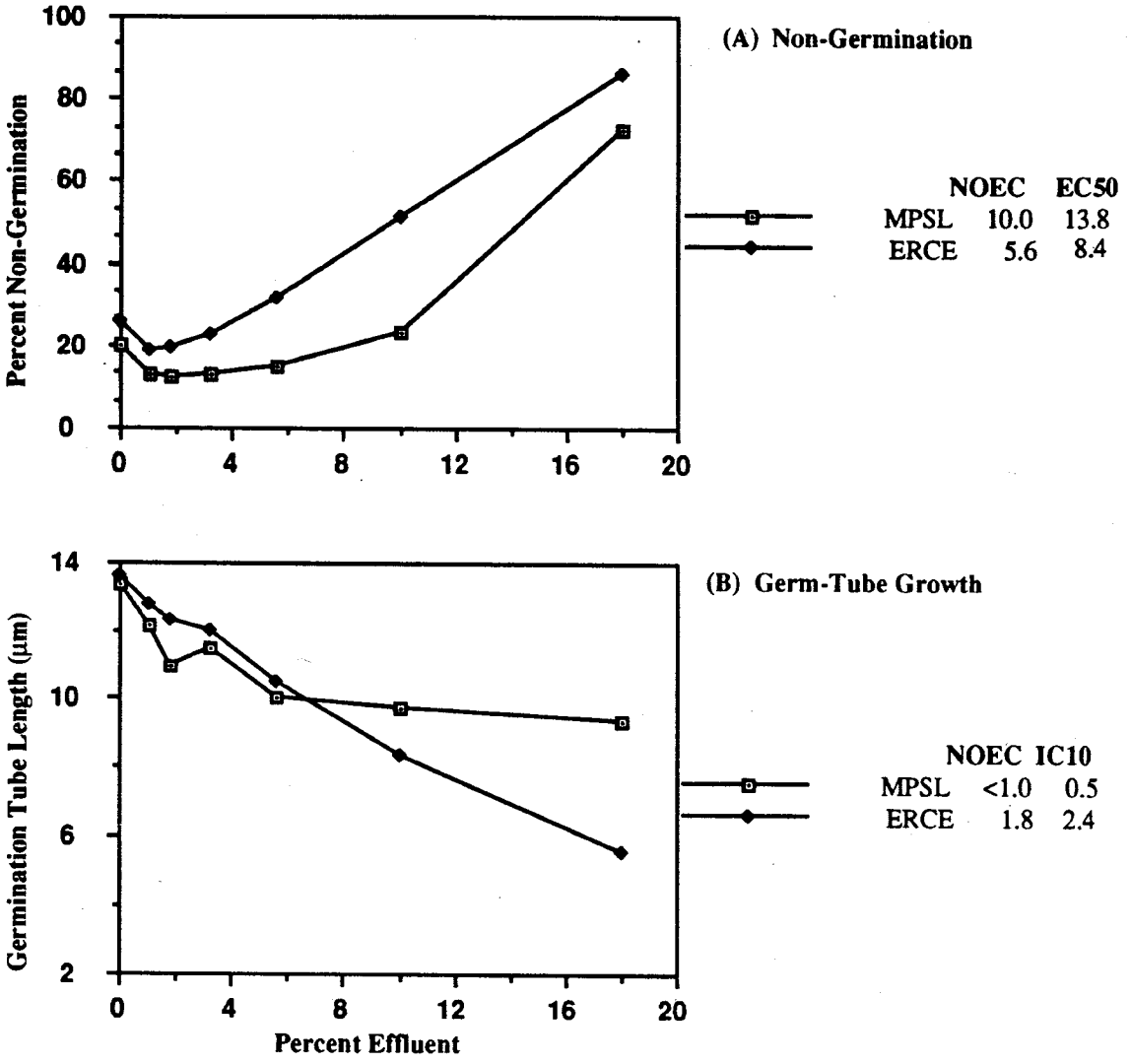
Figure 16. Results of interlaboratory comparison between MPSL, ERCE, and Hyperion laboratories using copper reference toxicant (concurrent with JWPCP effluent test). A = Non-Germination; B = Germ- Tube Growth.



Results of interlaboratory tests between MPSL and ERCE were comparable with San Diego effluent (Figure 17). The response curves for germination from the two laboratories were similar, but the resulting NOEC's were different by one dilution concentration. The coefficient of variation of the germination EC_{50}'s was 34.5% (Table 9).

The response curves for germ-tube growth from the two laboratories were similar at lower concentrations but diverged at higher concentrations (Figure 17 B). MPSL had a lower IC_{10} for germ-tube growth (0.5 % for MPSL vs 2.4% for ERCE), reflecting a steeper response to the effluent at lower concentrations. Germ-tube growth rates at ERCE were lower at higher concentrations. The coefficient of variation of the germ-tube growth IC10's was 89.7% (Table 10).

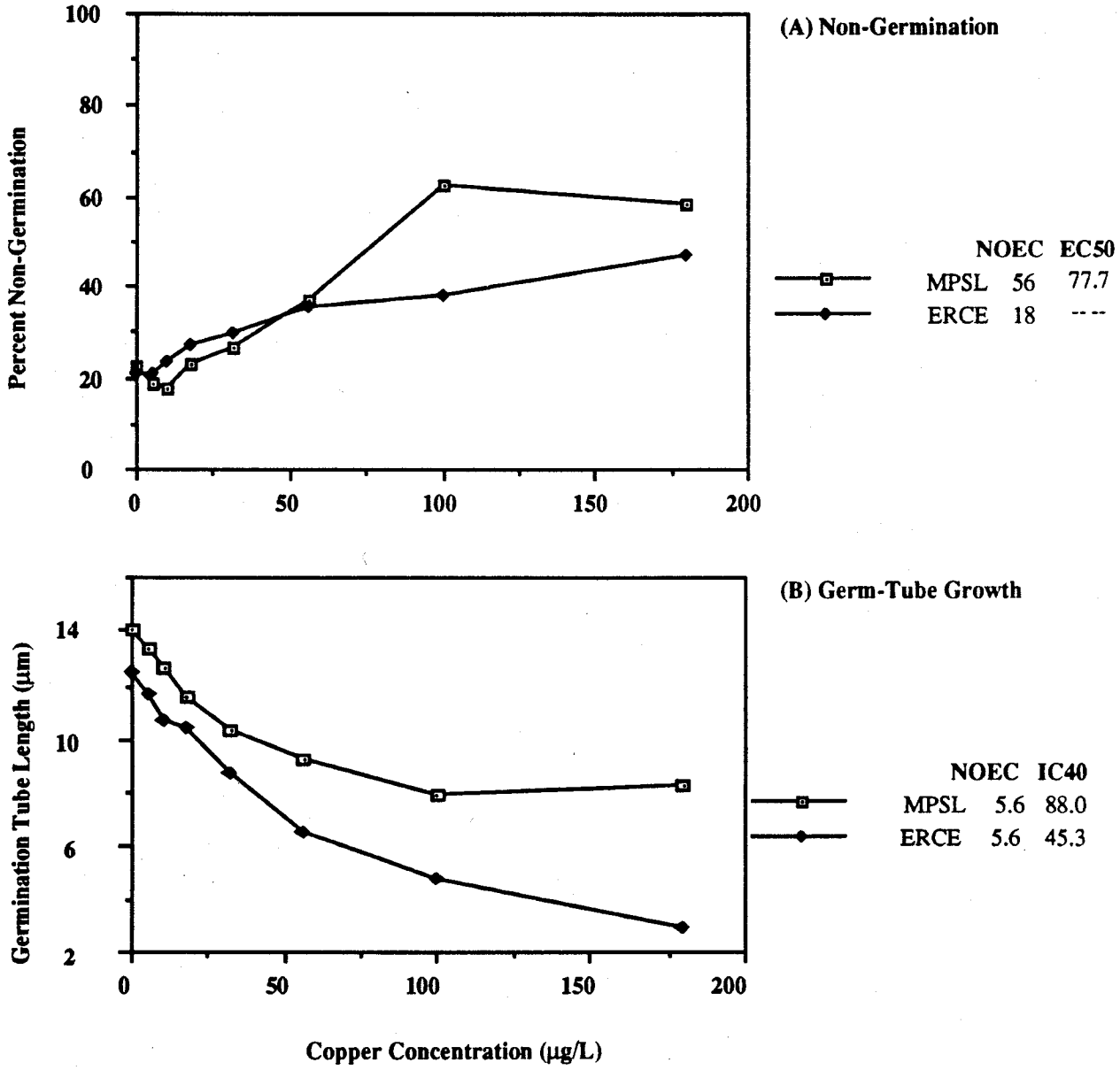
Figure 17. Results of interlaboratory comparison between MPSL, and ERCE laboratories using City of San Diego effluent. A = Non-Germination; B = Germ- Tube Growth.



The concurrent San Diego interlaboratory reference toxicant tests showed some variation in germination response to copper between the two laboratories (Figure 18 A). The NOEC for germination at MPSL was 56 µg/liter; the NOEC for germination at ERCE was 18 µg/liter. No EC_{50} could be calculated for the ERCE germination data.

The response curves for germ-tube growth at the two laboratories were quite similar (Figure 18 B); the NOEC's were the same (5.6 µg/liter). The coefficient of variation of the $IC_{40's}$ was 45.3% (Table 10).

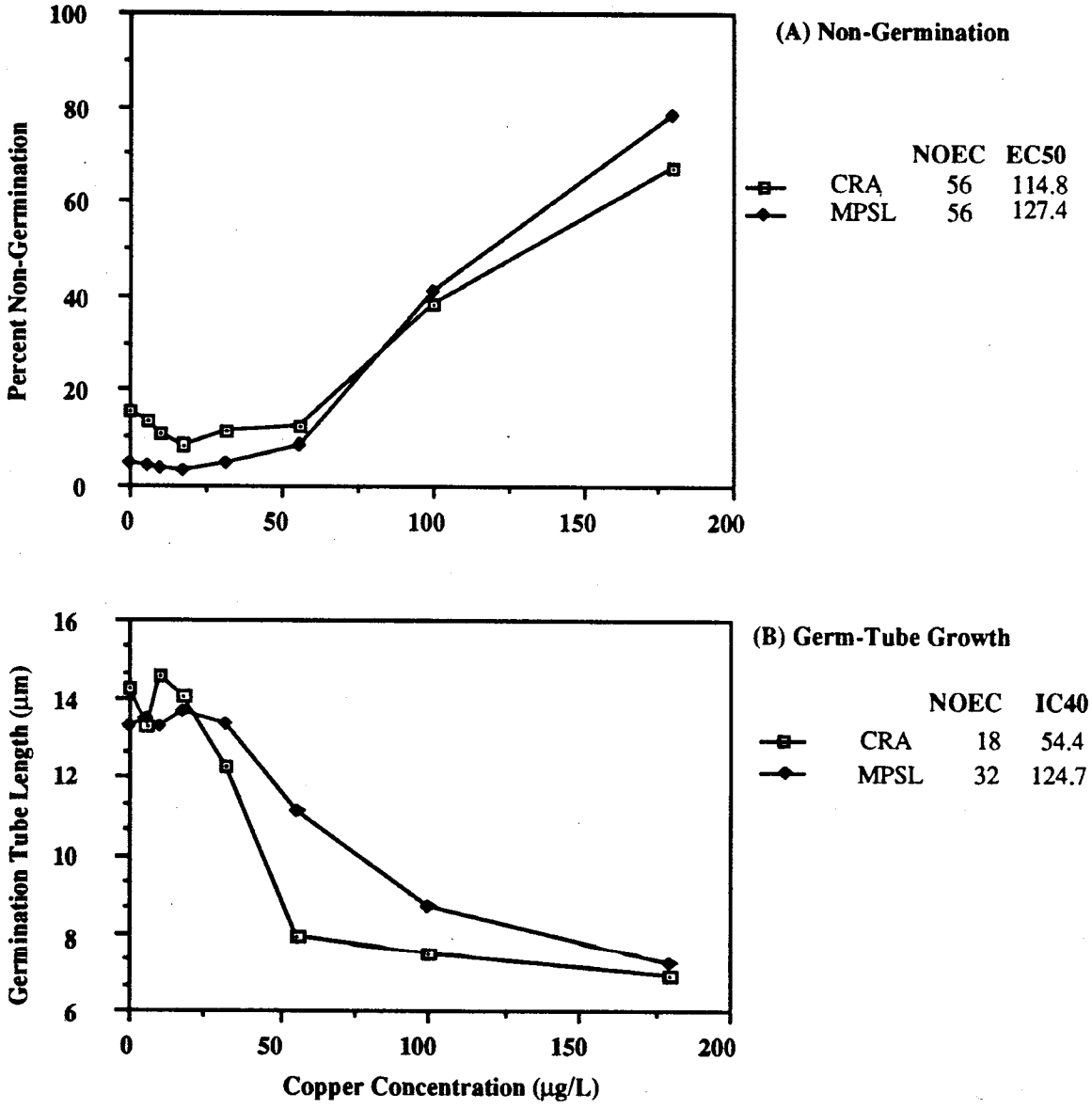
Figure 18. Results of interlaboratory comparison between MPSL, and ERCE laboratories using copper reference toxicant (concurrent with San Diego effluent test). A = Non-Germination; B = Germ- Tube Growth.



A separate copper interlaboratory test between MPSL and CRA showed similar responses in terms of germination (Figure 19 A). The NOEC's for this endpoint were the same at both laboratories, and the coefficient of variation of the $EC_{50's}$ was 7.4% (Table 9).

The NOEC for germ-tube growth was lower at CRA reflecting a steeper response curve than at MPSL (Figure 19 B). The coefficient of variation of the $IC_{40's}$ was 55.5% (Table 10).

Figure 19. Results of interlaboratory comparisons between MPSL and CRA using copper reference toxicant. A = Non-Germination; B = Germ- Tube Growth.



In previous Marine Bioassay Project reports we have described several sources of variability in interlaboratory toxicity tests. These include variations in experimental procedure, differences in endpoint analysis, differences in chemistry of dilution waters used at the various laboratories, subtle differences in the physical conditions of the test environment, and differences in handling of test organisms.

A strict interlaboratory comparison would require that all sources of variability other than those attributed to personnel conducting the tests be controlled. Although it may be possible to do this, we feel that by allowing some flexibility to the participating laboratories, the results better reflect the laboratory environment in which these toxicity tests will be routinely used. The purpose of these experiments was to demonstrate that laboratories with little or no previous experience could successfully use the 48-hour *Macrocystis* protocol to measure effluent and reference toxicant toxicity. Although there were some differences in results between the laboratories, the 48-hour kelp toxicity test was successfully completed in all experiments, and, except for germination in two tests, the results were within acceptable limits. The results of the copper reference toxicant experiments were within the range of previous test results. Rather than reflecting a lack of refinement of the test, or inherent variability in the organism, some of the differences reflect a lack of control over the above mentioned factors.

In addition to variability between laboratories, there are other potential sources of variability inherent in laboratory toxicity testing. As suggested in past reports, measuring total copper concentrations may not accurately reflect bioavailable concentrations because it is the free divalent metal ion that is the form of copper toxic to algae. Divalent ion concentrations may vary with concentrations of organic chelators present in the dilution water (Kuwabara, 1980; Sunda and Guilliard, 1976). Another source of variability is temporal variation in the sensitivity of the test organism. There is evidence to suggest that this contributes to between-test variability in kelp tests (Anderson et al., 1990a).

Rue et al. (1988) compared the precision of analytical chemistry methods to the precision of effluent toxicity tests at concentrations near EPA acute water quality criteria concentrations . They found that acceptable CV's for trace metals ranged from18% to 129%. The mean interlaboratory coefficient of variation of the germination endpoint in the copper reference tests was approximately 27%, although this may underestimate variability because in the Orange County copper test an EC_{50} could not be calculated for the ENSECO data, and in the San Diego copper test an EC_{50} could not be calculated for the ERCE data. The mean interlaboratory coefficient of variation of the germ-tube growth endpoint in the copper tests was 36%. Interlaboratory effluent test coefficients of variation were considerably higher and ranged between 18 and 90% for germ-tube growth IC10's (mean = 67%). Coefficients of variation could not be calculated for the effluent germination data because in most cases inhibition of germination was less than 50%.

Intralaboratory precision of the copper tests at MPSL was comparable to that of the interlaboratory tests. A summary of the 1990 copper reference toxicant tests conducted at MPSL is given in Table 3. The coefficient of variation of the germination EC_{50}'s was 32.9%. The coefficient of variation of the germ-tube

growth response IC_{40}'s was 38.8%. These precision estimates are comparable to intralaboratory precision estimates reported for the red macroalga *Champia parvula* tested with copper (CV = 38.6%; Morrison et al., 1989).

Table 11. Summary of 1990 kelp copper reference toxicant tests conducted at MPSL.

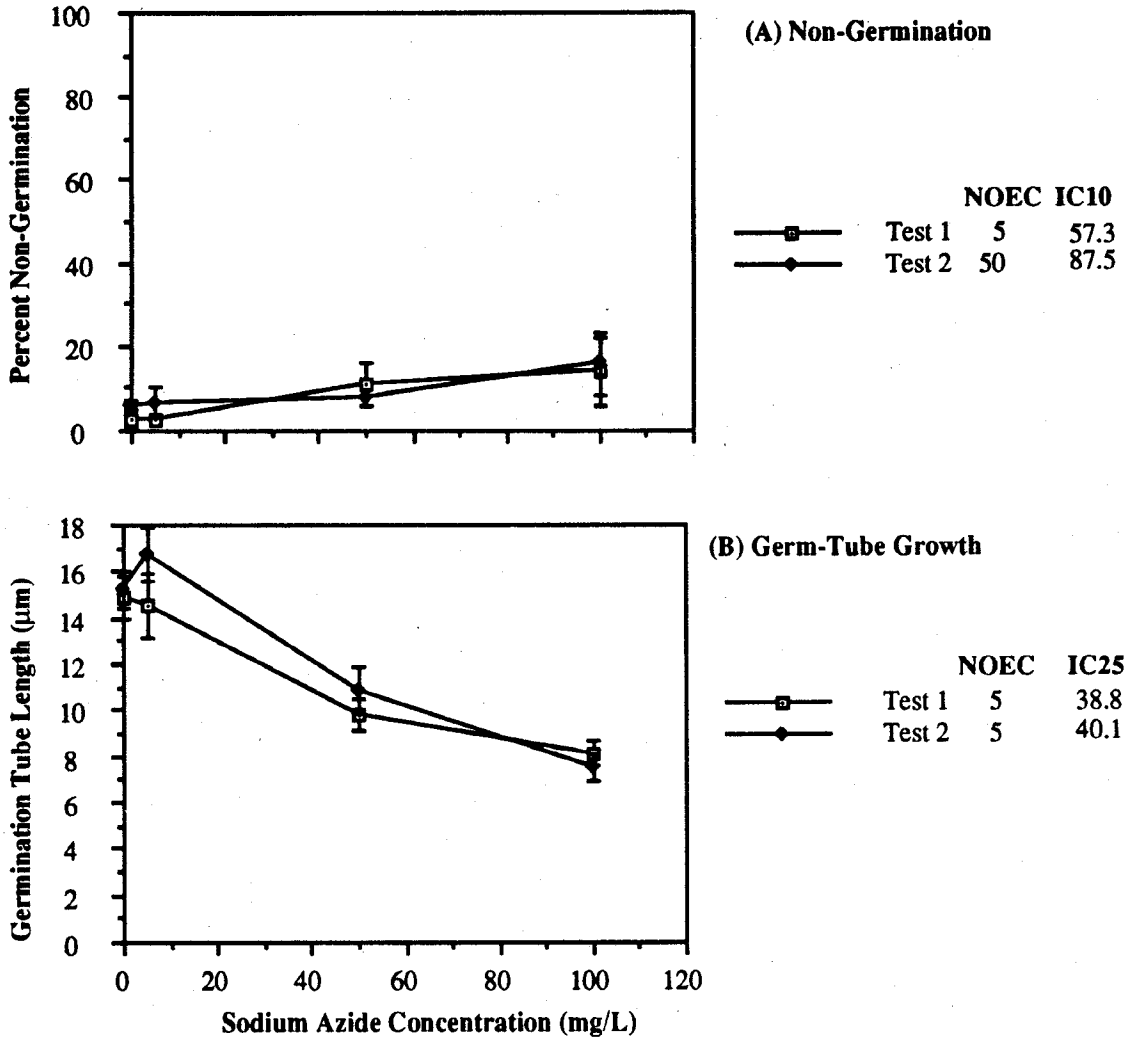
Test Number	Germination-Tube Length (µg/l)			Germination (µg/l)		
	NOEC	IC40	(95% CI)	NOEC	EC50	(95% CI)
1	<5.6	122.7	(83.1,173.2)	10.0	67.5	(55.6,74.7)
2	10.0	43.1	(28.8, 64.1)	18.0	73.5	(62.6, 78.7)
3	18.0	70.7	(52.3, 111.3)	18.0	124.3	(112.3, 134.5)
4	5.6	88.0	(66.1, 99.7)	56.0	101.6	(87.6, 154.5)
5	32.0	124.7	(95.8, 149.2)	56.0	122.9	(110.0, 137.8)
Mean		89.8			90.7	
CV		38.8%			32.9%	

Two reference toxicant tests with sodium azide were conducted to investigate it's use as an alternative reference toxicant to copper chloride. The results of these tests are presented in Figure 10. The response curves for the germination endpoint were quite similar. The NOEC's varied by one concentration; the NOEC for test number one was 5 mg azide/liter, the NOEC for test number two was 50 mg azide/liter (Note the wide intervals between concentrations in this preliminary test; Figure 10 A). The IC_{10} for germination in test number one was 57.3 mg azide/liter; the IC_{10} for test number two was 87.5 (coefficient of variation = 29.5%).

The response curves for germ-tube growth were also similar, and the NOEC's were the same, 5 mg azide/liter (Figure 10 B). The IC_{25} for test number one was 38.8 mg azide/liter; the IC_{25} for test number two was 40.1 (coefficient of variation = 2.3%).

Sodium azide is a promising alternative reference toxicant to copper chloride because it's 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. This will hopefully reduce between-test variability associated with temporal and spatial differences in the chelation capacity of dilution waters. We plan to conduct multiple experiments using azide to establish an adequate database to measure between test precision. The between-test precision of tests using sodium azide will then be compared to the precision of tests using copper. Future azide tests will include a greater number of concentrations bracketing the NOEC's for germination and germ-tube growth.

Figure 20. Sodium azide toxicity to kelp germination and germ-tube growth
A = Non-Germination; B = Germ- Tube Growth.



Summary

1. Out of 26 separate kelp experiments reported here, four did not meet the test acceptability criteria (three because they had low control germination rates; one because the brine and reference controls were significantly different). This gives a success rate of 85% for all of the interlaboratory tests.

2. The precision of the interlaboatory reference tests was within limits commonly attained by EPA analytical chemistry methods. The approximate mean interlaboratory coefficient of variation was 27% for germination and 36% for germ-tube growth. The coefficient of variation of the interlaboratory effluent tests was higher: 67% for germ-tube growth; not calculated for germination.

3. Intralaboratory precision of the copper reference tests conducted at MPSL was 33% for germination and 38% for germ-tube growth.

4. Although the results should be considered preliminary, the two tests using sodium azide had high precision. The coefficients of variation were 30% and 2% for germination and germ-tube growth, respectively.

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Section 4
Red Abalone Experiments

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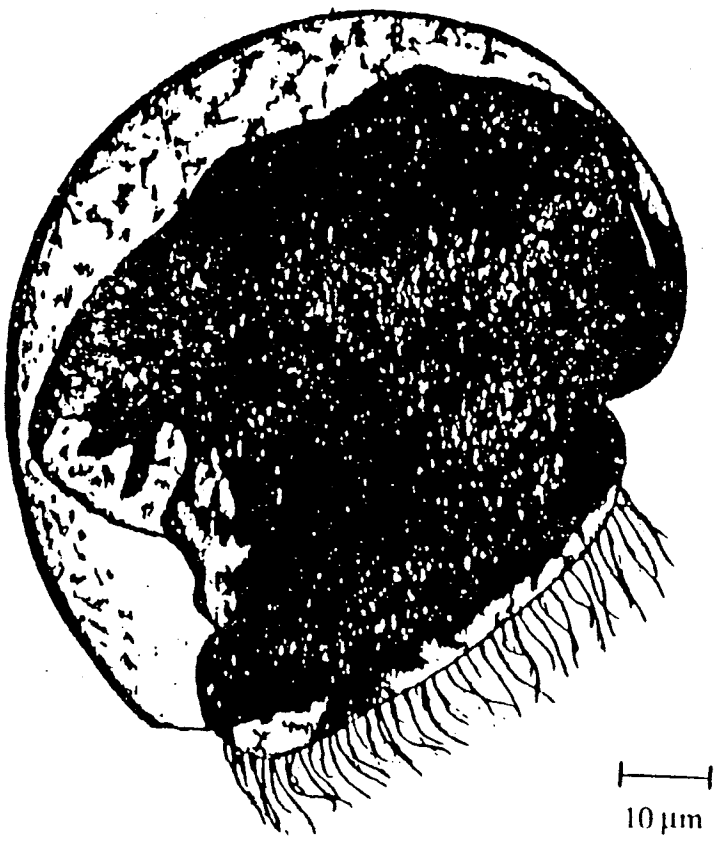


Haliotis rufescens - veliger larva

Introduction

The red abalone, *Haliotis rufescens*, was selected for toxicity testing by the Marine Bioassay Project for several reasons. This large gastropod mollusc is indigenous to California and is distributed throughout the State's coastal waters. It is an ecologically important herbivore and important prey for the sea otter, lobster, octopus, and many species of fish. The red abalone is highly valued for human consumption, and supports a commercial fishery in southern California and a popular recreational fishery throughout the State. There is evidence that past effluent discharges have caused declines in abalone populations near large municipal sewage outfalls (Grigg and Kiwala, 1970).

Most aspects of abalone biology and reproduction have been extensively researched for mariculture purposes (Hahn, 1989; Morse et al., 1977, 1979; Ebert and Houk, 1984), and abalone are easily cultured and spawned in the laboratory. Of 41 spawnings attempted in our laboratory over the past four years, 39 have been successful (95%), with unsuccessful spawnings occurring in March and December. The reproductive season is long, and spawnable abalone can generally be obtained throughout the year, though laboratory conditioning may be necessary to assure supply during winter months (December through February). Year-round spawning is routinely induced at the three major California commercial abalone hatcheries, although some culturists tend to concentrate spawning in winter, and some in summer, depending on optimum local conditions for larval culture (Frank Oakes, Michael Machuzak, Peter Scrivani, personal communications). Fertilization success is generally greater than 95%, and embryos and larvae are large enough to be seen with the naked eye, which facilitates counting and handling. Previous toxicity studies have shown abalone larvae to be sensitive to trace metals and a variety of organic toxicants (Martin et al., 1977, 1986; Morse et al., 1979; Hunt and Anderson, 1989).

The 48-hour abalone toxicity test protocol is similar to methods developed for mussels (Dimick and Breese, 1965) and oysters (Woelke, 1972). These protocols use molluscan embryo/larval development in short term tests to estimate the chronic toxicity of effluents. Embryos from laboratory spawnings are incubated in static toxicant solutions for 48 hours, then examined microscopically to determine the percentage that develop into larvae with abnormal shells. The abalone test has also been extended into a longer-term (9-day) flow-through test that uses inhibition of larval metamorphosis to indicate toxicity. Past trials of this metamorphosis test indicated that zinc concentrations causing abnormal shell development also affected the larvae's ability to metamorphose into juvenile abalone (Hunt and Anderson, 1989; Conroy et al., 1991).

Over the course of the project we have conducted 45 short term toxicity tests using a variety of toxicants. Copper, tributyltin, sodium pentachlorophenate and endosulfan were tested once each, zinc sulfate was tested 15 times, and complex effluents from various sources were tested 15 times. The remaining tests were used for range-finding or investigating brine toxicity. Control response has been acceptable in all tests (>80% normal development), and in most cases >90% of the control larvae developed

normally. The test is sensitive to a variety of toxicants, with mean No Observed Effect Concentrations (NOEC's) and EC_{50} values, respectively, of 28 µg/liter and 44 µg/liter for zinc (n = 15), <6 µg/liter and 9 µg/liter for copper, 180 µg/liter and 252 µg/liter for endosulfan, and 32 µg/liter and 59 µg/liter for pentachlorophenate (Hunt et al., 1989; Martin et al., 1986). Intralaboratory between-test precision is indicated by a coefficient of variation of 25% for EC_{50} values of all zinc tests (n = 15).

This section describes further experiments using the red abalone protocol for determining effluent toxicity under varying test conditions. The primary focus is on interlaboratory testing of complex effluents. Four separate interlaboratory comparisons were conducted using effluent from major Southern California municipal waste treatment facilities. Zinc reference toxicant tests were conducted concurrently with every effluent test.

Methods

All experiments were conducted between January 1990 and February 1991, at the California Department of Fish and Game's Marine Pollution Studies Laboratory at Granite Canyon. The facilities are described in detail by Martin et al. (1981). Methods for the red abalone tests reported in this section are given in the previously published red abalone protocol (Hunt et al., 1990). Detailed methods for interlaboratory tests are given in Anderson et al. (1990).

Interlaboratory Tests

The purpose of these experiments was to determine whether the abalone toxicity test protocol was sufficiently detailed to allow different investigators to produce acceptable test results under varying laboratory conditions. Four interlaboratory tests of the 48-hour protocol were conducted using split effluent samples and reference toxicants. The first comparisons were conducted in March, 1990 using effluent from the Orange County Sanitation Districts waste treatment facility. Participating laboratories were MPSL, ENSECO Chemical Research Laboratory (ENSECO), and ERC Environmental and Energy Services Company Inc. (ERCE). Orange County effluent concentrations tested were: 0, 0.18%, 0.32%, 0.56%, 1.0%, 1.8%, and 3.2%. The second set of tests was conducted in May, 1990 using effluent from the City of Los Angeles' Hyperion waste treatment facility. Participating laboratories were MPSL, ERCE, and Hyperion's biology laboratory. Effluent concentrations tested were: 0, 1.0%, 1.8%, 3.2%, 5.6%, and 10%. The third and fourth interlaboratory comparisons were conducted simultaneously in January 1991, using effluents from the Los Angeles County Joint Water Pollution Control Plant (JWPCP), and the City of San Diego waste treatment facility. Participating laboratories were MPSL and ERCE. Slightly different concentrations were inadvertantly tested at MPSL and ERCE in the JWPCP tests: JWPCP effluent concentrations tested at MPSL were: 0, 0.56%, 1.0%, 1.8%, 3.2%, and 5.6%; JWPCP effluent

concentrations tested at ERCE were: 0%, 0.31%, 0.62%, 1.25%, 2.5%, 5.0%, and 10.0%. San Diego effluent concentrations tested at both laboratories were: 0%, 0.32%, 0.56%, 1.0%, 1.8%, and 3.2%. The San Diego and JWPCP interlaboratory comparisons were repeats of two earlier experiments which failed. The San Diego tests were repeated because of temperature control failure at MPSL. The JWPCP tests were repeated because of test container toxicity at MPSL in the first round of tests. The test containers that apparently caused excessive larval abnormalities were polystyrene tissue culture flasks (250 ml). Tissue culture flasks are useful because larvae can be analyzed microscopically within the flasks, eliminating the need for screening and transfer. However, subsequent experiments at MPSL demonstrated that significantly more abalone larvae develop abnormally in polystyrene tissue culture flasks than in borosilicate glass beakers containing the same dilution water (23% ± 25% abnormal vs. 5% ± 2% abnormal, respectively, for tissue culture flaks vs. beakers; n = 5). Tissue culture flasks pre-soaked in deionized water or pre-washed with detergent then soaked with deionized water, produced 100% abnormal larvae. Different lots or different brands of tissue culture flasks probably have different toxicities. If you plan to use these flasks as test containers, we recommend pre-testing subsamples from each lot.

Zinc reference toxicant tests were conducted by all participating laboratories concurrent with the effluent tests. Zinc concentrations tested in each reference toxicant test were: 0, 18, 32, and 56 µg /liter.

For all but one set of experiments, 24-hour composite effluent samples were collected the day before the experiments. The JWPCP experiments used a grab effluent sample. Split samples of each effluent were shipped the day of sampling to each participating laboratory. Samples were shipped on ice via overnight couriers in polyethylene plastic containers. For two of the comparisons (Orange County and Hyperion), laboratories used their own equipment, dilution water, and broodstock suppliers. For the JWPCP and San Diego tests, participating laboratories used dilution water supplied by MPSL, and abalone broodstock from the same supplier. Although participating investigators were experienced in toxicity testing, all except MPSL had limited experience with the red abalone protocol. As such, the objective of these tests was not to determine test precision under strictly controlled conditions, but rather the suitability of the protocol for use in a wide range of laboratory situations.

No Observed Effect Concentrations (NOEC's) in these tests were calculated using ANOVA followed by Dunnett's multiple comparison test (Sokal and Rohlf, 1969; Zar, 1974). In effluent tests where brines were used, Dunnett's comparisons were made as follows: lower effluent concentrations (in which no brine was needed to adjust salinity) were compared to dilution water controls; higher effluent concentrations (that used brines to adjust salinity) were compared to brine controls. Median Effect Concentrations (EC_{50}'s) were calculated using the trimmed Spearman-Karber method (Hamilton et al., 1977). Precision of intra- and interlaboratory data was determined by calculating coefficients of variation of EC_{50} values from all tests.

Data for reference toxicant tests are reported as nominal concentrations. Test solutions in all tests were sampled for chemical analysis at the beginning and end of each test, and analyzed on a Perkin Elmer model 5000 atomic absorption spectrometer following the methods of Bruland et al. (1979).

Results and Discussion

Interlaboratory Tests

Results of the Phase 5 abalone interlaboratory tests are summarized in Table 12; results are given as NOEC's and EC_{50}'s and 95% Confidence Limits for all effluent and concurrent reference toxicant tests for all participating laboratories. Coefficients of variation between EC_{50} values from each set of comparisons are also included to indicate interlaboratory precision.

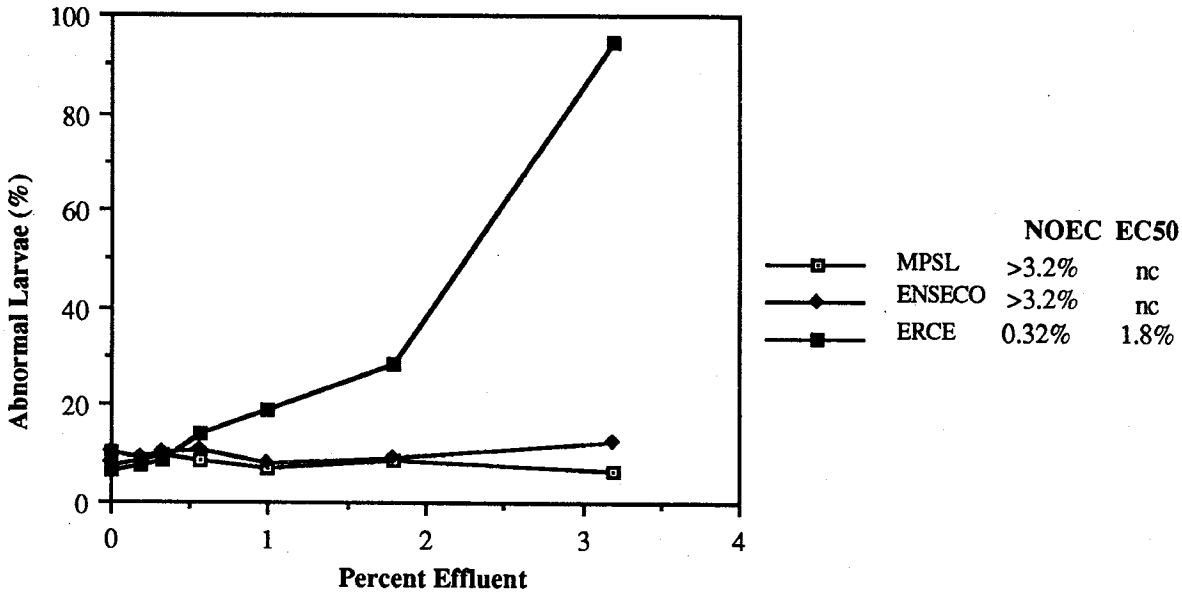
Table 12. Summary of Phase 5 abalone interlaboratory test results. All effluent concentrations are given as percent effluent; all zinc concentrations are given as µg/liter.

Test Date	Toxicant Comparison	Laboratory	NOEC	EC_{50} (95% C.L.)	Coefficient of Variation
March 90	Orange County Effluent (%)	MPSL	>3.20	*	
		ENSECO	>3.20	*	
		ERCE	0.32	1.83 (1.7, 2.0)	nc
	Orange County Zinc (µg/L)	MPSL	32.0	41.5 (40.5, 42.5)	
		ENSECO	18.0	28.7 (26.6, 30.9)	
		ERCE	18.0	31.1 (28.8, 33.6)	20.1%
May 90	Hyperion Effluent (%)	MPSL	3.20	4.70 (4.3, 5.2)	
		Hyperion	1.80	3.50 (3.3, 3.8)	
		ERCE	3.20	3.80 (3.3, 4.3)	16.0%
	Hyperion Zinc (µg/L)	MPSL	32.0	39.0 (37.3, 40.7)	
		Hyperion	32.0	45.7 (43.3, 48.2)	
		ERCE	32.0	36.9 (33.4, 40.8)	11.3%
January 91	JWPCP Effluent (%)	MPSL	<0.56	1.48 (1.4, 1.6)	
		ERCE	1.25	1.78 (1.7, 1.9)	13.0%
	JWPCP Zinc (µg/L)	MPSL	18.0	33.5 (31.2, 35.6)	
		ERCE	32.0	48.1 (45.0, 51.4)	25.4%
January 91	San Diego Effluent (%)	MPSL	1.00	2.72 (2.3, 2.9)	
		ERCE	1.80	2.79 (2.6, 3.0)	1.8%
	San Diego Zinc (µg/L)	MPSL	18.0	33.5 (31.2, 35.6)	
		ERCE	32.0	48.1 (45.0, 51.4)	25.4%

* No EC_{50} calculated because response was less than 50%; na = not applicable (insufficient numbers to calculate a coefficient of variation).
nc = not calculated (insufficient data to calculate a coefficient of variation).

Interlaboratory experiments between MPSL, and ENSECO showed similar results with the split Orange County effluent sample; results from ERCE with this sample were considerably more sensitive than those of the other two laboratories (Figure 21). There was no significant inhibition of abalone embryonic development by the effluent at MPSL or ENSECO. ERCE found significant inhibition at 0.56% effluent; the NOEC at ERCE was 0.32%, and the EC_{50} was 1.83% (Table 12).

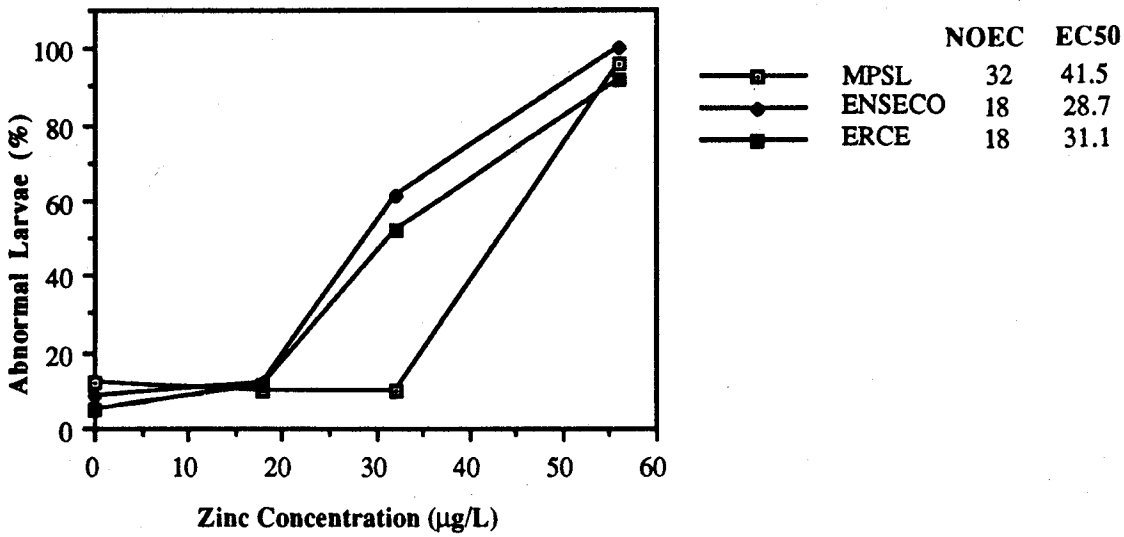
Figure 21. Abalone larval development in an interlaboratory comparison between MPSL, ENSECO, and ERCE using effluent from Orange County waste treatment facility (n = 5 for all laboratories); nc = not calculated.



It is not clear why the results of the ERCE effluent test were more sensitive than those of the other two laboratories; however, the difference in results could be attributed to any one of several factors affecting test variability (see discussion on page 79).

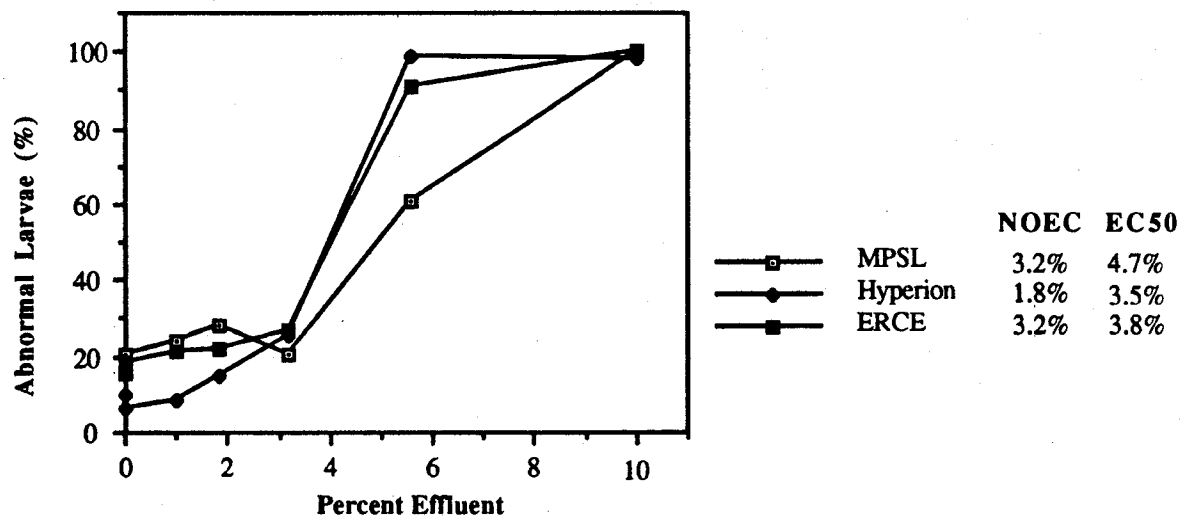
Results of the concurrent zinc reference toxicant tests were comparable between all laboratories. ERCE and ENSECO had nearly identical response curves, and the NOEC's at both laboratories were 18 µg/liter (Figure 22). The NOEC at MPSL was 32 µg zinc/liter; all tests met the protocol test acceptability requirements. The EC_{50}'s were 41.5, 28.7, and 31.1 at MPSL, ENSECO, and ERCE, respectively. The coefficient of variation of the EC_{50}'s was 20.1% (Table 12).

Figure 22. Abalone larval development in an interlaboratory comparison between MPSL, ENSECO, and ERCE using zinc (concurrent to Orange County effluent test; n = 5 for all laboratories).



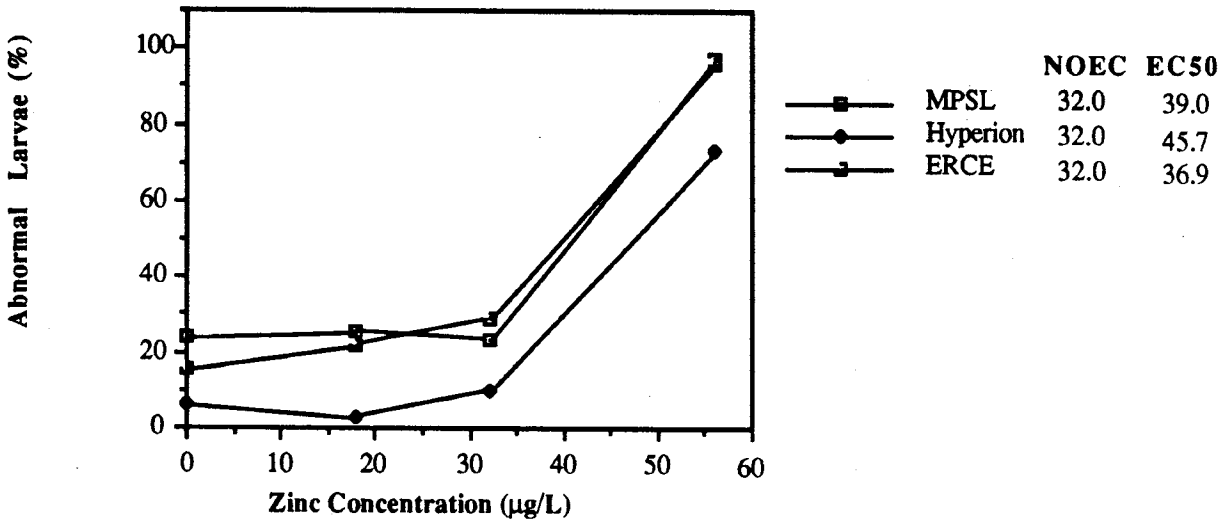
Results of the interlaboratory comparison between MPSL, Hyperion, and ERCE were comparable using split samples of effluent from the City of Los Angeles Hyperion waste treatment facility. The NOEC's were 3.2%, 1.8%, and 3.2%, and the EC50's were 4.7%, 3.5%, and 3.8% at MPSL, Hyperion, and ERCE, respectively (Figure 23). The coefficient of variation of the three EC_{50}'s was 16% (Table 12).

Figure 23. Abalone larval development in an interlaboratory comparison between MPSL, Hyperion, and ERCE using split samples of effluent from Hyperion waste treatment facility (n = 5 for all laboratories).



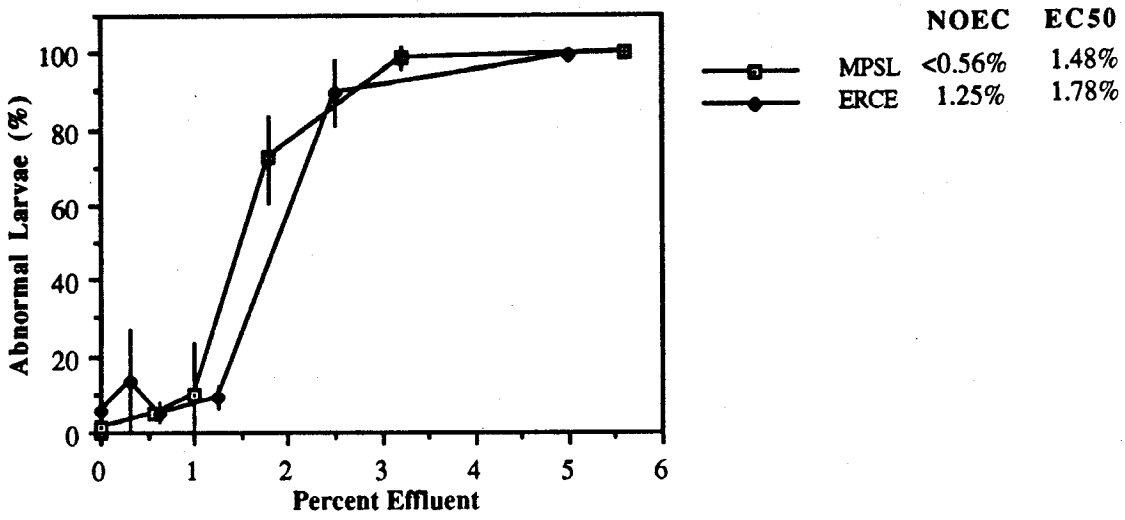
The NOEC's and EC50's for all participants were also similar in the zinc reference toxicant test conducted concurrently with the Hyperion effluent test. Results at all laboratories produced NOEC's of 32 µg zinc/liter (Figure 24). The EC50's were 39.0, 45.7 and 46.9 µg zinc/liter at MPSL, Hyperion and ERCE, respectively. The coefficient of variation of the three EC50's was 11.3% (Table 12). The control response at MPSL was 24%, below the acceptable level specified for this protocol.

Figure 24. Abalone larval development in an interlaboratory comparison between MPSL, Hyperion, and ERCE using zinc (concurrent to Hyperion effluent test; n = 5 for all laboratories).



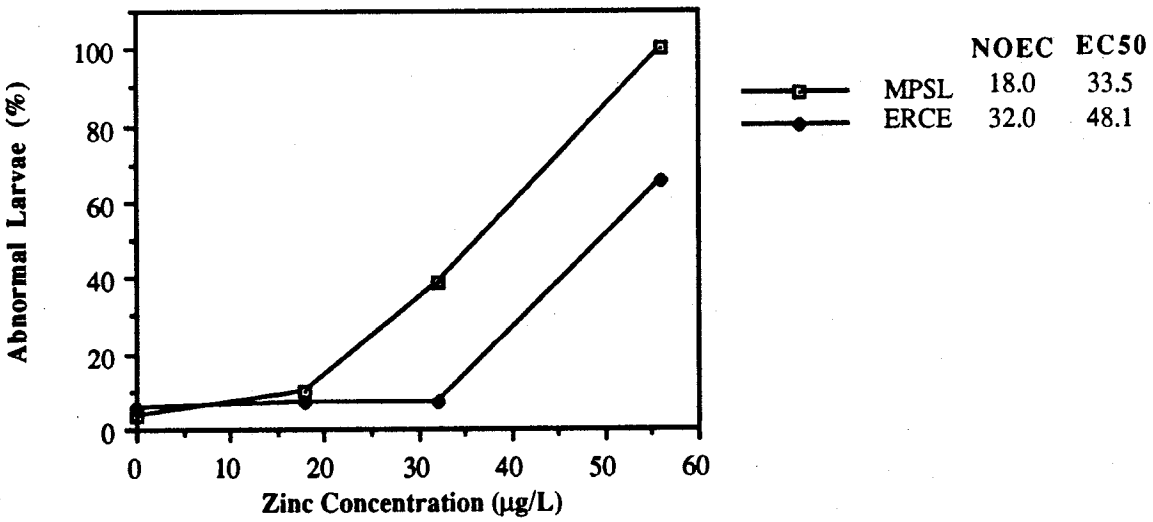
Interlaboratory tests between ERCE and MPSL using effluent from the City of Los Angeles (JWPCP) waste treatment facility produced similar response curves, despite the fact that slightly different effluent concentrations were tested (Figure 25). The NOEC's were different, apparently due to differences in between-replicate variability between the two laboratories. The Mean Square Error (MSE) value from the ANOVA table is a measure of between-replicate variability. The combined between-replicate variability was higher in the ERCE test; the MSE value from the MPSL test was 0.01; the MSE value from the ERCE test was 0.027. The different NOEC's could also be attributed to a difference in larval development in the controls at the two laboratories. MPSL found 1% abnormal control development, while ERCE found 5% abnormal control development. The response in the lowest test concentration in the MPSL test (0.56% effluent) was significantly different from the control response (NOEC <0.56%). The NOEC at ERCE was 1.25% effluent. The EC_{50}'s for these tests were 1.48% and 1.78% at MPSL and ERCE, respectively. The low coefficient of variation (13%) of these EC_{50}'s reflects the similarity in response to the effluent (Table 12).

Figure 25. Inhibition of abalone embryonic development in interlaboratory comparison between MPSL, and ERCE using split samples of effluent from JWPCP waste treatment facility (n = 5 for all laboratories; standard deviations are included for comparison of variability).



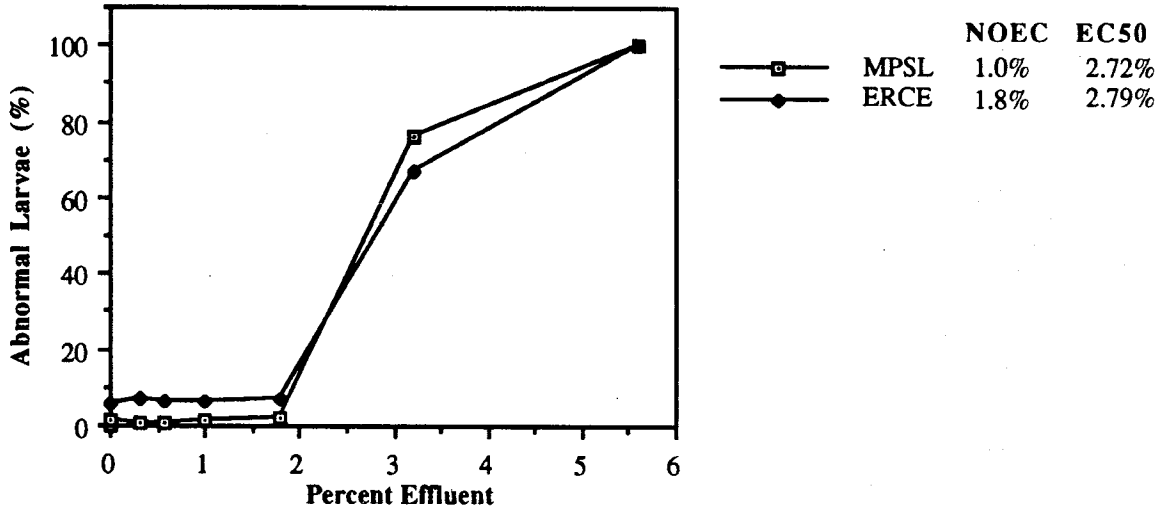
The zinc reference toxicant test conducted concurrent with the JWPCP effluent test was somewhat more variable (Figure 26). The test at MPSL was more sensitive resulting in a NOEC of 18 µg zinc/liter; the NOEC at ERCE was 32 µg zinc/liter. The $EC_{50's}$ at MPSL and ERCE were 33.5, and 48.1, respectively; the coefficient of variation of the $EC_{50's}$ was 25.4% (Table 12).

Figure 26. Abalone larval development in an interlaboratory comparison between MPSL, and ERCE using zinc (concurrent to JWPCP and San Diego effluent tests; n = 5 for both laboratories).



Results of the interlaboratory test between MPSL and ERCE using split samples of effluent from the City of San Diego were nearly identical (Figure 27). The NOEC in the MPSL test was 1.0%, and the NOEC in the ERCE test was 1.8%. As in previous interlaboratory tests where similar response curves yielded different NOEC's, the difference is a result of differences in between-replicate variability and control response. The MSE at MPSL was 0.005; the MSE at ERCE was 0.016. MPSL found 1% abnormal control development; ERCE found 5% abnormal control development. The close agreement in EC_{50}'s reflects the similarity in response curves. The EC_{50} at MPSL was 2.72% effluent, and the EC_{50} at ERCE was 2.79%. The coefficient of variation of the EC_{50}'s was 1.8%, the lowest CV observed in all of the comparisons reported here. This interlaboratory comparison was concurrent with the JWPCP tests and used the same zinc reference test (see Figure 26).

Figure 27. Abalone larval development in an interlaboratory comparison between MPSL, and ERCE using split samples of effluent from City of San Diego waste treatment facility (n = 5 for both laboratories).



In previous Marine Bioassay Project reports we have described the several possible sources of variability inherent in interlaboratory toxicity tests. These include variations in experimental procedure, differences in endpoint analysis, differences in chemistry of dilution waters used at the various laboratories, subtle differences in the physical conditions of the test environment, and differences in test organism condition and handling.

We did not attempt to control all of the possible sources of variability (eg., dilution water, test organism supplier, personnel experience, etc.) at each laboratory in these comparisons. Although it may be possible to do this, we feel that by giving some flexibility to the participating laboratories, the results better reflect the laboratory environment in which these toxicity test protocols will be routinely used. The purpose of these comparisons was to determine whether laboratories with little previous experience with the

test could successfully use the 48-hour red abalone protocol to measure effluent and reference chemical toxicity. Except for the Orange County effluent tests, most of the results of these experiments were in close agreement. The coefficients of variation in the effluent tests ranged from 1.8% to 16.0% (mean CV = 10.3%; n = 3).

The results of the zinc reference toxicant experiments were within the range of previous test results. Except for one test at MPSL, all reference tests met protocol test acceptability requirements. The coefficients of variation in the reference tests ranged from 11.3 to 25.4% (mean = 19.8%; n = 3).

The interlaboratory precision measurements from the comparisons reported here are comparable to the lowest reported for molluscan species. For example, in interlaboratory comparisons involving four laboratories using the Eastern Oyster (*Crassostrea virginica*) Zaroogian (1981) reported a mean coefficient of variation of 88% using 7 different toxicants; the range of CV's was 23% to 158% in these tests. Using the Pacific Oyster (*Crassostrea gigas*) Anderson et al. (1991) reported an interlaboratory coefficient of variation of 37% in tests involving four laboratories and one toxicant.

We attempted to further limit interlaboratory variability in the San Diego and JWPCP effluent tests between MPSL and ERCE by using the same dilution water and abalone broodstock from the same supplier. Results from these experiments, however, did not appear to be any less variable than the other comparisons (Table 1). For example, comparisons of the ERCE and MPSL EC_{50}'s in tests where different dilution waters and broodstock were used did not show greater interlaboratory variability. The coefficients of variation for EC_{50}'s from MPSL and ERCE were 20.2% and 3.9%, respectively, for the zinc reference tests where different dilution waters and abalone suppliers were used (eg., Orange County and Hyperion interlaboratory tests); the CV for the EC_{50}'s for the zinc reference tests where the same dilution waters and abalone suppliers were used was 25.4% (eg., JWPCP/San Diego interlaboratory tests) .

Rue et al. (1988) used the precision of analytical chemistry methods at concentrations near EPA acute water quality criteria levels as a standard to compare the precision of effluent toxicity tests. They found that acceptable CV's for trace metal ranged from 18% to 129%. Using this as a standard, the red abalone 48-h test shows minimal variability. The intralaboratory coefficient of variation for the three zinc tests conducted at MPSL during Phase 5 is 10.2%, comparable to the interlaboratory precision reported above.

Chemistry and Water Quality

Measured zinc concentrations varied somewhat from nominal concentrations in these experiments. The mean of the measured 18 µg/liter test solutions was 25.44 µg zinc/liter; the mean difference between all measured and nominal values at 18 µg zinc/liter was 25.5%. The mean of the measured concentration for the 32 µg/liter test solutions was 43 µg zinc/liter (mean difference between measured and nominal values was 24.6%). The mean of the measured concentration for the 56 µg/liter test solutions was 66.3 µg zinc/liter (mean difference between measured and nominal values was 18.5%). Overall, the mean

variation between measured and nominal zinc concentrations was 22.9%. It is unknown whether this difference resulted from errors in serial dilution, instrument calibration, sorption, or contamination.

Water quality measurements (DO, pH, temperature, and salinity) in all tests were within normal parameters, and met QA/QC requirements specified for the protocol.

Summary

1. Of 18 separate toxicity tests reported here, one test did not meet protocol test acceptability criteria for control response (MPSL - Figure 4). The remaining tests were successful and met all acceptability criteria. One test was repeated at MPSL due to test container toxicity; a second test at MPSL was repeated due to failure of a temperature control unit.

2. Results of interlaboratory comparisons showed good agreement between laboratories (except for the Orange County effluent interlaboratory tests). The mean coefficient of variation for EC_{50}'s for effluent tests at all participating laboratories was 10.3%. The mean coefficient of variation of EC_{50}'s for zinc reference tests for all participating laboratories was 19.8%. The precision measurements compare favorably to CV's reported for interlaboratory tests using other molluscan species.

3. Intralaboratory precision for the zinc reference tests conducted at MPSL during the Phase 5 research was 10.2%, lower than previously reported coefficients of variation.

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APPENDICES

The following appendices contain updated versions of the Marine Bioassay Project toxicity test protocols, as well as a quality assurance/quality control document developed to accompany the protocols. These protocols include minor changes from the protocols included in the Procedures Manual for Conducting Toxicity Tests Developed by the Marine Bioassay Project (State Water Resources Control Board, 1990). For regulatory purposes, the protocols presented here do not supercede those in the Procedures Manual. The Procedures Manual will remain as the official version of this set of protocols until the next triennial review of the Ocean Plan, which will specify the version to be used in regulatory applications.

APPENDIX I

TOPSMELT TOXICITY TEST PROTOCOL

TOPSMELT 7-DAY LARVAL GROWTH AND SURVIVAL TOXICITY TEST PROTOCOL

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1.0 Introduction

This protocol gives step-by-step instructions for performing a 7-day static renewal toxicity test using larval growth and survival to determine the toxicity of chemical compounds, complex effluents, or impacted marine and estuarine waters. In this procedure, topsmelt larvae are exposed to test solutions for 7 days. The percentage of larval mortality is tabulated and the remaining live larvae are dried then weighed to give a mean weight per larva for each treatment. These data are used to derive No Observed Effect Concentrations (NOECs) which give a numerical indication of toxicity. Because the test measures effects on larval stages of an ecologically important species possessing relatively stringent water quality requirements, the results constitute a good basis for decisions concerning either hazard evaluation or the suitability of marine waters for aquatic life (ASTM, 1987). A step by step summary of the protocol is provided in Section 13.

2.0 Equipment

2.1 Collection and Culture

- one-cm-mesh beach seine
- 100-liter clean plastic trash cans with lids
- 5-liter plastic buckets
- compressed oxygen or air with air stones
- recirculating pump
- cool-white lights
- water filter system

•1500-watt immersion heater

•(2) 1000-liter tanks

•fish food (Tetramin™ flake food)

•cotton or polyester fiber spawning substrate

2.2 Toxicity Testing

•meter and probes to measure pH, and dissolved oxygen

•pipets: (volumetric: 1 each 1, 10, 25, 50, 100-ml; graduated: 1 each 1-ml and 10-ml fire-polished wide bore)

•volumetric flasks: one-liter glass and polyethylene

•plastic squirt bottles

•thermometer and thermograph (for continuous temperature measurement)

•analytical balance

•salinity refractometer

•environmental chamber or water bath

•10-liter polyethylene plastic carboy

•10 liters 0.2-µm-filtered dilution seawater per test

•0.2-µm cartridge water filter

•cleaning liquids (2N HCl, 2N HNO_3, reagent grade acetone)

•500ml glass beakers (~30)

•300ml polyethylene plastic test containers (~30)

•data sheets

3.0 Experimental Design

3.1 Effluent Tests

To determine effluent toxicity, organisms are exposed to a series of effluent concentrations. The number of effluent concentrations should be based on study requirements or NPDES permit conditions. All concentrations must be replicated five times. Every test must contain five replicates of dilution water controls (see Quality Assurance Section 2.1.2 for a discussion of effluent dilution water). Tests that use hypersaline brine to adjust salinity must also contain five replicates of brine controls (see Section 4.2). Effluent concentrations should be assigned in a geometric sequence, with each concentration being at least 56% that of the next highest concentration (for example, 0% (control), 0% (brine or low salinity control) 0.56%, 1.0% 1.8%, 3.2%, 5.6%, and 10% effluent). Effluent treatments bracketing the concentration found at the edge of the outfall zone of initial dilution (ZID) may be most appropriate for evaluating

chronic toxicity. A preliminary range-finding test using a wider range of concentrations may be necessary for testing specific substances of unknown toxicity.

3.2 Reference Toxicant Tests

A reference toxicant test must be conducted concurrently with every effluent test. Reagent grade copper chloride ($CuCl_2 \cdot 2H_20$) should be used as the reference toxicant for topsmelt tests, unless another toxicant is specified by the Regional Water Quality Control Board. Stock solutions must be made on the day of the test. Prepare a 10,000 µg/liter copper stock solution by adding 0.0268 g $CuCl_2$ to one liter of distilled water in a one-liter volumetric flask (the attached dilution schedule gives the appropriate weights and volumes for making copper stocks and dilutions). Sample the reference toxicant stock solution at the beginning of the test for chemical verification of the copper concentration. Store samples in new, acid-washed 30 ml polyethylene vials. Preserve samples with 1% by volume double quartz distilled nitric acid (14N). Analyze samples within two months using atomic absorption spectroscopy.

Use five replicates of the following copper concentrations: 0, 56, 100, 180, and 320 µg/liter. Prepare one liter of each concentration by adding 0, 5.6, 10.0, 18.0, and 32 ml of 10,000 µg/liter stock solution, respectively, to a one-liter volumetric flask and fill with 0.2-µm-filtered reference dilution water (See Quality Assurance Section 2.1.2 for a discussion of reference toxicant dilution water). Start by mixing the control solution first and progress to the highest concentration to minimize contamination. Salinity adjustment and brine controls are not necessary in reference toxicant tests. The reference toxicant test must be conducted with larvae from the same cohort as those used in the effluent test.

4.0 Test Solutions

Prepare test solutions by combining effluent, hypersaline brine (or distilled water; see Section 4.2), and dilution water using volumetric flasks and pipets. Clean all glassware prior to use (see Section 7.0). Mix test solutions from the lowest concentration (control) to the highest concentration to avoid contamination.

4.1 Dilution Water

See Quality Assurance Section 2.1.2 for a discussion of dilution water.

4.2 Salinity Adjustment

Topsmelt larvae can tolerate a relatively wide range of salinities (10 through 35 ‰) if adequate acclimation is provided. In situations where the test salinity is significantly lower than

the salinity at which the larvae were cultured, it may be necessary to acclimate the larvae to the test salinity. Topsmelt larvae can tolerate a 5 ‰ per day change in salinity. When testing high effluents concentrations that result in test solution salinities of 28 ‰ or less (≥ 5 ‰ less than the larval culture salinity), advance planning for salinity acclimation may be necessary. Larvae should be acclimated by slowly dripping distilled water into an aerated (static) larval culture container to result in a 5 ‰ salinity daily decrease. Daily 5 ‰ adjustments can be made until the desired salinity is reached (not lower than 10 ‰).

The 7-day topsmelt protocol should be conducted at the salinity of the receiving water. Salinity adjustment is necessary where effluent concentrations are high enough to reduce test solution salinity below that of the receiving water, or in situations where the receiving water salinity is below that tolerable to topsmelt larvae (10 ‰). To maintain acceptable salinity, these test solutions must be adjusted to dilution water salinity by adding hypersaline brine. See Section 10.0 for statistical treatment of tests using brines.

If brine use is necessary, brines should be made by freezing 0.2-µm-filtered reference seawater (Anderson *et al.*, 1990). Clean, covered (not sealed) polyethylene containers should be used for freezing. One liter of brine can be made by freezing four one-liter containers of seawater in a conventional freezer (approximately minus 12°C). Freeze for approximately six hours, then separate the ice (composed mainly of fresh water) from the remaining liquid (which has now become hypersaline). Combine the liquid (brine) from the original four containers into two one-liter containers, place them back into the freezer overnight, then again separate the ice from the liquid brine. If the brine appears completely frozen, allow it to thaw; but check it often because the ice block can thaw quickly and liquid brine is often trapped inside. Confirm that the brine salinity is between 60 and 80 ‰ Brine can be refrozen or diluted to adjust its salinity.

Check the pH of all brine mixtures and adjust to within 0.1 units of dilution water pH by adding, dropwise, dilute hydrochloric acid or sodium hydroxide.

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

$$VB = VE\frac{(34 - SE)}{(SB - 34)}$$

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

4.2.1 Brine Controls

Use brine controls in all tests where brine is used. Brine controls contain the same amount of brine as does the highest effluent concentration using brine, plus distilled water equal to the volume of effluent in the highest concentration, plus dilution water to fill the mixing flask to one liter. For tests in which effluent salinity is greater than 10 ‰ or effluent dilutions above 10% are used, calculate the amount of distilled water to add to brine controls by using the above equation, setting SE = 0, and solving for VE.

See the example below and the attached dilution schedule worksheet for further details on making test solutions.

4.3 Example Test Solution

Two hundred milliliters of test solution are needed for each test container. To make a test solution at a concentration of 1% effluent, add 10 ml of effluent to the 1-liter volumetric flask using a volumetric pipet. Fill the volumetric flask to the 1-liter mark with dilution water, stopper it, and shake to mix. Pour equal volumes into the 5 replicate containers.

To make a test solution at a concentration of 80 % effluent, hypersaline brine must be used. Add 100 ml of effluent to a 1-liter volumetric flask. Then, assuming an effluent salinity of 2 ‰ and a brine salinity of 70 ‰, add 89 ml of brine (see equation above) and top off the flask with dilution water. Stopper the flask, shake well, and pour equal volumes into the 5 replicate containers.

4.4 Test Solution Renewals

The test duration is 7 days. Because effluent toxicity may change over short time periods in test containers, the test solutions must be renewed daily. Test solutions for the renewals should be prepared as described in Section 4.0. The water temperature of the new solution must be within 1 °C of the test container temperature to prevent thermal shock. The old solution is carefully siphoned out, leaving enough water so that all of the larvae can still swim freely (approximately 50 ml). Siphon from the bottom of the test containers so that dead *Artemia* nauplii are removed with the old test solution. It is convenient to siphon old solutions into a small (~500 ml) container in order to check to make sure that no larvae have been inadvertantly removed during solution renewals. If a larva is siphoned, return it to the test container and note it on the data sheet. New solution is siphoned into the test containers using a U-shaped glass rod attached to plastic tubing to minimize disturbance to the larvae.

5.0 Test Containers

For tests using complex effluents or organic reference toxicants, use 600 ml borosilicate glass beakers as the test containers. For tests using metal toxicants use 1000 ml polyethylene plastic food storage containers.

5.1 Randomization

To randomize placement of test containers and to eliminate bias in the analysis of test results, label the test containers using random numbers from 1 to 25 (for reference toxicant tests, or one to N for effluent tests, with N being the total number of containers). Record these numbers on a separate data sheet together with the concentration and replicate numbers to which they correspond. Identify this sheet with the date, test organism, test number, laboratory, and investigator's name, and safely store it away until after the larvae have been examined and weighed at the end of the test. **Note:** Loss of this randomization sheet would invalidate the test by making it impossible to analyze the data afterwards.

Arrange the test containers randomly in the water bath or controlled temperature room. Take care to follow the numbering system exactly while filling containers with the test solutions.

6.0 Physical/Chemical Measurements of Test Solutions

Prior to testing, consult the container randomization sheet (Section 5.0) to compile a list of containers to be sampled for measurement. One randomly chosen replicate from each test concentration should be measured as follows: measure salinity, pH, and dissolved oxygen at the beginning and end of the test; measure test solution temperature daily; and monitor water bath or environmental chamber temperature continuously. See Quality Assurance Section 5.0 for specifications and instrumentation for physical/chemical measurements.

7.0 Cleaning Procedure

New glass test containers should be scrubbed with a laboratory detergent and deionized water, then rinsed with deionized water, and soaked with dilution water overnight. Used containers should be cleaned as described below. New polystyrene tissue culture containers should not be washed, but should be soaked overnight in dilution water before use.

7.1 Effluents and organic toxicants

All test chambers used in organic toxicant and complex effluent tests should be cleaned as follows: 1) rinse three times with hot tap water, 2) rinse three times with new reagent grade methyl chloride, 3) rinse three times with deionized water, 4) soak 24 hours in 2N HCL, 5) rinse three times with deionized water, 6) soak 24 hours with HNO_3, 7) rinse three times with deionized water, 8) soak 24 hours in deionized water, 9) rinse three times with deionized water, 10) dry in a clean area. Acids may be re-used three times.

7.2 Trace metal toxicants

All test chambers used in testing trace metals should be cleaned as follows: 1) rinse three times with deionized water, 2) rinse three times with 2N HCl, 3) rinse three times with deionized water, 4) soak for 24 hours in 2N HCL, 5) rinse three times with deionized water, 6) soak 24 hours in 2N HNO_3, 7) rinse three times with deionized water, 8) soak 24 hours in deionized water, 9) rinse three times with deionized water, 10) dry in a clean area. Acids may be re-used three times.

7.3 Other Glassware

All volumetric flasks, pipets, and other labware used for handling effluent test solutions must be cleaned as described in Section 7.1. All volumetric flasks, pipets, and other labware used for handling trace metal reference toxicant solutions must be cleaned as described in Section 7.2.

8.0 Test Organism

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

8.1 Species Identification

Topsmelt, *Atherinops affinis*, often co-occur with jacksmelt, *Atherinopsis californiensis*. The two species can be distinguished based on several key characteristics. Jacksmelt have 10-12 scales between their two dorsal fins; topsmelt have 5-8 scales between the two fins. Jacksmelt teeth are arranged in several bands on each jaw and the teeth are not forked; topsmelt teeth are arranged in one band and the teeth are forked. In jacksmelt, the insertion of the first dorsal fin occurs well in advance of the origin of the anal fin. In topsmelt, the origin of the anal fin is under the insertion of the first dorsal fin. Consult Miller and Lea (1972) for a guide to the taxonomy of these two fishes.

8.2 Collection

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

Collection of topsmelt is regulated by California law. Collectors must obtain a scientific collector's permit from the California Department of Fish and Game and observe any regulations regarding collection, transfer, and maintenance of fish broodstock.

8.3 Broodstock Transport

Various containers can be used to transport fish; 100-liter covered plastic trash cans have been used successfully to transport topsmelt. New plastic containers should be leached in seawater for 96 hours prior to transporting fish. Each container can maintain approximately 20 adult fish for six to eight hours if adequate aeration is provided. Use compressed oxygen or air to supply aeration to the tanks during transport. Water temperature should not exceed 22 ° C during transport. See Section 8.6 for transport of larvae.

8.4 Broodstock Culture

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

maintained at greater than 6.0 mg/liter using aeration. Salinity should be checked periodically using a refractometer accurate to the nearest 0.5 ‰; tank salinity should be 34 ± 2 ‰.

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

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

8.5 Spawning Induction

Spawning is induced by a combination of three environmental cues: lighting, 'tidal' cycle, and temperature.

The photoperiod is 14 hours of light followed by 10 hours of darkness (14L:10D) with lights on at 0600 and off at 2000 hours. Use two cool white 40-watt fluorescent lamps suspended 1.25 meters above the surface of each tank to provide illumination. Light levels at the surface of the tanks should be 12 to 21 $\mu E\ m^{-2}\ s^{-1}$.

A 'tidal signal' of reduced current velocity is produced once daily in each tank, from 2400 to 0200 hrs, by turning off the circulating pump (Middaugh and Hemmer, 1984).

Temperature spikes are used as the final cue to induce spawning. A 1500-watt immersion heater is used to maintain constant temperature at 18 ° C and to provide temperature spikes. For spiking, the temperature is raised from 18 °C to 21 °C over a 12 h period, then allowed to return to 18 °C overnight. The temperature should be checked to the nearest 0.1 °C at 1 to 4 hour intervals on days when the temperature spikes are introduced. It is common for the fish to appear stressed during the temperature increase and one or two fish may die. If significant mortality begins to occur, the temperature should be lowered immediately. Significant egg production usually begins within five days of the temperature spike.

8.7 Larval Culture

Newly fertilized embryos should be placed in screen tubes set in aquaria and equipped with gently flowing seawater at 20 ±1 °C. The embryos can be left attached to the spawning substrates but care should be taken to ensure the substrates are relatively clean and free of food; strands of embryos should not overlap each other on the substrates, and gentle aeration must be provided. Beginning about day 9, check the screen tubes daily for the presence of larvae. Isolate newly-hatched larvae into a separate screen-tube at 21 °C by slow siphoning. Provide larvae with

newly-hatched *Artemia* nauplii (in excess) at 24-h post-hatch; supply gently flowing seawater, and aeration. Larvae aged 9 to 15 days are used in toxicity tests. For information about topsmelt larva suppliers call the Marine Pollution Studies Laboratory (408) 624-0947.

8.8 Larval Transport

Larvae can be transported in 1-liter ziplock plastic bags (double-bagged). No more than approximately 100 larvae should be transported in any one bag; do not include food. The seawater in the bags should be aerated with pure oxygen for 30 seconds prior to introduction of the larvae. The bag should be packed in an ice chest with one or two blue ice blocks (insulated by newspaper) for transport. The temperature during transport should be held between 15 and 18 °C. Larvae should be shipped via air-express overnight couriers.

9.0 Toxicity Test Procedure

9.1 Exposure of Test Organism

9.1.1 Randomized Placement of Larvae into Test Containers

Larvae must be randomized before placing them into the test containers. Pool all of the test larvae into a 1-liter beaker by slow siphoning from the screen-tube. The larvae in the screen-tube can be concentrated into the bottom by lifting the tube during siphoning. Using a fire-polished glass tube, place one larva into as many plastic cups as there are test containers (including reference toxicant containers). These cups should contain enough reference seawater to maintain water quality and temperature during the transfer process (approx. 50 ml). When each of the cups contains one larva, repeat the process, adding one larva at a time until each cup contains 5 animals.

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

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

9.1.2 Incubation and Feeding

The test period is 7 days and effluent solutions are renewed daily (see Section 4.4). The test temperature is 20°C. The feeding rates in the test containers must be closely controlled to

minimize over feeding and reduce variability. Add 40 newly hatched *Artemia* nauplii per larva twice daily: once in the morning and once in the afternoon. *Artemia* should be well rinsed with reference seawater and concentrated so that no more than one ml of seawater is added during feeding. Adjust feeding rates per larva as test animal numbers decline. Remove the majority of dead *Artemia* with every test solution renewal.

9.2 Endpoint

Survival and growth are analyzed at the end of 7 days. Death is defined as lack of response to stimulus such as prodding with a glass rod; dead larvae are generally opaque and curled. All surviving larvae in each replicate are dried together and the dry weight per larva is determined. The larvae are poured from the test container onto a screen (~ 400 µm) and rinsed thoroughly to remove *Artemia* and salt. They are then placed with forceps onto a pre-weighed and labelled foil. The foil numbers and corresponding weights are recorded on a separate data sheet prior to terminating the test; the foils should be dried for at least 24 hours prior to weighing. All larvae from each replicate are dried together for 24 hours at 55 °C. The larvae are then weighed together and the mean weight per larvae is determined by dividing the total weight per replicate by the number of larvae present. Take only a few foils from the drying ovens at a time for measuring or use a dessicator to prevent rehydrating the foils. If necessary, the larvae can be preserved in 5% buffered formalin, transferred to ethanol after 48 hours, and weighed at a later date for convenience.

10.0 Data Analysis

Use Analysis of Variance and Dunnett's multiple comparison test to determine No Observed Effect Concentrations (NOECs), as follows:

Divide the number of dead larvae in each replicate by the total (five) to get the proportion of dead larvae in each replicate. Proportional data must then be transformed for parametric statistical analysis. To transform the data, take the arcsine of the square root of each proportion value. Assign this transformed value to the proper test concentration using the original test container randomization sheet (see Section 5.1). Compare responses between concentrations using an analysis of variance (ANOVA). If a significant difference between concentrations is detected, use a Dunnett's multiple comparison test to compare each concentration against the control (Sokal and Rohlf, 1969; Zar, 1974). Derive the NOEC as the highest concentration that is not significantly different from the control. Concentrations above the NOEC for survival are excluded from the analysis of the growth data. Use an alpha level of $p = .05$ to determine statistical significance.

If brines were used in the effluent test, include all data in the ANOVA, and use Dunnet's multiple comparison test to deteremine the NOEC using the appropriate control (brine vs reference

toxicant). Use dilution water controls for the comparison with effluent treatments that had no brine added, and use brine controls for comparison with salinity adjusted effluents (see Section 4.2).

Use the trimmed Spearman-Karber method to calculate LC_{50}'s and EC_{50}'s and 95 % confidence intervals (Hamilton et al., 1977).

11.0 Test Acceptability

For tests to be considered acceptable, the following requirements must be met:

1) The mean survival of larvae must be at least 80% in the reference and effluent test controls. If the test starts with 9 day old larvae, the mean weight per larva must exceed 0.85 mg in the reference and brine controls; the mean weight of preserved larvae must exceed 0.72 mg. **Note:** Mean control weights for the other larva ages (10 - 15 day old) are now being established, please call the Marine Pollution Studies Laboratory for this information (408) 624-0947.
2) Results from the refence and brine controls must not be significantly different, as determined by a t-test with an alpha level of 0.05.
3) The response from the 180 µg/liter copper treatment for percent survival of larvae must be significantly different from the control response (see Section 10.0 for discussion of data analysis).
4) The between-replicate variability must be low enough that the ANOVA Error Mean Square (MS) does not exceed 10.00 in the reference toxicant test (using arcsine transformed proportion mortality data in degrees).

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13.0 Topsmelt Toxicity Test Protocol Summary

13.1 Preparation of Test Solutions

A. Determine effluent test concentrations and appropriate dilution water based on NPDES permit conditions and guidance from the appropriate regulatory agency (Section 3.1).

B. Prepare effluent test solutions by diluting well-mixed, unfiltered effluent using volumetric flasks and pipets (Section 4.0).

C. Prepare a copper chloride reference toxicant stock solution (10,000 µg/liter) by adding 0.0268g of reagent grade copper chloride ($CuCl_2 \cdot 2H_2O$) to 1 liter of distilled water (Section 3.2).

D. Prepare copper reference toxicant solutions of 0 (control), 56, 100, 180, and 320 µg/liter by adding 0, 5.6, 10.0, 18.0, and 32 ml of stock solution, respectively, to a one-liter polyethylene plastic volumetric flask and filling to one liter with 0.2-µm-filtered reference dilution seawater (Section 3.2).

E. Sample effluent and reference toxicant solutions for physical and chemical analysis. Measure salinity, pH, and dissolved oxygen from each test concentration (Section 6.0).

F. Sample stock solution for chemical verification of copper concentrations. Acidify sample vials with 1% by volume 14N double quartz-distilled nitric acid, and store in a refrigerator for no more than three months before analysis (Section 3.2).

G. Order the test containers from 1 - 25 and pour 200 mls of the appropriate test solution into each container. Randomize the test containers, and place five, 9 day-old larvae into each (Section 5.1). Place test containers in an environmental chamber, and allow temperature to equilibrate (Section 5.1).

H. Measure the temperature daily in one random replicate of each test concentration. Monitor the temperature of the water bath or environmental chamber continuously (Section 6.0).

I. At the end of the test, measure salinity, pH, and dissolved oxygen from each test concentration (Section 6.0).

13.2 Preparation and Analysis of Test Organisms

A) Slow-siphon 9 to 15 day-old larvae from culture screen-tube into a 1000 ml beaker (Section 9.1.1).

B) Randomize five larvae into as many randomization cups as there are test containers (Section 9.1.1).

C) Pour 200 ml of the appropriate test solution into each test container (Section 4.0).

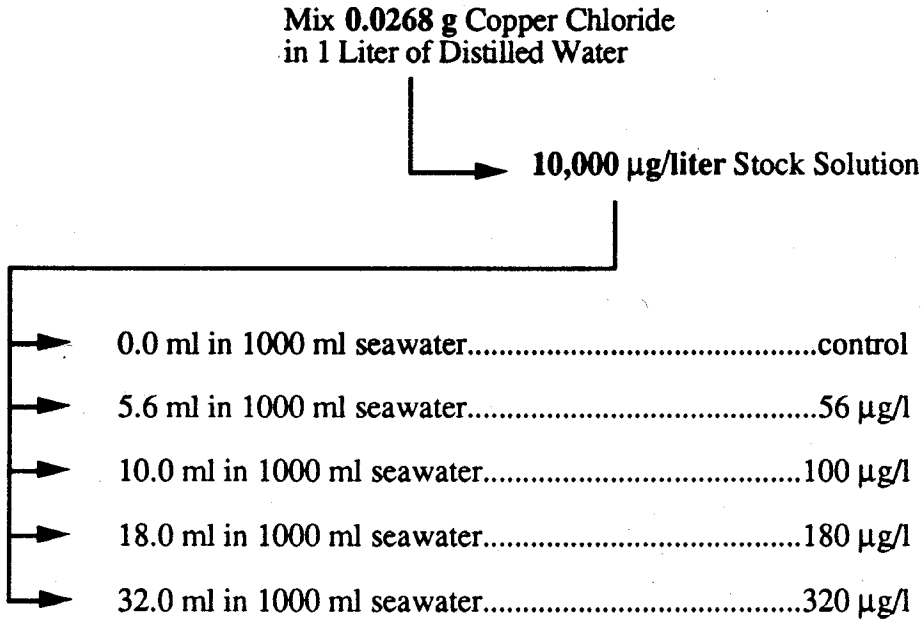
D) Randomize test containers and pipet five larvae into each test container (Section 9.1.2).

E) Assuming the test starts on day 0, renew effluent test solutions on days 2, 4, 6, and 7 (Section 4.4).

F) After 7 days determine the percentage larval survival and dry the remaining live larvae for 24 hours. Weigh all of the larvae form each replicate and divide by the total number per replicate to determine the men weight per larva for each replicate (Section 9.2).

G) Use ANOVA followed by either Dunnett's multiple comparison test to determine the NOEC for effluent and reference toxicant tests ($p = 0.05$; refer to Sokal and Rohlf 1969 for statistical methods, Section 11.0). Determine EC_{50}'s using the trimmed Spearman-Karber method.

Copper Dilutions
for Topsmelt Tests



Effluent Dilution Sheet

100% Effluent is the Stock Solution			Corresponding Beaker Numbers
→	0.0 ml in 1000 ml flask	→ Control	
→	0.0 ml in 1000 ml flask	→ Brine Control	
→	_____	→ Other	
→	_____	→ Other	
→	5.6 ml in 1000 ml flask	→ 0.56%	
→	10.0 ml in 1000 ml flask	→ 1.0%	
→	18.0 ml in 1000 ml flask	→ 1.8%	
→	32.0 ml in 1000 ml flask	→ 3.2%	
→	56.0 ml in 1000 ml flask	→ 5.6%	
→	100.0 ml in 1000 ml flask	→ 10.0%	
→	_____	→ Other	

Date Organism Investigator

Notes

Salinity Adjustment Using Hypersaline Brine

Add hypersaline brine to those concentrations in which test solution salinity would otherwise fall below the minimum acceptable test salinity (32‰).
The equation to calculate the volume of brine to add for each of these concentrations is: $VB = VE\left(\frac{34 - SE}{SB - 34}\right)$

Quantities known from dilution schedule: VE = Volume of Effluent added for each concentration (ml).

Quantities to be measured: SB = Salinity of Brine (‰), and SE = Salinity of Effluent (‰). SB = _____‰ SE = _____‰

Note: Always adjust the pH of the brine to equal that of the dilution water. Brine salinity should be 60 to 80 ‰.

Calculate the volume of brine to be added, VB, for each concentration that requires salinity adjustment using the above equation.

Example: If dilution water salinity is 33.5‰ and effluent salinity is 0‰, a test solution of 5.6% effluent would have a salinity of 31.6‰, which is below the acceptable salinity range. This test solution and any with a higher effluent concentration would have to be adjusted with hypersaline brine, and brine controls would be necessary. If the dilution schedule above is used, and the highest effluent concentration is 10%, then these test solutions and the brine control would be made up as follows:

Measure SB and SE. Use these to calculate the quantity $\frac{34 - SE}{SB - 34}$ = _____

Effluent Concentration	VE		$\frac{34 - SE}{SB - 34}$		VB	Final Test Solution Mixture
5.6%	56 ml	x	_____	=	_____ ml Brine	+ 56 ml effluent in a one liter flask; fill with seawater
10.0%	100 ml	x	_____	=	_____ ml Brine	+ 100 ml effluent in a one liter flask; fill with seawater
Brine Control	100 ml	x	_____	=	_____ ml Brine	+ 100 ml distilled water " " " ; fill with seawater

Marine Pollution Studies Laboratory

Data Sheet for Larval Fish Toxicity Test

Test Start Date: Start Time: Fish Species :
Test End Date: End Time: Station Codes for Env. Samples:
Fish Age at Start (days post-hatch): Sample Type
Toxicant: (Solid, Elutriate, Pore Water, Water):
Sample Source: Collection Date:
Ref. Tox. Chem. Sample No's: Analyzed By:

Test Cont. #	Tox. Conc.	Number Dead							Total Number Dead	Total Number at Start	Notes
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7			

Computer Data Storage
Disk:
File:

Note: See larval weight data on separate sheet.

Marine Pollution Studies Laboratory

Data Sheet for Larval Fish Toxicity Test

Test Start Date: Start Time: Fish Species :
Test End Date: End Time: Station Codes for Env. Samples:
Fish Age at Start (days post-hatch): Sample Type
Toxicant: (Solid, Elutriate, Pore Water, Water):
Sample Source: Collection Date:
Ref. Tox. Chem. Sample No's: Analyzed By:

Test Container Number	Toxicant Concentration	Foil Number	Foil Weight (mg)	Total Weight (mg)	Weight of Larval Fish (mg)	Number of Fish Larvae	Weight per Larval Fish (mg)

Computer Data Storage
Disk:
File:

Notes

Note: See larval mortality data on separate sheet.

APPENDIX II

MYSID TOXICITY TEST PROTOCOL

HOLMESIMYSIS COSTATA, JUVENILE MYSID GROWTH AND SURVIVAL TOXICITY TEST PROTOCOL (TENTATIVE)

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1.0 Introduction

This protocol gives step-by-step instructions for performing a 7-day static renewal toxicity test using 3-day-old juvenile mysids to determine the toxicity of chemical compounds, complex effluents, or marine waters. In this procedure, juvenile mysids are exposed to test solutions for seven days, after which the percentage mortality and total length are determined in each toxicant concentration. These data are used to derive No Observed Effect Concentrations (NOECs) or median effective concentrations (EC_{50}'s), which give a numerical indication of toxicity. Because the test measures effects on an early life-stage of an ecologically important species possessing relatively stringent water quality requirements, the results constitute a good basis for decisions concerning either hazard evaluation or the suitability of marine waters for aquatic life (ASTM, 1987). A summary of test procedures is given in Section 13.0.

2.0 Equipment

2.1 Collection and Culture

- 500-µm-mesh hand nets (~ 25-cm diameter opening)
- 20-liter plastic buckets with tight fitting lids
- airstones and portable aeration (pumps or compressed air or oxygen)
- aerated culture tanks
- nylon screening (100-µm, 150-µm, 500-µm, 2-mm)

- 20-µm-filtered and 1-µm-filtered seawater (15°C, see Quality Assurance Section 4.6)
- brine shrimp *Artemia* cysts for producing *Artemia* nauplii (see Section 8.3)
- Tetramin (or other flake fish food with > 5% lipid content)
- fronds of kelp (*Macrocystis*) for habitat in culture

2.2 Toxicity Testing

- 20- to 80-liter aquarium (static, recirculating, or flow-through; 150-µm mesh screened outflow)
- 2-mm-mesh screened compartment to separate juveniles from adults (see Section 8.4)
- *Artemia* nauplii (see Section 8.3)
- fronds of kelp (*Macrocystis*) for habitat in release aquarium
- smooth glass tubes [5-mm-bore, 15-cm length, with suction bulbs (for handling adults)]
- wide-bore 10-ml pipet or glass tubes [3- to 4-mm-bore (for handling juveniles)]
- 1000-ml glass beaker
- plastic randomization cups (~ 100 ml, one for each test container)
- 2 plastic, screen-bottom tubes (150-µm-mesh, 25 cm dia. for mysids; and 100-µm-mesh for *Artemia*)
- meter and probes to measure pH, and dissolved oxygen (and ammonia if specified in permit)
- refractometer to measure salinity
- thermometer and thermograph (for continuous temperature measurement)
- water bath or environmental chamber
- test containers (see Section 5.0)
- sample vials for trace metal reference toxicant analysis (polyethylene 30 ml, new, acid washed)
- volumetric pipets: 1-, 5-, 10-, 25-, and 100-ml
- graduated pipets: 1- and 10-ml
- volumetric flasks: 1 liter (glass for effluents and organics, plastic for trace metals)
- analytical balance
- plastic squirt bottles
- 10-liter polyethylene water bottle
- 10 liters of 0.2-µm-filtered dilution seawater per renewal (see Quality Assurance Section 2.1.2)
- Microscopes and micrometers for measuring mysid length (see Section 9.3.2)
- Reagent grade acetone (for cleaning, 1 liter per test; see Section 7.1)

- 2N hydrochloric acid (for cleaning, 15 liters/test, can be reused 3 times; Section 7.0)
- 2N nitric acid (for cleaning, 15 liters per test, can be reused 3 times; see Section 7.0)
- data sheets

3.0 Experimental Design

3.1 Effluent Tests

To determine effluent toxicity, organisms are exposed to test solutions of different effluent concentrations. The number and concentration of effluent treatments should be based on study requirements or NPDES permit conditions. All treatments must be replicated five times. Every test must contain five replicates of dilution water controls (see Quality Assurance Section 2.1.2 for a discussion of effluent dilution water). Tests that use hypersaline brine to adjust salinity must also contain five replicates of brine controls (see Section 4.2). Effluent concentrations should be assigned in a geometric sequence, with each concentration being at least 56% that of the next highest concentration (for example, 0% (control), 0% (brine control) 0.56%, 1.0% 1.8%, 3.2%, 5.6%, and 10% effluent). Effluent treatments bracketing the concentration found at the edge of the outfall zone of initial dilution (ZID) may be most appropriate for evaluating chronic toxicity. A preliminary range-finding test using a wider range of concentrations may be necessary for testing specific substances of unknown toxicity.

3.2 Reference Toxicant Tests

A reference toxicant test must be conducted concurrently with every effluent test to indicate the sensitivity of the organisms and the suitability of the test methodology. Reagent grade zinc sulfate should be used as the reference toxicant for mysid tests, unless another toxicant is specified by the Regional Water Quality Control Board or other appropriate regulatory agency. Stock solutions should be made on the day of the test. Prepare a 10,000 µg/liter zinc stock solution by adding 0.0440 g of zinc sulfate ($ZnSO_4 \cdot 7H_2O$) to one liter of distilled water in a polyethylene volumetric flask. Sample the reference toxicant stock solution at the beginning of the test for chemical verification of the zinc concentration. Acidify samples in clean sample vials (Section 7.2) with 1% by volume 14N double-quartz-distilled nitric acid, and refrigerate samples for no more than three months before analysis.

Reference toxicant solutions should be five replicates of 0 (control) 10, 18, 32, 56, and 100 µg/liter. Prepare one liter of each concentration by adding 0, 1.0, 1.8, 3.2, 5.6, and 10.0 ml of stock solution, respectively, to a one-liter volumetric flask and fill with 0.2-µm-filtered reference dilution water (see attached dilution schedule; also see Quality Assurance Section 2.1.2 for a discussion of reference dilution water). Start with the control solutions and progress to the

highest concentration to minimize contamination. Salinity adjustment and brine controls are not necessary in reference toxicant tests.

The effluent and reference toxicant tests must use juveniles released on the same day from the same pool of gravid females. They must be handled in the same way and delivered to the test containers at the same time.

4.0 Test Solutions

Prepare test solutions by combining effluent, hypersaline brine (see Section 4.2), and dilution water using volumetric flasks and pipets. Clean all glassware prior to use (see Section 7.0). Mix test solutions from the lowest concentration (control) to the highest concentration to avoid contamination.

4.1 Dilution Water

See Quality Assurance Section 2.1.2 for a discussion of dilution water.

4.2 Salinity Adjustment

The salinity of sewage effluents is generally lower than that tolerated by *H. costata*. Salinity adjustment is necessary where effluent concentrations are high enough to reduce test solution salinity below the acceptable range (34 ± 2‰). To maintain acceptable salinity, these test solutions must be adjusted to dilution water salinity by adding hypersaline brine. See Section 10.0 for statistical treatment of tests using brines.

If brine use is necessary, brines should be made by freezing 0.2-µm-filtered reference seawater (Anderson *et al.*, 1990). Clean, covered (not sealed) polyethylene containers should be used for freezing. One liter of brine can be made by freezing four one-liter containers of seawater in a conventional freezer (approximately minus 12°C). Freeze for approximately six hours, then separate the ice (composed mainly of fresh water) from the remaining liquid (which has now become hypersaline). Combine the liquid (brine) from the original four containers into two one-liter containers, place them back into the freezer overnight, then again separate the ice from the liquid brine. If the brine appears completely frozen, allow it to thaw; but check it often because the ice block can thaw quickly and liquid brine is often trapped inside. Check the salinity; brine salinity should be 60 to 80 ‰ Brine can be refrozen or diluted to adjust its salinity.

Check the pH of all brine mixtures and adjust to within 0.1 units of dilution water pH by adding, dropwise, dilute hydrochloric acid or sodium hydroxide.

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

$$VB = VE\frac{(34 - SE)}{(SB - 34)}$$

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

4.2.1 Brine Controls

Use brine controls in all tests where brine is used. Brine controls contain the same amount of brine as does the highest effluent concentration, plus distilled water equal to the volume of effluent in the highest concentration, plus dilution water to fill the mixing flask. For tests in which the effluent salinity is greater than 10 ‰, or if effluent dilutions above 10% are used, calculate the amount of distilled water to add to brine controls by using the above equation, setting SE = 0, and solving for VE.

See the example below and the attached dilution schedule worksheet for further details on making test solutions.

4.3 Example Test Solution

Two hundred milliliters of test solution are needed for each test container. Five replicates can be mixed in a 1-liter volumetric flask. To make a test solution at a concentration of 1% effluent, add 10 ml of effluent to the 1-liter volumetric flask using a volumetric pipet. Fill the volumetric flask to the 1-liter mark with dilution water, stopper it, and shake to mix. Pour equal volumes into the five replicate containers.

To make a test solution at a concentration of 10% effluent, hypersaline brine must be used. Add 100 ml of effluent to a 1-liter volumetric flask. Then, assuming an effluent salinity of 2 ‰ and a brine salinity of 70 ‰, add 89 ml of brine (see equation above) and top off the flask with dilution water. Stopper the flask, shake well, and pour equal volumes into the 5 replicate containers.

4.4 Renewal

The test duration is 7 days. Because effluent toxicity may change over short time periods in test containers, the test solutions must be renewed after 48 hours. Prepare new test solutions exactly as above. Remove three quarters of the original test solution from each container, taking care to avoid losing or damaging mysids. This can be done by siphoning with a small-bore (2 to

3 mm) fire-polished glass tube or pipet. Attach the glass tube to clear plastic tubing fitted with a pinch clamp so that the siphon flow can be stopped quickly if necessary to release entrained mysids. Follow the container randomization sheet (Section 5.1) to siphon first from the controls, then work sequentially to the highest test concentration to avoid cross-contamination. Glass tubes or pipets should be cleaned as in Section 7.0.

To minimize disturbance to the juvenile mysids, refill the containers to the 200-ml mark by carefully siphoning new test solution into the test containers using small diameter plastic tubing attached to a U-shaped clean glass rod that directs incoming upward to slow the current and minimize turbulence.

5.0 Test Containers

Test containers must hold 200 ml of test solution and should provide ample flat surface area to separate individual mysids. Recommended containers are glass stacking dishes (350-ml capacity) for complex effluents and organic toxicants, and polyethylene food containers (one pint, ~300-ml capacity) for trace metals.

5.1 Randomization

To randomize placement of test containers and to eliminate bias in the analysis of test results, label the test containers using random numbers from 1 to 30 (for reference toxicant tests, or 1 to N for effluent tests, with N being the total number of containers). Record these numbers on a separate data sheet together with the concentration and replicate numbers to which they correspond. Identify this sheet with the date, test organism, test number, laboratory, and investigator's name, and safely store it away until after the mysids have been examined at the end of the test.

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

Arrange the test containers randomly in the water bath or controlled temperature room. It is convenient to make a map of container placement for reference during water changes.

6.0 Physical/Chemical Measurements of Test Solutions

Prior to testing, consult the container randomization sheet (Section 5.1) to compile a list of containers to be sampled for measurement. One randomly chosen replicate from each test concentration should be measured as follows: measure salinity, pH, and dissolved oxygen concentration at the beginning and end of the test and before each renewal; measure test solution temperature daily; and monitor water bath or environmental chamber temperature continuously. See Quality Assurance Section 5.0 for specifications and instrumentation for physical/chemical measurements.

7.0 Cleaning Procedure

New food containers and stacking dishes should be scrubbed with a laboratory detergent and hot tap water, then rinsed with deionized water, and soaked with dilution water overnight. Used containers should be cleaned as described below.

7.1 Effluents and organic toxicants

All test chambers used in organics and complex effluent tests should be cleaned as follows: 1) rinse three times with hot tap water, 2) rinse three times with new reagent grade acetone, 3) rinse three times with deionized water, 4) soak 24 hours in 2N HCL, 5) rinse three times with deionized water, 6) soak 24 hours in 2N HNO3, 7) rinse three times with deionized water, 8) soak 24 hours in deionized water, 9) rinse three times with deionized water, 10) dry in a clean area. Acids may be reused three times.

7.2 Trace metal toxicants

All test chambers used in testing trace metals should be cleaned as follows: 1) rinse three times with deionized water, 2) rinse three times with 2N HCL, 3) rinse three times with deionized water, 4) soak for 24 hours in 2N HCL, 5) rinse three times with deionized water, 6) soak 24 hours in 2N HNO_3, 7) rinse three times with deionized water, 8) soak 24 hours in deionized water, 9) rinse three times with deionized water, 10) dry in a clean area. Acids may be reused three times.

7.3 Other Glassware

All volumetric flasks, pipets, and other labware used for handling effluent test solutions must be cleaned as described in Section 7.1. All volumetric flasks, pipets, and other labware used for handling trace metal reference toxicant solutions must be cleaned as described in Section 7.2.

8.0 Test Organism

The test organism for this protocol is the juvenile mysid crustacean *Holmesimysis costata* (Holmes 1900; previously refered to as *Acanthomysis sculpta*). *H. costata* occurs in the surface canopy of the giant kelp *Macrocystis pyrifera* where it feeds on zooplankters, kelp, epiphytes, and detritus. There are few references to the ecology of this mysid species (Holmquist, 1979; Clutter, 1967, 1969; Green, 1970). *H. costata* is numerically abundant in kelp forest habitats and is considered to be an important food source for kelp forest fish (Clark 1971, Mauchline 1980). Mysids are called oppossum shrimp because females brood their young in an abdominal pouch, the marsupium. *H. costata* eggs develop for about 20 days in the marsupium before the young are released as juveniles; broods are released at night during molting. Females release their first brood at 55 to 70 days post-release (at 12° C), and may have multiple broods throughout their approximately 120-day life.

H. costata has been used in previous toxicity studies with a variety of toxicants (Tatem and Portzer, 1985; Davidson et al., 1986; Machuzac and Mikel, 1987; Reish and Lemay, 1988; Asato, 1988; Martin et al., 1989; Singer et al., 1990; 1991). Mysids are useful as toxicity test organisms because of their widespread availability, ecological importance, sensitivity to toxicants, and amenability to laboratory culture (Nimmo et al., 1977; Mauchline, 1980; Gentile et al., 1982; Lussier et al., 1985).

8.1 Species Identification

All mysids must be identified to species; refer to Holmquist (1979, 1981). There have been recent revisions to the taxonomy of *H. costata*. Previous authors have referred to this species as *Acanthomysis sculpta*. However, Holmquist's (1979) review considers previous references to *Acanthomysis sculpta* in California to be synonymous with *Holmesimysis costata;* we consider Holmquist's designation to be definitive.

8.2 Collection and Transport

H. costata can be collected by sweeping a small-mesh (0.5 - 1 mm) hand net through the water just under the surface canopy blades of giant kelp *Macrocystis pyrifera*. Although this method collects mysids of all sizes, attention should be paid to the number of gravid females collected because these are used to produce the juvenile mysids used in toxicity testing. Mysids should be collected from waters remote from sources of pollution to minimize the possibility of physiological or genetic adaptation to toxicants (see Quality Assurance Section 3.4).

Mysids can be transported for a short time (< 3 hours) in tightly covered 20 liter plastic buckets. The buckets should be filled to the top with seawater from the collection site, and should

be gently aerated or oxygenated to maintain dissolved oxygen above 60% saturation (see Section 2.1). Transport temperatures should remain within 3° C of the temperature at the collection site.

For longer transport times of up to 36 hours, mysids can be shipped in sealed plastic bags filled with seawater. The following transport procedure has been used successfully: 1) fill the plastic bag with one liter of 1-μm-filtered seawater, 2) saturate the seawater with oxygen by bubbling pure oxygen for at least 10 minutes, 3) place 25-30 adult mysids, or up to 100 juvenile mysids in each bag, 4) for adults add about 20 *Artemia* nauplii per mysid, for 100 juveniles add a pinch (10 to 20 mg) of ground Tetramin® flake food and 200 newly-hatched *Artemia* nauplii, 5) seal the bag securely, eliminating any airspace, then 6) place it within a second sealed bag in an ice chest. A well insulated ice chest should be cooled to approximately 15 °C by adding one 1-liter blue ice block for every five 1-liter bags of mysids (a temperature range of 12 to 16 °C is tolerable). Wrap the ice in newspaper and a plastic bag to insulate it from the mysid bags. Pack the bags tightly to avoid shifting within the cooler.

8.3 Culture and Handling

After collection the mysids should be transported directly to the laboratory and placed in seawater tanks or aquaria equipped with flowing seawater. Initial flow rates should be adjusted so that any temperature change occurs gradually (0.5 °C per hour). The water temperature should be held at 15° ± 1° C. **Note:** Mysids collected north of Pt. Conception should be held and tested at 13° ± 1° C.

Mysids can be cultured in tanks ranging from 4 to 1000 liters. Tanks should be equipped with gentle aeration and blades of *Macrocystis* to provide habitat. Static culture tanks can be used if there is constant aeration, temperature control, and frequent water changes (one half the water volume changed at least twice a week). Maintain culture density below 20 animals per liter by culling out adult males or juveniles.

Adult mysids should be fed 100 *Artemia* nauplii per mysid per day. Juveniles should be fed 5 to 10 newly released *Artemia* nauplii per juvenile per day and a pinch (10 to 20 mg) of ground Tetramin® flake food per 100 juveniles per day. Static containers should be carefully monitored and rations adjusted to prevent overfeeding and fouling of culture water. Refer to Weber et al. (1988) for a discussion of *Artemia* culture and quality control.

8.3.1 Culture Materials

Refer to Quality Assurance Section 4.6 and 4.7 for a discussion of suitable materials to be used in laboratory culture of mysids. Be sure all new materials are properly leached in seawater

before use. After use, all culture materials should be washed in soap and water, then rinsed with seawater before re-use.

8.4 Isolation of Test Animals

Approximately 150 gravid female mysids should be isolated to provide approximately 400 juveniles for each set of toxicity tests (5 juveniles/container x 30 reference toxicant containers and aproximately 35 effluent containers, plus additional mysids so that only healthy active juveniles are used in the test). Gravid females can be identified by their large, extended marsupia filled with (visible) eyed juveniles; ripe females have grey marsupia.

Gravid females are easily isolated from other mysids using the following technique: 1) use a small dip net to capture about 100 mysids from the culture tank, 2) transfer the mysids to a screen-bottomed plastic tube (150 µm-mesh, 25-cm diam.) partly immersed in a water bath or bucket, 3) lift the screen-tube out of the water to immobilize mysids on the damp screen, 4) gently draw the gravid females off the screen with a suction bulb and fire-polished glass tube (5-mm bore), 5) collect the gravid females in a separate screen tube. Re-immerse the screen constantly during the isolation process to keep the mysids healthy.

Four days before a toxicity test begins, transfer gravid females into a removable, 2-mm-mesh screened cradle suspended within an aerated 80-liter aquarium. Before transfer, make sure there are no juveniles in with the adult females. Extraneous juveniles are excluded to avoid inadvertantly mixing them with the soon-to-be released juveniles used in testing. Provide the gravid females with newly hatched *Artemia* nauplii (approx. 200 per mysid) to help stimulate juvenile release. *Artemia* can be provided continuously throughout the night from an aerated reservoir holding approximately 75,000 *Artemia*. Direct the flow from the feeder into the screened compartment with the females, and add a few blades of *Macrocystis* for habitat. The females are placed within the screened compartment so that as the juveniles are released, they can swim through the mesh into the bottom of the aquarium. Outflows on flow-through aquaria should be screened (150-µm-mesh) to retain juveniles and allow some *Artemia* to escape.

Juveniles are generally released at night, so it is important to turn off all lights at night to promote release. In the morning, the screened compartment containing the females should be removed and placed in a separate aquarium. Juveniles should be slowly siphoned through a wide-diameter hose into a 150-µm-mesh screen-bottom tube (25 cm diam.) immersed in a bucket filled with clean seawater. Once the release aquarium is emptied, it should be washed with hot fresh water to eliminate stray juvenile.

After collection, the number of juveniles should be estimated visually or by counting subsamples with a small beaker. If there are not enough juveniles to conduct the necessary tests,

they should be discarded so they are not mixed with juveniles from subsequent releases. Initial experiments indicate that there may be differences in toxicant sensitivity among mysids of different ages (Hunt et al., 1989; Martin et al., 1989).

If there are enough juveniles to conduct the necessary tests, they should be transferred to additional screen-tubes (or to 4-liter static beakers if flowing seawater is unavailable). The screen-tubes are suspended in a 15-liter bucket so that 1-µm-filtered seawater (0.5 liter/min.) can flow into the tube, through the screen, and out the bucket. Check water flow rates to make sure that juveniles or *Artemia* nauplii are not forced down onto the screen. The height of the bucket determines the level of water in the screen tube. About 200 to 300 juveniles can be held in each screen-tube (200 juveniles per static 4-liter beaker). Juveniles should be fed 40 newly hatched *Artemia* nauplii per mysid per day and a pinch (10 to 20 mg) of ground Tetramin® flake food per 100 juveniles per day. A blade of *Macrocystis* (well rinsed in seawater) should be added to each container. Containers should be gently aerated and temperature controlled at 15° ± 1°C (or 13° ± 1°C if collected north of Pt. Conception). Half of the seawater in static containers should be changed at least once between isolation and test day.

The day juveniles are isolated is considered day 0 (the morning after their nighttime release). The toxicity test should begin on day three. For example, if juveniles are isolated on Friday, the toxicity test should begin on the following Monday.

9.0 Toxicity Test Procedure

9.1 Randomized Placement of Mysids into Test Containers

The juvenile mysids must be randomized before placing them into the test containers. Pool all of the test juveniles into a 1-liter beaker. Using a 10-ml wide-bore pipet or polished glass tube (approximately 4 mm inside diameter), place one or two juveniles into as many plastic cups as there are test containers (including reference toxicant containers). These cups should contain enough reference seawater to maintain water quality and temperature during the transfer process (approx. 50 ml). When each of the cups contains one or two juveniles, repeat the process, adding mysids until each cup contains 5 animals.

Carefully pour or pipet off excess water in the cups, leaving less than 5 ml with the test mysids. This 5 ml volume can be estimated visually after initial measurements. Carefully pour or pipet the juveniles into the test containers immediately after reducing the water volume. Gently rocking the water back and forth before pouring may help prevent juveniles from clinging to the walls of the randomization cups. Juveniles can become trapped in drops; have a squirt bottle ready to gently rinse down any trapped mysids. If more than 5 mls of water are added to the test

solution with the juveniles, report the amount on the data sheet. Be sure that all water used in culture, transfer, and test solutions is within 1°C of the test temperature. Because of the small volumes involved in the transfer process, temperature control is best accomplished in a constant temperature room.

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

9.2 Incubation and Feeding

The mysids must be exposed to the toxicant for 7 days. About sevety-five percent of the test solution must be renewed every 48 hours (see Section 4.4). Test temperature should be 15° C. (**Note:** the test temperature should be 13° C for mysids collected north of Pt. Conception.) Photoperiod should be 16 hours light and 8 hours dark. Light intensity should be no more than that provided by normal overhead laboratory lighting (10 to 20 microeinsteins m^{-2} sec^{-1} = 100 to 200 lux). Aerate the test containers only if dissolved oxygen concentrationss drop below 60% saturation (any use of aeration must be noted on the data sheet). Measure physical/chemical parameters as described in Section 6.0.

The feeding rates in the test beakers should be closely controlled to avoid overfeeding and fouling of test solutions. Add 40 newly hatched *Artemia* nauplii per test animal every 24 hours. *Artemia* nauplii should be well rinsed with reference seawater and concentrated so that no more than one ml of seawater is added during feeding. (Use a 100-µm-mesh screen tube for rinsing and concentrating the nauplii).

9.3 Endpoint Determination

9.3.1 Mortality

The two toxicity test endpoints are growth inhibition and mortality. Assess mortality by counting any dead mysids each day as the test progresses. The total number dead in each container at the end of the test is used in subsequent statistical analyses (see Section 10.0). Immobile mysids that do not respond to a stimulus are considered dead. The stimulus should be two or three gentle prods with a disposable pipet. Mysids that exhibit any response clearly visible to the naked eye are considered living. The most commonly observed movement in moribund mysids is a quick contraction of the abdomen. This or any other obvious movement qualifies a mysid as alive.

Test containers should be inspected each day, and any dead mysids should be removed with a pipet. This is necessary to avoid cannibalism and to prevent fouling of test solutions. Avoid cross-contamination by using a separate disposable pipet for each container.

9.3.2 Growth

Even when five 10-day-old mysids are pooled, their weight is too low to accurately measure using standard equipment. Growth is therefore measured as growth in length. (Future test modifications may involve increasing the number of replicates per treatment and pooling mysids from all replicates to measure weight. At present such pooled weight data is not available, and corresponding statistical techniques have not yet been evaluated.

To prepare mysids for measurement at the end of the exposure period, carefully siphon at least half the test solution from the test container, taking care not to lose any mysids. Make sure mortality counts have already been recorded. Pipet mysids from each test container into corresponding labelled test tubes (at least 10 ml capacity).

When all mysids have been placed in test tubes with at least 5 ml of seawater, the test tubes are cooled to relax the mysids before fixing. If preserved improperly, mysids will flex and curl their abdomenal segments, and the resulting dorsal extension confounds length measurements. To avoid abdomenal curling, place the test tubes in a conventional freezer and allow them to cool until ice begins to form at the surface of the seawater. (Mysids can be frozen solid without destruction of the exoskeleton, but more testing is necessary to determine if solid freezing affects length measurements.) Once the mysids have been cooled, remove them from the freezer, allow any ice to thaw, and add formalin to fix them in a final 5% formalin solution. Preserved mysids can be held for up to three months before measuring.

Position mysids so they are lying on their sides on a surface suitable for measurement under a microscope. It may be convenient to place individual mysids into individual wells on a multi-well depression slide, so that each slide holds all mysids from one replicate test container.

To determine mysid growth, measure the total length of each mysid. Total length is defined as the length of the dorsal edge from the base of the eyestock to the tip of the telson (see Figure 1). Even mysids that have been cooled before preservation will seldom have perfectly straight dorsal edges. To be accurate, the total length measurement must account for any curvature. The dorsal edge of curved mysids should be measured as the sum of a few relatively straight segments rather than as one simple line from head to tail.

Measurement of curved mysids is facillitated with a projecting microscope or a compound microscope with a *camera lucida* attachment, and measuring the outline using a fine scale odometer (available from architect supply stores). Any instrument used to measure tracings or projected

outlines is calibrated against the image of a stage micrometer projected and traced onto the paper at the same magnification. Other acceptable equipment for length measurement include compound microscopes (at ≤40x) with ocular micrometers, dissecting microscopes with a fine grid underlying the mysid, and compound microscopes with video and digitizer attachments.

Mysids should be measured to the nearest 0.05 mm. Total length of 10-day-old *Holmesimysis* averages about 2.50 to 3.00 mm.

10.0 Data Analysis

Appropriate statistical methods for Marine Bioassay Project protocols are under ongoing review. Check to be sure that the protocol being used is the most recent version.

To determine No Observed Effect Concentrations (NOECs), use Analysis of Variance and Dunnett's multiple comparison test as follows:

For mortality data, divide the number of dead mysids in each replicate by the total (five) to get the proportion of dead mysids in each replicate. Proportional data must then be transformed for parametric statistical analysis. To transform the data, take the arcsine of the square root of each proportion value. Assign this transformed value to the proper test concentration using the original test container randomization sheet (see Section 5.1). Compare responses between concentrations using an analysis of variance (ANOVA). If a significant difference between concentrations is detected, use a Dunnett's multiple comparison test to compare each concentration against the control (Sokal and Rohlf, 1969; Zar, 1974). Derive the NOEC as the highest concentration that is not significantly different from the control. Use an alpha level of $p = .05$ to determine statistical significance.

If brines were used in the effluent test, conduct two separate ANOVA's: one for treatments without brine (including the dilution water control), and one for treatments with brine (including the brine control). Follow each ANOVA with a Dunnett's comparison using the appropriate control. Use dilution water controls for the comparison with effluent treatments that had no brine added, and use brine controls for comparison with salinity adjusted effluents (see Section 4.2).

For growth data, derive a total length value for each replicate by taking the mean of all mysids measured in that replicate. Use the untransformed mean length per replicate data in the appropriate ANOVA comparison, as above.

To determine point estimates of effluent toxicity with mortality data, use the trimmed Spearman-Karber technique to generate an LC_{50} and 95% confidence intervals. Computer programs for the trimmed Spearman-Karber are available from the EPA Environmental Monitoring

and Support Laboratory in Cincinnati, Ohio. Alternative techniques, such as the probit or moving average techniques, may be used, but any such use must be specifically stated when reporting LC_{50}'s to avoid errors in manipulation of the data base. A number of computer programs are available to generate probit values (see Weber et al., 1988). Test the acceptability of available programs by comparison with known data sets.

To determine point estimates of effluent toxicity with growth data, use the Inhibition Concentration percentage (ICp) technique to generate an IC_{50}, IC_{25}, and/or other ICp recommended by the regulatory agency. Computer programs for the ICp are available from the EPA Environmental Monitoring and Support Laboratory in Cincinnati, Ohio.

11.0 Test Acceptability

For tests to be considered acceptable, the following requirements must be met:

1) Mean mortality in dilution water controls cannot exceed 25% in either effluent or reference toxicant tests.
2) Results from controls and brine controls must not be significantly different, as determined by a t-test with an alpha level of 0.05.
3) The response from the 100 µg/liter zinc treatment must be significantly different from the control response (see Section 10.0 for discussion of data analysis).
4) The between-replicate variability must be low enough that the ANOVA Error Mean Square (MS) does not exceed 200 in the reference toxicant test (using arcsine transformed proportion mortality data in degrees). (Many data analysis programs produce arcsin transformed proportion data in radians. Multiply these radian values by $360/2\pi = 57.3$ to convert them to degrees).

12.0 References

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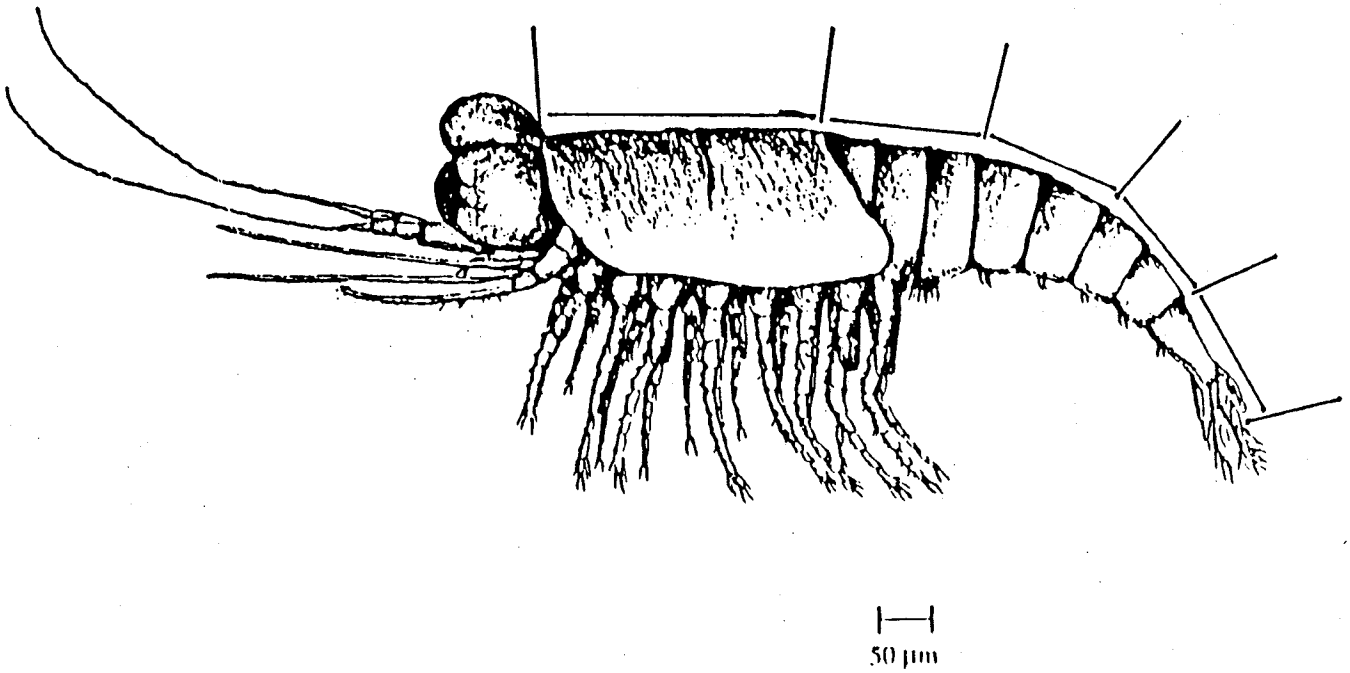
Figure 1. Illustration of a juvenile mysid. Broken line and hatch marks along the dorsal edge indicate the division of the total length into straight lines for measurement with an ocular micrometer or other straight rule. If a projecting microscope or *camera lucida* attachment are available, the rounded dorsal edge can be traced directly with a fine scale odometer wheel or other image tracing equipment. Total length is measured from the base of the eye stock to the tip of the telson (see Section 9.3.2).



Holmesimysis costata - mysid juvenile

13.0 Mysid Toxicity Test Protocol Step-by-Step Summary

13.1 Preparation of Test Solutions

A. Determine effluent test concentrations and appropriate dilution water based on NPDES permit conditions and guidance from the appropriate regulatory agency (Section 3.1).

B. Prepare effluent test solutions by diluting well mixed unfiltered effluent using volumetric flasks and pipets (Section 4.0). Use brines and brine controls where necessary to maintain test solution salinity at 34 ± 2 ‰ (Section 4.2)

C. Prepare a zinc reference toxicant stock solution (10,000 µg/liter) by adding 0.0440 g of zinc sulfate ($ZnSO_4 \cdot 7H_2O$) to one liter of distilled water. Sample stock solution for chemical verification. Acidify sample vials with 1% by volume 14N double quartz distilled nitric acid, and store in a dark refrigerator for no more than three months before analysis (Section 3.2)

D. Prepare zinc reference toxicant solutions of 0 (control) 10, 18, 32, 56, and 100 µg/liter by adding 0, 1.0, 1.8, 3.2, 5.6 and 10.0 ml of stock solution, respectively, to a one-liter volumetric flask and filling to one-liter with 0.2-µm-filtered reference dilution seawater (Section 3.2).

E. Sample effluent and reference toxicant solutions for physical/chemical analysis. Measure salinity, pH, and dissolved oxygen concentration from each treatment (Section 6.0).

F. Randomly label test containers, and record the container numbers with their respective test concentrations on a randomization sheet, to be used at the end of the test (Section 5.1).

G. Place test containers in a water bath or environmental chamber, cover, and allow to temperature equilibrate (Section 5.1).

I. Measure the temperature daily in one random replicate of each test concentration. Monitor the temperature of the water bath or environmental chamber continuously. At the end of the test, measure salinity, pH, and dissolved oxygen concentration from each test concentration (Section 6.0).

13.2 Preparation and Analysis of Test Organisms

A. Four days prior to the beginning of the toxicity test, isolate approximately 150 gravid female mysids in a screened (2-mm-mesh) compartment within an aerated 80-liter aquarium (15 °C). (Section 8.4). Add a surplus of *Artemia* nauplii (200 per mysid, static; 500 per mysid, flow-through) to stimulate overnight release of juveniles. Add blades of kelp as habitat (Section 8.4).

B. Isolate the newly released juveniles by slowly siphoning into a screen-tube (150-µm-mesh, 25 cm diam.) immersed in a bucket of clean seawater. Transfer juveniles into additional screen-tubes or static 4-liter beakers at a density of ~50 juveniles per liter. Juveniles should be fed five to ten newly released *Artemia* nauplii per juvenile per day and a pinch (10 to 20 mg) of ground Tetramin® flake food per 100 juveniles per day. Maintain the juveniles for three days at 15° C. (or 13°C), changing the water at least once in static containers (Section 8.4).

C. After three days, begin randomized introduction of juveniles into the test containers. Place one or two mysids at a time into as many plastic cups as there are test containers. Repeat the process until each cup has exactly five juvenile mysids (Section 9.1).

D. Eliminate excess water from the cups (no more than 5 ml should remain) and pipet the mysids into the test containers using a wide bore glass tube or pipet (~ 4 mm ID). Make sure no mysids are left in the randomization cups. Count the number of juveniles in each test container to verify that each has five (Section 9.1).

E. Remove all dead mysids daily, and add 40 newly hatched *Artemia* nauplii/mysid/day; Section 9.2).

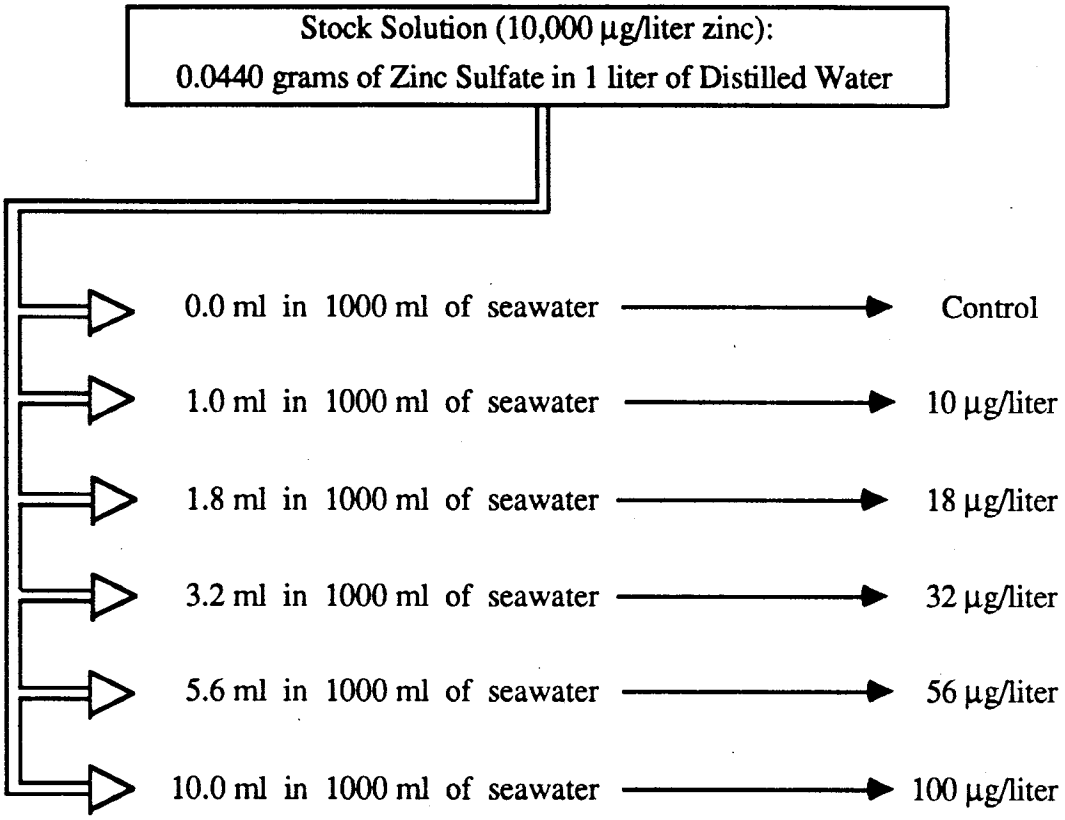
F. After each 48 hours, renew 75% of the test solution in each container (Section 4.4).

G. After 7 days, count the number of live and dead mysids in each container and record. After counting, use the randomization sheet to assign the correct test concentration to each container (Section 9.3.1). Transfer surviving mysids into labeled test tubes (Section 9.3.2).

H. Cool mysids to prevent abdominal flexing, then fix in 5% formalin. Measure total length of each mysid from the base of the eyestock to the tip of the telson (Section 9.3.2)

I. Calculate NOECs using Analysis of Variance and Dunnett's test. Calculate LC_{50}'s and 95% confidence intervals for mortality data using trimmed Spearman-Karber analysis. Calculated ICp for growth data using EPA ICp program (Section 10.0).

Zinc Dilution Schedule for Mysid Tests



Effluent Dilution Sheet

100% Effluent is the Stock Solution

Corresponding Beaker Numbers

Date Organism Investigator

Notes

→	0.0 ml in 1000 ml flask →	Control
→	0.0 ml in 1000 ml flask →	Brine Control
→	_____ →	Other
→	_____ →	Other
→	5.6 ml in 1000 ml flask →	0.56%
→	10.0 ml in 1000 ml flask →	1.0%
→	18.0 ml in 1000 ml flask →	1.8%
→	32.0 ml in 1000 ml flask →	3.2%
→	56.0 ml in 1000 ml flask →	5.6%
→	100.0 ml in 1000 ml flask →	10.0%
→	_____ →	Other

Salinity Adjustment Using Hypersaline Brine

Add hypersaline brine to those concentrations in which test solution salinity would otherwise fall below the minimum acceptable test salinity (32‰).
The equation to calculate the volume of brine to add for each of these concentrations is: $VB = VE\left(\frac{34 - SE}{SB - 34}\right)$

Quantities known from dilution schedule: VE = Volume of Effluent added for each concentration (ml).

Quantities to be measured: SB = Salinity of Brine (‰), and SE = Salinity of Effluent (‰). SB = _____‰ SE = _____‰

Note: Always adjust the pH of the brine to equal that of the dilution water. Brine salinity should be 60 to 80 ‰.

Calculate the volume of brine to be added, VB, for each concentration that requires salinity adjustment using the above equation.

Example: If dilution water salinity is 33.5‰ and effluent salinity is 0‰, a test solution of 5.6% effluent would have a salinity of 31.6‰, which is below the acceptable salinity range. This test solution and any with a higher effluent concentration would have to be adjusted with hypersaline brine, and brine controls would be necessary. If the dilution schedule above is used, and the highest effluent concentration is 10%, then these test solutions and the brine control would be made up as follows:

Measure SB and SE. Use these to calculate the quantity $\frac{34 - SE}{SB - 34}$ = _____

Effluent Concentration	VE		$\frac{34 - SE}{SB - 34}$		VB	Final Test Solution Mixture
5.6%	56 ml	x	_____	=	_____ ml Brine	+ 56 ml effluent in a one liter flask; fill with seawater
10.0%	100 ml	x	_____	=	_____ ml Brine	+ 100 ml effluent in a one liter flask; fill with seawater
Brine Control	100 ml	x	_____	=	_____ ml Brine	+ 100 ml distilled water " " " ; fill with seawater

Marine Pollution Studies Laboratory

Data Sheet for Juvenile Mysid Toxicity Test

Test Start Date: Start Time: Species :
Test End Date: End Time: Station Codes for Env. Samples:
Mysid Age at Start (days post-hatch): Sample Type
Toxicant: (Solid, Elutriate, Pore Water, Water):
Sample Source: Collection Date:
Ref. Tox. Chem. Sample No's: Analyzed By:

Test Cont. #	Tox. Conc.	Number Dead							Total Number Dead	Total Number at Start	Notes
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7			

Computer Data Storage
Disk:
File:

Note: See juvenile length data on separate sheet.

APPENDIX III

GIANT KELP TOXICITY TEST PROTOCOL

GIANT KELP GERMINATION AND GROWTH
SHORT-TERM TOXICITY TEST PROTOCOL

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1.0 Introduction

This protocol gives step-by-step instructions for performing a 48-hour static toxicity test using germination of kelp spores and growth of embryonic gametophytes to determine the toxicity of chemical compounds, complex effluents, or 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 both the percentage of spores that fail to germinate and the length of embryonic gametophytes. These data are used to derive No Observed Effect Concentrations (NOECs) which give numerical indications of toxicity. Because the test measures effects on developmental stages of an economically and ecologically important species possessing relatively stringent water quality requirements, the results constitute a good basis for decisions concerning either hazard evaluation or the suitability of marine waters for aquatic life (ASTM, 1987).

2.0 Equipment

- 350 ml polyethylene plastic food containers
  or 600 ml borosilicate glass beakers
- microscope slides and cover slips
- hemacytometer (bright-line rbc)
- one-liter plastic or glass beaker
- one-liter polyethylene volumetric flasks
- pH meter
- dissolved oxygen meter (w/NH_3 electrode)
- thermometer
- salinity refractometer

•microscope (w/ocular micrometer)

•light meter (irradiance meter w/ cosine corrected sensor)

•pipets: (volumetric: 1 ea. 1, 2, 5, 10, 100 ml; graduated 1, 10 ml)

•cool white fluorescent lights

•analytical balance

•waterbath, incubator, or constant temperature room

•hand counters

•hydrochloric and nitric acids (2N, for cleaning)

•petroleum ether (reagent grade, for cleaning)

3.0 Experimental Design

3.1 Effluent Tests

The number and concentration of effluent treatments should be based on study requirements or NPDES permit conditions. All treatments must be replicated five times. Every test must contain five replicates of dilution water controls (see Quality Assurance Document for a discussion of effluent dilution water). Tests that use hypersaline brine to adjust salinity must also contain five replicates of brine controls (see Section 4.2). Effluent concentrations should be assigned in a geometric sequence, with each concentration being at least 56% that of the next highest concentration (for example, 0% (control), 0% (brine control) 0.56%, 1.0% 1.8%, 3.2%, 5.6%, and 10% effluent). Effluent treatments that bracket the concentration found at the edge of the outfall zone of initial dilution may be most appropriate for evaluating chronic toxicity. A preliminary range finding test using a wider range of concentrations may be necessary for testing specific substances of unknown toxicity.

3.2 Reference Toxicant Tests

A reference toxicant test must be conducted concurrently with every effluent test. Copper chloride ($CuCl_2 \cdot 2H_2O$) should be used as the reference toxicant for kelp tests, unless another toxicant is specified by the Regional Water Quality Control Board. Stock solutions must be made on the day of the test. (The attached dilution schedule gives the appropriate weights and volumes for making copper stocks and dilutions.) Prepare a 10,000 µg/liter copper chloride stock solution by adding 0.0268 g of copper chloride to one liter of distilled water in a polyethylene plastic volumetric flask. Use five replicates of the following total copper concentrations: 0, 5.6, 10, 18, 32, 56, 100, and 180 µg/liter. Prepare one liter of each concentration by adding 0, 0.56, 1.0, 1.8, 3.2, 5.6, and 10 ml of stock solution, respectively, to a one-liter volumetric flask and fill with 0.2-µm-filtered reference dilution water (see Quality Assurance Section 2.1.2 for a discussion of reference toxicant dilution

water). Start with the control solutions and progress to the highest concentration to minimize contamination. Salinity adjustment and brine controls are not necessary. Reference toxicant solutions should be prepared before or during zoospore release. The reference toxicant test must be conducted in plastic containers with the same spores used in the effluent test.

Sample reference toxicant stock solutions at the beginning of each test to verify copper test concentrations. Store samples in new, acid-washed 30 ml polyethylene vials. Preserve samples with 1% by volume double quartz distilled nitric acid (14N). Analyze samples within two months using atomic absorbtion spectroscopy (or other approved method) at a certified analytical laboratory.

3.3 Lighting and Temperature

The kelp toxicity test must be done under controlled temperature and lighting in either an environmental chamber or water bath. The test chamber should provide adequate uniform lighting and cooling and allow easy access to all test containers. The lights used in this protocol are cool white fluorescent lights adjusted to give 50 $\mu E\ m^{-2}s^{-1}$ at the top of each test container. It is important that each test container receive the same quanta of light ($\pm$ 10 $\mu E\ m^{-2}\ sec^{-1}$). Areas of increased light can be eliminated by taping the outside of the light diffuser or wrapping the fluorescent bulbs with aluminum foil. The photoperiod for this protocol must be 16 hours light: 8 hours dark.

This protocol is conducted at 15 °C. If a water bath is used, adjust the level of the water bath for maximum cooling, but low enough to prevent floating the test containers. The test containers should be covered to prevent excessive evaporation, preferably with thin acrylic sheets or clear plastic food storage wrap attached to the individual containers.

4.0 Test Solutions

Prepare test solutions by combining effluent, hypersaline brine (see Section 4.2), and dilution water using volumetric flasks and pipets. Clean all glassware prior to use (see Section 3.1). Mix test solutions from the lowest concentration (control) to the highest concentration to avoid contamination.

4.1 Dilution Water

See Quality Assurance Section 2.1.2 for a discussion of dilution water.

4.2 Salinity Adjustment

The salinity of sewage effluents is generally lower than that tolerated by kelp gametophytes. To maintain acceptable salinity, test solutions containing more than 2% effluent must be adjusted to dilution water salinity by adding hypersaline brine. See Section 10 for statistical treatment of tests using brines.

If brine use is necessary, brines should be made by freezing 0.2-µm-filtered reference seawater (Anderson *et al.*, 1990). Clean, covered (not sealed) polyethylene containers should be used for freezing. Brine salinity should be 60 - 80 ‰. One liter of brine can be made by freezing two, one-liter containers of seawater for approximately six hours in a conventional freezer (approximately minus 12°C). Check the pH of all brine mixtures and adjust to within 0.1 units of dilution water pH by adding, dropwise, dilute hydrochloric acid or sodium hydroxide.

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

$$VB = VE\frac{(34 - SE)}{(SB - 34)}$$

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

4.2.1 Brine Controls

Use brine controls in all tests where brine is used. Brine controls contain the same amount of brine as does the highest effluent concentration plus distilled water equal to the volume of effluent in the highest concentration plus dilution water to fill the mixing flask. (If effluent salinity is greater than 10 ‰ or effluent dilutions above 10% are used, calculate the amount of distilled water to add to brine controls by using the above equation, setting SE = 0, and solving for VE).

See the example below and the attached dilution schedule worksheet for further details on making test solutions.

4.3 Example Test Solution

Two hundred milliliters of test solution are needed for each test container. Five replicates can be mixed in a one-liter volumetric flask. To make a test solution at a concentration of 1% effluent, add 10 ml of effluent to the one-liter volumetric flask using a volumetric pipet. Fill the volumetric flask to the one-liter mark with dilution water, stopper it, and shake to mix. Pour equal volumes into the five replicate containers.

To make a test solution at a concentration of 10 % effluent, hypersaline brine must be used. Add 100 ml of effluent to a one-liter volumetric flask. Then, assuming an effluent salinity of 2 ‰ and a brine salinity of 70 ‰, add 89 ml of brine (see equation above) and top off the flask with dilution water. Stopper the flask, shake well, and pour equal volumes into the five replicate containers.

5.0 Test Containers

For tests using complex effluents or organic toxicants, use 600 ml borosilicate glass beakers as the test containers. For tests using metal toxicants, use 350 ml polypropylene or polyethylene food storage containers. With both container types, place one standard microscope slide (flat) in each test container to serve as the substratum upon which the zoospores will settle. The microscope slide will be removed at the end of the experiment.

Note: Other test containers have been used successfully with this protocol. For example, smaller volume polystyrene or glass tissue culture containers or petri dishes may be substituted for the above containers as long as the reference toxicant test results using alternative containers conform to those specified in the quality assurance document appended to this protocol.

5.1 Randomization

To randomize placement of test containers and to eliminate bias in the analysis of test results, label the test containers using random numbers from 1 to 40 (for reference toxicant tests, or one to N for effluent tests, with N being the total number of containers). Record these numbers on a separate data sheet together with the concentration and replicate numbers to which they correspond. Identify this sheet with the date, test organism, test number, laboratory, and investigator's name, and safely store it away until after the gametophytes have been examined at the end of the test. **Note:** Loss of this randomization sheet would invalidate the test by making it impossible to analyze the data afterwards.

Arrange the test containers randomly in the water bath or controlled temperature room. Take care to follow the numbering system exactly while filling containers with the test solutions.

6.0 Physical/Chemical Measurements of Test Solutions

Prior to testing, consult the container randomization sheet (Section 5.0) to compile a list of containers to be sampled for measurement. One randomly chosen replicate from each test concentration should be measured as follows: measure salinity, pH, and dissolved oxygen at the beginning and end of the test; measure test solution temperature daily; and monitor water bath or environmental chamber temperature continuously. See Quality Assurance Section 5.0 for specifications and instrumentation for physical/chemical measurements.

7.0 Cleaning Procedure

New beakers should be scrubbed with a laboratory detergent and deionized water, then rinsed with deionized water, and soaked with dilution water overnight. Used containers should be cleaned as described below. New tissue culture containers should not be washed, but should be soaked overnight in dilution water before use.

7.1 Effluents and organic toxicants

All test chambers used for organic chemicals and complex effluent tests should be cleaned as follows: 1) rinse three times with hot tap water, 2) rinse three times with new reagent grade acetone or petroleum ether, 3) rinse three times with deionized water, 4) soak 24 hours in 2N HCL, 5) rinse three times with deionized water, 6) soak 24 hours in 2N HNO_3, 7) rinse three times with deionized water, 8) soak 24 hours in deionized water, 9) rinse three times with deionized water, 10) dry in a clean area.

7.2 Metal toxicants

All test chambers used in testing trace metals should be cleaned as follows: 1) rinse three times with deionized water, 2) rinse three times with 2N HCL, 3) rinse three times with deionized water, 4) soak for 24 hours in 2N HCL, 5) rinse three times with deionized water, 6) soak 24 hours in 2N HNO_3, 7) rinse three times with deionized water, 8) soak 24 hours in deionized water, 9) rinse three times with deionized water, 10) dry in a clean area.

7.3 Other Glassware

All volumetric flasks, pipets, and other labware used for handling effluent test solutions must be cleaned as described in Section 7.1. All volumetric flasks, pipets, and other labware used for handling trace metal reference toxicant solutions must be cleaned as described in Section 7.2.

8.0 Test Organism

The test organisms for this protocol are the zoospores of the giant kelp *Macrocystis pyrifera*. *Macrocystis* is the dominant canopy forming Laminarian alga in southern and central California and forms extensive subtidal forests along the coast. Giant kelp forests support a rich diversity of marine life and provide habitat and food for hundreds of invertebrate and vertebrate species (North, 1971; Foster and Schiel, 1985). It is an appropriate bioassay species because of its availability, economic and ecological importance, history of successful laboratory culture (North, 1976; Luning, 1980; Kuwabara, 1981; Deysher and Dean, 1984; Linfield, 1985), and previous use in toxicity testing (Smith and Harrison, 1978; James *et al.*, 1987; Anderson and Hunt, 1988; Hunt *et al.*, 1989; Anderson *et al.*,

1990). Other Laminarian alga species have proven to be useful for laboratory toxicity testing (Chung and Brinkhuis, 1986; Thompson and Burrows, 1984; Hopkin and Kain, 1978)

Like all kelps, *Macrocystis* has a life cycle that alternates between a microscopic gametophyte stage and a macroscopic sporophyte stage. It is the sporophyte stage that forms kelp forests. These plants produce reproductive blades (sporophylls) at their base. The sporophylls develop patches (sori) in which biflagellate, haploid zoospores are produced. The zoospores are released into the water column where they swim and eventually settle onto the bottom and germinate. The dioecious spores develop into either male or female gametophytes. The male gametophytes produce flagellated gametes which may fertilize eggs produced by the female gametophytes. Fertilized eggs develop into sporophytes within 12- 15 days, completing the lifecycle.

The bioassay protocol described here focuses on germination of the zoospores and the initial growth of the developing gametophytes. It involves the controlled release of zoospores from the sporophyll blades, followed by the introduction of a spore suspension of known density into the test containers. The zoospores swim through the test solution and eventually settle onto glass microscope slides. The settled spores germinate by extruding the cytoplasm of the spore through the germ-tube into the first gametophytic cell. This stage is often referred to as the "dumbell" stage. The two endpoints measured after 48 hours are germination success and growth of the embryonic gametophytes (germ-tube length, Figure 1).

8.1 Species Identification

Although there is some debate over the taxonomy of the genus *Macrocystis*, Abbott and Hollenberg (1976) consider only two species in California: *M. pyrifera*, and *M. integrifolia*. The two are distinguished from each other based on habitat and the morphology of their holdfasts. *Macrocystis pyrifera* occurs subtidally while *M. integrifolia* occurs in the low intertidal and shallow subtidal zones. *Macrocystis pyrifera* has a conical holdfast while *M. integrifolia* has a more flattened, creeping holdfast. Consult Abbott and Hollenberg (1976) for a more detailed taxonomic discussion of the two species.

8.2 Collection

Macrocystis zoospores are obtained from the reproductive blades (sporophylls) of the adult plant. The sporophylls, are located near the base of the plant just above its conical holdfast. Sporophylls must be collected subtidally and should be collected from at least five different plants in any one location to give a good genetic representation of the population. The sporophylls should be collected from areas free of point and non-point source pollution to minimize the possibility of genetic or physiological adaptation to pollutants. In situations where a thermocline is present at the collection

site, the sporophylls should be collected from below the thermocline to ensure adequate spore release. Sporophylls are identified in the field by the presence of darkened patches called sori. The zoospores develop within the sori. In addition, the sporophylls are distinguished from vegetative blades by their thinner width, basal location on the adult plant, and general lack of pneumatocysts (air bladders). Collection of algae is regulated by California law. Collectors must obtain a scientific collector's permit from the California Department of Fish and Game and observe any regulations regarding collection and transport of kelp. For further information regarding sporophyll collection, contact the Marine Pollution Studies Laboratory, Coast Route 1, Granite Canyon, Monterey CA, 93940. (408) 624-0947.

8.3 Sporophyll Transport

After collection, the sporophylls should be kept damp and not exposed to direct sunlight. Avoid immersing the blades in seawater, however, to prevent premature spore release. The sporophylls should be rinsed thoroughly in 0.2 µm filtered seawater to remove diatoms and other epiphytic organisms. The individual blades can be gently rubbed between fingers under running filtered seawater or brushed with a soft bristled brush. The blades are stored between moist paper towels (lasagna style so that the sporophylls do not overlap each other, and each layer of sporophylls are separated by a layer of paper towels) at approximately 9-12 °C until needed. The zoospores must be released within 24 hours of collection to insure their viability. Preliminary data indicate that prolonged storage times may reduce germination rates (Bottomley et al., 1991); however as long as germination rates meet control acceptability criteria this should not affect test results. Sporophylls should be kept shaded to prevent damage to the spores. For holding or transport times longer than approximately six hours, the sporophylls should be placed in an ice chest with blue ice. The blue ice should be wrapped in newspaper (10 layers) for insulation, then plastic to prevent leaking.

8.4 Zoospore Release

Zoospores are released by slightly desiccating the sporophyll blades, and then placing them in filtered seawater. To desiccate the sporophyll, blot the blades with paper towels and expose them to air for 1 hour. The number of sporophyll blades needed depends upon their maturity; usually 25-30 blades (~ 100 grams wet weight) are sufficient. After 1 hour the blades should be rinsed again thoroughly using 0.2 µm-filtered seawater, then placed in a one L glass or plastic beaker filled with 0.2 µm filtered seawater at 15-16°C. The release water should never exceed 18 °C. After one hour, a sufficient number of zoospores should be present to conduct the test. The presence of zoospores is indicated by a slight cloudiness in the water. To verify whether zoospores are present, periodically sample the solution and observe the sample microscopically (100x). To insure that the zoospores are

viable and have not begun to germinate before they are exposed to the toxicant, the zoospore release process should not be longer than two hours. If it takes longer than two hours to get an adequate density of zoospores (~7,500 zoospores / ml of water), repeat the release process with a new batch of sporophylls.

8.5 Zoospore Density

After the zoospores are released, remove the sporophylls and let the spore mixture settle for 30 minutes. After 30 minutes, decant 250 mls from the top of the spore solution into a separate clean glass beaker. Sample the spore solution and determine the spore density using a bright-line hemacytometer (100x). To obtain an accurate count, fix a sample of spores by mixing nine milliliters of spore solution with 1-ml of 37 % buffered formalin in a test tube. Shake the sample well before placing it on the hemacytometer. After counting, the density is multiplied by 1.111 to correct for the dilution caused by adding 1 ml of formalin to the sample (see attached work sheet). Use at least five replicate counts. After the density is determined, calculate the volume of zoospores necessary to give approximately 7,500 spores / ml of test solution. To prevent over-dilution of the test solution, this volume should not exceed 1 % of the test solution volume. If this volume exceeds 1% of the test solution volume, it should be noted in the results.

Test solutions must be prepared while the zoospores are releasing from the sporophylls. Test solutions must be mixed, sampled, and temperature equilibrated in time to receive the swimming zoospores as soon as they are counted. Zoospore release and counting should be done in a room separate from that use for toxicant preparation, and care should be taken to avoid contaminating the zoospores prior to testing.

9.0 Toxicity Test Procedure

9.1 Exposure of Test Organisms

9.1.1 Delivery of Zoospores

After determining the zoospore density and calculating the volume yielding 7,500 zoospores/ml test solution, add this volume to each test container (this is the start time of the test). Observe a sample of zoospores microscopically to verify that they are swimming before adding them to the test containers.

9.1.2 Incubation

Incubate the developing gametophytes for 48 hours in the test containers at 15 °C under 50 μE m^{-2} sec^{-1}. The zoospores germinate and develop to the "dumbell" gametophyte stage during the exposure period.

9.2 Endpoint Determination

After 48 hours, the test is terminated. Because it takes a considerable amount of time to read the test, reading can begin after 45 hours. Remove the slide without decanting the test solution. The test slide can be lifted from the bottom of the test container with a separate clean microscope slide. Blot the bottom on a towel paper and place an 18-mm square cover slip on the slide. Blot the excess water around the edge of the cover slip to eliminate the flow of water under the cover slip.

The endpoints measured for the 48 hour *Macrocystis* bioassay are germination success and germination tube length. Germination is considered successful if a germ-tube is present on the settled zoospore. Germination is considered to be unsuccessful if no germination tube is visible. To differentiate between a germinated and non-germinated zoospore, observe the settled zoospores at 400x magnification and determine whether they are circular (non-germinated) or have a protuberance that extends at least one spore diameter (about 3.0 μm) from the edge of the spore (germinated). Spores with a germination tube less than one spore diameter are considered non-germinated. The first 100 spores encountered while moving across the microscope slide are counted for each replicate of each treatment. It is not necessary to measure germination in the copper reference toxicant test; only the growth endpoint is quantified in the copper reference test. Use the receiving water control to establish control germination rates for test acceptability criteria. **Note:** Sewage effluents may contain certain objects, such as ciliates, which look similar to non-germinated kelp spores. It is important to ensure that only kelp spores are counted for this endpoint. Kelp spores are green-brown in color, spherical, and lack mobility. Also, components of the cytoplasm of kelp spores appear to fluoresce a light green color when the spore is slightly out of focus. If a particular object cannot be identified, it should not be counted.

The growth endpoint is the measurement of the total length of the germination tube from the edge of the original spore membrane (Figure 1). Only germinated spores with straight germination tubes and within the same focal plane are measured; if a spore is not completely in focus from tip to tip it should not be measured. The spores to be measured are randomly selected by moving the microscope stage to a new field of view without looking through the ocular lens. Measure the germination tube length of the spore whose spore case center is nearest the micrometer in each field; the spores case can be distinguished from the growing tip because it is usually clear (empty) at 48

hours, and it is more circular than the growing tip. If more than one spore case is touching the micrometer, both (or all) germinated spores are measured. A total of 10 spores for each replicate of each treatment are measured. It is easier to measure germ-tube length with a micrometer having a 10 mm linear scale (0.1 mm subdivisions); measure lengths to the nearest micron. In situations where germination is significantly inhibited it may be difficult to find germinated spores for germ-tube growth measurement using the random search technique. To expedite reading, the slide can be scanned to find germinated spores if germination is 30% or less. In this situation the first 10 spores encountered are measured for germ-tube length

9.3 Preservation of Cultures

In some cases it may be convenient to preserve the kelp cultures for later analysis. Preliminary work at MPSL has indicated that cultures can be preserved in 0.1% glutaraldehyde and that preservation has no significant effect on germination or germ-tube growth. Other researchers have used higher glutaraldehyde concentrations and found adequate preservation with no effect on spore germination or gametophyte growth (K. Goodwin, Calif. Inst. of Tech., unpublished data). Because data on the effects of preservation are preliminary, we recommend that anyone interested in preserving kelp cultures for later analysis first demonstrate that preservation does not affect test results. This can be done by comparing germination and germ-tube growth in preserved vs non-preserved kelp cultures. We also recommend that if it is necessary to preserve kelp cultures for later analysis, a complete test should be preserved so that if any replicates are read preserved, all of the replicates should be read preserved. In the case where concurrent reference toxicant and complex effluent tests are conducted, it may be convenient to fix one test in glutaraldehyde and read the other test immediately. When fixing kelp cultures, it is important to minimize disturbance to the gametophytes. Make sure that the culture slides are fixed and stored horizontally. We have used disposable petri dishes for preservation chambers; these allow individual replicate slides to be labelled and preserved separately to avoid mixing replicates. **Note:** Glutaraldehyde is a potent carcinogen. If you intend to use this material as a preservative, study the material data safety sheets from the supplier and follow strict safety precautions. Make sure test containers and solutions contaminated with this material are disposed of properly.

10.0 Data Analysis

Add the number of germinated and non-germinated spores together to obtain the total number of spores counted for each replicate. Calculate the number of non-germinated spores as a percentage of this total for each replicate. Transform the proportion data to the arcsine of their square root. (This transformation is standard practice for percentage data used in parametric statistics). Check the original test container randomization sheet and assign the correct concentration and replicate number to

the transformed percentage data. Perform an analysis of variance (ANOVA) to compare concentrations. If a significant difference is detected, use Dunnett's multiple comparison test to compare each concentration against the control (Zar, 1974; Sokal and Rohlf, 1969). Derive the No Observed Effect Concentration (NOEC) as the highest concentration that is not significantly different from the control ($p = 0.05$).

No data transformation is necessary for the length data. Calculate the mean length measurement (in microns) for each replicate and perform an analysis of variance to compare concentrations. Derive the NOEC using Dunnett's multiple comparison as above.

If brines were used in the effluent test, include all data in the ANOVA, and use the appropriate control for the Dunnett's comparison. Use dilution water controls for comparison with effluent treatments that had no brine added, and use brine controls for comparison with salinity adjusted effluent treatments (see Section 4.2).

11.0 Test Acceptability

For tests to be considered acceptable, the following requirements must be met:

1) Mean control germination must be at least 70% in the effluent test controls.

2) Mean germination-tube length in the controls must be at least 10 µm in the reference toxicant test; mean control germination-tube length must be at least 10 µm in the effluent test controls.

3) Brine control results must not be significantly different from dilution water control results in the effluent test, using a t-test and an alpha level of 0.05.

4) The germination-tube growth NOEC must be below 35 µg/liter (chemically verified copper concentrations).

5) The between-replicate variability for germ-tube growth data must be low enough that the ANOVA Error Mean Square (MS) does not exceed 12.00 in the reference toxicant test (using untransformed length data). This corresponds to a Dunnett's Standard Error (SE) of 2.19 (with $n = 5$ replicates).

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13.0 Macrocystis Toxicity Test Protocol Summary

13.1 Preparation of Test Solutions

A. Determine effluent test concentrations and appropriate dilution water based on NPDES permit requirements and guidance from the appropriate regulatory agency (Section 3.1).

B. Prepare effluent test solutions by diluting unfiltered effluent using volumetric flasks and pipets (Section 4.0).

C. Prepare copper chloride reference toxicant stock solution (10,000 µg/liter) by adding 0.0268 g of copper chloride ($CuCl_2 \cdot 2H_2O$) to one liter of distilled water. Sample reference toxicant stock solution for chemical verification of copper concentration. Acidify sample vials with 1% by volume 14 N double quartz--distilled nitric acid, and refrigerate (Section 3.2).

D. Prepare copper reference toxicant solutions of 0 (control), 5.6, 10, 18, 32, 56, 100, and 180 µg/liter by adding 0, 0.56, 1.0, 1.8, 3.2, 5.6, 10.0, and 18.0 ml of stock solution, respectively, to a one-liter polyethylene plastic volumetric flask and filling to one liter with distilled water (Section 3.2).

E. Sample effluent and reference toxicant solutions for physical/chemical analysis. Measure salinity, pH, and dissolved oxygen from each test concentration (Section 6.0).

F. Randomize labelling of test containers, and record the container numbers with their respective test concentrations on a randomization sheet. Store the sheet safely until after the test samples have been analyzed (Section 5.1).

G. Place test containers in a water bath or environmental chamber, cover, and allow to temperature equilibrate (Section 5.1).

H. Measure the temperature daily in one random replicate of each test concentration. Monitor the temperature of the water bath or environmental chamber continuously (Section 6.0).

I. At the end of the test, measure salinity, pH, and dissolved oxygen concentration from each test concentration (section 6.0).

13.2 Preparation and Analysis of Test Organisms

A) Collect sporophylls and rinse in 0.2 µm filtered seawater. Store at 9-12 °C for no more than 24 hours before zoospore release (Section 8.2).

B) Blot sporophylls and leave exposed to air for one hour (Section 8.4).

C) Place 25-30 sporophylls one liter of 0.2 µm filtered seawater for no more than two hours. The presence of zoospores is indicated by a slight cloudiness in the water (Section 8.4).

D) Take a sample of the zoospore solution from the top 5 centimeters of the beaker and determine the spore density using a hemacytometer. Determine the volume of water necessary to give 7,500 spores / ml of test solution. This volume should not exceed one percent of the test solution volume (Section 8.5).

E) Verify that the zoospores are swimming, then pipet the volume of water necessary to give 7,500 spores / ml into each of the test containers. Take zoospores from the top 5 centimeters of the release beaker so that only swimming zoospores are used (Section 9.1).

F) After 48 hours, count the number of germinated and non-germinated spores of the first 100 spores encountered in each replicate of each concentration (**Note:** it is not necessary to quantify the germination endpoint in the copper reference test). Measure the length of 10 randomly selected germination tubes (Section 9.2).

G) Calculate the percentage of germinated spores for each replicate of each concentration. Transform proportion to the arcsine of the square root, and conduct an analysis of variance (ANOVA) to discern differences between concentrations. Compare each concentration to the control using Dunnett's multiple comparison test. Determine the NOEC value as the highest concentration that is not significantly different from the control (at $p \leq 0.05$). Do an ANOVA on the (untransformed) length data and determine the NOEC using the Dunnett's test as above (Section 10.0).

Copper Dilutions
for Kelp Tests

Mix **0.0268** g Copper Chloride
in 1 Liter of Distilled Water

→ **10,000** µg/liter Stock Solution

- 0.00 ml in 1000 ml seawater.....control
- 0.56 ml in 1000 ml seawater.....5.6 µg/l
- 1.00 ml in 1000 ml seawater.....10.0 µg/l
- 1.80 ml in 1000 ml seawater.....18.0 µg/l
- 3.20 ml in 1000 ml seawater.....32.0 µg/l
- 5.60 ml in 1000 ml seawater.....56.0 µg/l
- 10.00 ml in 1000 ml seawater.....100.0 µg/l
- 18.00 ml in 1000 ml seawater.....180.0 µg/l

Effluent Dilution Sheet

100% Effluent is the Stock Solution		Corresponding Beaker Numbers
→	0.0 ml in 1000 ml flask →	Control
→	0.0 ml in 1000 ml flask →	Brine Control
→	_____ →	Other
→	_____ →	Other
→	5.6 ml in 1000 ml flask →	0.56%
→	10.0 ml in 1000 ml flask →	1.0%
→	18.0 ml in 1000 ml flask →	1.8%
→	32.0 ml in 1000 ml flask →	3.2%
→	56.0 ml in 1000 ml flask →	5.6%
→	100.0 ml in 1000 ml flask →	10.0%
→	_____ →	Other

Date Organism Investigator

Notes

Salinity Adjustment Using Hypersaline Brine

Add hypersaline brine to those concentrations in which test solution salinity would otherwise fall below the minimum acceptable test salinity (32‰).
The equation to calculate the volume of brine to add for each of these concentrations is: $VB = VE\left(\frac{34 - SE}{SB - 34}\right)$

Quantities known from dilution schedule: VE = Volume of Effluent added for each concentration (ml).

Quantities to be measured: SB = Salinity of Brine (‰), and SE = Salinity of Effluent (‰). SB = _____‰ SE = _____‰

Note: Always adjust the pH of the brine to equal that of the dilution water. Brine salinity should be 60 to 80 ‰.

Calculate the volume of brine to be added, VB, for each concentration that requires salinity adjustment using the above equation.

Example: If dilution water salinity is 33.5‰ and effluent salinity is 0‰, a test solution of 5.6% effluent would have a salinity of 31.6‰, which is below the acceptable salinity range. This test solution and any with a higher effluent concentration would have to be adjusted with hypersaline brine, and brine controls would be necessary. If the dilution schedule above is used, and the highest effluent concentration is 10%, then these test solutions and the brine control would be made up as follows:

Measure SB and SE. Use these to calculate the quantity $\frac{34 - SE}{SB - 34}$ = _____

Effluent Concentration	VE		$\frac{34 - SE}{SB - 34}$		VB	Final Test Solution Mixture
5.6%	56 ml	x	_____	=	_____	ml Brine + 56 ml effluent in a one liter flask; fill with seawater
10.0%	100 ml	x	_____	=	_____	ml Brine + 100 ml effluent in a one liter flask; fill with seawater
Brine Control	100 ml	x	_____	=	_____	ml Brine + 100 ml distilled water " " " ; fill with seawater

Kelp Sporophyll Release

Date:
Test:
Investigator:

Condition of majority of blades used: poor fair good
Number of blades used: _____ Weight of blades: _____ Volume of release water: _____
Time of spore release: _____
Temperature of spore solution: _____
Check for zoospore motility on microscope: _____
Fix a 9 ml spore samples with 1 ml formalin.
Determine spore density on the hemacytometer.
Determine density with 5 counts.

1. _____
2. _____
3. _____
4. _____
5. _____

Mean _____ S. D. _____

Mean x 10,000 x 1.11 = _____ spores/ml. This is the density of spore release.
1.11 is the dilution factor for 1ml formalin + 9 ml spore solution.

Volume of test container: _____
The desired final density of zoospore solution is 7,500 spores/ml of test container.
To determine volume of spores to deliver to test containers:

7,500 spores/ml x _____ ml/test container = _____ spores/ test container

_____ spores/test container ÷ density of spore release _____ spores/ml = _____ ml/test container

Temperature of spore solution: _____ Temperature of test containers: _____
Time test containers are inocculated: _____

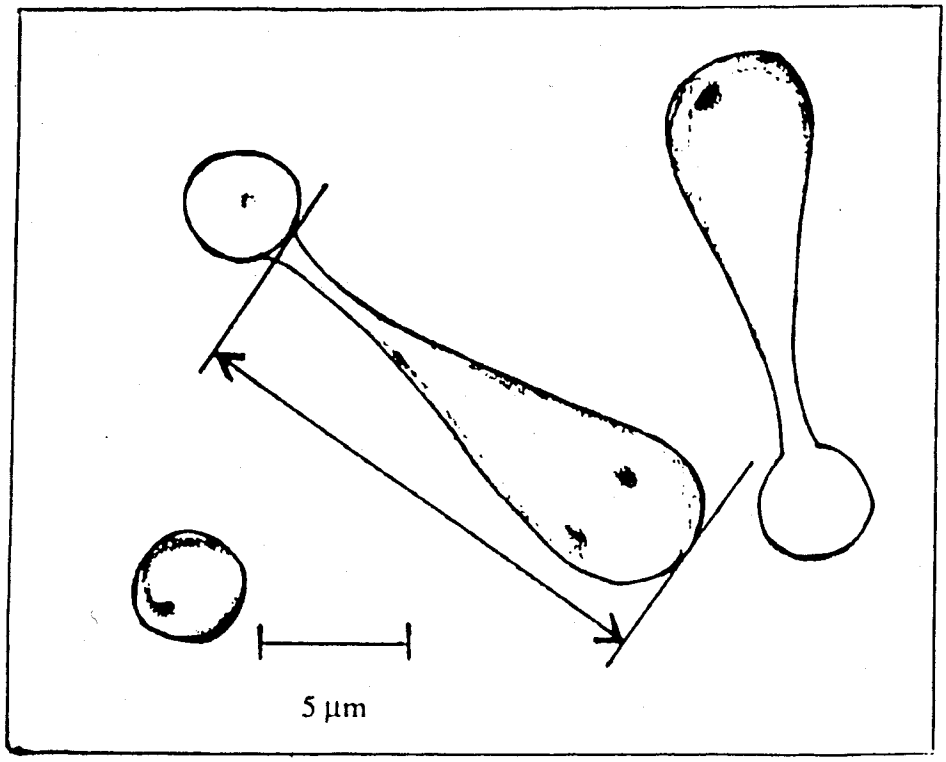

Figure 1. Microscopic view of non-germinated *Macrocystis* spore (bottom left) and two germinated spores. Arrow indicates length measured for germ-tube growth endpoint.

APPENDIX IV

RED ABALONE TOXICITY TEST PROTOCOL

ABALONE LARVAL DEVELOPMENT
SHORT-TERM TOXICITY TEST PROTOCOL

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1.0 Introduction

This protocol gives step-by-step instructions for performing a 48-hour static test using early development of abalone larvae to determine the toxicity of chemical compounds, complex effluents, or ambient marine waters. In this procedure, abalone develop from embryos into veliger larvae while exposed to test solutions. After a 48-hour exposure, larval shells are examined microscopically to determine the percentage of abnormally developed larvae in each toxicant concentration. These data are used to derive No Observed Effect Concentrations (NOECs) or median effective concentrations (EC_{50}'s), which give numerical indications of toxicity. Because the test measures effects on developmental stages of an economically and ecologically important species possessing relatively stringent water quality requirements, the results constitute a good basis for decisions concerning either hazard evaluation or the suitability of marine waters for aquatic life (ASTM, 1987). A step by step summary of the protocol is provided in Section 13.

2.0 Equipment

Equipment requirements depend on the techniques used to incubate and analyze larvae. Two techniques are acceptable. In the first technique, abalone are exposed to test solutions in open 250 ml beakers. After exposure the larvae are screened, concentrated into vials, fixed, and analyzed in Sedgewick Rafter slides under a standard compound microscope. Any equipment used only in this first technique is marked with a bold superscript **a**.

The second technique is preferred. It is quicker and more cost effective, and reduces contamination and volatilization of toxicant solutions. It also reduces worker exposure to hazardous fumes (formalin) because fixed larvae are contained within sealed flasks during analysis. In this technique, larvae are exposed to toxicants and fixed in closed disposable tissue culture flasks, then analyzed in the flasks using an inverted microscope. Equipment used only in this

second technique is marked with a bold superscript[b]. Protocol variations for each technique are also marked in the text with the appropriate superscript [a, b].

NOTE: Some brands or batches of tissue culture flasks may contain toxic residues. Each batch should be tested by exposing abalone in clean seawater in samples of the new containers.

2.1 Equipment for Culture and Transport

- transportation equipment (1 to 4 hours)
  - 20 liter plastic buckets with tight fitting lids
  - airs stones
  - compressed air or portable air pumps
- transportation equipment (to 30 hours)
  - compressed oxygen
  - polyfoam sponges
  - large plastic bags
  - blue ice
  - ice chest
- 2 or more aquaria
- supply of *Macrocystis* or other macroalgae (if holding broodstock longer than 5 days)
- stainless steel butter knife, rounded smooth-edged blade (for handling adult abalone)
- flowing 20-µm-filtered seawater (for static or recirculated seawater, see Section 8.4)

2.2 Equipment for Toxicity Testing

- ultraviolet water sterilization unit (4 to 5 foot UV bulb) for UV spawning method
  - or hydrogen peroxide (fresh, refrigerated, reagent grade H_2O_2, 30%)
  - and Tris biological buffer [reagent grade, Tris (hydroxymethyl) aminomethane] for H_2O_2 spawning method
- 15-liter polyethylene buckets (3)
- 1000-ml beaker (tall form)
- perforated plunger (a perforated plastic [for example, poly(vinyl chloride)] disk fastened perpendicularly to a rod, used for vertical stirring within the tall beaker.)
- wide-bore pipets: 1-ml, 10-ml (1 each)
- 1-µm-filtered seawater: 60 liters for UV spawning, 40 liters for H_2O_2 spawning (15°)
- constant temperature water bath or environmental chamber ($15 \pm 1^{\circ}C$)
- compound light microscope (100x)[a]
  - or inverted microscope (100x)[b]

- meter and probes for dissolved oxygen, pH, and ammonia
- salinity refractometer
- thermometer
- analytical balance
- 1-liter volumetric flasks (2)
- volumetric pipets: 1-ml, 5-ml, 10-ml, 25-ml, 50-ml, 100-ml (1 each)
- graduated pipets: 1-ml, 10-ml (1 each)
- 10 liters of 0.2-µm-filtered dilution seawater for reference toxicant test
- 10 liters of dilution seawater per effluent test (see Quality Assurance Section 2.1.2)
- 10-liter polyethylene water bottle
- test containers (see Section 3.0)
- 37-µm-mesh sieve[a]
- 25-ml screw-capped test tubes (30 - 40 per test)[a]
- polyethylene funnel (with spout to fit into test tubes)[a]
- Sedgewick-Rafter counting cell microscope slide[a]
- hand counters (2)
- buffered formalin [formaldehyde 37% (1 liter), sodium borate (3 g), and glycerin (50 ml)] either 200 ml per test[a] or 2 liters per test[b]
- reagent grade acetone (1 liter per test)[a]
- 3N hydrochloric acid (15 liter per test; can be reused three times)[a]
- reference toxicant (zinc sulfate $ZnSO_4 \cdot 7H_2O$, unless otherwise specified in the NPDES permit)
- data sheets

3.0 Experimental Design

3.1 Effluent Tests

To determine effluent toxicity, organisms are exposed to test solutions of different effluent concentrations. The number and concentration of effluent treatments should be based on study requirements or NPDES permit conditions. All treatments must be replicated five times. Every test must contain five replicates of dilution water controls (see Quality Assurance Section 2.1.2 for a discussion of effluent dilution water). Tests that use hypersaline brine to adjust salinity must also contain five replicates of brine controls (see Section 4.2). Effluent concentrations should be assigned in a geometric sequence, with each concentration being at least 56% that of the next highest concentration (for example, 0% (control), 0% (brine control) 0.56%,

1.0% 1.8%, 3.2%, 5.6%, and 10% effluent). Effluent treatments that bracket the concentration found at the edge of the outfall zone of initial dilution may be most appropriate for evaluating chronic toxicity. A preliminary range finding test using a wider range of concentrations may be necessary for testing substances of unknown toxicity.

3.2 Reference Toxicant Tests

A reference toxicant test must be conducted concurrently with every effluent test to indicate the sensitivity of the organisms and the suitability of the test methodology. Zinc sulfate ($ZnSO_4 \cdot 7H_2O$) should be used as the reference toxicant for abalone tests, unless another toxicant is specified by the Regional Water Quality Control Board or other appropriate regulatory agency. Stock solutions should be made on the day of the test. Prepare a 10,000 µg/liter zinc stock solution by adding 0.0440 g of zinc sulfate ($ZnSO_4 \cdot 7H_2O$) to one liter of distilled water in a polyethylene volumetric flask.

Sample each reference toxicant stock solution at the beginning of the test to chemically verify zinc concentrations. Acidify samples in clean sample vials (Section 7.2) with 1% (by volume) 14N double quartz distilled nitric acid, and store in a dark refrigerator for no more than three months before analysis.

Reference toxicant solutions should be five replicates of 0 (control) 18, 32, and 56 µg/liter. Prepare one liter of each concentration by adding 0, 1.8, 3.2, and 5.6 ml of stock solution, respectively, to a one-liter volumetric flask and fill with 0.2-µm-filtered reference dilution water (see attached dilution schedule; see also QA/QC Section 2.1.2 for information on reference dilution water). Start with the control solutions and progress to the highest concentration to minimize contamination. Salinity adjustment and brine controls are not necessary in reference toxicant tests.

The effluent and reference toxicant tests must use embryos from the same spawn. They must be handled in the same way and delivered to the test containers at the same time.

4.0 Test Solutions

Prepare test solutions by combining effluent, hypersaline brine (see Section 4.2), and dilution water using volumetric flasks and pipets. Clean all glassware prior to use (see Section 7.0). Mix test solutions from the lowest concentration (control) to the highest concentration to avoid contamination.

4.1 Dilution Water

See Quality Assurance Section 2.1.2 for a discussion of dilution water.

4.2 Salinity Adjustment

The salinity of sewage effluents is generally lower than that tolerated by abalone larvae. Salinity adjustment is necessary where effluent concentrations are high enough to reduce test solution salinity below the acceptable range (34 ± 2 ‰). To maintain acceptable salinity, these test solutions must be adjusted to dilution water salinity by adding hypersaline brine. See Section 10.0 for statistical treatment of tests using brines.

If brine use is necessary, brines should be made by freezing 0.2-µm-filtered reference seawater (Anderson *et al.*, 1990). Clean, covered (not sealed) polyethylene containers should be used for freezing. One liter of brine can be made by freezing four one-liter containers of seawater in a conventional freezer (approximately minus 12°C). Freeze for approximately six hours, then separate the ice (composed mainly of fresh water) from the remaining liquid (which has now become hypersaline). Combine the liquid (brine) from the original four containers into two one-liter containers, place them back into the freezer overnight, then again separate the ice from the liquid brine. If the brine appears completely frozen, allow it to thaw; but check it often because the ice block can thaw quickly and liquid brine is often trapped inside. Check the salinity; brine salinity should be 60 to 80 ‰ Brine can be refrozen or diluted to adjust its salinity.

Check the pH of all brine mixtures and adjust to within 0.1 units of dilution water pH by adding, dropwise, dilute hydrochloric acid or sodium hydroxide.

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

$$VB = VE\frac{(34 - SE)}{(SB - 34)}$$

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

4.2.1 Brine Controls

Use brine controls in all tests where brine is used. Brine controls contain the same amount of brine as does the highest effluent concentration, plus distilled water equal to the volume of effluent in the highest concentration, plus dilution water to fill the mixing flask. For tests in which the effluent salinity is greater than 10 ‰, or if effluent dilutions above 10% are used,

calculate the amount of distilled water to add to brine controls by using the above equation, setting SE = 0, and solving for VE.

See the example below and the attached dilution schedule worksheet for further details on making test solutions.

4.3 Example Test Solution

Two hundred milliliters of test solution are needed for each test container. Five replicates can be mixed in a 1-liter volumetric flask. To make a test solution at a concentration of 1% effluent, add 10 ml of effluent to the 1-liter volumetric flask using a volumetric pipet. Fill the volumetric flask to the 1-liter mark with dilution water, stopper it, and shake to mix. Pour equal volumes into the five replicate containers.

To make a test solution at a concentration of 10% effluent, hypersaline brine must be used. Add 100 ml of effluent to a 1-liter volumetric flask. Then, assuming an effluent salinity of 2 ‰ and a brine salinity of 70 ‰, add 89 ml of brine (see equation above) and top off the flask with dilution water. Stopper the flask, shake well, and pour equal volumes into the 5 replicate containers.

5.0 Test Containers

Two types of containers can be used, depending on whether a standard[a] or inverted[b] microscope is used to analyze the samples at the end of the test. For tests using a standard microscope, 250 ml borosilicate glass beakers[a] are used for complex effluents and organic toxicants, and 250 ml polypropylene beakers[a] are used for trace metals. For tests using an inverted microscope, 250 ml polystyrene tissue culture flasks[b] are used with all toxicants (See Section 2.0).

5.1 Randomization

To randomize placement of test containers and to eliminate bias in the analysis of test results, label the test containers using random numbers from 1 to 20 (for reference toxicant tests, or 1 to N for effluent tests, with N being the total number of containers). Record these numbers on a separate data sheet together with the concentration and replicate numbers to which they correspond. Identify this sheet with the date, test organism, test number, laboratory, and investigator's name, and safely store it away until after the larvae have been examined at the end of the test. **Note:** Loss of this randomization sheet would invalidate the test by making it

impossible to correctly analyze the data afterwards. Take care to follow the numbering system exactly while filling containers with the test solutions.

Arrange the test containers by random number in the water bath or controlled temperature room.

6.0 Physical/Chemical Measurements of Test Solutions

Prior to testing, consult the container randomization sheet (Section 5.1) to compile a list of containers to be sampled for measurement. One randomly chosen replicate from each test concentration should be measured as follows: measure salinity, pH, and dissolved oxygen concentration at the beginning and end of the test; measure test solution temperature daily; and monitor water bath or environmental chamber temperature continuously. See Quality Assurance Section 5.0 for specifications and instrumentation for physical/chemical measurements.

7.0 Cleaning Procedure

New beakers should be scrubbed with a laboratory detergent and hot tap water, then rinsed with deionized water, and soaked with dilution water overnight. Used containers should be cleaned as described below. New tissue culture flasks should not be washed, but should be soaked overnight in dilution water before use. Discard or recycle used tissue culture flasks.

7.1 Effluents and organic toxicants

All test chambers used for organic toxicant and complex effluent tests should be cleaned as follows: 1) rinse three times with hot tap water, 2) rinse three times with new reagent grade acetone, 3) rinse three times with deionized water, 4) soak 24 hours in 2N HCL, 5) rinse three times with deionized water, 6) soak 24 hours in 2N HNO_3, 7) rinse three times with deionized water, 8) soak 24 hours in deionized water, 9) rinse three times with deionized water, 10) dry in a clean area. Acids may be reused three times.

7.2 Trace metal toxicants

All test chambers used in testing trace metals should be cleaned as follows: 1) rinse three times with deionized water, 2) rinse three times with 2N HCL, 3) rinse three times with deionized water, 4) soak for 24 hours in 2N HCL, 5) rinse three times with deionized water, 6) soak 24 hours in 2N HNO_3, 7) rinse three times with deionized water, 8) soak 24 hours in

deionized water, 9) rinse three times with deionized water, 10) dry in a clean area. Acids may be reused three times.

7.3 Other Glassware

All volumetric flasks, pipets, and other labware used for handling effluent test solutions must be cleaned as described in Section 7.1. All volumetric flasks, pipets, and other labware used for handling trace metal reference toxicant solutions must be cleaned as described in Section 7.2.

8.0 Test Organism

The species used in this test is *Haliotis rufescens*, the red abalone. This large gastropod mollusc is harvested commercially in southern California and supports a popular recreational fishery throughout the state. It consumes a variety of seaweeds and small incidental organisms, and is an important food source for sea otters, lobsters, and octopods (Hines and Pearse 1982). Abalone are "broadcast" spawners that reproduce by ejecting large numbers of gametes into the water column, where fertilization takes place externally. Free-swimming larvae hatch as trochophores, then undergo torsion while passing through a veliger stage. Abalone larvae do not feed during their one to three weeks in the plankton, but exist on energy stored in a yolk sack, supplemented perhaps by the uptake of dissolved amino acids. Once larvae come into contact with suitable substrate, they metamorphose and begin to consume benthic algae using a rasp-like tongue (the radula). Red abalone become reproductive after about two years at a length of about 7 cm, and can live for at least 25 years, growing to 30 cm in length. Refer to Hahn (1989) for a review of abalone life history and culture.

The red abalone is recommended for marine toxicity testing by the State Water Resources Control Board because it is ecologically and economically important, it has a history of successful laboratory culture, and it naturally occurs along the entire California coast (including areas impacted by effluents; Grigg and Kiwala, 1970).

8.1 Species Identification

Broodstock should be positively identified to species. Epipodial characteristics provide the best means of identification. All California haliotids have a lacey epipodial fringe, except for the red and black abalone, which have smooth, lobed epipodia. The red abalone can be distinguished from the black by shell coloration and by the number of open respiratory pores in the shell (reds have 3 to 4, blacks have 5 to 8). For further information on abalone taxonomy consult Owen *et al.* (1971), and Morris *et al.* (1980).

8.2 Collection

Mature red abalone broodstock can be collected from rocky substrates from the intertidal to depths exceeding 30 meters. They are found most commonly in crevices in areas where there is an abundance of macroalgae. (Collection of abalone is regulated by California law. Collectors must obtain a scientific collector's permit from the California Department of Fish and Game and observe any regulations regarding collection, transfer, and maintenance of abalone broodstock.)

While abalone captured in the wild can be induced to spawn, those grown or conditioned in the laboratory have been more dependable. Commercial mariculture facilities in California produce large numbers of abalone, and distribution systems exist to supply live spawners to a number of market areas. Contact the Marine Bioassay Project for a list of broodstock suppliers. In any case, broodstock should be obtained from sources free of contamination by toxic substances to avoid genetic or physiological preadaptation to pollutants (See Quality Assurance Section 3.2).

8.3 Broodstock Transport

Abalone broodstock can be transported for short time periods from the field or supply facility in clean covered plastic buckets filled with seawater. Use compressed air, or battery powered pumps to supply aeration. Compressed oxygen is not recommended because bubbled oxygen may induce unintended spawning (Morse et al. 1977). Maintain water temperatures within 3° C of the temperature at the collecting site. Four abalone in a 15-liter bucket should remain healthy for up to four hours under these conditions.

Abalone can be transported for up to 30 hours in sealed, oxygen-filled plastic bags containing moist (seawater) polyfoam sponges (Hahn, 1989). Cut the polyfoam into sections (about 20 x 40 cm), and allow them to soak in clean seawater for a few minutes. New sponges should be leached in seawater for at least 24 hours. Rinse the sponges in fresh seawater and wring them out well. Place the polyfoam inside double plastic trash bags, then place the abalone on the moist foam. It is important that there is no standing water in the bags. Put the abalone bag into an ice chest (10 to 15 liter), fill the bags with pure oxygen, squeeze the bags to purge all the air, then refill with oxygen (approximately three liters of oxygen gas will support eight abalone). Seal the bags (air-tight) with a tie or rubber band. Wrap two small (one-liter) blue ice blocks in sections of newspaper (about 15 pages thick) for insulation, and place the wrapped blue ice in a sealed plastic bag in the chest on top of the abalone bags. Fill any remaining space with packing and seal the box for shipping. Avoid transporting the ice chest in temperatures below freezing or above 30°C (86° F).

8.4 Broodstock Culture and Handling

At the testing facility, place the abalone in aerated tanks with flowing seawater (1 to 2 liter/min). With high water quality, water flow, and aeration, abalone 8 to 10 cm long can be kept at a density of one per liter of tank space or one per 100 cm^2 of tank surface area, whichever provides the lower density. Density should be cut to a maximum of 0.5 per liter in recirculating systems and to a maximum of 0.25 per liter in static tanks. Tanks should be covered for shade and to prevent escape. Drain and rinse culture tanks twice weekly to prevent build-up of detritus. Remove any dead abalone immediately, and drain and scrub its tank.

Ideal maintenance temperature is 15^{o} C, the toxicity test temperature (see also Leighton, 1974). If broodstock are to be held for longer than 5 days at the testing facility, feed broodstock with blades of the giant kelp *Macrocystis*. Feed to slight excess; large amounts of uneaten algae will foul culture water. If *Macrocystis* is unavailable, other brown algae (*Nereocystis, Egregia, Eisenia*) or any fleshy red algae can be substituted (Hahn, 1989).

Recirculating tanks should be equipped with biological or activated carbon filtration systems and oyster shell beds to maintain water quality. Measure the ammonia content of static or recirculating seawater daily to monitor the effectiveness of the filtration system. Un-ionized ammonia concentrations should not exceed 20 µg/liter and total ammonia concentrations should not exceed 1.0 mg/liter. Supply constant aeration and temperature control. Add only a few blades of algal food at each cleaning to prevent its accumulation and decay.

When handling abalone, use a rounded, dull-bladed stainless-steel butter knife to release the animal's grip on the substrate. Gently slide the flat dull blade under the foot at the posterior end near the beginning of the shell whorl, and slide it under about two-thirds of the foot. Apply constant pressure to keep the front edge of the blade against the substrate and not up into the foot. Quickly and gently lift the foot off the substrate. A smooth deliberate motion is more effective and less damaging than repeated prying.

Assess the reproductive condition of the broodstock by examining the gonads, located under the right posterior edge of the shell. An abalone placed upside down on a flat surface will soon relax and begin moving the foot trying to right itself. Take advantage of this movement and use the dull blade to bend the foot away from the gonad area for inspection. The female ovary is jade green, male testes are cream-colored. When the gonad fully envelopes the dark blue-gray conical digestive gland and is bulky along its entire length, the abalone is ready for spawning (Hahn, 1989). Ripe (recrudescent) spawners have a distinct color difference between the gray digestive gland and the green or cream-colored gonad. Less developed gonads appear gray (in females) or brown (in spent males).

Abalone 7 to 10 cm in shell length are recommended as broodstock. They are easier to handle than larger ones, and can be spawned more often (approximately every four months under suitable culture conditions; Ault, 1985). Though spawning fewer eggs than larger abalone, 10 cm abalone will produce over 100,000 eggs at a time (Ault, 1985). Twenty to thirty-five thousand eggs are needed for a single toxicant test, depending on test design. For further information on red abalone culture, see Ebert and Houk (1984) or Hahn (1989).

8.4.1 Culture Materials

Refer to Quality Assurance Section 4.6 and 4.7 for a discussion of suitable materials to be used in laboratory culture of abalone. Be sure all new materials are properly leached in seawater before use. After use, all culture materials should be washed in soap and water, then rinsed with seawater before reuse.

8.5 Spawning Induction

Note: Before beginning the spawning induction process, be sure that test solutions will be mixed, sampled, and temperature equilibrated in time to receive the fertilized embryos. Spawning induction generally takes about three hours, but if embryos are ready before test solutions are at the proper temperature, the delay may allow embryos to develop past the one-cell stage before transfer to the toxicant. Transfer can then damage the embryos, leading to unacceptable test results.

Culture work (spawning, etc.) and toxicant work should be done in separate laboratory rooms, and care should be taken to avoid contaminating organisms prior to testing.

Ripe abalone can be induced to spawn by stimulating the synthesis of prostoglandin-endoperoxide in the reproductive tissues (Morse et al., 1977). This can be done in two ways: addition of hydrogen peroxide to seawater buffered with Tris (Morse et al., 1977), or irradiation of seawater with ultraviolet light (Kikuchi and Uki, 1974). The first method is preferable for small laboratories because it avoids the cost and maintenance requirements of a UV system. If a UV system is available, this method may be preferable because it is simple, does not use chemicals that could accidentally harm larvae, and is considered to be less likely to force gametes from unripe adults.

If broodstock are shipped to the laboratory by a supplier, allow two days or more for laboratory acclimation before spawning induction; this should increase the probability of acheiving a successful spawn of viable gametes.

8.5.1 Hydrogen Peroxide Method.

Select four ripe male abalone and four ripe females. Clean their shells of any loose debris. Place the males in one clean polyethylene bucket and the females in another. Cover the buckets with a tight fitting perforated lid, supply the containers with flowing or recirculating (1 liter/minute) 20 µm-filtered seawater (15° C), and leave the animals without food for 24 to 48 hours to acclimate and eliminate wastes. If flowing seawater is unavailable, keep the spawners in larger (>30 liter) aquaria with aeration at 15° C for 24 hours without food to eliminate wastes. Three hours prior to the desired spawning time, drain the buckets, wipe and rinse out mucus and debris, and refill with 6 liters of 1 µm-filtered seawater. If abalone have been kept in larger aquaria, put them in the buckets at this time. Check the abalone from time to time to make sure they remain underwater. Add air stones to the buckets and keep them aerated until spawning begins.

Dissolve 12.1 g of Tris into 50 ml of distilled water. When the Tris has dissolved completely, mix the hydrogen peroxide (H_2O_2) solution in a separate flask by pouring 10 ml of fresh* refrigerated H_2O_2 (30%) into 40 ml of refrigerated distilled water (1:5 dilution). Pour 25 ml of Tris solution and 25 ml of H_2O_2 solution into each of the spawning buckets (male and female). Stir well to mix; the final concentrations in the spawning buckets will be approximately 6 mM Tris (pH = 9.1) and 5 mM H_2O_2. Allow the abalone to remain in contact with the chemicals for 2.5 hours at 15° C. The chemical reaction is temperature dependent; allow three hours of contact with H_2O_2 at 11°C. Temperatures higher than 15°C are not recommended for spawning. Maintain constant aeration.

* Note: Hydrogen peroxide loses potency over time. Purchase reagent or certified grade H_2O_2 in small containers (100 ml). Store unopened containers for no more than one year, and discard open containers after one month. Mark the purchase date and opening date on all containers, and keep all containers refrigerated.

After 2.5 hours, empty the spawning buckets, rinse them well, and refill them to the top with fresh 1 µm-filtered seawater at the same temperature (15°C). Keep the containers clean by siphoning away mucus and debris. Maintain constant aeration until spawning begins, then remove the airstones. The abalone begin spawning about three hours after the introduction of the chemicals (at 15°C). Eggs are dark green and are visible individually to the naked eye, sperm appear as white clouds emanating from the respiratory pores.

If spawning begins before the chemicals have been removed, drain the both buckets immediately, discarding any gametes. Rinse the buckets thoroughly and refill with clean, 1-µm-filtered seawater (15° C). Use only the gametes subsequently spawned in clean water for testing.

8.5.2 UV Irradiation Method

Select four ripe male abalone and four ripe females. Clean their shells of any debris. Place the males in one clean polyethylene bucket and the females in another. Cover the buckets with a tight fitting perforated lid, supply the containers with flowing or recirculating (1 liter/minute) 20 µm-filtered seawater (15°C), and leave the animals without food for 24 to 48 hours to acclimate and eliminate wastes. If flowing seawater is unavailable, keep the spawners in larger (>30 liter) aquaria with aeration at 15° C for 24 hours. Three hours prior to the desired spawning time, drain the buckets, wipe and rinse out mucus and debris, and refill with just enough water to cover the abalone (which should all be placed in the bottom of the bucket). Begin slowly filling the buckets with 1 µm-filtered seawater (15°C) that has passed through the UV sterilization unit. Flow rates to each of the buckets should be 150 ml/min. A low total flow rate (300 ml/minute) in the UV unit is necessary to permit sufficient seawater irradiation. (The sterilization unit should be cleaned and the UV bulb replaced at least once annually.) Place the buckets in a water bath at 15°C to counter the temperature increase caused by the slow passage of water past the UV lamp. Check the containers periodically, and keep them clean by siphoning out any debris. After three hours (± about 1/2 hour), abalone should begin spawning by ejecting clouds of gametes into the water. Eggs are dark green and are visible individually to the naked eye, sperm appear as white clouds emanating from the respiratory pores.

Note: If past experience or other factors indicate difficulties in achieving synchronous spawning, it may be helpful to induce a second group of females about an hour after the first. This will increase the chances of providing fresh eggs (less than one hour old) for fertilization if males spawn late (see below). Senescence of sperm is seldom a problem because males continue spawning over a longer period of time.

8.5.3 Pooling Gametes

Although it is not necessary, it is preferable to have more than one abalone of each sex spawn. To increase the probability of multiple spawners without risking senescence of the gametes, allow one-half hour after the first individual of the second sex begins to spawn before initiating fertilization. For example, if males spawn first, wait one-half hour after the first female spawns before fertilizing eggs. In most cases this will provide time for more than one of each sex to spawn. More important than multiple spawning, however, is avoiding delay of fertilization. Eggs should be fertilized within one hour of release (Uki and Kikuchi, 1974). All sperm should be pooled, and all eggs should be pooled prior to fertilization. This can be accomplished by gentle swirling within the spawning buckets. **Note:** Take care to avoid contaminating eggs with sperm

prior to the intended fertilization time. It is important that development is synchronous among all test embryos.

8.6 Fertilization

As the females spawn, allow the eggs to settle to the bottom. If necessary, gently stir to evenly distribute the eggs. Siphon out and discard any eggs that appear clumped together. Eggs are ready to transfer to a third (fertilization) bucket when either: 1) one-half hour has passed since the first individual of the second sex has spawned (see Section 8.5.3), 2) multiple individuals of each sex have spawned, or 3) there are too many eggs on the bottom of the bucket to allow evenly distributed eggs to avoid touching each other. Slowly siphon eggs into a third clean polyethylene bucket containing one or two liters of 1-µm-filtered seawater (15°C). Siphon carefully to avoid damaging the eggs and to avoid collecting any debris from the spawning container. Siphon about 100,000 eggs, enough to make a single even layer on the container bottom. Each egg should be individually distinguishable, and not touching other eggs. If excess eggs are available, siphon them into a second fertilization bucket to be used as a reserve. Keep all containers at 15° C. Make sure that water temperatures differ by no more than 1° C when transferring eggs or sperm from one container to another.

As the males spawn, siphon sperm from directly above the respiratory pore and collect this in a 500 ml flask with 1-µm-filtered seawater. Keep the flask at 15°C, and use it as a back-up in case the males stop spawning. If spawning continues renew this reserve every 15 minutes. Usually the males will continue spawning, turning the water in the bucket milky white. As long as the males continue spawning, partially drain and refill the bucket every 15 minutes, replacing old sperm-laden water with fresh seawater (15°C). Use the freshest sperm possible for fertilization.

Make sure eggs are fertilized within one hour of release (Uki and Kikuchi, 1974; see Note after Section 8.5.2). To fertilize the eggs, collect about 200 ml of sperm-laden water in a small beaker. The sperm concentration in the beaker does not have to be exact, just enough to give a slightly cloudy appearance (approximately 1 to 10 x 10^6 cells/ml; giving a concentration of 0.5 to 5 x 10^5 cells/ml in the fertilization bucket). See Hahn (1989) for further information on sperm concentrations and the protocol for fertilization. Pour the sperm solution into the fertilization bucket containing the clean isolated eggs. Using a hose fitted with a clean glass tube, add 1-µm-filtered seawater to the fertilization bucket at a low flow rate (<1 liter/min; 15°C). Use the water flow to gently roil the eggs to allow them to mix with the sperm and fertilize. When the bucket is about half-full and eggs are evenly mixed, stop the water flow and allow the eggs to settle to the bottom of the bucket (about 15 minutes). Fertilization is then complete.

Note: Once fertilized eggs have settled to the bottom of the bucket (15 minutes after addition of the sperm), the following steps (rinsing, concentrating, and counting the embryos) must proceed without delay to assure that they are transferred into the test solutions within about one hour. Embryos must be delivered to the test containers before the first cell division takes place. (Multicellular embryos are more susceptible to damage in handling, and test endpoint analysis assumes that the first cell division takes place in the toxicant solution).

After embryos have settled, carefully pour or siphon off the water from above the settled embryos to remove as much of the sperm laden water as possible without losing substantial numbers of embryos. Slowly refill the bucket with 1 µm-filtered seawater (15°C). Allow the embryos to settle, and siphon them into a tall 1000 ml beaker for counting. Siphon at a slow flow rate, and move the siphon along the bottom of the bucket quickly to pick up a large number of embryos in the short amount of time it takes to fill the beaker. Examine a sample of the embryos at 100x magnification. One to one hundred sperm should be visible around the circumference of each embryo, 15 sperm per egg is optimal. If sperm are so dense that they appear fuzzy (>>100 sperm/egg), the abalone will develop abnormally and cannot be used.

8.7 Estimation of Embryo Density

Evenly mix the embryos in the 1000 ml beaker by gentle vertical stirring with a clean perforated plunger. Never allow embryos to settle densely in the bottom of the beaker, and take care not to crush embryos while stirring. Take 5 samples of the evenly suspended embryos using a 1 ml wide bore graduated pipet. Hold the pipet up to the light and count the individual embryos using a hand counter. Alternatively, empty the contents of the pipet onto a Sedgewick-Rafter slide and count embryos under low magnification on a compound scope. Discard the sampled embryos after counting. Take the mean of five samples to estimate the number of embryos per milliliter. Density of embryos in the beaker should be between 200 and 300 embryos/ml. Dilute if the concentration is to high, let embryos settle and pour off excess water if concentration is too low.

9.0 Toxicity Test Procedure

9.1 Exposure of Test Organisms

9.1.1 Delivery of Fertilized Embryos

Using the estimate of embryo density in the 1000 ml beaker, calculate the volume of water that contains 1000 embryos (See attached worksheet). Remove 1000 embryos by drawing the appropriate volume of water from the well mixed beaker using a 10 ml wide bore pipet. Deliver the embryos into the test containers directly from the pipet making sure not to touch the pipet to the test solution. Stir the embryo beaker with the plunger between taking aliquots. The temperature of the embryo suspension must be within 1° C of the temperature of the test solution. (As above, all solutions are kept at 15°C). Record the volume of water delivered into the test containers with the embryos.

Embryos must be delivered into the test solutions within one hour of fertilization. Immediately after the embryos have been delivered, take a sample from the embryo beaker and examine it under 100x magnification. All embryos should still be in the one-cell stage; record any observations to the contrary on the data sheet.

9.1.2 Incubation

Incubate test organisms for 48 hours in the test containers at 15° C under low lighting (approximately 10 microeinsteins m^{-2} sec^{-1}; or 100 lux) with a 16L:8D photoperiod. Fertilized embryos become trochophore larvae, hatch, and develop into veliger larvae in the test solutions during the exposure period.

9.2 Sample Preservation

After the 48 hour exposure, the abalone larvae are fixed in formalin. Two methods for sample preservation are described. Be sure that samples for physicochemical measurements and reference toxicant chemical verification have been taken before further processing of test solutions.

The first method is for use with open beakers and a standard compound microscope[a]. At the end of the 48-hour incubation period, remove each test container, swirl the solution to suspend all the larvae, and pour the entire contents through a 37 µm-mesh screen. The test solution is discarded and the larvae are retained on the screen. Using streams of filtered seawater from a squeeze bottle, rinse the larvae from the screen through a funnel into 25 ml screw cap vials. Be careful not to hit the larvae directly with the streams of water; rough handling during transfer may cause fragmentation of the larvae, making counting more difficult and less accurate. Add enough buffered formalin to preserve larvae in a 5% solution. Addition of formalin is more accurate if the

vials are premarked with lines showing the volume of sample and the volume of formalin to be added. Larvae should be counted within two weeks.

For tissue culture flasks using an inverted microscope[b], add formalin directly to each flask, and fill the flask to the top with dilution water so that the final formalin concentration is between 4 and 5%. In a 250 ml flask with 200 ml of test solution, add 30 ml of 37% buffered formalin and fill the flask to the top with dilution seawater. Reseal the flask, shake gently to mix, and store the flasks away from direct sunlight with the broad side down for counting on the inverted microscope.

9.3 Counting

To count the larvae using a standard compound microscope[a], pipet all the larvae from the bottom of the preservation vial onto a Sedgewick-Rafter counting cell. Examine 100 larvae from each vial under 100x magnification. To best characterize the sample and to avoid bias, select groups of larvae one field of vision at a time, moving to the next field without looking through the lens. Be careful to work across the slide in one direction to avoid recounting the same areas. Count the number of normal and abnormal larvae using hand counters. After counting, use a funnel to return the larvae to the vial for future reference.

Note[a]: Preserved larvae will give off formalin fumes from the counting slide[a]. Study and follow all recommended safety precautions to avoid exposing laboratory personnel. Ventilation and safety equipment, such as respirators and gloves, should be carefully considered and used where necessary. See Quality Assurance Section 9.0 for further safety information.

To count the larvae using an inverted microscope[b], set the tissue culture flasks broad side down (the same way they were stored) on the stage of the inverted microscope. Examine the first 100 larvae encountered in each flask under 100x magnification. Count the number of normal and abnormal larvae using hand counters.

9.4 Endpoint

Examine the shape of the larval shell to distinguish normal from abnormal larvae. Count veliger larvae as normal if they have smoothly curved larval shells that are striated with calcareous deposits and somewhat opaque. It is common for normal larvae to have a slight curved indentation near the leading edge of the shell. A single indentation in this area is thus counted as normal.

Larvae with both multiple indentations and an obvious lack of calcification (ie. clear appearance in at least part of the shell) are counted as abnormal. The combination of these two features indicates inhibition of a biological process (lack of calcification) and actual damage to the

organism (indentations) allowed by the thin shell. Refer to the accompanying photographs (Figure 1) for classification of marginally deformed larvae. The following types of larvae are also counted as abnormal: 1) larvae that have arrested development (from one cell through trochophore stage), 2) larvae with obvious severe deformations, 3) larvae with broken shells, 4) larval shells separated from the rest of the animal, and 5) larvae found remaining in the egg membrane (however, take care to distinguish these from larvae that may have come in contact with loose egg cases, especially in the tissue culture flasks).

Record all counts and the test container number on the data sheet.

10.0 Data Analysis

Determine the proportion of abnormal larvae in each replicate container. Transform the proportion data to the arcsine of their square root. (This transformation is a standard requirement for proportion data that are analyzed using parametric statistics). Check the original test container randomization sheet (see Section 4.5), and assign the correct concentration and replicate number to the transformed proportion data. Perform an analysis of variance (ANOVA) to compare concentrations. If a significant difference is detected, use a Dunnett's multiple comparison test to compare each concentration against the control (Sokal and Rohlf, 1969; Zar, 1974). Derive the No Observed Effect Concentration (NOEC) as the highest concentration that is not significantly different from the control. Use an alpha level of $p = 0.05$ to determine statistical significance.

If brines were used in the effluent test, include all data in the ANOVA, and use the appropriate control for Dunnett's comparison. Use dilution water controls for comparison with effluent treatments that had no brine added, and use brine controls for comparison with salinity adjusted effluent treatments (see Section 4.2).

11.0 Test Acceptability

For tests to be considered acceptable, the following requirements must be met:

1) Control larval abnormality cannot exceed 20% in the reference toxicant test.
2) Brine control results must not be significantly different from dilution water control results in the effluent test, using a t-test and an alpha level of 0.05.
3) The response from the 56 µg/liter zinc treatment must be significantly different from the control response (see Section 10.0 for discussion of data analysis).
4) The between-replicate variability must be low enough that the ANOVA Error Mean Square (MS) does not exceed 100.00 in the reference toxicant test (using arcsine transformed percentage abnormality data in degrees).

12.0 References

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13.0 Abalone Toxicity Test Protocol Step-by-Step Summary

13.1 Preparation of Test Solutions

A. Determine effluent test concentrations and appropriate dilution water based on NPDES permit conditions and guidance from the appropriate regulatory agency (Section 3.1).

B. Prepare effluent test solutions by diluting well mixed unfiltered effluent using volumetric flasks and pipets. Use hypersaline brine where necessary to maintain all test solution salinities at 34 ± 2 ‰. Include brine controls in tests where brine is used (Section 4.0).

C. Prepare a zinc reference toxicant stock solution (10,000 µg/liter) by adding 0.0440 g of zinc sulfate ($ZnSO_4 \cdot 7H_2O$) to 1 liter of distilled water. Sample the stock solution for chemical verification of the zinc concentration. Acidify sample vials with 1% by volume 14N double quartz distilled nitric acid, and store in a dark refrigerator for no more than three months before analysis (Section 3.2).

D. Prepare zinc reference toxicant solutions of 0 (control) 18, 32, and 56 µg/liter by adding 0, 1.8, 3.2, and 5.6 ml of stock solution, respectively, to a 1-liter volumetric flask and filling to 1 liter with 0.2-µm-filtered reference dilution seawater (Section 3.2).

E. Sample effluent and reference toxicant solutions for physical/chemical analysis. Measure salinity, pH, and dissolved oxygen from each test concentration (Section 6.0).

F. Randomize labelling of test containers, and record the container numbers with their respective test concentrations on a randomization sheet. Store the sheet safely until after the test samples have been analyzed (Section 5.1).

G. Place test containers in a water bath or environmental chamber and allow to temperature equilibrate (Section 5.1).

H. Measure the temperature daily in one random replicate of each test concentration. Monitor the temperature of the water bath or environmental chamber continuously (Section 6.0).

I. At the end of the test, measure salinity, pH, and dissolved oxygen concentration from each test concentration (Section 6.0).

13.2 Preparation and Analysis of Test Organisms

A. Induce four male and four female abalone to spawn using either H_2O_2 and Tris or UV irradiated seawater (300 ml /min flow rate through the UV unit). All solutions should be maintained at 15°C (Section 8.5).

B. Siphon eggs into a fertilization bucket. Add 200 ml of sperm-laden water to fertilize the eggs. Wash the fertilized eggs at least twice by slowly decanting and refilling the container with fresh filtered seawater. Temperatures should vary by no more than 1°C between waters used in mixing and refilling (Section 8.6).

C. Suspend the embryos evenly in a 1000 ml beaker and count five samples in a 1 ml pipet to estimate embryo density (Section 8.7).

D. Pipet 1000 fertilized embryos into each test container. Be sure temperatures in the embryo beaker and the test solutions are at 15° ± 1° C. Incubate for 48 hours (Section 9.1).

E. At the end of the 48 hour period, pour the entire test solution with larvae through a 37 µm-mesh screen[a]. Wash larvae from the screen into 25 ml vials[a]. Add buffered formalin to preserve the larvae in a 5% solution. If an inverted microscope and 250 ml tissue culture flasks are used[b], add 30 ml of 37% formalin directly to the flask, cap the flask and shake gently to mix (Section 9.2).

F. Pipet a sample from each vial onto a Sedgewick-Rafter counting slide[a] and examine 200 larvae. Return the larvae to the vials for future reference. If tissue culture flasks are used[b], place the flask directly on the stage of the inverted microscope (Section 9.3).

G. Count the number of normal and abnormal larvae in each replicate container. Use larval shell development as the test endpoint (Section 9.4).

H. Calculate the proportion of normal larvae for each replicate, transform this percentage value to the arcsine of its square root, then assign each value to the proper test concentration using the container randomization sheet (Section 10.0 and Section 5.1).

I. Analyze the data from each test using an Analysis of Variance, then compare each concentration to the appropriate control group using a Dunnett's multiple comparison test. Determine the NOEC value as the highest concentration that is not significantly different from the control (Section 10.0).

[a] For use with a compound microscope. [b] For use with an inverted microscope (Section 2.0).

14.0 Legend for Abalone Endpoint Photographs

The following three pages show 12 photographs of 48-hour-old abalone veliger larvae from effluent toxicity tests. All larvae were taken from intermediate effluent concentrations and were chosen to represent "borderline" cases (ie. larvae that were slightly affected and are therefore difficult to categorize as normal or abnormal). In most cases, larvae from lower and higher effluent concentrations are more easily categorized than those shown here; in the lower concentrations they are obviously without shell abnormalities and in the higher concentrations they are severely deformed. These photographs are presented as a visual reference to help standardize test analysis and eliminate bias in the interpretation of marginally deformed larvae. All larvae on the left-hand side of these pages were counted as normal, all larvae on the right-hand side were counted as abnormal. Refer to Section 9.4 for a written description of the test endpoint.

A. Normal larva with well calcified (striated) shell but slight uneven shell outline.

B. Obviously abnormal larva with transparent shell and numerous shell deformities.

C. Normal larva with some shell thinning and mild flattening of shell curvature near the leading edge (left side of photograph).

D. Abnormal larva with multiple slight indentations and transparency near the leading edge of the shell (right side of photograph).

E. Normal larva with well calcified (striated) shell but uneven shell outline.

F. Abnormal larva with transparent shell and large indentation.

G. Normal larva, anterior (rather than lateral) view. Well striated, smooth rounded shell outline.

H. Abnormal larva, anterior (rather than lateral) view. Transparent irregular shell with indentations.

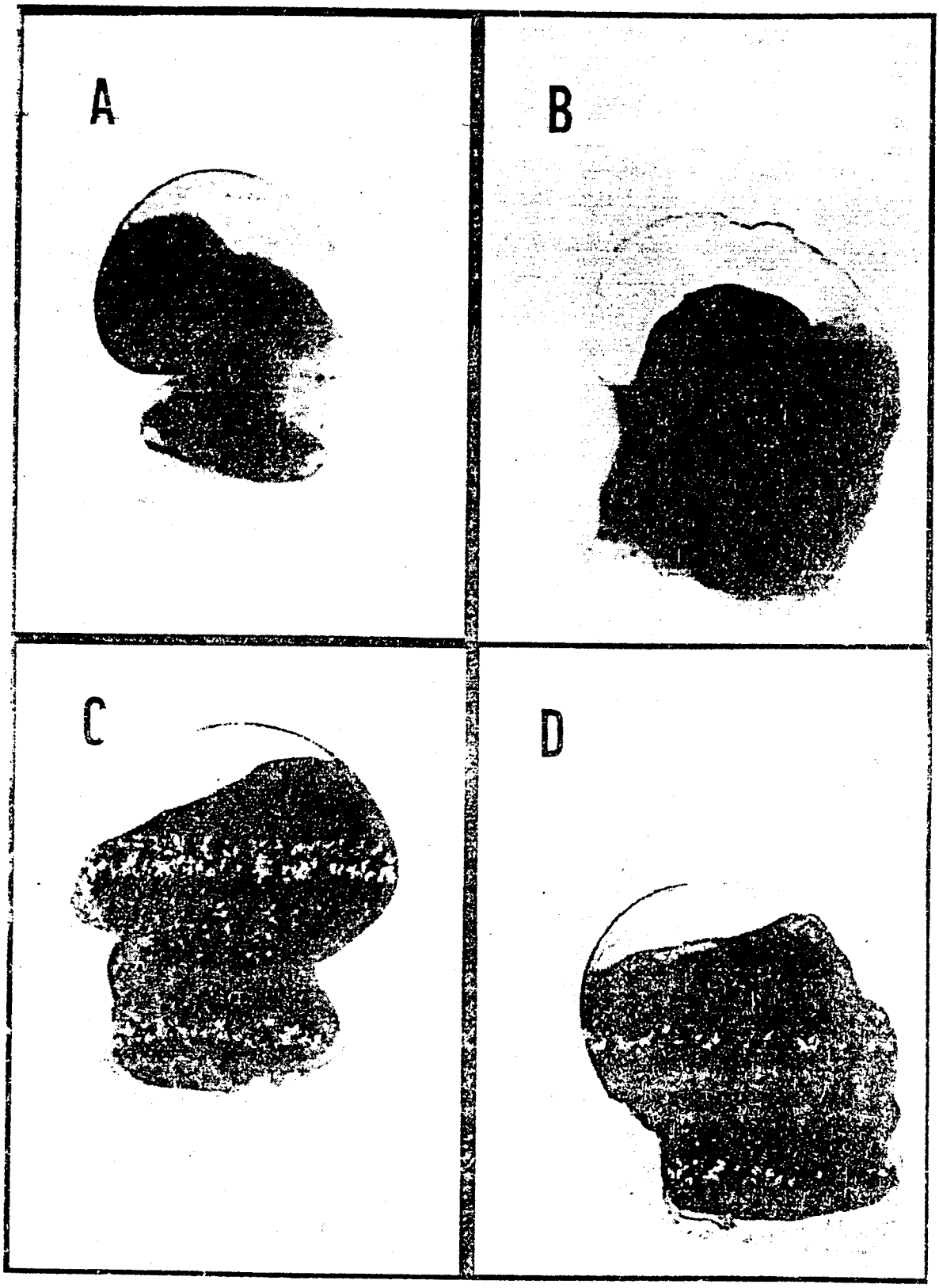
I. Normal larva with well calcified shell and one small indentation at leading edge.

J. Abnormal larva with shell transparencies, indentations and irregular shape.

K. Three normal larvae, all well calcified with small indentations at the leading edge.

L. Abnormal larva with arrested development at an early stage. Any larva found within the egg membrane, no matter how well developed, is counted as abnormal.

Laboratories wishing to receive higher quality photographic reproductions of these figures should contact the Marine Bioassay Project, Coast Route 1 Granite Canyon, Monterey, CA 93940.


A
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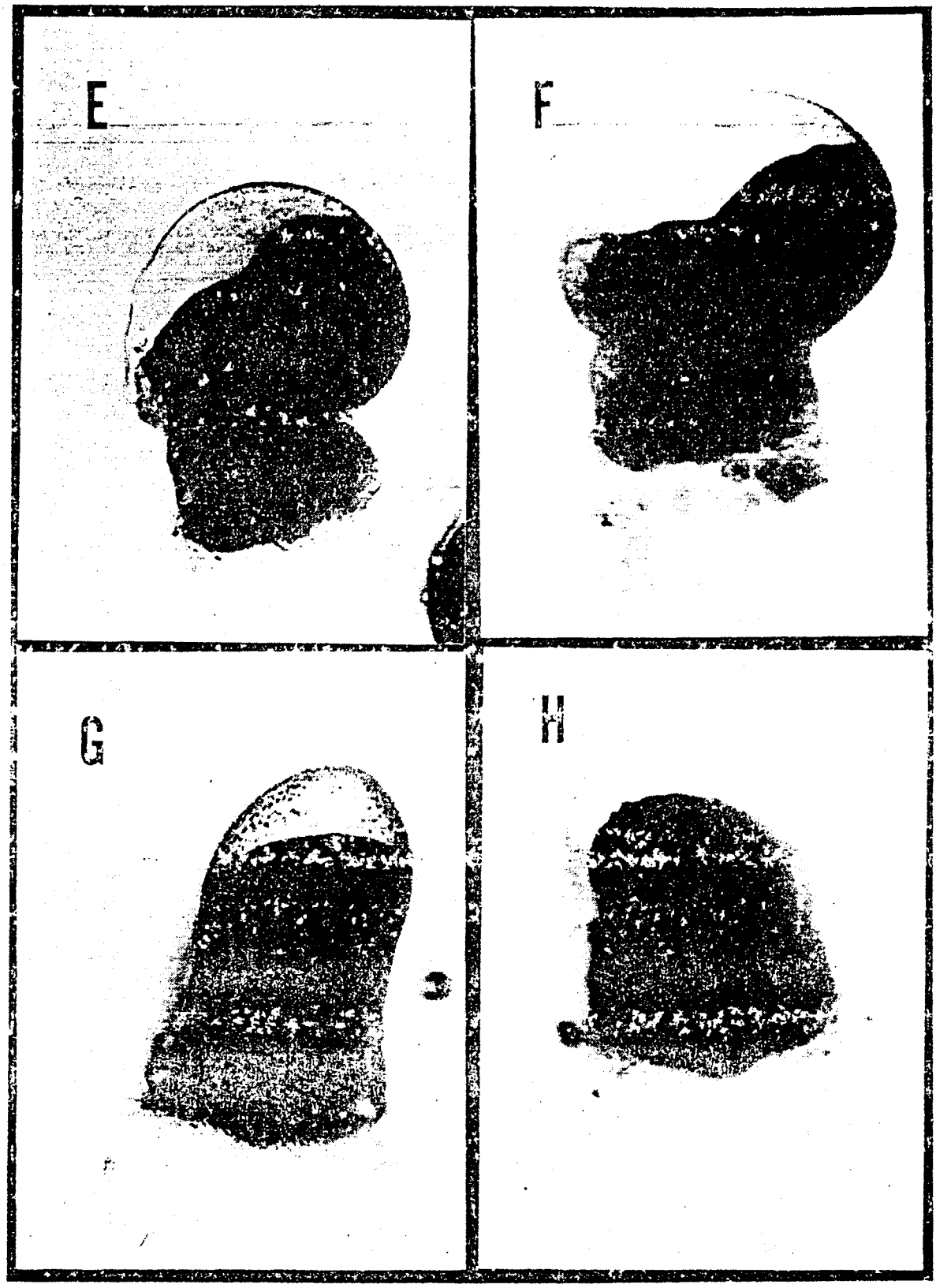
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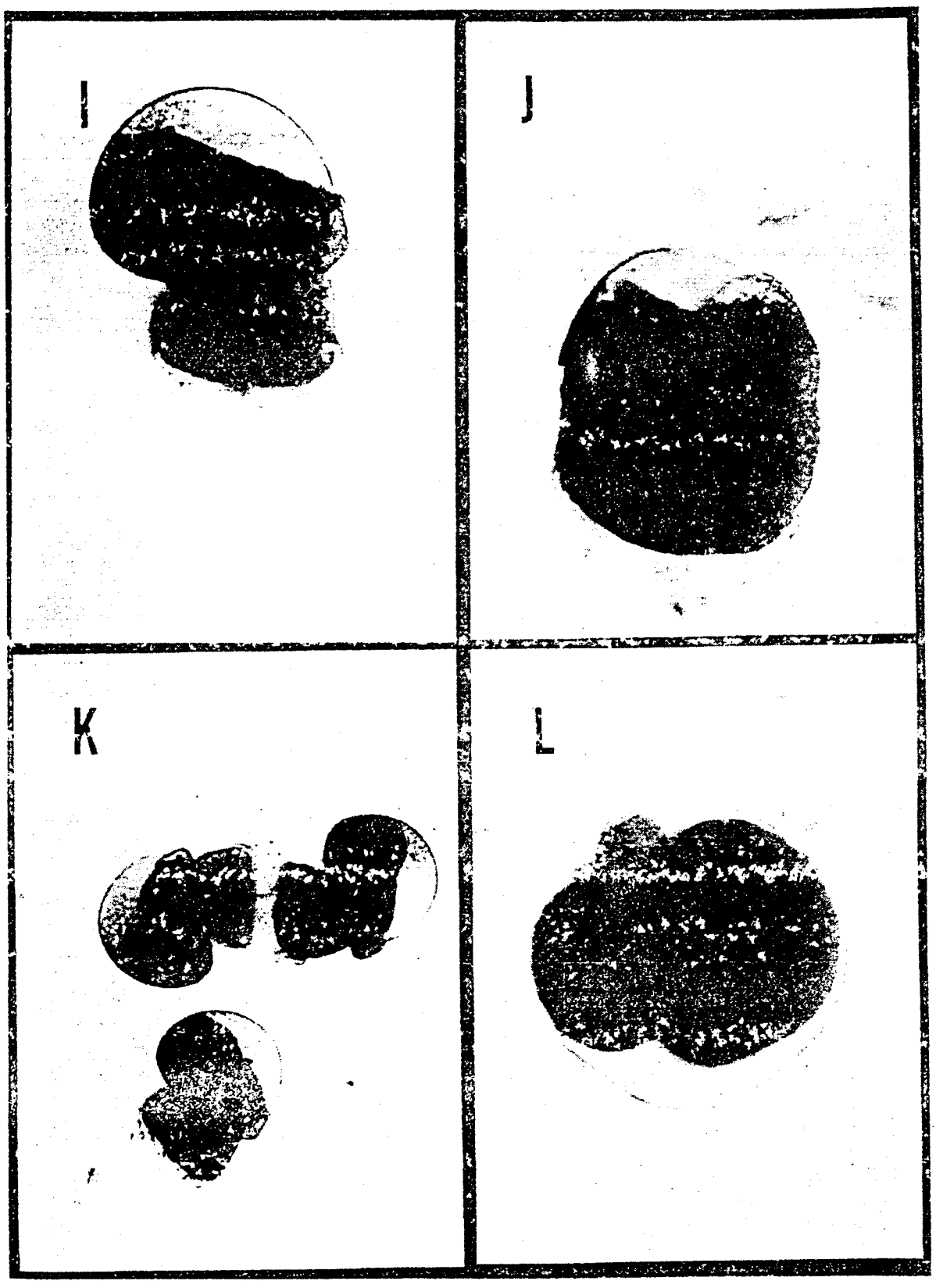
I
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Abalone Spawning Worksheet

Date:

Toxicant and test number:

Investigator:

Condition of abalone spawners:

	abalone	gonad index	broodstock record
Male	1.		
	2.		
	3.		
	4.		
Female	1.		
	2.		
	3.		
	4.		

Method of spawning (circle one): H_2O_2 UV irradiated seawater Other _____

	Time	Temperature
Beginning of spawning treatment:		
Taken out of H_2O_2 (if applicable):		
First male abalone spawns:		
First female abalone spawns:		
Multiple spawners, male:	female:	
Fertilization:		

Condition of spawn (circle) Males: light moderate heavy Females: light moderate heavy

Condense the fertilized eggs (embryos) into a beaker and determine the density (in embryos/ml). Counts:

1. _____ 2. _____ 3. _____ 4. _____ 5. _____

Mean _____ S.D. _____

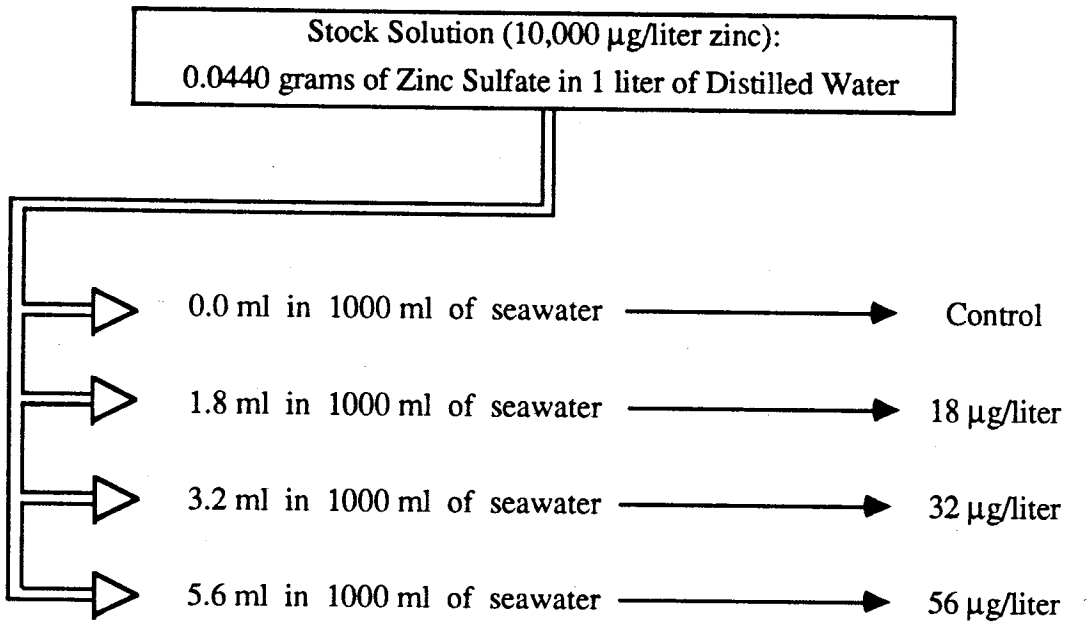
Add 1000 embryos to each test container by pipetting the volume calculated below from the well-mixed beaker sampled above.

1000 embryos/test container + number of embryos/ml _____ = _____ ml/test container

Temperature of embryos: _____ Temperature of test containers: _____

Time embryos are added to test containers: _____.

Zinc Dilution Schedule for Abalone Tests



Effluent Dilution Sheet

100% Effluent is the Stock Solution

Corresponding Beaker Numbers

Date Organism Investigator

Notes

→	0.0 ml in 1000 ml flask →	Control
→	0.0 ml in 1000 ml flask →	Brine Control
→	_____ →	Other
→	_____ →	Other
→	5.6 ml in 1000 ml flask →	0.56%
→	10.0 ml in 1000 ml flask →	1.0%
→	18.0 ml in 1000 ml flask →	1.8%
→	32.0 ml in 1000 ml flask →	3.2%
→	56.0 ml in 1000 ml flask →	5.6%
→	100.0 ml in 1000 ml flask →	10.0%
→	_____ →	Other

Salinity Adjustment Using Hypersaline Brine

Add hypersaline brine to those concentrations in which test solution salinity would otherwise fall below the minimum acceptable test salinity (32‰).
The equation to calculate the volume of brine to add for each of these concentrations is: $VB = VE\left(\frac{34 - SE}{SB - 34}\right)$

Quantities known from dilution schedule: VE = Volume of Effluent added for each concentration (ml).

Quantities to be measured: SB = Salinity of Brine (‰), and SE = Salinity of Effluent (‰). SB = _____‰ SE = _____‰

Note: Always adjust the pH of the brine to equal that of the dilution water. Brine salinity should be 60 to 80 ‰.

Calculate the volume of brine to be added, VB, for each concentration that requires salinity adjustment using the above equation.

Example: If dilution water salinity is 33.5‰ and effluent salinity is 0‰, a test solution of 5.6% effluent would have a salinity of 31.6‰, which is below the acceptable salinity range. This test solution and any with a higher effluent concentration would have to be adjusted with hypersaline brine, and brine controls would be necessary. If the dilution schedule above is used, and the highest effluent concentration is 10%, then these test solutions and the brine control would be made up as follows:

Measure SB and SE. Use these to calculate the quantity $\frac{34 - SE}{SB - 34}$ = _____

Effluent Concentration	VE		$\frac{34 - SE}{SB - 34}$		VB	Final Test Solution Mixture
5.6%	56 ml	x	_____	=	_____ ml Brine	+ 56 ml effluent in a one liter flask; fill with seawater
10.0%	100 ml	x	_____	=	_____ ml Brine	+ 100 ml effluent in a one liter flask; fill with seawater
Brine Control	100 ml	x	_____	=	_____ ml Brine	+ 100 ml distilled water " " " ; fill with seawater

Data Sheet for Abalone Larval Development Toxicity Test

Test Start Date: | Start Time: | Broodstock Source:
Test End Date: | End Time: | Analyzed By:

Toxicant: | Effluent Source: | Collection Date:

Ref. Tox. Chem. Sample No's:

Test Cont. #	Storage Vial #	Toxicant Concentration	Number of Abnormal Larvae	Number of Normal Larvae	Notes

Computer Data Storage | Notes
Disk:
File:

Physical/Chemical Measurements of Test Solutions

Date	Test Container Number	Nominal Concentration ($\mu g\ l^{-1}$ or %)	Temperature (°C)	Dissolved Oxygen ($mg\ l^{-1}$)	pH	Salinity (°/oo)	Ammonia ($mg\ l^{-1}$)

Notes

APPENDIX V

TOXICITY TEST
QUALITY ASSURANCE and QUALITY CONTROL

MARINE TOXICITY TEST
QUALITY ASSURANCE and QUALITY CONTROL

1.0 Introduction

This manual provides a detailed description of quality assurance and quality control (QA/QC) procedures for effluent toxicity testing with topsmelt embryos, red abalone larvae, giant kelp zoospores, and mysid juveniles. These procedures address all aspects of toxicity testing that can potentially affect data quality and interpretation, including sampling and handling of effluents and dilution waters, collection and conditioning of test organisms, test conditions and equipment, calibration of instruments, replication, reference toxicants, record keeping, and statistical evaluation of the data.

Quality assurance programs are dependent on timely and accurate record keeping. Records of QA checks and procedures provide proof of performance and a reference to guide future work.

This section describes the types of records to be kept and assigns responsibility for record keeping. It is based on a compilation of quality assurance guidelines for toxicity testing from the American Public Health Association, American Society for Testing and Materials, U.S. Army Corps of Engineers, U.S. Environmental Protection Agency, and private consultants. Selected source documents are listed in Section 10.0.

Each laboratory should prepare its own internal quality assurance/quality control document to ensure that acceptable practices are followed and complete records are kept.

2.0 Sampling and Handling of Effluents and Dilution Water

2.1 Sampling Locations

The locations for collecting effluents and dilution waters should be the same as those specified in the NPDES discharge permit. The exact sampling location will be determined on an individual basis for each discharge and for the type of test being conducted. The sampling locations must be fully described in the toxicity test reports.

2.1.1 Effluents

Record the date, time, and duration (e.g. grab, or 24-hour composite, etc.) of effluent sample collection. Record the sample volume. The effluent sampling point and the type of sample collected should be the same as that specified in the NPDES permit.

Collect samples within 36 hours of initiating the bioassay test. Do not filter effluent. Agitate samples to evenly suspend particles before subsampling or preparing test solutions.

Effluent samples should be shipped on ice and stored in the laboratory at 4°C. Precautions should be taken to ensure that methods for collection and storage of effluent samples (including materials used) do not contribute to effluent toxicity.

Effluent samples may be shipped in glass or plastic (polyethylene or polypropylene) bottles, or in disposable Cubitainers®. New containers should be leached to ensure that container materials do not contribute to effluent toxicity. After use, glass or plastic bottles should be cleaned as follows: 1) rinse with a reagent grade organic solvent (e.g. petroleum ether or acetone), 2) triple rinse with deionized water (18 M ohm), 3) soak for 24 hours in 2N hydrochloric acid, 4) triple rinse with deionized water, 5) soak for 24 hours with deionized water, and 6) triple rinse with deionized water. Disposable containers must be punctured after use to prevent reuse.

2.1.2 Dilution Water

Collect dilution water as specified in the NPDES discharge permit. The type of dilution water used should be determined on an individual basis for each discharge.

Dilution and control water for effluent tests shall be obtained from an unaffected area of the receiving waters. If this receiving water produces an unacceptable control response (see Section 11.0 of the test protocol), use seawater from a reference site as test dilution water (see below).

Dilution and control water for reference toxicant tests shall be obtained from reference sites that are remote from pollution sources and acceptable to the Regional Water Quality Control Board. The minimum requirement for reference dilution water is that the test organisms survive, develop, and reproduce normally in it.

Collect receiving water within 96 hours of initiating the bioassay test. Store receiving water at 4°C, unless it will be used for testing within 24 hours, in which case it can be stored at 15°C to avoid the inconvenience of cooling and reheating. Gently agitate receiving water to evenly suspend particulates before subsampling or preparing test solutions. Do not filter receiving water, except in the case of kelp tests where particulate concentrations interfere with accurate analysis of the kelp endpoint. In this case, the receiving water may be filtered through a 30 µm screen, or through smaller mesh bag filters if necessary.

Reference seawater should be filtered through a 1 µm filter prior to storage and through a 0.2 µm filter prior to testing. Store reference seawater at 4°C. Filtered reference seawater may be held for periods of one week or longer, as long as acceptable control responses are obtained.

Dilution water salinity must be 34 ± 2 ‰. If dilution water salinity is below 32 ‰, increase salinity by addition of hypersaline brine (see protocol Section 4.2). Do not use dilution waters with salinity greater than 36 ‰. If dilution water dissolved oxygen concentration is less than 60% of saturation at test temperature, gently aerate the dilution water to raise the DO to between 60% and 100%. Precautions should be taken to ensure that methods for collection and

storage of dilution water (including materials used) do not increase dilution water toxicity.

2.3 Chain-of-Custody Procedures

2.3.1 Introduction

The purpose of these procedures is to maintain an accurate written record that can be used to trace the possession of the sample from the moment of its collection through its final analysis. In addition, these procedures insure that the samples are handled only by authorized and properly trained personnel.

2.3.2 Guidelines for Sample Collection

Collect each sample according to established guidelines for the type of sample and the sampling location. Each sample must have a sample tag or label securely attached to the sample container at the time the sample is collected. This tag must contain the following items: 1) serial number of the tag, 2) station name and location, 3) date and time the sample was collected, 4) type of sample, 5) sequence number for multiple samples at the same location, and 6) name of the sample collector. Write labels legibly with waterproof ink. Log all field measurements, records and notes (including temperature, salinity, etc. as required by the Regional Board) in bound field notebooks. Record sufficient detail in the field notebook to completely reconstruct the sampling procedure.

The sample collector is responsible for the care and custody of the samples until they are transferred to the appropriate laboratory or given to an assigned custodian.

2.3.3 Transfer of Custody

Samples must be accompanied by a chain of custody record (see attached) that includes the name of the study, location of collection (or station number and location), date and time of collection, type of sample, sequence number, number of containers, analysis required, and the collectors' signatures. When turning over possession of samples, the transferor and the transferee sign, date, and record the time on the record sheet. The record sheet allows the transfer of a group of samples at a time. If the samples arrive at the laboratory when the designated personnel are not there to receive them, the samples must be put into a secure location and the transfer conducted when the appropriate personnel are present.

2.3.4 Laboratory Custody Procedures

Samples should be handled by the minimum possible number of people. Designate a sample custodian at each laboratory and an alternative custodian to act in their absence. Store samples in a secure area at the appropriate temperature. Discard samples only under the direction

of the Laboratory Director when it is certain that all tests have been properly performed and recorded.

3.0 Test Organisms

3.1 Giant Kelp

Collect kelp sporophyll blades from areas unaffected by local sources of pollution. The sporophyll collection site must be acceptable to the Regional Water Quality Control Board.

Sporophylls must be collected no longer than 24 hours prior to beginning the toxicity test. Keep the sporophylls moist during transportation and storage by packing them with moist (seawater) paper towels in an ice chest. Do not immerse them in seawater until spore release is desired. Maintain sporophylls between 10° and 15° C during transport and storage at the laboratory.

Refer to the *Macrocystis* protocol for details on spore release, handling and toxicity testing.

3.2 Red Abalone

Spawnable red abalone can be obtained through commercial facilities that distribute live abalone grown at commercial mariculture operations. Contact the Marine Bioassay Project for a list of potential broodstock suppliers. Abalone may also be collected from rocky intertidal and subtidal areas unaffected by local sources of pollution. The collection site must be acceptable to the Regional Water Quality Control Board. Consult the California Department of Fish and Game for regulations on abalone collection.

For details on handling, transport and storage of abalone, refer to the abalone protocol.

3.3 Topsmelt

Collect topsmelt using a small mesh (one centimeter stretch measure) seine. Identify specimens using an appropriate taxonomic key (eg. Miller and Lea, 1973). Transfer fish immediately to a large (>100 liter) container filled with seawater from the collection site. Use air diffusers to bubble compressed air or oxygen to maintain high levels of dissolved oxygen in the seawater during transport to the laboratory. Maintain temperature within 3° C of the temperature at the collection site. At the laboratory, hold fish in a large (3000 – 4000 liter) quarantine tank, treat with a general antibiotic for two days, and monitor their survival closely for one week. Remove dead specimens immediately. Refer to the topsmelt protocol for details of treating, handling, and conditioning broodstock.

Topsmelt are found in bays and estuaries throughout California. Broodstock for toxicity testing must be collected from a site acceptable to the Regional Water Quality Control Board. Topsmelt used in compliance monitoring should be collected from the same site as those used during the initial screening period.

3.4 Mysids

Holmesimysis costata are collected from among the canopy fronds of the giant kelp, *Macrocystis*, using a small mesh (~1 mm) dip net. Mysid collection sites should be remote from sources of pollution and must be acceptable to the Regional Water Quality Control Board. Large numbers of adult mysids (~250) can be held for 2 hours in 20 liter buckets filled with water from the collection site. Buckets must be aerated or oxygenated, and temperature must remain within 3° C of that at the collection site during transport. For longer transport times (up to 24 hours), mysids (10 per liter) may be transported in sealed plastic bags filled with oxygen saturated 1-µm-filtered seawater at temperatures within one degree of the collection (or culture) water. Refer to the mysid protocol for further details on transport and culture.

Mysids must be identified to species. Use Holmquist (1979, 1981) as a guide for identification. There have been recent changes in the taxonomy of this crustacean. Most previous authors have used the name *Acanthomysis sculpta*. However, Holmquist (1979) established the genus *Holmesimysis* to include all known species of the genus *Acanthomysis* from the Pacific coast of North America. Kathman et al. (1986) stated that the genus *Acanthomysis* does not occur in the Pacific Ocean. We consider Holmquist's designation as *Holmesimysis costata* to be definitive.

4.0 Facilities, Equipment, and Test Conditions

4.1 General

Tests may be performed in either fixed or mobile laboratories, the same specifications for materials, equipment, and test conditions apply to both. Toxicity testing areas should be well ventilated.

Laboratory temperature control equipment must be adequate to maintain test water temperatures within the recommended ranges. Water baths, heat exchangers, or environmental chambers may be used.

4.2 Giant Kelp

Refer to the kelp protocol for details.

4.3 Red Abalone

Refer to the abalone protocol for details.

4.4 Topsmelt

Refer to the topsmelt protocol for details.

4.5 Mysid

Refer to the mysid protocol for details.

4.6 Culture Facilities

Controlled temperature seawater tanks or aquaria must be available for holding and acclimating test organisms or broodstock. These may be static if aquaria are large (> 50 liter for abalone and mysids, 3000 - 4000 liter for topsmelt) and water is changed at least every 96 hours. Ammonia concentrations should be monitored daily in static and recirculating tanks to assure adequate water quality. Un-ionized ammonia concentrations should not exceed 20 µg/liter and total ammonia should not exceed 1.0 mg/liter. Dissolved oxygen concentrations should be maintained between 60 and 100% saturation. Tanks with flowing or recirculating natural seawater are preferable. Recirculating systems should use particle filters and either activated carbon or biological filters to remove metabolites. Nitrogenous waste levels should be checked twice weekly in recirculating tanks to ensure that filters are effective. Holding tanks must be aerated. Artificial or reconstituted seawater is not recommended for the test species discussed in this manual.

Culture facilities may be necessary to produce sufficient test organisms for a large testing program. A central culture facility can supply numerous testing laboratories. Culture facilities should be supplied with flowing natural seawater drawn from unpolluted areas. Seawater system design should be carefully considered (see Huguenin and Colt, 1989, for further information). Well designed recirculating systems may be adequate for culturing large numbers of test organisms if water quality is carefully monitored. The minimum requirement for culture water quality is that test organisms, survive, develop, and reproduce normally in it.

Aeration should be provided to all culture tanks. Protect airlines from contamination by using either low-pressure air blowers or air compressors with water seals. Place appropriate filters on airlines to remove moisture, oil, or toxic vapors. Air intakes should not be located in shops or furnace rooms, or near outlets from fume hoods, chemical laboratories, or vehicle exhausts.

Protect organisms from outside disturbances such as noise, vibration, or sudden changes in lighting. Culture facilities should be designed for effective control of temperature and photoperiod, and should be physically separated from laboratories in which toxic substances are used.

4.7 Materials

Materials for culture and toxicity testing must be carefully chosen. Non-contaminating materials must be used wherever surfaces come into contact with organisms, samples, dilution water, or culture water. Fiberglass reinforced polyester and epoxy resins, borosilicate glass, and perfluorocarbon (Teflon®) plastics are suitable for culture materials. Polypropylene and polyethylene plastics are also acceptable. These materials should be soaked in seawater for one week prior to use. Concrete leached with flowing seawater for one month is suitable for use in culture tanks. Polyvinyl chloride (PVC) pipe and fittings are acceptable for air and seawater systems, though these should be leached with slowly flowing air or seawater for one month prior to use. Leach all new cartridge filters for at least one-half hour at low flow rates (100 to 200 ml/minute). Copper, lead, brass, stainless-steel, galvanized metal, or rubber should not be used, except that stainless-steel, titanium, or non-toxic plastic impellors should be used on all pumps that contact culture or dilution water. All questionable materials should be assayed for toxicity to the test organism before purchasing significant quantities.

Test containers should be borosilicate glass beakers for effluent tests, or plastic (polyethylene or polypropylene) beakers or food containers for tests using trace metals. Polystyrene tissue culture containers may be used with effluents or reference toxicants. Test containers should be cleaned as indicated in the protocols for each species (Attachments A – D). Deionized water used in cleaning should be continuously monitored using an in-line conductivity meter. Resin columns should be changed when conductivity exceeds 18 mega-ohms. Silicone adhesive absorbs many organic compounds and should be used carefully and sparingly in constructing any test containers or toxicant delivery systems.

5.0 Instrument Calibration and Standardization

5.1 Temperature

Measure temperature to the nearest degree Centigrade using digital or mercury thermometers. Calibrate laboratory thermometers semi-annually against a Standard Thermometer that has been certified factory calibrated against the National Institute of Standards and Technology (NIST) thermometer.

5.2 Salinity

Measure salinity to the nearest part per thousand (‰) using a hand-held refractometer. Calibrate the refractometer before reading and after each 20 measurements using a seawater standard measured by salinometer at a qualified laboratory. Keep standards refrigerated in clean, sealed glass

bottles. Minimize handling of standards by using subsamples for multiple calibrations. Unless a temperature compensated refractometer is used, make sure standard seawater is at the same temperature as the sample.

5.3 pH

Measure pH to the nearest 0.1 pH unit using an appropriate meter and probe. Maintain the meter and probe according to factory specifications. Calibrate the probe before each use using buffer solutions that bracket the pH range of the samples (pH 7.0 and 10.0 buffers for seawater samples).

5.4 Dissolved Oxygen

Measure dissolved oxygen to the nearest 0.1 ppm with an appropriate meter and probe. Maintain the meter and probe according to factory specifications. Calibrate before each set of measurements using water saturated air or oxygen saturated seawater as specified in the manufacturers instructions for the probe. Zero the probe using a 0 ppm oxygen solution (e.g. 3.81 g analytical grade sodium borate in a liter of distilled water saturated with crystalline sodium sulphite).

5.5 Irradiance

Measure irradiance (for the *Macrocystis* protocol) using an appropriate meter and a cosine corrected quantum irradiance sensor that measures photosynthetically active radiation (PAR, photons) in units of microeinsteins m^{-2} sec^{-1}. Have the meter factory calibrated at intervals recommended by the manufacturer. Meters that read in lux units are acceptable for tests with abalone, topsmelt and mysids.

5.6 Weights and Volumes

Weigh reference toxicants using an analytical balance accurate to the nearest 0.1 mg. Calibrate the balance monthly using weights traceable to NIST standards. Inspect weights at each calibration and discard if corroded. Check weights against NIST certified weights annually. Make effluent and reference toxicant dilutions using volumetric flasks and pipets. Calibrate flasks and pipets annually by weighing volumes of distilled water on an analytical balance.

6.0 Test Acceptability

The consistency and precision of laboratory results for a given species must be demonstrated by conducting at least five reference toxicant tests that meet acceptability criteria (see

below and Section 11.0 of the protocol) before effluent test results can be accepted. Record and report any deviation from test specifications.

6.1 Reference Toxicants

Reference toxicant tests indicate the sensitivity of the organisms being used and the suitability of the test methodology. Reference toxicant tests must be conducted simultaneously with each effluent test. A single reference toxicant test is acceptable for comparison with multiple effluent tests if: 1) all tests are conducted concurrently, 2) test conditions are the same for all tests, and 3) all organisms are from a single group spawned or released at the same time.

Each reference toxicant stock solution must be sampled for chemical verification at the beginning of each exposure period and at each water change. For trace metal toxicants, preserve samples for up to two months by addition of 1% by volume 14N double quartz distilled nitric acid, and store in clean acid washed containers in a dark refrigerator. These samples must be chemically measured to verify the reference toxicant concentration by a laboratory acceptable to the Regional Water Quality Control Board.

6.2 Acceptability of Test Organisms

Refer to Section 11.0 of the individual protocols to determine the acceptability of control and reference toxicant test responses by test organisms.

6.3 Acceptability of Physical/Chemical Properties of Test Solutions

For test results to be considered acceptable:

all salinity measurements must be between 32‰ and 36‰, and

all temperature measurements must be within 1°C of the test temperature designated in the protocol;

6.4 Brine Controls

Brine controls must be included in all tests that use hypersaline brine to adjust the salinity of effluent dilutions (see Protocol Section 4.2). Make brine controls as described in the protocol (see Protocol Section 4.3) using the same volume of brine as is used in the highest effluent concentration. Brines produced by freezing natural seawater are preferable to brines made by evaporation or by addition of commercial sea salt formulations.

Compare control and brine control results using a t-test or ANOVA. The effluent toxicity test is acceptable only if there is no significant difference at the $p = 0.05$ level. It may be advisable to test the organism's response to specific brines before attempting their use in a full effluent toxicity test.

7.0 Record Keeping

7.1 Effluents and Dilution Waters

Maintain a field notebook that records the dates, locations, and procedures used for collecting effluent samples and dilution water. The notebook should be hardbound and all entries must be made in waterproof ink.

Maintain a laboratory file of all chain-of-custody forms.

7.2 Test Organisms

Record in a hardbound notebook information on the collection and maintenance of test organisms. Record the location and time of collection. In the case of abalone obtained from mariculture or distribution facilities, record the source, length, sex, and a qualitative description of gonadal condition. If known, record the age and parentage.

Record the method and duration of transportation to the laboratory, including the size of the container, medium (seawater, air, or oxygen gas), temperature, and method of aeration.

Record the water quality conditions of holding aquaria. Include temperature, aeration, ammonia concentration, and either seawater change schedule or turnover time. Note food supplied, if any.

Make all entries in waterproof ink.

7.3 Dilutions and Standards

Record the procedures used in making reference toxicant standards, reference toxicant dilutions, and effluent dilutions in duplicate, with one copy in a laboratory notebook, and a second copy to be kept in laboratory files. Include all weights and volumes measured.

7.4 Test Conditions

At the beginning and end of every test, and before each water change, record the temperature, salinity, pH, and dissolved oxygen concentration from one random replicate of each test concentration in a hardbound laboratory notebook and on the data sheet used to record test results. Photocopy data sheets at the end of the test and store copies separately.

7.5 Test Results

Record the results of each toxicity test on preprinted data forms designed specifically for each type of test. Make no erasures on the original data sheets. Mistakes may be crossed out (one line only), and must be initialed, with a note indicating why the change was made. Photocopy all

original data sheets. Store the originals in one laboratory file and the copies in a separate file. Use only the photocopies for statistical analysis or other work.

A standard file format for computer data storage and transmission to a central data base has not yet been designated. Please contact the Marine Bioassay Project for further information on computerized data storage and transmission.

8.0 Report Preparation

Follow the format designated by the Regional Water Quality Control Board when reporting the results of toxicity tests to satisfy permit conditions. The following is an outline of relevant information.

8.1 Introduction

1. Permit number
2. Toxicity testing requirements of permit
3. Station location
4. Name of receiving water body
5. Contract laboratory (if the test was performed under contract)

8.2 Plant Operations

1. Products/Function
2. Raw materials
3. Operating schedule
4. Description of waste treatment
5. Schematic of waste treatment
6. Retention time (if applicable)
7. Rate of waste flow (volume /time)

8.3 Source of effluent and dilution water

1. Effluent samples
    a. Sampling locations
    b. Collection dates and times
    c. Sample collection method
    d. Physical/chemical data, including known contaminant concentrations
2. Surface water samples
    a. Sampling locations

b. Collection dates and times

c. Sample collection method

d. Physical/chemical data, including known contaminant concentrations

e. Tide stages

3. Dilution water samples

a. Sampling location

b. Collection dates and times

c. Pretreatment

d. Physical/chemical data, including known contaminant concentrations

8.4 Test Methods

1. Toxicity test method and species used (including reference citation)
2. Endpoint(s) of test
3. Deviation(s) from reference method, if any, and reason(s)
4. Date and time test started
5. Date and time test terminated
6. Type of test containers
7. Volume of test solution used per test container
8. Number of organisms used per test container
9. Number of replicate test containers per treatment
10. Acclimation of test organisms (time held, temperature and salinity; give means and ranges)
11. Test solution temperature, salinity, pH, and D.O. (means and ranges)
12. Specify if aeration was needed to maintain D.O. above 60% saturation

8.5 Test Organism

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

8.6 Quality Assurance

1. Complete formulation and source of reference toxicant
2. Dilution water used in reference toxicant test
3. Results (NOEC, ANOVA Error Mean Square, control effect [%], $EC_{50} \pm 95\%$ CI where applicable)
4. Calibration values for physical and chemical methods used, including temperature, salinity, D.O., pH, and blanks and standards used in chemical measurement of reference toxicant solutions

8.7 Test Results

1. Raw test data in tabular form
2. Graphical plots of test data, including means and standard deviations
3. NOEC, and where applicable, $EC_{50} \pm 95\%$ CI.
4. Summary table of physical and chemical data

9.0. Health and Safety Precautions

9.1 Toxic Materials

Most toxic agents can adversely affect laboratory personnel if appropriate precautions are not taken. Contact with all toxic agents and test solutions should be minimized. Fume hoods are necessary for testing volatile substances. Information on toxicity to humans and recommended handling procedures (Walters and Jameson, 1984; ITII, 1977) should be studied before working with any toxic substance.

Personnel collecting or testing effluents should take all necessary precautions to prevent ingestion or invasion (as through broken skin) of infectious agents. Prohibit eating, drinking, or smoking in laboratories where toxic or infectious materials are used. Personnel handling samples suspected to contain human waste should be immunized against tetanus, typhoid fever, and polio.

Provide sufficient and organized storage space for toxic materials. Review all applicable Material Safety Data Sheets, and do not store incompatible materials (e.g. acids and bases) together. Store flammable solvents in cabinets approved by the National Fire Protection Association. All containers should be adequately labeled to indicate their contents and potential hazards.

9.2 Waste Disposal

All persons conducting toxicity tests must know, understand, and comply with the local, state, and federal regulations applicable to waste disposal at their testing facility. Dispose of reference toxicants, effluents, and other laboratory chemicals according to established guidelines

(ASTM, 1987). Health and safety precautions should be checked prior to disposal (Walters and Jameson, 1984; ITII, 1977).

Because of possible toxicant or pathogen contamination, destroy all test organisms and dispose of them along with other contaminated materials.

9.3 Cleaning Solutions

Glassware and test apparatus should be rinsed with acetone, petroleum ether, or other volatile solvents only in well ventilated areas. Face shields, gloves, and other protective clothing should be worn when working with acids.

9.4 Safety Equipment

Prior to sample collection and laboratory work, determine that all necessary safety equipment and materials have been obtained and are in good condition. Personnel should use safety equipment, as required, such as rubber aprons, laboratory coats, respirators, gloves, safety glasses, and face shields. Each laboratory (including mobile laboratories) should be provided with safety equipment such as first aid kits, fire extinguishers, fire blankets, emergency showers, eye fountains, and chemical spill clean-up kits.

All electrical circuits in wet "laboratories" and mobile laboratories must be properly grounded. Ground-fault interrupters must be installed in all "wet" laboratories where electrical equipment is used.

Maintaining a clean and organized laboratory contributes to both safety and reliable results.

10.0 References

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THE MARINE BIOASSAY PROJECT

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