MARINE BIOASSAY PROJECT

FIFTH REPORT

PROTOCOL DEVELOPMENT AND INTERLABORATORY TESTING WITH COMPLEX EFFLUENTS

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Marine Bioassay Project Fifth Report

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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 currently in use are relatively insensitive because the adverse effect measured is lethality to adult organisms. In contrast, a newer generation of tests is being 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.

This report, the fifth in a series, describes the work performed during Phase Four, which occurred from January 1, 1989 to December 31, 1989. During this phase, the giant kelp, red abalone, and topsmelt toxicity tests developed in previous phases were refined by testing complex effluents. In addition to performing toxicity testing of complex effluents, a draft 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. The manual was available from the State Board in late 1990.

The fifth report is organized into four sections. Section I 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 nearshore marine ecosystem. Section II 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. Section III focuses on topsmelt, *Atherinops affinis*, one of the most abundant fish species in central and southern California estuaries. Section IV reviews the evaluations of two one-day workshops conducted by the MBP staff. These workshops were conducted to familiarize discharger biologists with the red abalone and giant kelp toxicity tests. 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); the fifth appendix provides general quality assurance procedures to be followed by testing laboratories. Two of these protocols, the giant kelp and red abalone toxicity tests, have undergone sufficient development and testing to be used now. The crustacean (mysid shrimp) protocol is considered a test of acute toxicity, with a sublethal endpoint still under development. The fish protocol should be considered tentative until further testing has been completed.

Complex effluents tested in Phase Four included discharges from a large municipal wastewater treatment plant and from a pulp mill. Each complex effluent was tested concurrently by the MBP laboratory and a second laboratory. For municipal wastewater testing, the participant was the on-site laboratory of the water treatment plant. For pulp mill effluent, the participating laboratory was the University of California's Bodega Marine Laboratory. A summary of the test results from this phase of the Marine Bioassay Project is presented in the discussion below.

The giant kelp toxicity testing experiments focused on refining the 48-hour germination and growth protocol. Interlaboratory tests were conducted on the both the sewage and pulp mill effluents, with a copper reference toxicant tested concurrently. All 14 kelp toxicity tests conducted met test acceptability requirements provided in the protocol. The kelp toxicity protocol was relatively insensitive to toxicity in the sewage plant effluent. At concentrations of effluent as high as 10 percent, only the MBP laboratory measured toxicity at the highest concentration for growth; neither laboratory detected toxicity with the germination endpoint. In contrast, in tests conducted with pulp mill effluent, the germination endpoint was more sensitive than growth. The demonstration that either of the germination or growth endpoints may be more sensitive to a given effluent suggests the value of retaining both endpoints in the kelp protocol.

The abalone toxicity test protocol was more sensitive than the kelp protocol to toxicity in both the pulp mill and sewage treatment plant effluents. Ten of the twelve abalone tests conducted met all test acceptability criteria outlined in the protocol.

Topsmelt research focused on developing methods for spawning adult topsmelt and measuring the response of several life-stages to a reference toxicant. A combination of environmental cues (lighting, temperature, and tidal) was used to induce topsmelt spawning in a laboratory culture system. Fertilization tests, embryo tests, and larval tests were conducted on topsmelt with copper. Of the early life stages (sperm, embryos, and larvae) topsmelt sperm were equally or more sensitive to copper chloride than embryos and both were more sensitive than larvae. Future

experiments will focus on the continued assessment and development of a 48-hour fertilization test, a 12-day embryo development test, and a seven day larval growth and survival test.

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.

PROJECT OVERVIEW & TECHNICAL SUMMARY

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 using a fish species. An acute effect of exposure in fish generally refers to mortality. For example, when an acute test is conducted on adult fish with an endpoint of mortality and a duration of 96 hours, acute describes both duration of exposure and toxic effect. However, the distinction between acute and chronic is less clear when referring to tests that utilize species with shorter reproductives cycles such as invertebrates and algae.

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 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 five years of intensive effort by two principal investigators and two laboratory technicians.

REGULATORY BACKGROUND

Development of toxicity test protocols to estimate the 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 will be required in their permits to use these toxicity test protocols for monitoring complex effluents by 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, the giant kelp and the red abalone. 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:

$$TU = 100/NOEL$$

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 designed 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, four phases of the Marine Bioassay Project have been completed; chapters of this report describe work performed during the fourth phase. The fifth phase is being carried out during calendar year 1990. A sixth and final phase has been planned for the period from January 1991 to June 1992. The fifth and sixth phases focus on two major areas: (1) completing protocols for fish and crustacean species and (2) ensuring that the toxicity testing program for marine discharges is properly implemented. The latter area includes training of technical staff, providing technical support for participating laboratories, and developing sound quality assurance/quality control testing procedures.

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 are static tests; that is, the test solutions are not changed during testing. Each protocol measures a different effect or endpoint. These protocols, designed to estimate chronic toxicity of discharge to ocean waters, utilize 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):

Phase Three was conducted from November 1987 to 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, the topsmelt (Atherinops affinis). The project's fourth report provides detailed descriptions of work completed in Phase Three.

Phase Four (January 1989 - December 1989):

This phase is discussed in the executive Summary.

Phase Five (January 1990 to December 1990)

Three major tasks have been proposed for Phase Five of the project:

1. Conduct complex effluent tests and develop protocols using a native fish, the topsmelt.

2. Develop a sub-lethal endpoint for the mysid shrimp protocol. Perform complex effluent testing and interlaboratory verification.

3. Provide technical support for laboratories using MBP protocols. Types of technical support include training of laboratory technicians, providing brookstock organisms, detailing specific requirements for test acceptability of individual protocols and performing interlaboratory tests with discharges exceeding 100 million gallons per day of complex effluent.

Phase Six (January 1991 to June 1992)

If funding is available, 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

Giant Kelp Experiments

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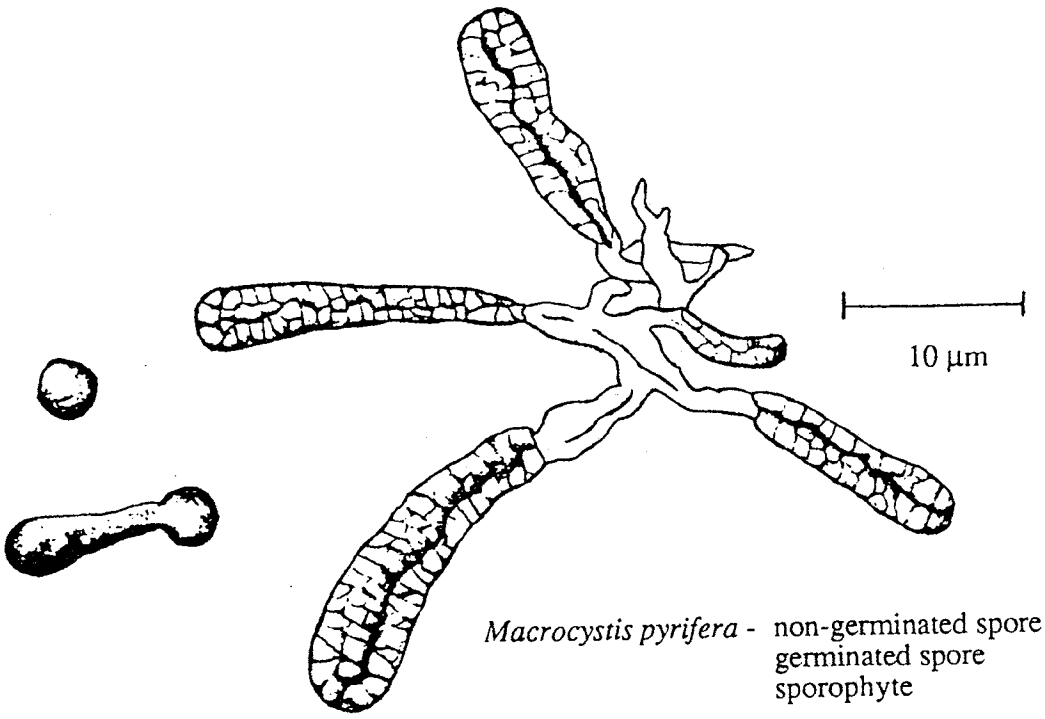


Macrocystis pyrifera - non-germinated spore
germinated spore
sporophyte

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 the continued testing of complex effluents and repeated replicate reference toxicant testing (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.*, in review). An interlaboratory comparison produced consistent results between laboratories using copper chloride as a reference toxicant.

Kelp research during Phase 4 of the Marine Bioassay Project has focused on the continued refinement of the *Macrocystis* 48-hour protocol. Interlaboratory experiments were conducted to ascertain whether 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.

Methods

Detailed methods for the kelp experiments discussed in this section are given in the *Macrocystis* protocol (Appendix I). All experiments were conducted between January, 1989 and December, 1989 at the Marine Pollution Studies Laboratory (MPSL).

Interlaboratory Testing

Two interlaboratory tests of the 48-hour protocol were conducted using split effluent samples and reference toxicants. Methods for both tests followed the procedures for interlaboratory tests given in Anderson *et al.* (1988). The first interlaboratory test was conducted in September, 1989 between MPSL and the City of Los Angeles Hyperion waste treatment facility. The second interlaboratory test was conducted in November, 1989 between MPSL and the Bodega Marine Laboratory (BML).

For all of the interlaboratory comparisons, *Macrocystis* sporophylls were collected from adult plants at Monastery Beach, Monterey County, the day before the experiments began. Half of the sporophylls were retained in coolers overnight for experiments at MPSL (temperature = 9 °C). The other half were shipped in coolers via overnight courier to the other participating laboratory.

For the MPSL - Hyperion interlaboratory experiment, a 24-hour composite effluent sample was collected the day before the experiment. Half of the sample was held in coolers at Hyperion and the other half was shipped in coolers to MPSL via overnight courier. Dilution water for the Hyperion experiments was supplied by the Southern California Edison Marine Laboratory at Redondo Beach. Dilution water for the MPSL experiments was taken from the MPSL seawater system. Both dilution waters were filtered to 0.2 µm. Brine used to adjust effluent salinity at both laboratories was from a split sample prepared at MPSL. This brine was a mixture of WIMEX® artificial sea salt and MPSL 0.2 µm-filtered seawater. The final brine had a salinity of 70 $^{o}/_{oo}$ and a pH of 8.0. The concentrations of effluent tested at both laboratories were 0 (dilution water), 0 (brine control), 0.56, 1.0, 1.8, 3.2, 5.6 and 10% effluent. A separate copper chloride reference toxicant experiment was conducted at both laboratories concurrently with the effluent tests, with copper chloride supplied by MPSL. The nominal copper concentrations tested were: 0, 10, 18, 32, 56, 100, and 180 µg/liter.

For the MPSL - BML interlaboratory experiment, a sample of lyophilized bleached kraft mill effluent (L-BKME) was prepared by BML using methods developed by Shenker *et al.* (1989). Lyophilized BKME is a powdered distillate which retains the toxicity of the original effluent, and is a convenient

complex effluent for interlaboratory testing. The L-BKME was split into two samples; half of the sample was shipped to MPSL, and the other half was retained for testing at BML. The L-BKME was dissolved directly in 0.2-µm-filtered seawater prior to testing and required no salinity adjustment. Both BML and MPSL used their own dilution waters (0.2-µm-filtered) for their respective experiments. The concentrations of effluent tested at both laboratories were 0, 0.5, 1, 2, 3, 4, and 5 % effluent. A copper chloride reference toxicant test was conducted at both laboratories concurrently with the effluent tests, with copper supplied by MPSL. The copper concentrations tested were 0, 10, 18, 32, 56, 100, and 180 µg/liter. Because of time constraints, the gametophytes in BML's copper reference toxicant test were fixed at 48 hours with 0.5 % glutaraldehyde. The preserved gametophytes were read the following day. All other tests were terminated and read at 48 hours. No Observed Effect Concentrations and EC_{50}'s from interlaboratory tests were compared.

Germination Study

There has been some question if non-germinated spores are lost from microscope slides when slides are removed from the test solution for analysis. This could result in an underestimation of the percentage of non-germination, especially at higher concentrations where greater numbers of non-germinated spores occur. To investigate this possibility, we conducted an experiment comparing the percentage of non-germinated spores on slides before and after the test solutions were decanted.

The test containers used in this experiment were Labtek® tissue culture chamber/slides. These are standard glass microscope slides fitted with a removable polystyrene chamber. Each chamber has a removable lid and holds 5 mls of test solution. A standard 48-hour copper experiment was conducted using these test chambers. At the end of the experiment, percent non-germination was quantified using an inverted microscope before pouring off the test solution. The test solution was then poured off, the polystyrene chamber was peeled from the slide, and the glass slide was fitted with a cover-slip. Percent non-germination was then counted using a standard brightfield microscope. Germination was compared on each slide before and after the test solution was poured off, and results were analyzed using a paired t-test.

Container Comparison

In the past, 200-ml polyethylene food containers or 600-ml glass beakers have been used as kelp test chambers. Larger volume containers are less convenient for static laboratory bioassays because they require more space and larger volumes of test solution. Smaller volume containers are more convenient but have larger surface-to-volume ratios, which increase the possibility of significant adsorption of toxicants onto container walls, thereby reducing toxicant bioavailability. To evaluate smaller test containers, two 48-hour copper experiments were conducted concurrently using the standard 200-ml polyethylene food containers and 5-ml Lab-Tek® chamber/slide test containers. Both experiments used spores from the same spore release.

Effects of Lighting on Copper Toxicity

The 48-hour kelp protocol was originally developed using constant lighting to promote accelerated development of the gametophytes in culture. However, constant lighting never occurs in nature and might result in experimental artifacts in laboratory toxicity tests. A photoperiod of 16 hours light followed by eight hours of darkness (16L:8D) more closely approximates natural conditions and is commonly used in toxicity tests with other organisms. We compared results of simultaneous tests, one using constant light and one using a 16L:8D photoperiod. Both experiments used spores from the same spore release.

Analysis of Copper Test Concentrations

In our previous studies, reference toxicant 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). Unfortunately, the analytical laboratory that normally conducts these analyses suffered considerable damage in the October 17, 1989 earthquake and was unable to measure the copper concentrations in time for the publication of this report. All copper concentrations are therefore reported as nominal concentrations.

Results and Discussion

Interlaboratory Tests

Interlaboratory experiments between MPSL and Hyperion produced similar results with the split Hyperion effluent sample. Although control germination was higher at Hyperion, neither laboratory found significant inhibition of kelp spore germination by the effluent (Figure 1a). Germ-tube growth was slightly greater at MPSL, where significant inhibition of germ-tube growth was found at 10 % effluent (Fig. 1b). There was no inhibition of germ-tube growth detected in the effluent test conducted at Hyperion.

The interlaboratory copper reference toxicant tests conducted concurrently with the effluent tests also produced similar results. Germination in the lower copper concentrations was similar, but diverged beyond 56 µg/liter copper (Figure 2 a). MPSL found greater inhibition of germination at the higher copper concentrations; the NOEC's for germination were 56 and 100 µg/liter at MPSL and Hyperion, respectively. The EC_{50} for percent germination in copper was 88.5 at MPSL; no EC_{50} could be calculated for the Hyperion data because no response was greater than 50 %. As in the effluent tests, control germ-tube growth was greater at MPSL; the NOEC's at both laboratories were less than 10 µg/liter (Figure 2 b).

Figure 1 a-b. Results of interlaboratory tests between MPSL and Hyperion using Hyperion effluent: (a) percent non-germination at both laboratories; (b) germ-tube growth at both laboratories.

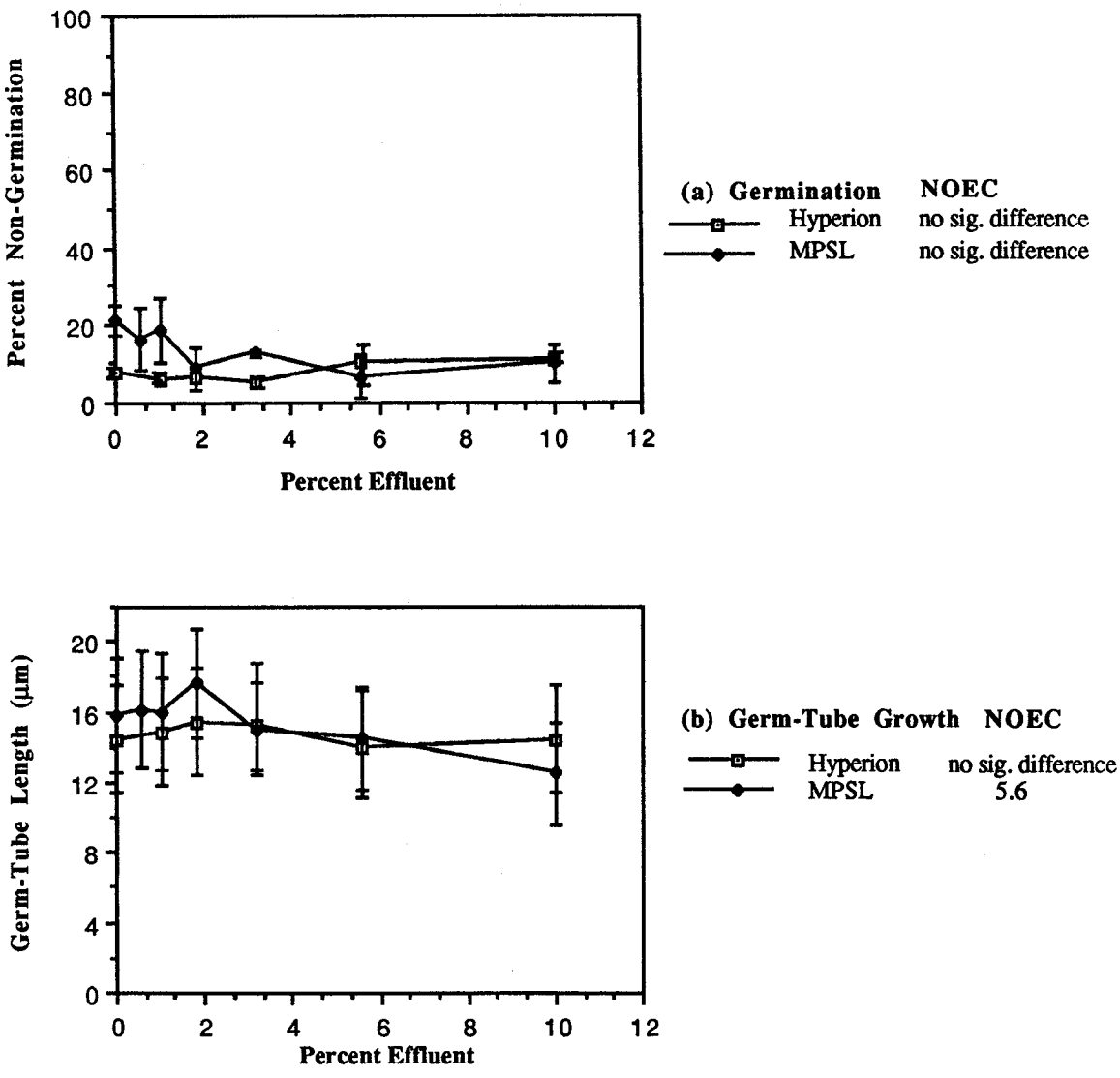
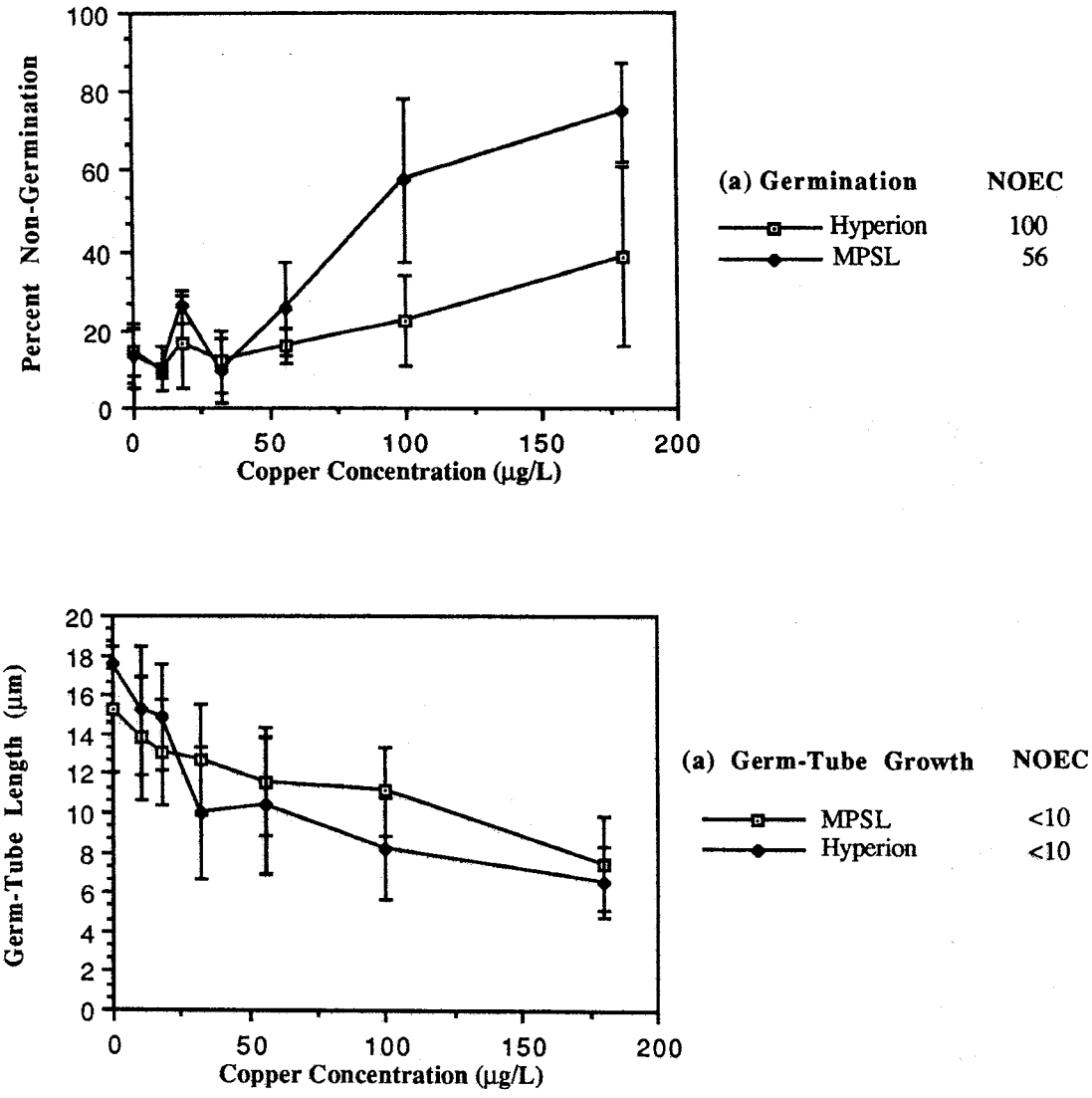


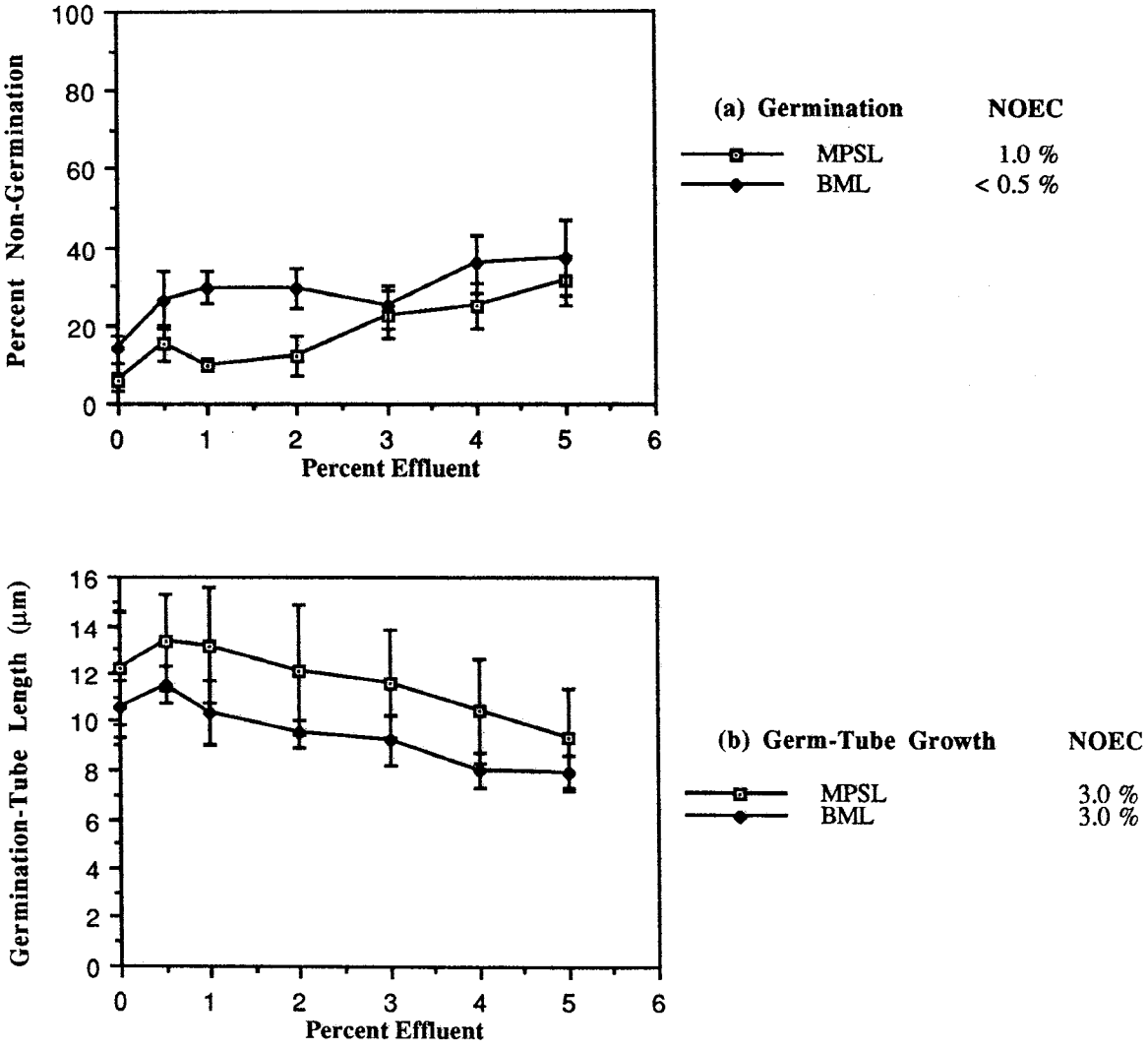
Figure 2 a-b: Results of interlaboratory tests between MPSL and Hyperion using copper chloride. (a) percent non-germination at both laboratories; (b) germ-tube growth at both laboratories.



Interlaboratory experiments between MPSL and Bodega Marine Laboratory (BML) produced similar results with a split sample of lyophilized bleached kraft mill effluent (L-BKME). Although the dose-reponse curves were similar at lower concentrations, the NOEC for germination was 1 % at MPSL and less than 0.5 % at BML. The different NOEC's reflect less germination at BML than MPSL (Figure 3 a). Germ-tube growth responded similarly to L-BKME at both laboratories (Figure 3 b), the NOEC for both laboratories was 3 %. Germination percentages and germ-tube growth were consistently greater at MPSL than BML. The reason for this difference is unknown, but may be due to differences in handling of the sporophylls or the use of different dilution waters at the two laboratories.

Germination was a more sensitive indicator of BKME toxicity than germ-tube growth. Although our previous research with metal reference toxicants have shown that germ-tube growth is generally a more sensitive toxicity indicator than germination (see following discussion), other researchers have found that germination is more sensitive to certain toxicants such as chlorine (personal communication, T. Dean, Coastal Resources Associates, Encinitas CA).

Figure 3 a-b. Results of interlaboratory tests between MPSL and Bodega Marine Laboratory using lyophilized BKME effluent: (a) percent non-germination at both laboratories; (b) germ-tube growth at both laboratories.



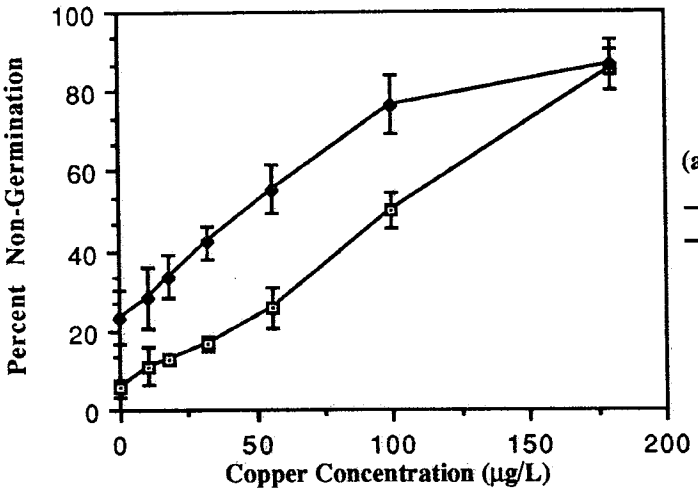
Results of the copper reference toxicant tests conducted concurrently with the L-BKME tests varied between laboratories. MPSL found significant germination inhibition at 10 µg/liter; the NOEC for germination was less than 10 µg/liter, and the EC_{50} was 100 µg/liter. BML found significant inhibition at 32 µg/liter (NOEC =18), and the EC_{50} for germination at BML was 45 µg/liter (Figure 4 a). The EC_{50} values were not consistent with the NOEC values; the NOEC at MPSL was lower than BML's, while the EC_{50} was higher. The difference in NOEC's is apparently due to higher control germination rates and lower between-replicate variability at MPSL, resulting in the detection of a statistical difference at a lower copper concentration in the MPSL experiment. The difference in EC_{50}'s probably reflects the fact that MPSL had greater germination overall, and that the BML response curve has a steeper slope (1.78 for BML vs 1.39 for MPSL), .

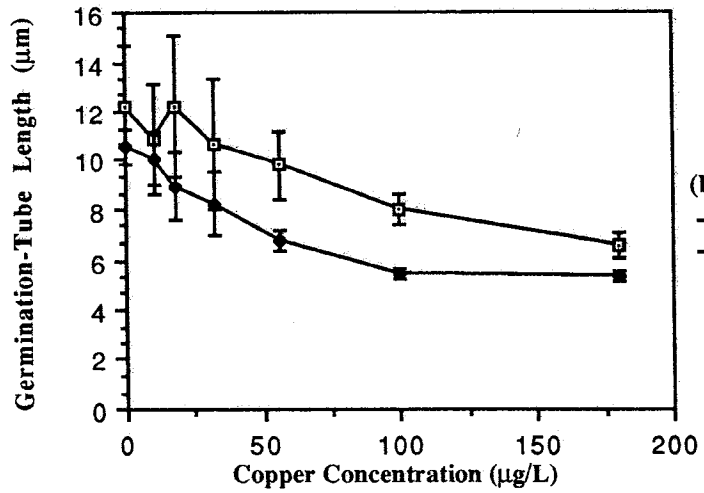
The germination endpoint was particularly sensitive to copper in these experiments. The MPSL germination NOEC was exceptionally low. Although germination was less sensitive than germ-tube growth at BML, the NOEC for germination in the BML test was at the low end of the range of our previous copper tests. The low copper germination NOEC's correspond with the low L-BKME germination NOEC's in the interlaboratory experiments. The greater apparent sensitivity of the germination endpoint may be due to temporal variability in the sensitivity of the kelp spores, or temporal variability in the dilution waters.

Germ-tube growth was apparently inhibited at a lower copper concentration at BML than MPSL. The copper NOEC's for germ-tube growth were 10 µg/liter at BML and 32 µg/liter at MPSL. Germ-tube growth in the MPSL copper test had greater between-replicate variability, especially between the 18 µg/liter replicates (Figure 4b). The germ-tube growth NOEC's fell within the range of previous NOEC's for this endpoint with copper.

The gametophytes in the BML copper test were preserved with 1% glutaraldehyde solution at the end of the test, then counted and measured the next day. Preservation of the gametophytes apparently did not affect the reading of the test or the results (personal communication, G. Cherr, BML). This is encouraging because if the gametophytes can be preserved, it will allow more flexibility in conducting and analyzing the kelp test. The effects of longer preservation times on the color and size of gametophytes will need to be investigated before this becomes accepted practice.

Figure 4 a-b. Results of interlaboratory tests between MPSL and Bodega Marine Laboratory using copper chloride. (a) percent non-germination at both laboratories; (b) germ-tube growth at both laboratories.





There are several sources of variability in interlaboratory toxicity experiments. These include variations in experimental procedure, 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 test protocols will be routinely used. Future interlaboratory tests will assess the the degree to which different sources of variability affect the results. The purpose of these interlaboratory 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 one test, the results of the copper reference toxicant experiments were within the range of previous test results.

In addition to variability between laboratories, there are other potential sources of variability inherent in laboratory toxicity testing. For example, because it is the free divalent metal ion that is the form of copper toxic to aquatic organisms, measuring total copper concentrations may not accurately reflect bioavailable concentrations. 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., in review).

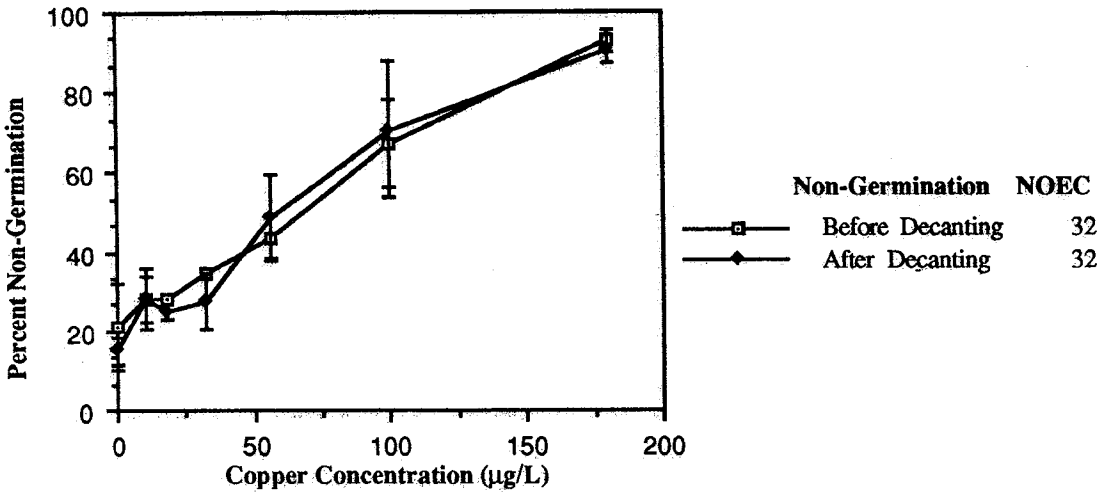
Between-test precision can be assessed by calculating a coefficient of variation (CV) for EC_{50} values from replicate tests (Weber *et al.*, 1988; CV = standard deviation ÷ mean of EC_{50}'s from replicate tests). The coefficient of variation for the germination endpoint from all of the copper experiments conducted at MPSL in 1989 is 21.84 % (n = 6). This does not include the interlab data from the Hyperion and BML laboratories. It is not possible to calculate a standard EC_{50} value for the growth endpoint in kelp 48-hour protocol because the data generated is continuous data. Calculating inter- and intralaboratory precision of growth data by comparing NOEC values is unsatisfactory because NOEC's do not adequately characterize data across the range of concentrations tested. We are now investigating ways to measure precision of the growth data from replicate tests. One possibility uses methods developed by the United States Environmental Protection Agency specifically for continuous data (eg., Inhibition Concentration Percentage or IC_P.).

Research on metal toxicity to kelp has shown that vegetative endpoints such as germination and growth are less sensitive indicators of toxicity than reproductive endpoints such as sporophyte production (Anderson et al., in review; Chung and Brinkhuis, 1986; Thompson and Burrows, 1984; and Hopkins and Kain , 1978). Although there is a certain amount of variability in response to copper using vegetative endpoints, particularly the germination endpoint, the NOEC ranges for both endpoints are within the range of copper concentrations that would significantly inhibit sporophyte production. Therefore, even the lowest NOEC's given by the 48-hour endpoints are conservative when compared to reproductive effects.

Germination Study

Decanting the test solutions from the microscope slides apparently does not remove a significant proportion of non-germinated spores. The percentage of non-germinated spores was the same on microscope slides before and after the test solutions were decanted (students t-test, $p = 0.39$; Figure 5). Assuming that decanting the test solution has the same effect as removing the slide from a test chamber, neither procedure appears to bias test results.

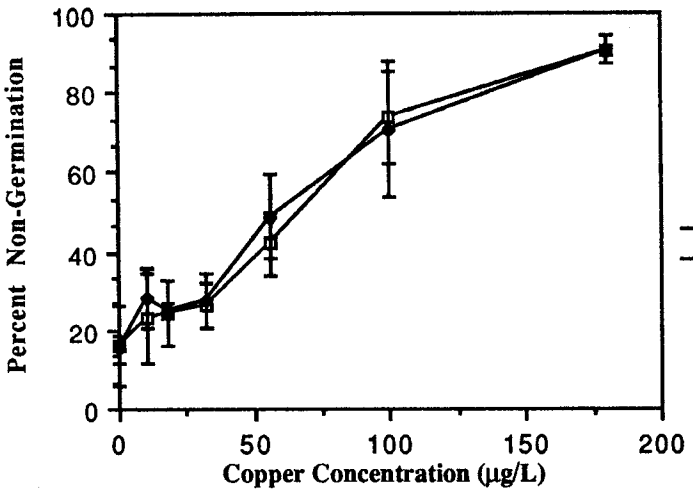
Figure 5. Percent non-germination on microscope slides before test solutions were decanted (Non-Germ Before), and on the same slide after the test solutions were decanted (Non-Germ After).



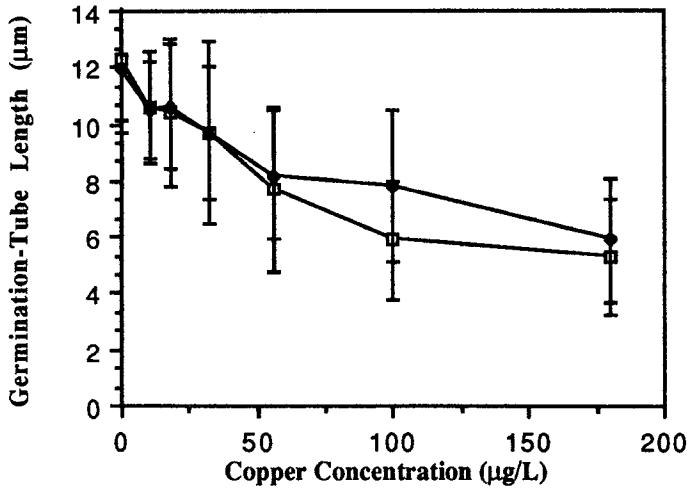
Container Comparison

There was no significant difference in response to copper between kelp gametophytes cultured in the standard 200-ml polyethylene food containers and the 5-ml Lab-Tek® polystyrene tissue culture containers (Figure 6 a-b). The NOEC's for germination were the same (32 µg/liter), and the EC_{50}'s were similar (64.5 and 57.5 for the polyethylene and Lab-Tek containers, respectively). The NOEC's for germ-tube growth were also the same (<10 µg/liter). This indicates that copper adsorption onto the sides of the smaller volume containers is insignificant. If this is also true for complex effluents, we can minimize the space and sample requirements of the protocol. Experiments are currently being designed to evaluate small-volume plastic and glass containers with organic toxicants and complex effluents.

Figure 6 a-b. Effects of copper on percent non-germination (a) and germ-tube growth (b) in polyethylene and Lab-Tek® containers.



(a) Non-Germination	NOEC
Polyethylene Container	32
LabTek Polystyrene Container	32



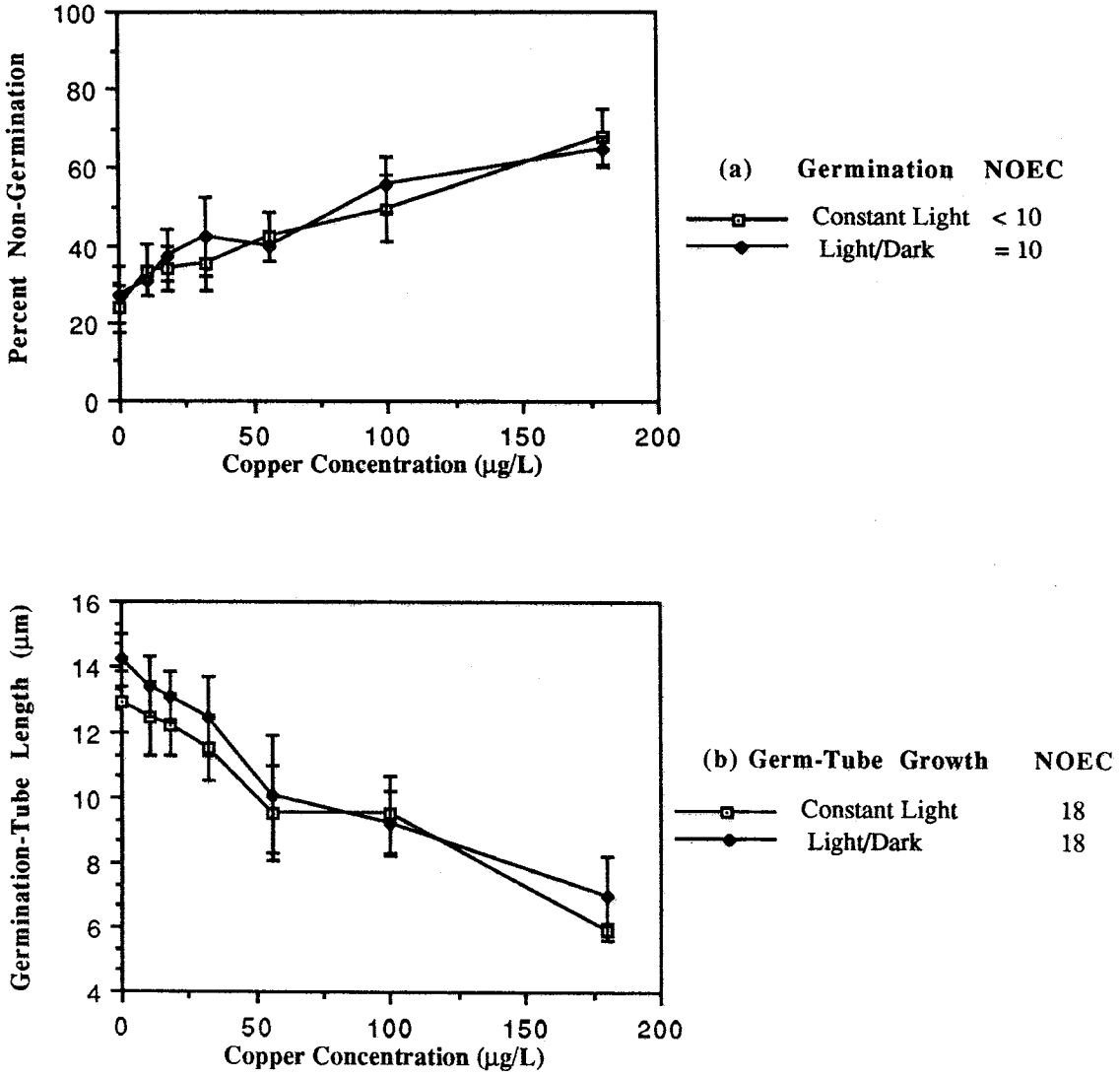
(b) Germ-Tube Growth	NOEC
Polyethylene Container	< 10
LabTek Polystyrene Container	< 10

Photoperiod Study

Kelp gametophytes incubated under constant light and under 16L:8D lighting had similar responses to copper (Figure 7 a-b). Although the dose-response curves were nearly identical, the NOEC for germination was lower under constant light. The NOEC's for germ-tube growth were the same. The different germination NOEC's appear to be caused by differences in between-replicate variability in the two experiments, there was greater variability under the light/dark light regime. The EC_{50}'s for germination

were 99.9 and 80.5 for constant and the light/dark photoperiods, respectively. The protocol has been modified to specify a 16L:8D photoperiod because this is closer to the natural lighting regime.

Figure 7 a-b. Copper toxicity to kelp under two different photoperiods: (a) percent non-germination under constant light and 16L:8D; (b) germ-tube growth under constant light and 16L:8D.



Summary

1. Out of fourteen separate 48-hour kelp toxicity tests conducted with reference toxicants and complex effluents during phase 4, all had adequate spore releases and acceptable germination rates.

2. MPSL and Hyperion had similar results in interlaboratory tests using sewage effluent and copper reference toxicant. The NOEC's for germination in copper differed by one concentration. MPSL and Bodega Marine Laboratory had similar results in interlaboratory experiments using lyophilized BKME. Results of concurrent copper reference tests varied between MPSL and BML.

3. Results showed that an insignificant proportion of non-germinated spores are removed during decanting of the test solution, indicating that removing the microscope slide from the test chamber does not bias test results.

4. An experiment designed to compare the performance of the 48-hour kelp test in small-volume polystyrene containers (5-ml) vs standard 200-ml polyethylene containers indicated that there was no difference in the effects of copper on gametophytes cultured in the two types of containers.

5. There was little difference between results of 48-hour kelp tests conducted under constant lighting and under a photoperiod of 16L:8D.

References

Anderson, B.S. and J.W. Hunt. 1988. Bioassay methods for evaluating the toxicity of heavy metals, biocides, and sewage effluent using microscopic stages of giant kelp *Macrocystis pyrifera* (Agardh): A preliminary report. Mar. Environ. Res. 26: 113-134.

Anderson, B.S., J.W. Hunt, S.L. Turpen, A.R. Coulon, and M. Martin. In Review. Copper toxicity to microscopic stages of *Macrocystis pyrifera*: interpopulation comparisons and temporal variability. Mar. Ecol. Prog. Ser.

Anderson, B.S., J.W. Hunt, M. Martin, S.L Turpen, and F.H. Palmer. 1988. Marine Bioassay Project third report, protocol development: reference toxicant and initial complex effluent testing. Division of Water Quality report no. 88-7. State Water Resources Control Board, Sacramento, CA. 151 pp.

Bruland, K.W., R.P Franks, G.A. Knauer, and J.H. Martin. 1979. Sampling and analytical methods for the determination of copper, cadmium, zinc, and nickel at the nanogram per liter level in seawater. Anal. Chem. Acta. 150: 233-245.

Chung, I.K., and B.H. Brinkhuis. 1986. Copper effects in early life stages of kelp, *Laminaria saccharina.* Mar. Poll. Bull. 17: 213-218.

Clendenning, K.A. 1958. Laboratory Investigations. In : The effects of waste discharges on kelp. Water Pollution Control Board Annual Progress Report, July 1958. IMR reference 58-11, 27-38.

Clendenning, K.A. 1959. Laboratory investigations. In : The effects of waste discharges on kelp. Water Pollution Control Board Quarterly Progress Report Oct.-Dec. 1958. IMR reference 59-4, 1-13.

Clendenning, K.A. 1960. In : The effects of waste discharge on kelp. Water Pollution Control Board Annual Progress Report, April 1960. IMR reference 60-4, 45.

Deysher, L.E. and T.A. Dean. 1984. Critical irradiance levels and the interactive effects of quantum irradiance and quantum dose on gametogenesis in the giant kelp, *Macrocystis pyrifera* . J. Phycol. 20, 520-524.

Foster, M.S. and D.R. Schiel. 1985. The ecology of giant kelp forests in California: a community profile. U.S. Fish Wildl. Serv. Biol. Rep. 85 (7.2), 1-152.

Hopkin, R. and J.M. Kain. 1978. The effects of some pollutants on survival, growth and respiration of *Laminaria hyperborea*. Estuar. and Coast. Mar. Science 7: 531-553.

Hunt, J.H., B.S. Anderson, S.L. Turpen, A.R. Coulon, M. Martin, F.H. Palmer, and J.J. Janik. 1989. Marine Bioassay Project fourth report: Experimental evaluation of effluent toxicity testing protocols with giant kelp, mysids, red abalone and topsmelt. Division of Water Quality report no. 89-5 WQ. State Water Resources Control Board, Sacramento, CA. 144 pp

James, D.F., S.L. Manley, M.C. Carter, and W.J. North. 1987. Effects of PCB's and hydrazine on life processes in microscopic stages of selected brown seaweeds. Hydrobiologia 151/152: 411-415.

Kuwabara, J. S. 1980. Micronutrient requirements for *Macrocystis pyrifera* (giant kelp) gametophytes determined by means of a chemically defined medium, Aquil. Doctoral Dissertation, California Institute of Technology, Pasadena, California, 1-185.

Kuwabara, J.S. 1981. Gametophytic growth by *Macrocystis pyrifera* (Phaeophyta) in response to various iron and zinc concentrations. J. Phycol. 17, 417-419.

Kuwabara, J.S. 1983. Effects of trace metals and natural organics on algae. In : The Effects of Waste Disposal on Kelp Communities. W. Bascom (ed.), Southern California Coastal Water Research Project, Long Beach, CA. 136-146.

Kuwabara, J.S., and W.J. North. 1980. Culturing microscopic stages of *Macrocystis pyrifera* (Phaeophyta) in Aquil, a chemically defined medium. J. Phycol. 16, 546-549.

Linfield, J.L.T., M. Martin, and J. Norton. 1985. Marine Bioassay Project first progress report: bioassay species selection and recommended protocols. Division of Water Quality. State Water Resources Control Board. 133 pp.

Luning, K. 1980. Critical levels of light and temperature regulating the gametogenesis of three *Laminaria* species (Phaeophyceae). J. Phycol. 16: 1-15.

Luning, K., and M. Neushul. 1978. Light and temperature demands for growth and reproduction of laminarian gametophytes in southern and central California. Mar. Biol. (Berl.) 45, 297-309.

Martin, M., K.E. Osborne, P. Billig, and N. Glickstein. 1981. Toxicities of ten metals to *Crassostea gigas* and *Mytilus edulis* embryos and *Cancer magister* larvae. Mar. Pollut. Bull. 12: 305-308.

North, W.J. 1971. *The Biology of Giant Kelp Beds (Macrocystis) in California.* Nova Hedwigia, 32: 1-600.

North, W.J. 1976. Aquacultural techniques for creating and restoring beds of giant kelp, *Macrocystis* spp. J. Fish. Res. Board Can. 33, 1015-1023.

Shenker, J.M., G.N. Cherr, R.M. Higashi, and D.G. Crosby. 1989. Toxicity of bleached kraft mill effluent (BKME) constituents in the marine environment. Society of Environ. Tox. and Chem. (SETAC) tenth annual meeting. Abstract 332.

Smith, B.M. and F.L. Harrison. 1978. Sensitivity of *Macrocystis* gametophytes to copper. Final report to US Nuclear Regulatory Commision, NRC FIN No. A0119: 1-34.

Sunda, W.G. and R.R.L. Guilliard. 1976. The relationship between cupric ion activity and the toxicity of copper to phytoplankton. J. Mar. Res. 35: 511-29.

Thompson, R.S. and E.M. Burrows. 1984. The toxicity of copper, zinc, and mercury to the brown macroalga *Laminaria saccharina.* In: *Ecotoxicological Testing For The Marine Environment.* G. Persoone, E. Jaspers, and C. Claus (Eds). State Univ. Ghent and Inst. Mar. Scient. Res., Brendene, Belgium. 2: 588 pp.

Weber, C.I., W.B. Horning, D.J. Klemm, T.W. Neiheisel, P.A. Lewis, and E.L. Robinson. 1988. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to marine and estuarine organisms. United States Environ. Protection Agency. EPA/600/4-87/028. Cincinnati, OH. 417 pp.

Section 2
Red Abalone Experiments

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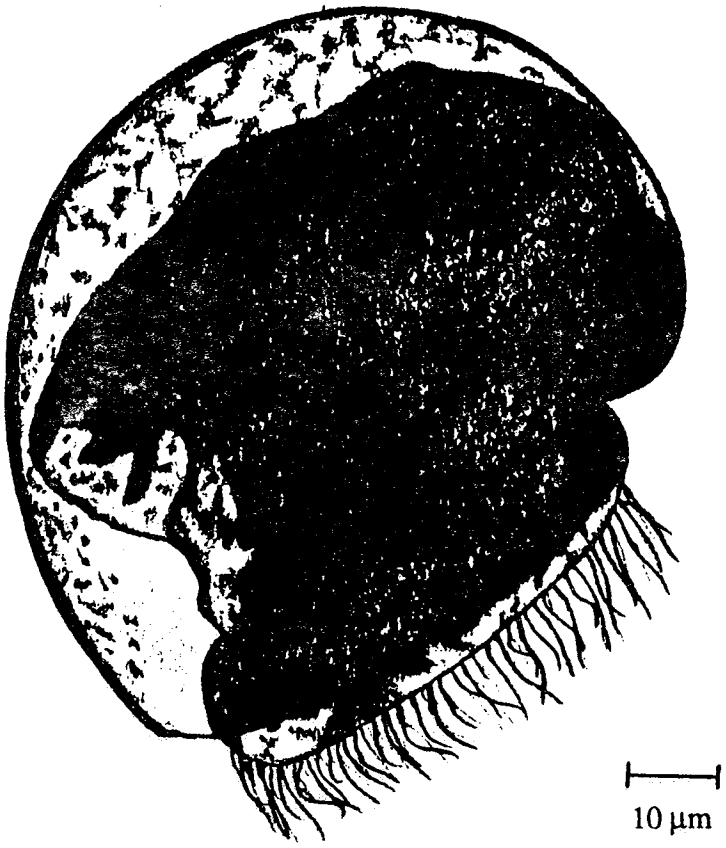


Haliotis rufescens - veliger larva

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 36 spawnings attempted in our laboratory over the past four years, 34 have been successful (97%), 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). 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. A past trial 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).

Over the course of the project we have conducted 38 short term toxicity tests using a variety of toxicants. Copper, tributyltin, sodium pentachlorophenate and endosulfan were tested once each, zinc sulfate was tested 13 times, and complex effluents from various sources were tested 11 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 = 13), <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 = 13).

This section describes experiments done during the past year to test the suitability of the red abalone protocol for determining effluent toxicity under varying test conditions. The primary focus has been on interlaboratory testing of complex effluents. Other experiments examined methods of effluent salinity adjustment, and compared interpretation of the test endpoint by a number of investigators viewing exposed larvae for the first time. The results of this work were combined with previous data and the comments of reviewers to revise the abalone protocol.

Methods

Methods for the red abalone tests reported in this section are given in the previous red abalone protocol (Hunt et al., 1989). Revisions of the protocol were made based on the work reported here, and the revised protocol is appended to this report (Appendix 2).

Interlaboratory Tests

Interlaboratory tests serve a variety of functions. The purpose of the interlaboratory testing described here was to determine whether the abalone test protocol was sufficiently detailed to allow different investigators to produce acceptable test results under varying laboratory conditions. Three sets of interlaboratory tests were conducted: one between the Marine Pollution Studies Laboratory (MPSL) and a sewage treatment plant laboratory using complex municipal effluent, one between MPSL and a university research laboratory using bleached kraft mill effluent, and one between MPSL and a private contract laboratory using a reference toxicant. In each case, laboratories used their own equipment and dilution water, and in two cases the test date and source of test organisms varied between MPSL and the participating laboratory. Although participating investigators were experienced in toxicity testing, all except MPSL were conducting the abalone test for the first or second time. 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.

The Hyperion Waste Treatment Plant laboratory (City of Los Angeles) and MPSL conducted the abalone test on the same date using a split sample of Hyperion effluent. Effluent was shipped on ice from Hyperion on the day of collection, and arrived at MPSL the next morning, when testing began at both laboratories. Abalone broodstock (four males and four females) were shipped from MPSL to Hyperion two days before testing, and were held in static aquaria until spawning induction. MPSL broodstock were packaged for shipment and held at MPSL overnight, then held in static aquaria prior to spawning induction

to simulate handling of the abalone shipped to Hyperion. Zinc sulfate was provided by MPSL for the concurrent reference toxicant tests at both laboratories. Hypersaline brine for both laboratories was made and pH-adjusted at MPSL using Wimex® sea salt. Each laboratory used its own dilution water: MPSL dilution water is pumped from a depth of five meters on the exposed coast of central California remote from large anthropogenic inputs; Hyperion dilution water is pumped from a depth of 14 meters near the head of the Redondo submarine canyon in Santa Monica Bay. Embryos were added to the test containers approximately two hours after fertilization at both laboratories. Abalone larvae were incubated in beakers at both laboratories, then screened and concentrated into vials for preservation (protocol method[a]). The larvae were analyzed three days after test termination at MPSL, but at Hyperion, scheduling difficulties resulted in a 12 week delay in larval analysis. Larvae were analyzed on Sedgewick-Rafter counting slides at MPSL (method[a]); at Hyperion they were transferred from the preservation vials into tissue culture flasks for analysis (a variation on method[b]).

A split sample of the same Hyperion complex effluent described above was used in an echinoderm sperm cell toxicity test conducted by the Southern California Coastal Water Research Project. This test used the sea urchin, *Strongylocentrotus purpuratus*, following the protocol by Dinnel et al. (1987). Results of this test were used to compare the relative sensitivity of the two test organisms to the complex effluent.

The Bodega Marine Laboratory (BML) participated in an interlaboratory test with MPSL using lyophilized samples of bleached kraft mill effluent (L-BKME). Lyophilized BKME is a powdered distillate that retains the toxicity of the original effluent and is a convenient complex effluent for interlaboratory testing. Both laboratories used L-BKME from a split sample, and zinc sulfate reference toxicant from the same source. Both laboratories used tissue culture flasks as test containers (protocol method[b]). The laboratories used abalone from different sources: MPSL used abalone from Granite Canyon, and BML used abalone from a private mariculture facility in southern California. Each laboratory used different dilution water from its own seawater system. The two laboratories conducted the tests on different dates approximately three weeks apart.

A third interlaboratory test was conducted using only a reference toxicant. A private independent contract laboratory, McCormick and Associates (MA), conducted the test three weeks after it was done at MPSL. Both laboratories used zinc sulfate from the same source, and both used open beakers as test containers (protocol method[a]). MPSL used abalone from Granite Canyon, and MA used abalone from their own facility. Each laboratory used different dilution water from its own seawater system.

No Observed Effect Concentrations (NOEC's) were derived for all tests using Analysis of Variance (ANOVA) and Dunnett's multiple comparison (Sokal and Rohlf, 1969; Zar, 1974). Median Effective Concentrations (EC_{50}'s) were calculated using angular transformation linear interpolation. Probit was not used because most of the tests had an insufficient number of data points intermediate between low (near zero) and high (near 100%) effect. Data from different laboratories were compared using two-factor ANOVA, with the two factors being toxicant concentration and laboratory.

Data for reference toxicant tests are reported as nominal concentrations. Test solutions in all tests were sampled for chemical analysis to verify test concentrations, but the two trace metal analytical facilities contracted to conduct the analyses were severely damaged in the October 17, 1989 earthquake, and we were unable to make other arrangements before preparing this report.

Comparison of Hypersaline Brines

Because most effluents have very low salinities, effluent tests that use marine organisms use hypersaline brines to adjust the salinity of effluent test solutions. Some brine mixtures are toxic to sensitive marine species, while others may contain compounds that interfere with effluent toxicity. Two experiments were done to assess the effects of different brines on abalone larvae.

The first experiment compared three commercial sea salts commonly used to make brines for effluent testing. The sea salt brands were Instant Ocean®, Forty Fathoms®, and Wimex®. Seventy grams of each salt were added to two liters of distilled water each and stirred for one hour. Precipitates were allowed to settle for 15 minutes, and then supernatant brine was decanted into a separate container. Small amounts of salt were added to each as needed to bring the salinity to 33‰, the salinity of MPSL natural seawater, which was used as a control. The pH of each was adjusted to the pH of natural seawater (7.9 ± 0.1 pH unit), using 2N hydrochloric acid. Abalone embryos were placed in five replicate 200-ml samples of each mixture, and into five replicates of natural seawater, and incubated and analyzed as in the toxicity test protocol. Each salt mixture was also diluted with natural seawater to make 10% brine solutions. Abalone embryos were placed in five replicates of each of these, and incubated and analyzed as in the toxicity test protocol. These 10% solutions were similar to "brine controls" that would be used in a toxicity test that used 10% effluent as the highest test concentration. No toxicants were used in this experiment. Data were compared using an ANOVA and Student Neuman Keuls multiple comparison.

A second experiment further compared the effects of different brines on effluent toxicity tests, and also examined the necessity of salinity adjustment at effluent concentrations up to 10%. In this experiment, four effluent tests were run concurrently, each using a different method of salinity adjustment. The complex effluent used was a fresh sample from the Hyperion Treatment Plant. Test solutions were adjusted to dilution water salinity (33‰) in three effluent tests using brines made from either Forty Fathoms® sea salt, Wimex® sea salt, or by freezing (the freezing process is described in the protocol, Appendix 2). Results of the three effluent tests with salinity adjustment were compared to each other and to a fourth effluent test without salinity adjustment. Each of the three brine tests had five replicates of a brine control, as described in the protocol (Appendix 2). The fourth test had five replicates of a "low salinity control", made by adding 100 ml of distilled water to 900 ml of dilution water, to simulate the reduced salinity of the highest effluent concentration (10% effluent). Each test had effluent concentrations of 0 (control), 0 (brine control), 0.56, 1.0, 1.8, 3.2, 5.6, and 10% effluent, each replicated five times. The differences between brines were analyzed using the following statistical tests: two-factor ANOVA on all test

data, with effluent concentration and salinity treatment as the two factors; one-factor ANOVA at each concentration to detect differences between salinity treatments; Student Neuman Keuls multiple comparisons to distinguish among individual treatments; t-tests between controls and brine controls; and calculation of effluent NOEC's for each test (Sokal and Rohlf, 1969; Zar, 1974).

Endpoint Comparisons

As part of a training workshop held August 30, 1989 at MPSL (see Section 5), representatives from several California waste treatment plants analyzed abalone larvae at the end of a standard 48-hour reference toxicant test. None of the participants had previously conducted the abalone test; but they studied photographs of test organisms and discussed the endpoint criteria with the MPSL staff prior to viewing the samples. Three test containers were sampled, one from each of the intermediate toxicant concentrations (18, 32, and 56 µg/liter zinc). Larvae from each of these test containers were screened and preserved in vials (method[a]). These three vials were sampled and analyzed by each of the participants. Samples from intermediate concentrations were chosen because these are the most difficult to read, each having a mixture of both normal and abnormal larvae. The results were analyzed using a randomized block ANOVA to detect significant differences in endpoint analysis between participants (Zar, 1974).

Results and Discussion

Interlaboratory Tests

Effluent toxicity tests conducted concurrently at the Marine Pollution Studies Laboratory (MPSL) and the Hyperion Treatment Plant produced similar results (ANOVA, $p = .35$). The No Observed Effect Concentrations (NOEC's) were 1.8% at MPSL and 3.2% at Hyperion. The EC_{50}'s were 3.1% at MPSL and 4.2% at Hyperion. The proportions of abnormal larvae in controls, brine controls, and lower effluent concentrations were higher at Hyperion than at MPSL (Figure 8), perhaps due to broodstock stress caused by shipping. The response at the 3.2% effluent concentration, though highly variable, was greater at MPSL than at Hyperion, resulting in the difference in NOEC's between laboratories.

In the echinoderm sperm cell test conducted concurrently with the same effluent, the NOEC was found to be 1.8%, the same as that from the MPSL abalone test (personal communication, S. M. Bay, Southern California Coastal Water Research Project).

The MPSL and Hyperion reference toxicant test results showed similar trends but were significantly different (ANOVA, $p<.01$). The differences were again the result of higher proportions of abnormal larvae at Hyperion, especially in the controls and lower zinc concentrations (Figure 9). There were significantly more abnormal larvae in the 32 µg/liter treatments at Hyperion than in controls, while

FIGURE 8. Results of complex effluent interlaboratory tests between the Marine Pollution Studies Laboratory and the Hyperion Treatment Plant. Data are means ± S.D. of five replicates per treatment.

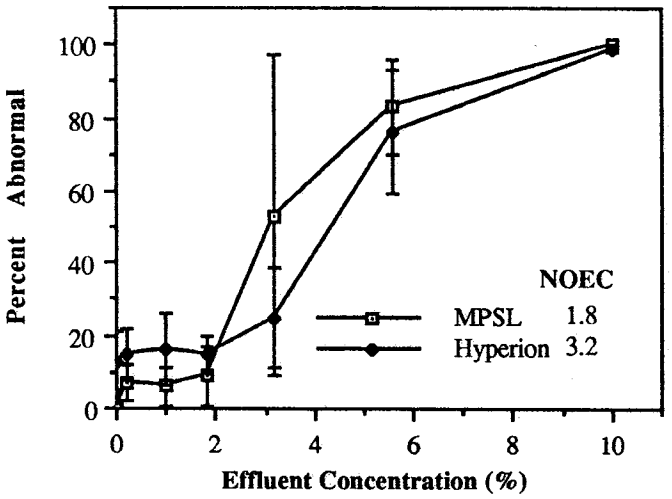
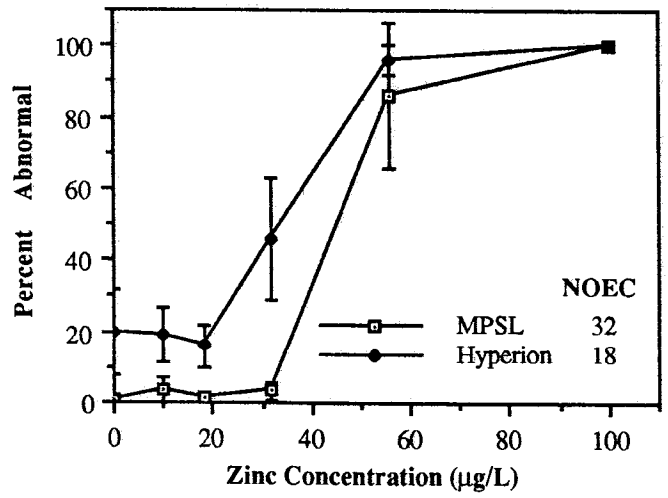


FIGURE 9. Results of concurrent reference toxicant interlaboratory tests between the Marine Pollution Studies Laboratory and the Hyperion Treatment Plant. Data are means ± S.D. of five replicates per treatment.

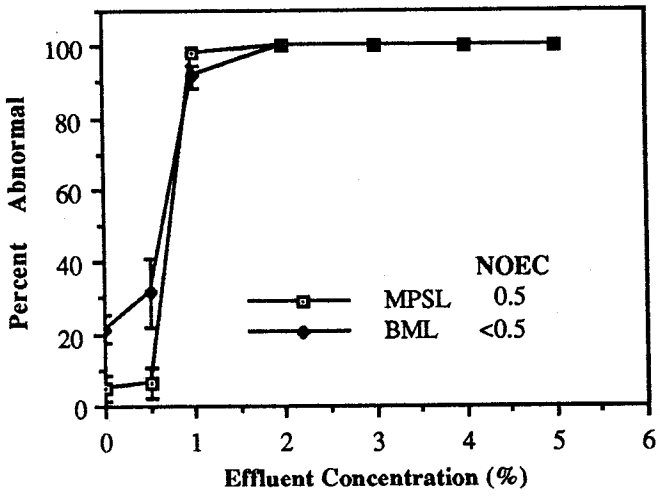


this was not the case at MPSL. The resulting NOEC's were 18 µg/liter at Hyperion and 32 µg/liter at MPSL, both within the protocol range of acceptability. The EC_{50} was 33 µg/liter at Hyperion and 45 µg/liter at MPSL. All zinc values are reported as nominal concentrations.

Differences between laboratories, especially at the 3.2% effluent concentration and the 32 µg/liter zinc concentration, may have been caused by differences in dilution waters, differences in handling broodstock (ie. shipping), or differences in handling larvae (length of preservation time and method of analysis, see Methods). Intermediate concentrations tend to be the most variable, and are most affected by factors influencing toxicity or toxicant bioavailability, such as organism stress or the chelation capacity of the dilution water. The relative effects of these factors are difficult to resolve, but differences between tests would probably decrease if all laboratories used the same dilution water and maintained their own broodstock. Despite variations in test conditions at the different laboratories, the tests met acceptability criteria for control response, between replicate variability, and reference toxicant NOEC value.

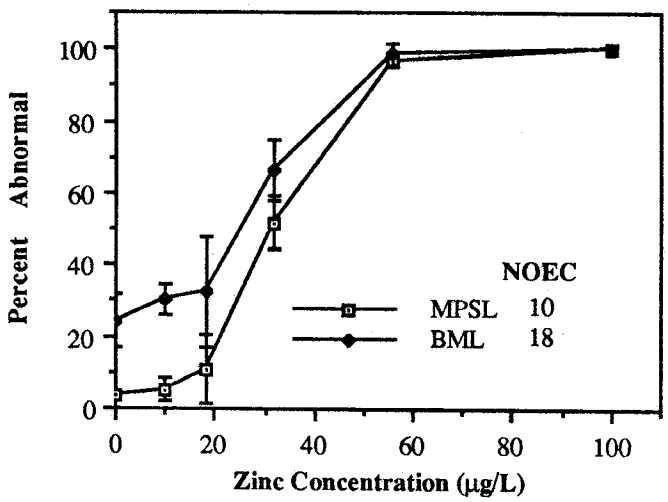
Interlaboratory tests with lyophilized bleached kraft mill effluent (L-BKME) at the Bodega Marine Laboratory and MPSL produced similar response curves but different control responses (Figure 10). As with the Hyperion tests, larval abnormality was higher at BML than at MPSL, resulting in significant differences between tests (ANOVA, $p < .01$). The NOEC was <0.5% effluent at BML, and 0.5% effluent at MPSL. The EC_{50} was 0.60% at BML and 0.68% at MPSL.

FIGURE 10. Results of lyophilized bleached kraft mill effluent interlaboratory tests between the Marine Pollution Studies Laboratory and the Bodega Marine Laboratory. Data are means ± S.D. of five replicates per treatment.



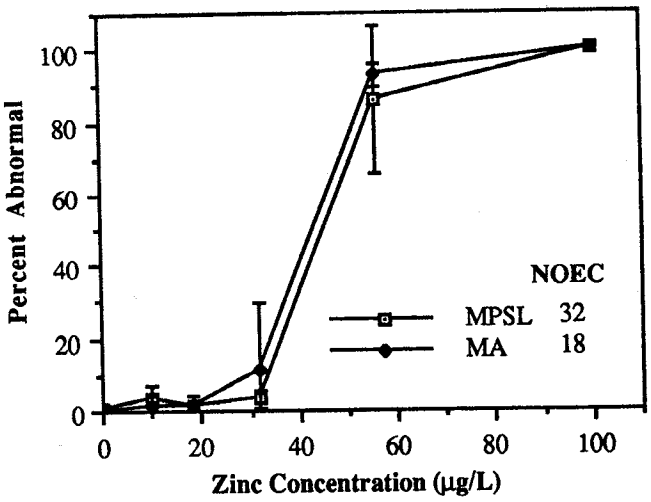
Concurrent zinc reference toxicant tests at the two laboratories again produced similar response curves and different control responses (Figure 11). Control abnormality was high at BML (>20%), beyond the range of acceptability established for the protocol. The NOEC was 18 µg/liter at BML, and 10 µg/liter at MPSL. The MPSL NOEC was low because of a small but significant difference in response between the control and 18 µg/liter treatments. The zinc EC_{50} was 24 µg/liter at BML and 31 µg/liter at MPSL. Differences in results between laboratories may reflect differences in abalone broodstock source, test date, or dilution water.

FIGURE 11. Results of reference toxicant interlaboratory tests between the Marine Pollution Studies Laboratory and the Bodega Marine Laboratory. Data are means ± S.D. of five replicates per treatment.



Interlaboratory tests with zinc reference toxicant produced good correlation between results from MPSL and a private contract laboratory, McCormick and Associates (MA). Response curves and control responses were similar (Figure 12), and there was no significant difference between tests (ANOVA, $p = .78$). The zinc NOEC was 32 µg/liter at MPSL, and 18 µg/liter at MA. The lower NOEC at MA is the result of one anomalous replicate at the 32 µg/liter concentration (44% abnormal), which caused this treatment to be significantly different from the control. The EC_{50} was 45 µg/liter at MPSL and 40 µg/liter at MA.

FIGURE 12. Results of reference toxicant interlaboratory tests between the Marine Pollution Studies Laboratory and McCormick and Associates. Data are means ± S.D. of five replicates per treatment.



Results from these three sets of interlaboratory tests suggest a relationship between broodstock handling and control response. Although control response criteria were met in all but two tests, the proportions of abnormal control larvae were significantly higher at the Hyperion and BML laboratories (which used broodstock sent from MPSL and MA, respectively) than at MPSL or MA (which used broodstock cultured at their own facilities). Past interlaboratory abalone tests have also shown slightly higher control response from laboratories using shipped broodstock (Anderson et al., 1988; Hunt et al., 1989). A comparison of data from five MPSL interlaboratory tests showed that control response was significantly higher for laboratories using shipped broodstock than for those using broodstock cultured at the testing facility (t-test, $p < .0001$). There are a number of alternative possible explanations. Stress from shipping, changes in culture environment, differences in techniques for handling and spawning, or some combination of these factors may have affected control response. Abalone used at MPSL were always packaged for shipment and held for 24 hours to simulate shipping stress, but the low temperature and cabin pressure experienced during airfreight were not simulated, and these may have affected the test organisms (Reish et al., 1978). Further experimentation is necessary, but differences in control response may be reduced if broodstock are acclimated for longer periods before spawning at the testing laboratory. It is also important to note that differences in control response did not correlate with test sensitivity; there was no significant difference in reference toxicant NOEC values between laboratories using shipped broodstock and those using their own (t-test, $p = 0.46$).

These three interlaboratory tests demonstrated that laboratories having limited experience with the abalone test could follow the protocol to produce acceptable results with different effluents. Except for control response at BML, as discussed above, all three laboratories produced results within the range of test acceptability proposed for the protocol (see Appendix 2). In all cases, NOEC values from participating laboratories were within one concentration of the respective MPSL value, dose response curves were similar, and EC_{50}'s varied between laboratories by an average of 16%.

Comparison of Hypersaline Brines

In the preliminary comparison of hypersaline brines made from commercial sea salts, there were no significant differences between brands in their effects on abalone larvae. In each of the 100% artificial seawaters made from sea salt and distilled water, all larvae developed abnormally. In the 10% artificial seawaters (diluted in natural seawater), mean larval abnormality ranged from 2% to 5%, and there were no significant differences between brands or controls. Control (natural seawater) abnormality was 6.8%. No toxicants were used in this comparison.

Some differences were apparent in the more extensive experiment comparing concurrent effluent tests using different methods of salinity adjustment. The most striking result was the difference in dose-response curves between the non-salinity-adjusted test and the three tests that used brines to adjust salinity (Figure 13). Curves in salinity-adjusted tests had the common sigmoidal shape, while the curve from the non-salinity-adjusted test increased linearly. The non-salinity-adjusted effluent was apparently more toxic than the salinity-adjusted effluents at lower concentrations, and less toxic at higher concentrations. This result is difficult to interpret for two reasons. First, it was expected that relative toxicity would increase in the non-salinity-adjusted effluent as its salinity decreased (with increasing effluent addition) below the abalone's accustomed range. Second, if brine addition suppressed effluent toxicity (relative to the non-salinity-adjusted effluent) at lower effluent concentrations, it would not be expected to increase effluent toxicity at higher concentrations. These apparent contradictions would be difficult to resolve without further investigations into the physiological mechanisms of effluent toxicity, but there were some useful comparisons made among the different treatments.

The anomalous shape of the non-salinity-adjusted test curve (Figure 13) indicated that changes in salinity did affect the organisms' response to the effluent. There were significant differences between salinity-adjusted and non-salinity-adjusted treatments at 3.2% and 5.6% effluent, but not at 1.8% effluent (ANOVA, SNK; $p = 0.004$, 0.0001, and 0.27, respectively). Addition of 3.2% effluent (with a salinity of 1‰) to dilution seawater (with a salinity of 33‰) resulted in a test solution salinity of less than 32‰, which is below the range of 34 ± 2‰ suggested in the protocol.

FIGURE 12. Results of reference toxicant interlaboratory tests between the Marine Pollution Studies Laboratory and McCormick and Associates. Data are means ± S.D. of five replicates per treatment.

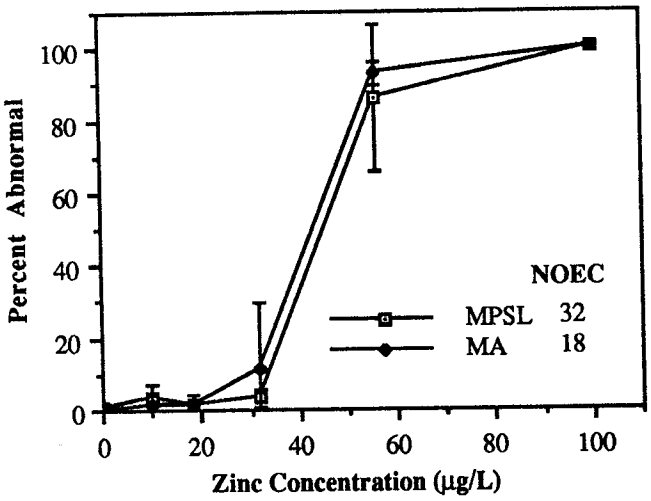


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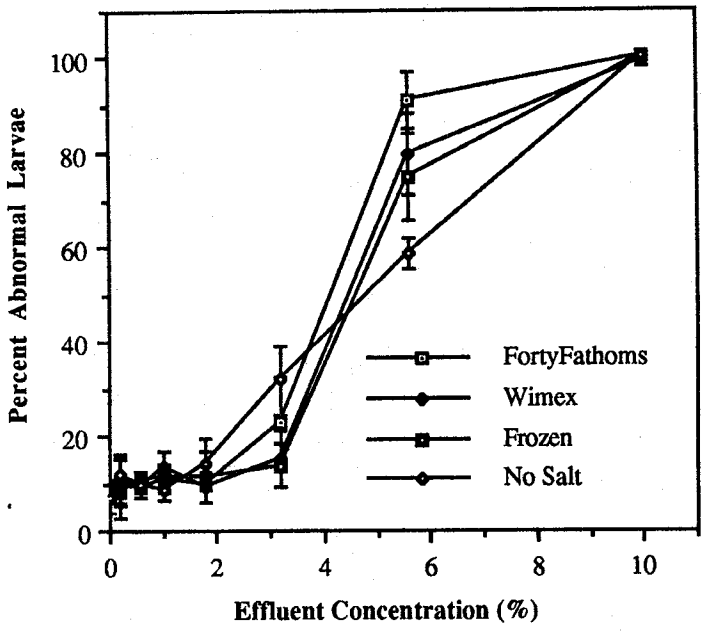
Figure 13. Results of four concurrent effluent tests, each using a different method of salinity adjustment. Forty Fathoms® and Wimex® are commercial sea salts used to make hypersaline brine, and "Frozen" refers to hypersaline brine made by freezing natural dilution seawater. Each of these brines was used to adjust effluent salinity to that of the dilution water in one of three effluent tests. "No Salt" indicates the fourth effluent test in which salinity was not adjusted. Data are means ± S.D. of five replicates per treatment.



There were also significant differences among the three types of brines in their effects on effluent toxicity. Effluent test solutions adjusted with Forty Fathoms® sea salt produced the greatest overall toxicity, while effluents adjusted with the brine made by freezing produced consistently lower toxicity at all concentrations. The difference between the two was statistically significant (ANOVA, SNK, $p < .05$).

There were no significant differences between dilution water controls and brine controls for any treatment, but the brine made by freezing had the lowest brine control response. The low salinity controls, with salinities as low as 28‰, were most different from dilution water controls, though not significantly (t–test, $p = .13$). The NOEC's for the four effluent tests were 1.8% for the non-salinity-adjusted effluent, 1.8% for the Forty Fathoms® effluent, 3.2% for the Wimex® effluent, and 3.2% for the effluent adjusted with brine made by freezing.

These results support the protocol guidelines for salinity adjustment using brines made by freezing to maintain test solution salinity within the range of 34 ± 2‰.

Endpoint Comparisons

There was no significant difference in the interpretation of the abalone test endpoint among investigators viewing the test for the first time (ANOVA, $p > .05$). Nine representatives from large California waste treatment plants and public utilities independently sampled and analyzed three test containers, and the coefficient of variation for the nine scores averaged 5.2% for the three test samples. The larvae analyzed were from intermediate zinc concentrations in a standard reference toxicant test, and had a mean of 62 abnormal larvae and 42 normal larvae per sample. Intermediate concentrations such as these are more difficult to analyze than low concentrations where most larvae are clearly normal, or high concentrations where most larvae are severely deformed. The low variability between inexperienced investigators in this test indicates a consistent interpretation of the abalone endpoint.

Summary

1. Of twelve separate abalone toxicity tests conducted with reference toxicants and complex effluents, ten met all test acceptability criteria outlined in the protocol. Two tests had less than 80% normal larvae in controls (77% and 76%).
2. In each of five interlaboratory tests using reference toxicants and complex effluents, NOEC's varied between laboratories by one concentration. Response curves were similar in all tests, and Median Effective Concentrations (EC50's) varied between laboratories by 8% to 22% (coefficients of variation), giving a mean interlaboratory precision of 16%.
3. The percentage of abnormal abalone in controls was significantly different between laboratories using their own broodstock and laboratories using broodstock shipped to them prior to testing. Further experimentation is recommended to determine adequate acclimation times to improve control response at laboratories dependent on outside sources for broodstock.
4. Use of brines made by freezing filtered dilution seawater is recommended to maintain test solution salinity at 34 ± 2‰ in abalone tests.
5. There was no significant difference in the interpretation of the abalone test endpoint among investigators viewing the test for the first time. Endpoint counts varied by an average of 5.2% among investigators.

References

Anderson, B.S., J.W. Hunt, M. Martin, S.L. Turpen, and F.H. Palmer. 1988. Protocol development: reference toxicant and initial complex effluent testing. Marine Bioassay Project 3rd Report. California State Water Resources Control Board Report No. 88-7WQ. Sacramento, Ca.

Dimmick, R.E. and W.P. Breese. 1965. Bay mussel embryo bioassay. In: Proc. 12th Pacific Northwest Industrial Waste Conference, University of Washington, Seattle. p. 165.

Dinnel, P. A., J. M. Link, and Q. J. Stober. 1987. Improved methodology for a sea urchin sperm cell bioassay for marine waters. Arch. Environ. Contam. Toxicol. 16: 23-32.

Ebert, E. E. and J. L. Houk. 1984. Elements and innovations in the cultivation of red abalone *Haliotis rufescens*. Aquaculture. 39: 375-392.

Grigg, R. W. and R. S. Kiwala. 1970. Some ecological effects of discharged wastes on marine life. Calif. Fish Game. 56: 145-155.

Hahn, K.O. 1989. *Handbook of culture of abalone and other marine gastropods*. CRC Press, Inc. Boca Raton, Florida. 348 pp.

Hunt, J.W. and B. S. Anderson. 1989. Sublethal effects of zinc and municipal effluents on larvae of the red abalone *Haliotis rufescens*. Marine Biology 101: 545 - 552.

Hunt, J.W., B.S. Anderson, S.L. Turpen, A.R. Coulon, M. Martin, F.H. Palmer, J.J. Janik. 1989. Experimental evaluation of effluent toxicity testing protocols with giant kelp, mysids, red abalone, and topsmelt. Marine Bioassay Project 4th Report. California State Water Resources Control Board Report No. 89-5WQ. Sacramento, Ca.

Martin, M., M. D. Stephenson, and J. H. Martin. 1977. Copper toxicity experiments in relation to abalone deaths observed in a power plant's cooling waters. Calif. Fish. Game. 63: 95-100.

Martin, M., Hunt, J., Anderson, B., Espinosa, L., and F. Palmer. 1986. Acute toxicity tests and protocols: mysid shrimp, red abalone, and giant kelp. Marine Bioassay Project 2nd Report. State Water Resources Control Board. Sacramento, Ca.

Morse, D. E., H. Duncan, N. Hooker, and A. Morse. 1977. Hydrogen peroxide induces spawning in molluscs, with activation of prostaglandin endoperoxide synthetase. Science. 196: 298-300.

Morse, D. E., N. Hooker, H. Duncan, and L. Jensen. 1979. Gamma aminobutyric acid, a neurotransmitter, induces planktonic abalone larvae to settle and begin metamorphosis. Science. 204: 409-410.

Reish, D.J., C.E. Pesch, J.H. Gentile, G. Bellan, and D. Bellan-Santini. 1978. Interlaboratory calibration experiments using the polychaetous annelid Capitella capitata. Marine Environ. Res. 1: 109 - 118.

Sokal, R. R. and F. J. Rohlf. 1969. Biometry. W.H. Freeman and Company, San Francisco.

Woelke, C.E. 1972. Development of a receiving water quality bioassay criterion based on the 48-hour Pacific oyster (Crassostrea gigas) embryo. Washington Depart. Fish., Tech. Rept. 9: 1 - 93.

Zar, J. H. 1974. Biostatistical Analysis. Prentice-Hall, Inc., Inglewood Cliff, N.J.

Section 3
Topsmelt Experiments

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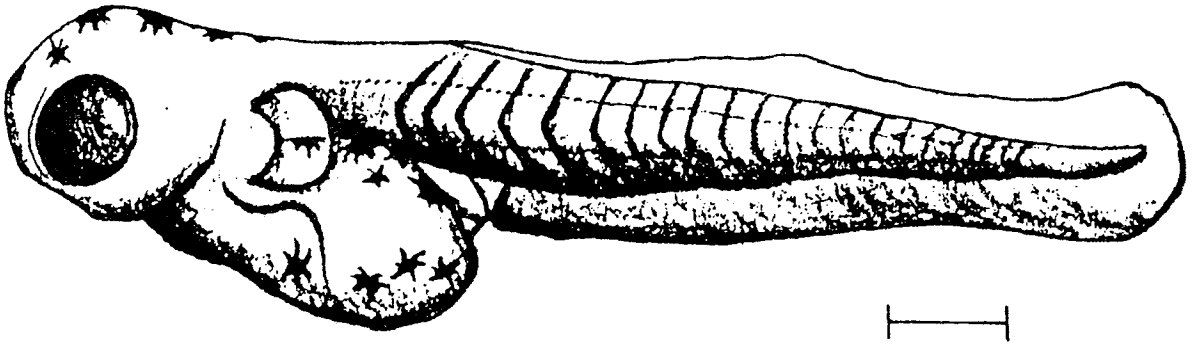


Atherinops affinis - larva

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). Allen (1982) found that topsmelt accounted for 85.1 percent of the total annual fish productivity in upper Newport Bay. 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 and potential susceptibility to coastal pollution. 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 these species can be easily adapted for topsmelt.

Early-life stage toxicity testing is dependent on the development of suitable techniques for laboratory spawning. Past attempts to spawn this species in the laboratory have been unsuccessful (Fronk, 1969). Larval topsmelt have been reared from field-collected embryos (McHugh and Walker, 1947; Ehrlich et al. 1979), and Middaugh et al. (1988) investigated the effects of salinity on growth and survival of larvae hatched from field-collected embryos. 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; these appeared to be relatively tolerant of zinc.

The 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, a metal found in high concentrations in sewage and power plant effluents (Young et al., 1977; Martin et al., 1977; Harrison, 1982). Copper has been used in similar toxicity tests with other marine fishes (Rice and Harrison, 1978; Engel and Sunda, 1979; Rice et al., 1980), allowing for direct comparison of bioassay results between species.

Methods

Facilities

All experiments were conducted from April to July, 1989, at the California Department of Fish and Game's Marine Pollution Studies Laboratory (MPSL) located on the Big Sur coast in Monterey County. Background concentrations of total copper in the laboratory seawater were below the detection limit of 3 µg/liter in this study.

Laboratory Spawning

Adult topsmelt were seined from Elkhorn Slough, Monterey County, California, in April, 1989 (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 (designated west [W] and east [E]; approximately 30 fish per tank). 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 into 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 14 hours light followed by 10 hours darkness (14L:10D) with lights commencing at 0600. Two 'cool white' 40 watt fluorescent lamps, suspended 1.25 m above the surface of each tank, provided illumination. Tanks were adjacent to a west-facing window and also received natural light. Midday light levels at the surface of the tanks ranged from 12 to 21 $\mu E\ m^{-2}\ s^{-1}$ (microeinsteins per meter squared per second; mean = 17.3 $\mu E\ m^{-2}\ s^{-1}$), measured with a Licor Model LI-185 B Quantum Photometer.

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 40 grams of chopped squid (maximum particle size 2 $mm^{2)}$ at 0900 to 1000 hrs, and approximately eight grams of freeze dried Krill (Superba®, First Choice), crumpled into 0.1 to 0.5-mm^2 pieces at 1100 to 1200. Each tank received approximately four grams of Tetramin ™ flake food at 1300-1500 hours daily. Tanks were siphoned clean twice weekly.

Temperature Spikes

Adult topsmelt were held at approximately 14 to 15 °C for 30 days (4 April until 6 May, 1989), then the water temperature was increased to 19.1 °C over a 14-hour period. This elevated temperature stressed the fish as evidenced by mortality. In the west tank, one male and one female died, and in the east tank three males and two females died. Temperature was then reduced to 18.1°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. These temperature spikes consisted of increasing the temperature from 18.0 to 20.0-20.5 °C during a 12-h period, then allowing water temperature to return to 18.0 °C overnight. Egg production was monitored for six temperature spike cycles during this study.

Toxicity Tests

Fertilization Tests

The fertilizatioin test consisted of exposing sperm to a toxicant, mixing eggs and sperm, and then measuring percent fertilization. Four toxicity tests were conducted. For each experiment, single adult male topsmelt were isolated, and milt was stripped into a 100-ml glass beaker containing 50 ml filtered seawater (19 °C). Five drops of this sperm solution were then pipeted into each test container. 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 and contained 10 ml of test solution. Sperm were exposed to total copper concentrations of 0, 18, 32, and 56 µg/liter in the initial test. For the second through fourth tests nominal copper concentrations were 0, 18, 32, 56, and 100 µg/liter. A no-sperm control was included in each experiment to verify that there was no inadvertant contamination of the eggs prior to exposure. Each treatment was replicated twice. Eggs were added after sperm were exposed to the test solution for 15 minutes. Eggs were stripped from one female fish into a polycarbonate plastic tray containing a small amount of water, then immediately pipeted with a 3-mm-bore fire-polished glass tube into the test containers.

Sperm densities were determined with a hemacytometer from samples of the initial sperm solution. Sperm densities were not manipulated in these experiments because of their relatively short span of viability. Egg densities were estimated and varied considerably (see results).

The sperm and eggs were left to incubate at 21 ± 1 ° C for 48 hours, with a 14L:10D photoperiod (50 µE 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. Percent fertilization data were transformed to the arcsine of their square root and compared using Analysis of Variance (ANOVA). The No Observed Effect Concentration (NOEC) was calculated as the highest copper

concentration not significantly different from the control using Dunnett's multiple comparison test (Sokal & Rohlf 1969). Probit analysis was used to calculate LC_{50}'s and 95 % confidence intervals for all tests.

Embryo Tests

Three embryo experiments were conducted to assess developmental toxicity. The procedures were similar to those described by Middaugh et al. (1988) for tests with embryonic inland silversides, *Menidia beryllina*. Single early blastula embryos (stage 8-9, after Lagler *et al.*, 1962) were placed in glass tissue culture tubes (94mm x 16mm) containing nominal copper solutions of 18, 32, 56, 100, or 180 µg/liter. Control and dilution water was 0.2 µm-filtered, UV-sterilized 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). 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, terata, mortality, and hatching sucess. Post-hatch larvae were also examined for terata at the end of the tests. Teratogenic expressions were quantified using the procedure described by Weiss and Weiss (1982). Three categories of developmental abnormalities were quantified: craniofacial, cardiovascular, and skeletal defects. Observations on the degree of malformation in each category were recorded daily for each embryo.

Upon completion of each experiment, three endpoints were compared: the number of embryos with terata, the number of hatched larvae, and the number of hatched larvae with terata. Terata were combined for the statistical analyses. A row by column test was employed to compare all data for each endpoint. This test of independence uses the G statistic and is based on the chi-squared distribution. NOEC's for each endpoint were calculated by pairwise comparisons using an adjusted alpha (alpha' = 0.05) to compare test concentrations to controls (Sokal and Rohlf 1969). Probit analysis was used to calculate EC_{50}'s and 95% confidence intervals for all tests.

Larval Tests

Larval topsmelt were cultured at 21-23 °C and 33 ‰ using the procedures described by Middaugh et al. (1990).

The larval test was a 96-hour static toxicity test that measured lethality. A total of three acute larval experiments were conducted. Eight day-old larval topsmelt (five larvae per replicate) were exposed to copper solutions in 250-ml, acid-washed, polyethylene plastic food containers. The first test used five replicates each of 0, 32, 56, 100, 180, and 320 µg/liter copper. The second and third tests used nominal

copper concentrations of 0, 100, 180, 320, 560 or 1000 µg/liter. Each container held 200 ml of solution (salinity 33 ‰).

Containers were placed in an incubator 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). Analysis of Variance followed by Dunnett's multiple comparison test were used to derive NOEC's, and probit analysis was used to calculate LC_{50}'s and 95 % confidence intervals for all tests.

Chemical and Physical Measurements

Physical parameters for the fertlization, embryo, and larval tests were measured in each test concentration at the beginning and end of each test. Dissolved oxygen was measured using a Lozar D.O. 166 microprobe. The pH was measured using an Orion® Model SA 50 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 at 48 and 96 hours in the larval tests.

One random sample of each test concentration was taken at the beginning and end of each test for chemical verification. For the fertilization tests, the combined contents of both well containers were measured. For the embryo tests, the combined contents of 5 glass tissue culture tubes were analyzed. Total copper concentrations were verified using a Perkin Elmer Model 6003 Atomic Absorption Spectrophotometer.

Results

Induced Spawning

The mean number and percentage viability of embryos produced after each temperature spike are summarized in Table 1. Embryo yield was greatest on the fourth day after each temperature spike, declining thereafter. Embryo viability was generally high (> 80 %) but tended to decline near the end of each cycle (days 8 and 9).

Table 1. Mean number of embryos produced and percentage viability on days 1-9 of six temperature spike cycles. East tank had 21 female fish and 16 male fish; West tank had 13 female fish and 20 male fish.

	EAST TANK		WEST TANK	
Days After Temperature Spike	N	Percent Viable	N	Percent Viable
1	258	88	361	92
2	364	99	141	88
3	322	95	239	95
4	710	95	1395	95
5	130	82	344	93
6	135	87	285	94
7	344	97	66	91
8	190	89	308	67
9	30	54	159	91

The developmental stages of all embryos were determined after collection, usually between 0700 and 0900 each morning. A comparison of these with known developmental rates at 18-20 °C indicated that all reproductive activity occurred during darkness. Approximately 70 percent of the reproductive activity occurred between 2000 and 2200, the period just after lights-out, and approximately 23 percent occurred between 2400 and 0200, the 'tidal signal' period of reduced current velocity. The remaining 7 percent of reproductive activity occurred randomly during the night. It is possible that this small percentage represented embryos that had arrested development at various embryonic stages.

Fertilization Tests

The No Observed Effect Concentrations (NOEC's) for the four fertilization tests ranged between 32 and $\geq$100 µg/liter (Table 2, Figure 14). The EC_{50}'s (the concentrations at which the percent fertilization was reduced to 50 percent) ranged between 17 and 178. Control fertilization rates were 97, 90, 82, and 62 percent for tests one through four respectively. There were no fertilized eggs in any of the no-sperm control replicates. Between replicate variability was high in some cases, particularly at 100 µg/liter in the second test (Fig. 14).

Table 2. Summary of results of Phase 4 topsmelt toxicity tests. The sensitivity of three different developmental stages were compared using copper chloride as a reference toxicant: sperm, embryos, and larvae. For each toxicity test, the No Observed Effect Concentration (NOEC) and Effective Concentration (EC_{50}) or Lethal Concentration (LC_{50}) were calculated. See text for test methods and an explanation of endpoints.

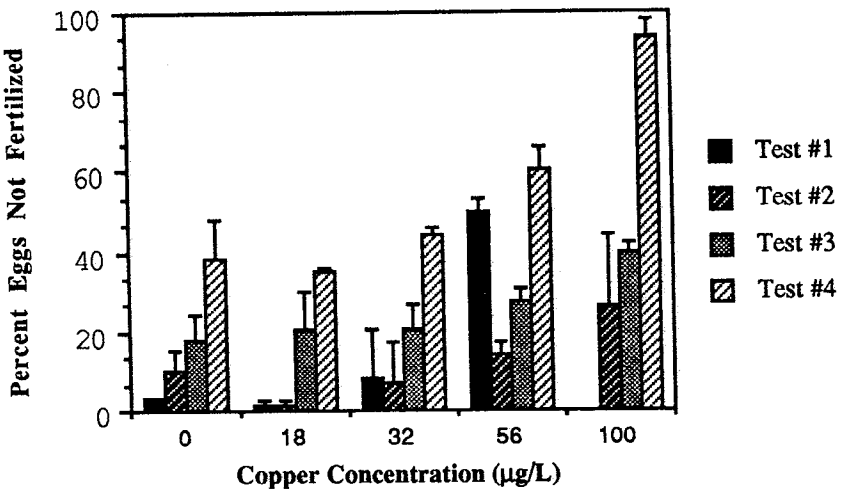
48-h Fertilization Tests	NOEC	EC50	(95% CI)
1	32	54	(48-65)
2	≥100	178	(121-534)
3	56	128	(76-903)
4	32	17	(11-21)
mean ± standard deviation	55.0 ± 32.1	94.3 ± 72.4	

12-d Embryo Tests	NOEC	EC_{50}	(95% CI)
Embryo Abnormalities			
1	100	150	(115-241)
2	100	175	(nd)
3	56	128	(108-153)
mean ± standard deviation	85.3 ± 25.4	152.3 ± 25.1	
Larval Hatching			
1	100	170	(123-331)
2	100	151	(105-309)
3	56	120	(102-142)
mean ± standard deviation	85.3 ± 25.4	147.0 ± 25.2	
Larval Abnormalities			
1	56	68	(62-75)
2	56	188	(162-230)
3	56	209	(177-268)
mean ± standard deviation	56.0 ± 0	155.0 ± 76.1	

96-h Larval Tests	NOEC	LC_{50}	(95% CI)
1	180	324	(252-536)
2	100	208	(177-242)
3	100	262	(229-300)
mean ± standard deviation	126.7 ± 46.2	264.7 ± 58.0	

nd = not determined

Figure 14. Effects of copper chloride on topsmelt fertilization in four, 48-h fertilization tests (mean ± s.d.; n = 2 for each test)



Variability in fertilization test results was associated with variability in gamete density in the test containers. Sperm densities in the test containers were 5.8×10^5, 4.4×10^5, and 14.1×10^5 in test two through four respectively. The sperm density was not measured in test number one. Mean egg numbers in the test containers were 56 ± 35 (n = 8) in test #1; 40 ±13 (n = 10) in test #2; 15 ± 4 (n = 10) in test #3; and 33 ± 7 (n=10) in test #4.

Embryo Tests

The NOEC's for embryo abnormalities ranged between 56 and 100 µg/liter in three tests (Table 2, Figure 15). The EC_{50}'s ranged between 128 and 175 µg/liter. The photomicrographs in Figures 16a (normal embryo) and 16b illustrates topsmelt embryo abnormality resulting from elevated copper concentrations. A 10-day-old topsmelt embryo in a control replicate just prior to hatching is of normal size and nearly fills the egg case (Figure 16a). A 10-day-old embryo in 180 µg/liter copper is stunted and has a bent notochord (scoliosis) (Figure 16b).

Figure 15. Effects of copper chloride on topsmelt embryo development in three tests. "Percent Abnormal Embryos" is the percentage of embryos with visible terata of the total number of embryos exposed (n = 20 for each test).

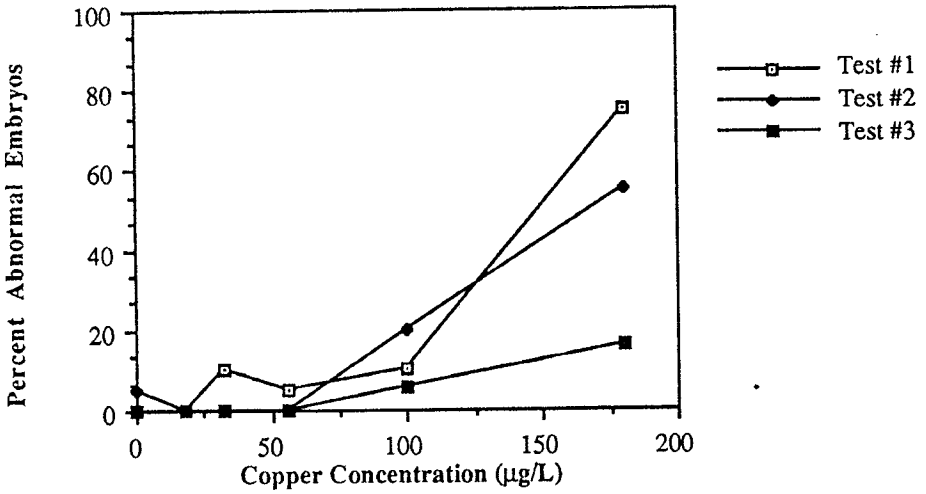
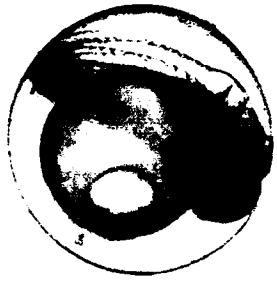


Figure 16 a-b. Photomicrographs of 10 day-old topsmelt embryos 2 days before hatching: (a) normal control embryo, (b) abnormal embryo in 180 µg/liter copper displaying stunting and scoliosis.

(a)

(b)

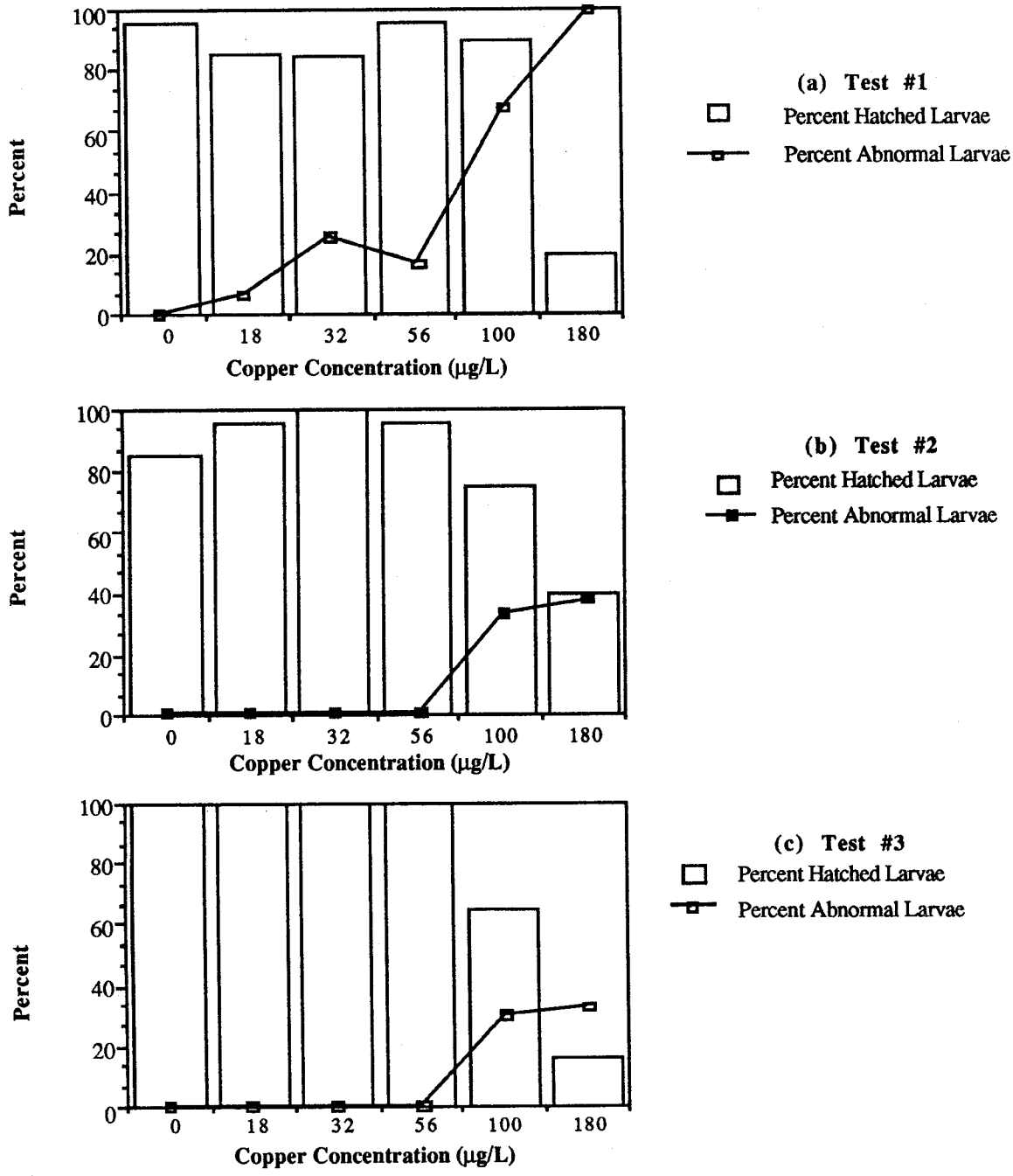


The majority of observed abnormalities at 100 µg/liter copper consisted of skeletal defects, usually stunting or scoliosis (Table 3). At 180 µg/liter, the embryos exhibited a variety of terata including slight convergence of the eyes, greatly reduced circulation or hemostasis, and stunting and/or scoliosis. The NOEC's for larval hatching success, the second endpoint for the embryo tests, paralleled those for embryo abnormalties. The NOEC's for larval hatching success were between 56 and 100 µg/liter (Figure 17 a-c Table 2). The EC_{50}'s for hatching success were between 120 and 170 µg/liter for the three tests. The NOEC's for larval abnormalities, the third endpoint, were consistently lower than the other endpoints in these experiments. The NOEC for larval abnormalities was 56 µg/liter for all three tests; the EC_{50}'s were between 68 and 209 µg/liter (Figure 17 a through c; Table 2).

Table 3. Summary of terata observed in embryos at the highest copper concentrations in three topsmelt embryo experiments. Percentages are the proportions of observed abnormalities tallied at the end of the experiment divided by the number of embryos exposed. Many embryos had multiple abnormalities, so percentages are not additive. Also given are the proportion of embryos that died, the proportion that hatched, and the proportion of hatched larvae with terata. CF = craniofacial abnormalities; CV = cardiovascular abnormalities; SK = skeletal abnormalities; After Weiss and Weiss (1982).

Concentration (µg/L)	CF	CV	SK	Dead	Hatched	Hatched with Terata
Test #1						
100	10%	10%	75%	10%	90%	67%
180	90%	90%	80%	80%	20%	100%
Test #2						
100	20%	30%	50%	25%	75%	33%
180	55%	60%	65%	60%	40%	38%
Test #3						
100	35%	35%	50%	35%	65%	30%
180	80%	85%	80%	85%	15%	33%

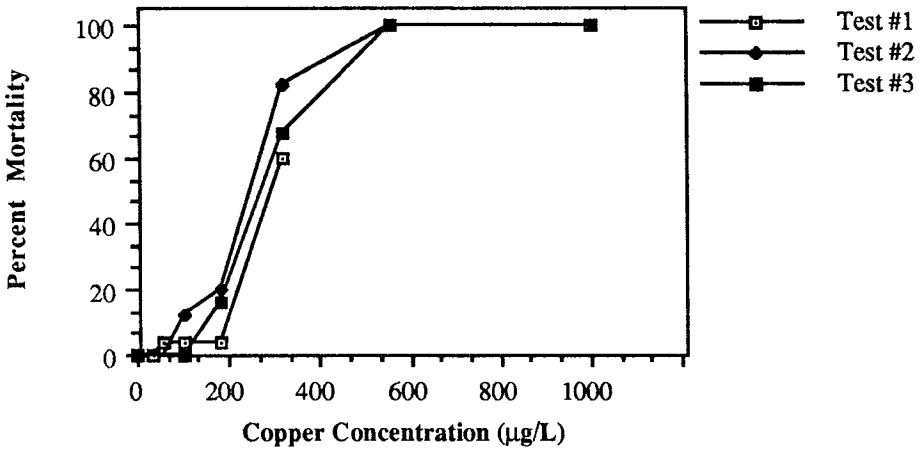
Figure 17 a-c. Effects of copper chloride on topsmelt embryos in 3 tests. "Percent Hatched Larvae" is the proportion of embryos developing and hatching as live larvae compared to the total number exposed. "Percent Abnormal Larvae" is the proportion of hatched larvae displaying visible terata compared to the total number hatched (n = 20 for each test).



Larval Tests

In the first larval test, the highest copper concentration used was 320 µg/liter. Because larval topsmelt mortality was relatively low between 180 and 320 µg/liter, higher copper concentrations were used in the second and third tests. Larval topsmelt mortality significantly increased at 320 µg/liter copper in Test #1 (NOEC = 180 µg/liter); mortality significantly increased at 180 µg/liter copper in Tests #2 and #3 (NOEC's = 100 µg/liter; Table 2, Figure 18). The LC_{50}'s were between 208 and 324 µg/liter in the three tests (Table 2).

Figure 18. Effects of copper chloride on survival of topsmelt larvae in three 96-h tests (n = 5 for each test).



Validation

Methods for using larval topsmelt in toxicity testing have been validated by two other laboratories. Singer *et al.* (in review) used these methods to evaluate the toxicity of the oil dispersant Corexit 9527™ in three separate trials. They found topsmelt mortality significantly increased above 12.3 ppm in each trial (mean NOEC = 13.5 ± 1.02 (s.d.), n = 3).

Researchers at the United States EPA have conducted over twenty-five 96-h mortality tests comparing the relative sensitivity of two larval topsmelt age classes to eleven separate pesticide compounds. These tests have used the same experimental methods described here. The results of these experiments indicate that larval topsmelt are equally or more sensitive than *Menidia beryllina* to the pesticides tested (personal communication, Michael Hemmer, U.S. EPA Environmental Research Laboratory, Gulf Breeze, FL).

Chemical and Physical Measurements

Water quality parameters were within normal ranges for all tests. In the four fertilization tests dissolved oxygen ranged from 5.0 to 6.9 mg/L; pH ranged from 7.9 to 8.0; temperature was 21 ± 1 °C; and salinity was 35 $^{o}/_{oo}$. In the three embryo tests, dissolved oxygen ranged from 5.6 to 6.8 mg/L; pH ranged from 7.1 to 7.7; temperature was 21 ± 1°C; and salinity was 33 $^{o}/_{oo}$. In the three 96-h larval tests, dissolved oxygen ranged from 5.9 to 7.2; pH ranged from 7.5 to 8.1; temperature was 21 ± 1 °C; and salinity ranged from 33 to 36 $^{o}/_{oo}$.

Analytically measured copper concentrations were close to nominal concentrations. Measured concentrations varied 9.6% ± 6.7% (mean variation ± standard deviation) from nominal concentrations, which is within the accuracy of the analytical technique.

Discussion

Induced Spawning

We were able to repeatedly induce laboratory spawning of topsmelt by providing a 14 to 18 hour increase in temperature ('spike' of 2 °C) at 7 to 9 day intervals. Our experimental design was based upon preliminary results provided by R. Hoffman. Hoffman induced limited reproduction of topsmelt in the laboratory by providing a single temperature 'spike' (unpublished observations, Marine Science Center, Oregon State University, Newport, OR).

Temperature fluctuations in combination with lighting schedules have also been used to spawn the red drum, *Sciaenops ocellatus*, and orangemouth corvina, *Cynoscion xanthurus* (Roberts, 1987; Prentice *et al.*, 1989). In red drum, spawning followed synchronized reductions in day length and temperature. Orangemouth corvina generally spawned 1 to 7 days after the temperature decline, most often in response to reductions of 2 to 6 °C. The photoperiod ranged from 10 to 14 hours of light. In nature, red drum and orangemouth corvina both spawn during the fall and winter months. Prentice *et al.* (1989) surmised that stable summer temperature and photoperiod regimes enhanced the effect of decreasing temperature on reproductive activity in orangemouth corvina.

Reproduction in topsmelt was enhanced by periodic increases in water temperature with fish maintained under a 14L:10D photoperiod. In California, topsmelt spawn from May through August, a period characterized by long days and elevated water temperatures in nearshore habitats (Carpelan, 1957; Carpelan, 1961). It seems likely that periodic temperature 'spikes' in this study mimicked the movement of topsmelt from cooler Pacific waters into warmer shallow waters to spawn. Typical surface water temperature during June at the mouth of the Elkhorn Slough is 14.9 °C, while June surface water temperature in the upper slough where topsmelt spawn is 19.5 °C (W. Hayden, unpublished observations,

Moss Landing Marine Laboratories, Moss Landing, CA; Broenkow and Smith , 1972). Croaker (1934) observed that topsmelt spawned during nighttime low tides in the upper reaches of Catalina Harbor.

Toxicity Tests

Our results indicate that topsmelt sperm cells are equally or more sensitive than embryos to copper, and embryos are more sensitive than larvae. Previous reports on the sensitivity of fish sperm to contaminants have focused primarily on freshwater fish. Shaw and Brown (1971), and Billard and Roubaud (1985) found no effect on rainbow trout (*Salmo gairdneri*) fertilization after exposing sperm to relatively high copper concentrations (1 and 5 mg copper/liter, respectively). Billard and Roubaud found that lower copper concentrations enhanced fertilization, possibly by controlling fungal infections. Khan and Weiss (1987) found that 0.05 mg mercury/liter reduced fertilization success in the estuarine killifish (*Fundulus heteroclitus*).

A fish sperm cell test provides an attractive alternative to embryo tests for routine toxicity testing because the test can be conducted quickly and requires less effort to obtain test organisms. Our results should be considered preliminary, however, because the copper NOEC's for the fertilization tests were variable, ranging between 32 and ≥100 µg/liter for the four tests (mean ± s.d. = 55.0 ± 32.1; n = 4). The highest calculated NOEC (> 100 µg/liter) was the result of high variability between the 100 µg/liter replicates (Figure 14, standard deviation = 17.7). Differences in sperm and egg density and the limited number of replicates used both contributed to statistical differences between tests. Because topsmelt sperm viability decreases after 15 minutes, standardization of gamete density is dependant on developing a rapid method for counting sperm. Sperm activation may be delayed by not exposing the sperm to seawater until after gamete density has been determined; this may extend sperm viability. In addition, between-test variability might be reduced by using mixtures of sperm from several male fish. By manipulating the sperm to egg ratio it may also be possible to increase the sensitivity of the test, as has been demonstrated with echinoderms (Dinnel et al. 1987).

Results of the three topsmelt embryo tests were relatively consistent. Of the three endpoints monitored, visible abnormalities in the hatched larvae were the most sensitive indicator of copper toxicity. Exposed embryos exhibited a significantly greater number of terata than the control embryos at copper concentrations above a mean NOEC of 85.3 ± 25.4 µg/liter (n = 3). The NOEC's for hatching success parallelled those for embryo abnormalities. Larval abnormalities resulted in a mean NOEC of 56 µg/liter ± 0 (n = 3). The mean $EC_{50's}$ for embryo abnormalities and larval hatching success were 152 (± 25.1) µg/liter and 147 (± 25.2) µg/liter respectively. The mean EC_{50} for larval abnormalities was 155 (± 76.1) µg/liter.

In all embryo experiments, skeletal and heart abnormalities were the first obvious terata to appear. These were usually manifested in the 100 and 180 µg/liter copper concentrations at five to six days after fertilization, when the embryos were at stages 27-28 (after Lagler 1962). Craniofacial abnormalities (eye

convergence) were apparent at stages 29-30. Increased mortalities began to occur two or three days before hatching, an observation similar to the findings of Engel and Sunda (1979) using silversides *Menidia menidia*. The most obvious terata displayed by the topsmelt larvae in our experiments were skeletal deformities (scoliosis and stunting).

Results of the three 96-h larval tests indicated that 10-day-old topsmelt larvae were less sensitive to copper than were sperm or embryos. The mean NOEC for the three experiments was 126.7 ± 46.2 µg/liter; the mean LC_{50} was 264.7 ± 58 µg/liter (n = 3).

Our results are consistent with research comparing the relative sensitivities of embryos and larvae of other marine species. Rice and Harrison (1978) found that embryos of herring (*Clupea harengus*) were more sensitive to copper than larvae. They found significant mortality in herring embryos at 38 µg/liter copper (NOEC = 28 µg/liter), and significant larval mortality at 274 µg/liter copper (NOEC < 274 µg/liter). Rice et al. (1980) also found embryos of northern anchovy (*Engraulis mordax*) to be more sensitive to copper than larvae; NOEC's for anchovy were less than 92 µg/liter and less than 277 µg/liter for embryos and larvae respectively . Topsmelt embryos appeared to be less sensitive than herring embryos, but more sensitive than anchovy. Copper LC_{50}'s for topsmelt larvae were comparable to values reported for other atherinids. Copper LC_{50}'s for *Menidia menidia* larvae ranged between 67 and 217 µg/liter (Cardin; 96-h flow-through tests, U.S. EPA, personal communication). A copper 96-h LC_{50} (static) of 140 µg/liter has been reported for *M. peninsulae* (Mayer, 1987).

Although copper concentrations in sewage effluents are sometimes within the range toxic to topsmelt, in general, ambient concentrations are considerably lower. Average annual copper concentrations compiled for 1988 for 5 major southern California municipal sewage effluents ranged between 32 and 58 µg/liter, with flow rates ranging between 704 and 1408 million liters per day (H. Schafer, Southern California Coastal Water Research Project, personal communication). Ambient concentrations for California coastal waters ranged between 0.5 and 6.0 µg/liter (Harrison 1982). We conclude that localized increases in copper concentration such as those resulting from discharge of treated cooling waters from power plants (Martin *et al.*, 1977) could impair topsmelt reproduction.

Summary

1. A combination of environmental cues (lighting, temperature, and tidal) were used to induce spawning of topsmelt held in a laboratory culture system. A 14 to 18 hour increase in temperature ('spike' of 2 ° C) was found to be the most important factor necessary for spawning induction. Spawning occurred at night.

2. Of three early life stages compared (sperm, embryos, and larvae), topsmelt sperm were more sensitive to copper chloride. The mean NOEC of four 48-hour sperm tests was 55 µg/liter copper.

3. In 12-day copper embryo exposure experiments, developmental abnormalities in embryos and hatching success of larvae were less sensitive endpoints than larval abnormalities. The mean NOEC for embryo abnormalities and hatching success was 85 µg/liter copper in three experiments. The mean NOEC for larval abnormalities was 56 µg/liter copper for the three tests.

4. Ten-day-old topsmelt larvae were the least sensitive stage tested. The mean NOEC for three 96-hour larval tests was 127 µg/liter copper.

5. Future experiments will focus on the continued development of a 48-hour topsmelt fertilization test, and a 12-day embryo development test.

References

Allen, L.G. and M.H. Horn. 1975. Abundance, diversity and seasonality of fishes in Colorado Lagoon, Alamitos Bay, California. Estuar. Coastal Mar. Sci. 3, 371- 380.

Allen, L.G. 1980. Structure and productivity of the littoral fish assemblage of upper Newport Bay, California. PhD. Dissert., Univ. of So. Calif. 175 p.

Allen, L.G. 1982. Seasonal abundance, composition, and productivity of the littoral fish assemblage in upper Newport Bay, California. Fish. Bull., 80 (4), 769-790.

Billard, R., and P. Roubaud. 1985. The effects of metal and cyanide on fertilization in rainbow trout (*Salmo gairdneri*). Water Res. 19 (2), 209-214.

Borthwick, P.W., J.M. Patrick, and D.P. Middaugh. 1985. Comparative acute sensitivities of early life stages of atherinid fishes to chlorpyrifos and thiobencarb. Arch. Environ. Contam. Toxicol. 14, 465-473.

Broenkow, W. W. and R .E. Smith. 1972. Hydrographic observations in Elkhorn Slough and Moss Landing harbor, California, October 1970 to November 1971. Office of Sea Grant Programs Annual Report, Part 3, July 1972. Moss Landing Marine Laboratories Technical Publication 72-3.

Carpelan, L. H. 1957. Hydrobiology of Alviso Salt Ponds. Ecology 38 (3), 375-390.

Carpelan, L. H. 1961. Salinity tolerances of some fishes of a southern California coastal lagoon. Copeia, 32-39.

Croaker, R. S.. 1934. The spawning of *Atherinops insularum insularum*, Copeia 1934:43.

Dinnel, P.A., J.M. Link, and Q.J. Stober. 1987. Improved methodology for a sea urchin sperm cell bioassay for marine waters. Arch. Environ. Contam. Toxicol. 16, 23-32.

Ehrlich, K.F., J.M. Hood, G. Muszynski, and G.E. McGowen. 1979. Thermal behavioral responses of selected California littoral fishes. Fish. Bull. 76 (4), 837-849.

Engel, D.W. and W.G. Sunda. 1979. Toxicity of cupric ion to eggs of the spot *Leiostomus xanthurus* and the Atlantic silverside *Menidia menidia*. Mar. Biol. 50, 121-126.

Fronk, R.H. 1969. Biology of *Atherinops affinis* Hubbs in Newport Bay. M.S. Thesis, Univ. of Calif., Irvine. 106p.

Harrison, F.L. 1982. A review of the impact of copper released into marine and estuarine environments. Report to the U.S. Nuclear Regulatory Commision. NUREG/CR-2823. UCRL-53042. pp 119.

Horn, M.H. 1980. Diel and seasonal variation in abudance and diversity of shallow-water fish populations in Morro Bay, California. Fish. Bull.. 78, 759-770.

Hose, J.E., R.J. Stoffel, and K. E. Zerba. 1983. Behavioral responses of selected marine fishes to chlorinated seawater. Mar. Env. Res. 9, 37-59.

Khan, A.T. and J.S. Weiss. 1987. Effects of methylmercury on sperm and egg viability of two populations of killifish (*Fundulus heteroclitus*). Arch. Environ, Contam. Toxicol. 16, 499-505.

Lagler, K.F., J.E. Bardach, and R.R. Miller. 1962. *Ichthyology.* John Wiley and Sons, New York. pp 545.

Martin, M., M.D. Stephenson, and J. H. Martin. 1977. Copper toxicity experiments in relation to abalone deaths observed in a power plant's cooling waters. Calif. Fish and Game. 63, 95-100.

Mayer, F. L. Jr. 1987. Acute toxicity handbook of chemicals to estuarine organisms. U. S. EPA Office of Research and Development. Environmental Research Laboratory, Gulf Breeze FL. EPA/600/8-87/017.

McHugh, J.L., and B.W. Walker. 1947. Rearing marine fishes in the laboratory. Calif Fish and Game. 34, 37-38.

Middaugh, D.P., and M.J. Hemmer. 1984. Spawning of the tidewater silverside *Menidia peninsulae* (Goode and Bean) in response to tidal and lighting schedules in the laboratory. Estuaries 7 (2), 139 - 148.

Middaugh, D.P., M.J. Hemmer, and L.R. Goodman. 1987. Methods for spawning, culturing and conducting toxicity tests with early life stages of four atherinid fishes: the inland silverside, *Menidia beryllina*, Atlantiic silverside, *M. menidia*, tidewater silverside, *M. peninsulae* and California grunion, *Leuresthes tenuis*. U.S. EPA 600-8-87-004. 56 pp.

Middaugh, D.P. and J.M. Shenker. 1988. Salinity tolerance of young topsmelt, *Atherinops affinis*, cultured in the laboratory. Calif. Fish Game, 74 (4), 232-235

Middaugh, D.P., M.J. Hemmer, and E.M. Lores. 1988. Teratological effects of 2,4- dinitrophenol, 'produced water' and naphthalene on embryos of the inland silverside *Menidia beryllina*. Dis. Aquat. Org. 4, 53-65.

Middaugh, D. P., M. J. Hemmer, J. M. Shenker, and T. Takita. 1990. Laboratory culture of jacksmelt, *Atherinopsis califoriensis*, and topsmelt, *Atherinops affinis* (Pisces:Atherinidae) with a description of larvae. Calif. Fish and Game. In Press.

Miller, D.J. and R.N. Lea. 1972. Guide to the coastal marine fishes of California. Calif. Dept. Fish Game, Fish Bull. 157, 1-235.

Prentice, J. A., R. L. Colura and B. W. Baumguardner. 1989. Observations on induced maturation and spawning of orangemouth corvina. Calif. Fish and Game. 75 (1), 27-32.

Quast, J. C. 1968. Observations on the food of the kelp-bed fishes. Calif. Dept. Fish and Game, Fish Bull. 139: 109-142.

Rice, D.W. and F.L. Harrison. 1978. Copper sensitivity of pacific herring *Clupea harengus pallasi* during its early life history. Fish. Bull. 76 (2), 347-356.

Rice, D.W., F.L. Harrison, and A. Jearld. 1980. Effects of copper on early life stages of northern anchovy *Engraulis mordax*. Fish. Bull. 78 (3), 675-683.

Roberts, D. E. , Jr. 1987. Photoperiod / temperature control in the commercial production of red drum (*Sciaenops ocellatus*) eggs. *In* G.W. Chamberlain, R. J. Miget, and M. G. Hardy, eds. Manual on red drum aquaculture. Texas Agricultural Extension Service and Sea Grant College Program, Texas A&M Univ. College Station. 470 pp.

Shaw, T.L. and V.M. Brown. 1971. Heavy metals and the fertilization of rainbow trout eggs. Nature 230, 251.

Singer, M. M., D.L. Smalheer, and M. Martin. In Press. Toxicity of an oil dispersant to the early life stages of four California marine species. Environ. Tox. and Chem..

Sokal, R.R. and F.J. Rohlf. 1969. *Biometry.* W.H. Freeman and Company, San Francisco. 859 pp.

Young, D.R, T-K. Jan, and M.D. Moore. 1977. Metals in power plant cooling waters discharge. In: W. Bascom (ed.) Coastal Water Research Project: Annual Report.

Weiss, P., and J.S. Weiss. 1982. Toxicity of methylmercury, mercuric chloride, and lead in killifish, *Fudulus heteroclitus*, from Southhampton, New York. Environ. Res. 28, 364-374.

Section 4

Training Workshops and Protocol Evaluation

Introduction

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

Regional Water Quality Control Board Workshops

Seminar-style workshops gave regional board staffs an overview of the two MBP protocols. One seminar, held at the offices of the Southern California Coastal Water Research Project in Long Beach, included representatives of Regions Three, Four, Eight, and Nine. A second seminar, held at the Santa Rosa Regional Water Quality Control Board, included Region One staff. Each seminar consisted of a one-hour slide presentation for each of the two toxicity tests, followed by a technical question and answer session addressing details of the protocols. Representatives from the State Water Resources Control Board also participated in the regional workshops to answer specific questions concerning the California Water Code, the California Ocean Plan, and details and timing of toxicity test implementation.

Hands-On Technical Workshops

Two one-day workshops, held at MPSL on August 30 and 31, 1989 familiarized discharger biologists with the red abalone and giant kelp toxicity tests. The workshop format allowed each participant to conduct every aspect of both protocols to facilitate a technical exchange between the researchers who developed the protocols and those who will conduct the tests on a routine basis once implementation proceeds. (A list of the workshop participants follows the end of this section).

Abalone Workshop

The workshop devoted one day to the red abalone toxicity test. The workshop began with a slide presentation of the protocol, so that participants would receive an overview and could ask questions on

general principles and underlying assumptions. Participants then divided into three small groups that proceeded to a different work-station where each person conducted a segment of the toxicity test. Each group spent approximately one-and-a-half hours at each station, then rotated to the next. The following agenda lists the tasks performed at each of the red abalone protocol stations:

Red Abalone Toxicity Test Workshop Stations

STATION 1: TEST SOLUTIONS
- Reference Toxicants
  - Stock solutions
  - Standard dilution series
- Preparation of Hypersaline Brine
  - Types of commercial sea salts
  - Mixing and decanting brine
  - pH adjustment and salinity measurement
- Effluent Dilution
  - Standard dilution series
  - Salinity adjustment worksheet
- Water Quality Measurements
  - Temperature, salinity, pH, dissolved oxygen, ammonia
  - Buffers and standards
- Equipment:
  - volumetric flasks and pipets, stirrers, sea salts, thermometer, refractometer, ion analyzer and probes, water quality data sheet, dilution worksheet, effluent/brine worksheet.

STATION 2: TEST ORGANISMS
- Handling and inspecting broodstock
- Spawning induction
- Fertilization
- Washing and concentrating embryos
- Density estimation
- Delivery into test containers
- Equipment:
  - abalone broodstock, smooth blade knife, polyethylene buckets, tall beaker, plunger, 1 and 10 ml wide-bore pipets, handcounter, 0.2 µ-filtered seawater, spawning worksheet

STATION 3: PRESERVATION AND ANALYSIS OF LARVAE
- Sampling test solutions for water quality and chemical analysis
- Collecting larvae on sieve
- Transferring larvae to test tubes
- Preservation in formalin
- Microscopic analysis
- Endpoint interpretation
- Alternative tissue culture flask method
- Equipment:
  - 37 µm mesh sieve, funnel, test tubes (numbered randomly), tissue culture flasks, formalin, squirt bottles, disposable pipets, Sedgewick-Rafter counting slides, compound microscope, inverted compound microscope (for tissue culture flasks), randomization sheet, data sheet.

At Station One, participants performed serial dilutions of stock reference toxicants and effluents, prepared hypersaline brines, set up a standard reference toxicant test, and measured water quality parameters. At Station Two, participants learned about maintenance of abalone broodstock, induced male and female

abalone to spawn using hydrogen peroxide, fertilized abalone eggs and then counted and delivered embryos to test containers. At Station Three, participants terminated a standard zinc toxicity test using two methods for isolation and preservation of the larval abalone. Participants analyzed representative test containers, and compared their results to previous zinc reference toxicant test results.

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

Kelp Workshop

The kelp workshop followed the same format as described above, except that instead of reiterating methods of mixing toxicant solutions, a station was devoted to data analysis and computer formats.

Giant Kelp Toxicity Test Work Stations

STATION 1: SPOROPHYLL PREPARATION and SPORE RELEASE
- Sporophyll collection
- Washing sporophylls
- Preparing sporophylls for storage and transport
- Zoospore release
- Test container set-up
- Determination of spore viability
- Fixing spore samples and estimating density
- Innoculation of test containers
- Equipment:
  - sporophylls, 1-liter beaker, 0.2 µm-filtered seawater, paper towels, clean tray, test tubes (each marked for 9 and 10 ml volumes), disposable pipets, hemacytometers, compound microscope (100x), sporophylls worksheet

STATION 2: ANALYSIS OF SPORES AND GAMETOPHYTES.
- Sampling test solutions for water quality and chemical analysis
- Removal of settling slides from test containers
- Microscopic analysis of spores and gametophytes
- Germination endpoint determination
- Growth endpoint determination
- Equipment:
  - compound microscope, data sheets.

STATION 3: STATISTICAL ANALYSIS OF TOXICITY TEST DATA.
- Data preparation and transformation
- Experimental design
- Statistical tests
  - ANOVA (Analysis of Variance)
  - Dunnett's Multiple Comparison
  - Probit
- Computer programs for Macintosh
- Computer formats for reporting raw data to central data base

At Station One, participants sorted and prepared *Macrocystis* reproductive blades (sporophylls) for zoospore release, used a hemacytometer to estimate spore density, and innoculated test solutions with zoospores. At Station Two, participants learned how to identify and count germinated and non-germinated spores, and measured gametophyte germ-tube growth. Station Three topics included experimental design, data input and transformation, statistical analysis (Analysis of Variance, Dunnett's test) and No Observed Effect Concentration (NOEC) calculation, and central data base design.

Workshop Evaluation

By the conclusion of the two workshops, each person had participated in all aspects of the two toxicity tests. They were each asked to complete a questionaire to provide the MBP with written comments on technical and general aspects of the abalone and kelp toxicity test protocols, and on format and utility of the workshops themselves. The specific questions and answers follow the end of this section. In general, responses to the questionaires were positive, and indicated that the hands-on approach of the workshops provided adequate training. Protocol changes reflect modifications suggested by the participants.

The MBP invited representatives of the eight entities currently discharging more than 100 million gallons per day to marine waters. The following dischargers received an invitation and agenda for the August 30 - 31 workshops:

City of Los Angeles Department of Public Works (Hyperion)
City of San Diego Water Utilities Department (Point Loma)
Los Angeles City Department of Water and Power
Los Angeles County Sanitation Districts (JWPCP)
Orange County Sanitation Districts
Pacific Gas and Electric
San Diego Gas and Electric
Southern California Edison.

We thank the workshop participants for their suggestions and cooperation:

Tom Gerlinger, Orange County Sanitation District
Sally Krenn and Ted Holmbeck, Pacific Gas and Electric
Mike Mullin, Hyperion Waste Treatment Plant
Ken Nakada and Tom Parker, JWPCP
Tim Rothans, City of San Diego Water Utilities Department

The following is a list of questions asked of the participants at the end of the workshop. The first questions concern the two toxicity test protocols; the final questions concern the format and contents of the workshops. All responses are verbatim.

Abalone Protocol

Is toxicity testing with red abalone ecologically or scientifically relevant to the ocean outfall that you monitor?

1. Yes to both. Seems like a very appropriate test. Unsure of sensitivity to effluents compared to other suggested test species.

2. Typical discharge area, ecologically, abalone never have occurred 2 miles off shore at 61 m on mud bottoms: so that's an issue. But as a nearshore, shallow water indicator it can serve as a good sentinel. Strength of this use will be improved when numerous complex effluents are run to measure variability of seasons and environmental changes. Also needs some measure of component toxicant as would be seen in TRE type program.

3. Yes, because Hyperion discharges into Santa Monica Bay. Testing a marine species is better than testing a freshwater species. However, abalone are not found on the sandy bottom in Santa Monica Bay.

4. Not really, we have no rocky substrate or kelp beds within 10 miles.

5. Yes, red abalone are common in the cooling water effluent at the Diablo Canyon Power Plant.

6. Black abalone are primarily the most prevalent species located near our outfall. I have no knowledge/data if the red abalone is more or less sensitive than the black abalone. Seasonal variability is a problem.

What changes would you make in the abalone protocol?

1. Possibly clean up distinction between abnormal (>1 dent) and normal.

2. Set up dilutions all at once -not as single flasks. Use tissue culture flasks and incubator for toxicity test. Use P.C. based system to record, measure and differentiate shell perimeter and tissue density irregularities. This eliminates transcription error of paper record, eases storage, analysis and transmission of ever growing data sets. Investigate elimination of formalin fixative to ease OSHA health issues.

3. Move toward glass culture tubes for ease of handling and avoiding formalin. Use slides or tubes with grids for easy counting; what about archiving and QA/QC of samples?

4. Refine inverted scope methods with a grid on container. The formalin smell on open slides is bad.

5. In the veliger counts, a grid placed under the slides would be helpful, or a grid under the tissue culture containers.

6. The number of replication! Do you need these replications for statistical reasons?

How difficult will it be to train technicians at your laboratory to make distinctions between normal and abnormal abalone larvae, based on the criteria described in the protocol?

1. Not difficult, especially after they run a few tests.

2. Not difficult. It may become advantageous to establish P.C.-image-analysis to automate and facilitate scoring, collecting and analysis of data. Initial cost of such a set-up would best be justified for QA of long term monitoring (NPDES) program.

3. It will be easy based on the criteria of one dent + thin shell = normal. Reference slides could be issued by MPSL along with photographs.

4. Should not be too difficult.

5. Many of our technicians have worked with larval invertebrates (incl. red abalone veligers).

6. Not difficult at all; pretty straight forward and not as time consuming as I thought.

Is toxicity testing with red abalone feasible at your laboratory at the present time? If not, what facilities, equipment, personnel or training would be needed to conduct this test on a routine basis (say monthly)?

1. Not feasible at this time. Limited on laboratory space. Equipment not really a problem. Space, personnel and costs (of new space) prohibitive to running these tests in-house.

2. Not feasible at present time. New building, supply of seawater and specimens would have to be established. At least two additional staff would be added to conduct testing.

3. We conducted an interlab bioassay under makeshift conditions. To conduct this test on a routine basis, we would need a reliable water bath or incubator, a large supply of 15°C 1µm filtered seawater, acid washing facilities.

4. No, we have a 2000 ft^2 lab in the works to be ready by Jan. 1992.

5. Yes, very feasible. We have around 6000 red abalone in our lab tanks.

6. We need clean sea water, storage, collection vehicle, temp. controlled room, animal holding facilities, more personnel; a bigger lab all in all.

What types of computer facilities are available at your laboratory for data analysis, storage and transmission?

1. Mainframe system (with SAS). Personal computers (IBM, XT, AT and PJ-Z's) with all necessary software. Macintosh. Modems and mainframe capability of producing tapes if this is necessary.

2. IBM mainframe, PC, slave terminals. Storage of data is not a problem. Data analysis includes canned and custom programs. Transmission by modem, discs, tape etc.

3. VAX mainframe linked to IBM 386 with 60 meg hard disk, with modem hook up.

4. Full data analysis available on disks. Soon to be equipped for tape. Network system, modems plus carbon copy system.

5. Alpha micro, MAC, IBM, SAS, you name it.

6. IBM pc and Apple II; no modem; IBM mainframe.

Kelp Protocol

Is toxicity testing with *Macrocystis* ecologically or scientifically relevant to the ocean outfall that you monitor?

1. Yes. Large kelp bed located both north and south (and on) outfall pipe. Bed is cultivated heavily by Kelco and used for recreational diving and sportfishing.

2. Ecologically *Macrocystis* was heavily impacted by sewage from 1950 to about 1975. Since 1975 kelp has returned and expanded to major commercial beds. So its sensitive but no longer impacted by discharge at Palos Verdes.

3. Yes, because it is a marine species. However, *Macrocystis* isn't usually found on the sand bottom in Santa Monica Bay.

4. No. Closest beds 10 miles away.

5. Yes, *Macrocystis* grows in our discharge plume.

6. Yes, beds of *Macrocystis* nearby.

What changes would you make in the *Macrocystis* protocol?

3. Acid wash slides.

4. Measuring is difficult.

5. The data sheet on kelp sporophyll release needs to be edited. Time of Release isn't accurate; should be stated as time of observation.

6. Unfamiliar with testing, dislike seasonal variability and number of replication.

How difficult will it be to obtain sporophylls for your laboratory?

1. Fairly simple unless heavy storm activity and kelp beds thinned. In this case, we would spend more time diving for blades.

2. Not difficult, if collection is permitted from Palos Verdes.

3. It will be difficult because it might be impossible under L.A. City rules about diving. Hopefully, it can be worked out. Palos Verdes and Catalina are the two localities with *Macrocystis.*

4. Would have to send out or contract for collection.

5. Not at all. *Macrocystis* grows in our intake cove.

6. Need additional qualified personnel for collection and equipment pertaining to this.

Is toxicity testing with *Macrocystis* feasible at your laboratory at the present time? If not, what facilities, equipment, personnel or training would be needed to conduct this test on a routine basis (say monthly)?

1. Space limitations, would need to hire additional personnel, obtain equipment and lease space. Since diving is presently not required in any of our monitoring we would need to set up a program to collect sporophylls to take care of the city's liability concerns.
2. No. New building, support, staff.
3. We have a small water bath (15° C). Everything else needs to be obtained.
4. No. New lab planned by 1992. I would have to staff for all these tests.
5. Yes, we have the facilities and personnel. We would probably have to invest some budget money to update our equipment; construct a water bath.
6. Need new lab with temp. control room, additional equipment in collection, vehicle for water collection and storage. More personnel, etc.

What species are presently used and/or under consideration for toxicity testing at your facility?

1. Acute toxicity testing weekly on fathead minnow (effluent).
2. At JWPCP facility no testing is currently conducted. At San Jose Creek facility fish and *ceriodaphnia* are used.
3. Presently: *Pimephales*, adult acute 96 hr. and larvae chronic 168hr. *Ceriodaphnia*, larvae chronic 168hr. Future: *Selenastrum, Haliotis, Macrocystis*, Mysid, *Menidia*.
4. Fathead minnows (adult 96hr., monthly). TU usually 0 or <1.
5. Red abalone around 20 mm in length.
6. Fathead minnow presently used for acute toxicity tests. We are considering not doing any chronic tests that show seasonal changes, and no background work with industrial effluents. Industrial effluents vary hr/hr; day/day; etc.

Workshop

Would it be useful to hold this type of workshop later in another location? If so where?

1. If a major aspect of the protocols change then another workshop would be important. Carmel location is excellent. Perhaps a workshop demonstrating the protocols for any contractors the dischargers may be using (local area).
2. This location is fine.

3. No, because this is where the test was developed.

4. No. I like coming up here. But I guess the urchin sperm test workshop would be held at SCCWRP. We need a fish test workshop up here when you get ready.

5. Granite Canyon is a great spot!

6. Yes, nearby an eatery! Los Angeles, San Francisco, San Diego, etc.

What aspects of the workshop were most informative?

1. Techniques, time lines, both of the protocol and labor (man hours needed to run tests). Experience in identifying end points, especially with the red abalone.

2. The actual hands-on demonstrations.

3. Overviews of tests in the morning, objectives and reasons for testing, hands-on work, written protocols, meeting other dischargers.

4. Actually doing some of the procedures.

5. Laboratory techniques (I was pretty rusty). The protocols were well written, will be an asset towards setting up our lab for these bioassays.

6. Hands-on work.

What aspects of the workshop were unnecessary or redundant?

1. All of it was useful.

2. None, as an introductory workshop.

4. None

6. Making water dilutions, brine, reference toxicant.

How would you improve on the presentation, organization or content of the workshop?

1. Have a group discussion of possible improvements in techniques or analysis. Any idea sharing could take place during this discussion.

2. Present more information on variability by season, or source of specimens and water.

3. Develop photographic key for criteria on abalone and kelp. Good use of rotating groups.

4. I would have samples from each discharger, and have everyone completely set up and take down an entire test with each species. That way I could spend at least a week or two up here.

Would your laboratory be interested in conducting interlaboratory tests of the two protocols as part of a training or quality assurance exercise?

1. This sounds like a good idea but would probably only be feasible if the request came from the State Board. I would like to require this of whatever contract lab performs our tests.

2. I think this will be necessary, not only as a start-up procedure, but also as an on going QA/QC.

3. Yes.

4. If we were set up to run them, yes, but it will be a few years.

5. Yes.

6. Yes!

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. The protocols and QA/QC document together comprise a standard operating procedures manual for all MBP toxicity tests. A complete toxicity testing manual can be obtained from the State Water Resources Control Board upon request.

APPENDIX I

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 ($CuCl_2 \cdot 2H_20$) 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. 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 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 250 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 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 3.1.1. All volumetric flasks, pipets, and other labware

used for handling trace metal reference toxicant solutions must be cleaned as described in Section 3.1.2.

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.*, in review). 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. 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. Avoid immersing the blades in seawater, however, to prevent premature spore release. The sporophylls should be rinsed 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. 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 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 not 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 equillibrated 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. 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 $\mu E\ m^{-2}\ sec^{-1}$. 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. Remove the slide without decanting the test solution. 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. **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 length of the germination tube that is nearest the micrometer in each field; if more than one spore is touching the micrometer, both (or all) 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).

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 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 reference toxicant test; mean brine control germination must be at least 70% in the effluent test.
2) Mean germination-tube length in the controls must be at least 10 µm in the reference toxicant test; mean brine control germination-tube length must be at least 10 µm in the effluent test.
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 NOEC in the copper reference toxicant test must be below 110 µg/liter; the germination-tube growth NOEC must be below 35 µg/liter (chemically verified copper concentrations).

5) The between-replicate variability for the germination data must be low enough that the ANOVA Mean Square (MS) does not exceed 70.00 in the reference toxicant test (using arcsine transformed percentage germination data in degrees). This corresponds to a Dunnett's Standard Error (SE) of 5.29 (with n = 5 replicates).

6) The between-replicate variability for germ-tube growth data must be low enough that the ANOVA 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).

12.0 References

Abbott, I. A. and G. J. Hollenberg. 1976. *Marine Algae of California.* Stanford University Press, Stanford, CA. 827 pp.

Anderson, B. S., J. W. Hunt, S. L. Turpen, and M. Martin. In Press. Copper toxicity to microscopic stages of *Macrocystis pyrifera*: interpopulation comparisons and temporal variability. Mar. Ecol. Prog. Ser.

Anderson, B.S., J.W. Hunt, S.L Turpen, F.H. Palmer, and M. Martin. 1990. Marine Bioassay Project fifth report: protocol development and interlaboratory testing with complex effluents. State Water Resources Control Board. Sacramento, CA.

Anderson, B. S. and J. W. Hunt. 1988. Bioassay methods for evaluating the toxicity of heavy metals, biocides, and sewage effluent using microscopic stages of giant kelp *Macrocystis pyrifera* (Agardh): a preliminary report. Mar. Environ. Res. 26:113-134.

American Society for Testing and Materials. 1987. Annual Book of ASTM standards; water and environmental technology. Vol 11.04: 382-388.

Chung, I. K. and B. H. Brinkhuis. 1986. Copper effects in early life stages of kelp *Laminaria saccharina.* Mar. Pol. Bull. 17: 213-218.

Deysher, L. E. and T. A. Dean. 1984. Critical irradiance levels and the interactive effects of quantum irradiance and quantum dose on gametogenesis in the giant kelp, *Macrocystis pyrifera.* J. Phycol. 20: 520-524.

Hopkin, R. and J. M. Kain. 1978. The effects of some pollutants on survival, growth and respiration of *Laminaria hyperborea.* Est. and Coast. Mar. Science 7; 531-553.

Hunt, J.W., B.S. Anderson, S.L. Turpen, A.R. Coulon, M. Martin, F.H. Palmer, J.J. Janik. 1989. Marine Bioassay Project fourth report: experimental evaluation of effluent toxicity testing protocols with giant kelp, mysids, red abalone, and topsmelt. DWQ report No. 89-5WQ, State Water resources Control Board, Sacramento, California.

James, D. E., S. L. Manley, M. C. Carter, and W. J. North. 1987. Effects of PCBs and hydrazine in life processes in microscopic stages of selected brown seaweeds. Hydrobio. 151/152: 411-415.

Foster, M. S. and D. R. Schiel. 1985. The ecology of giant kelp forests in California: a community profile. U.S. Fish and Wild. Serv. Biol. Rep. 85 (7.2) 152 pp.

Kuwabara, J. S. 1981. Gametophytic growth by *Macrocystis pyrifera* (Phaeophyta) in response to various iron and zinc concentrations. J. Phycol. 17: 417-419.

Linfield, J.L.T., M. Martin, and J. Norton. 1985. Marine Bioassay Project first progress report: bioassay species selection and recommended protocols. California State Water Resources Control Board, Sacramento, California.

Luning, K. 1980. Critical levels of light and temperature regulating the gametogenesis of three Laminaria species (Phaeophyceae). J. Phycol. 16: 1-15.

North, W.J., ed. 1971. The Biology of Giant Kelp Beds (*Macrocystis*) in California. Nova Hedwigia, 32:1-600.

North, W. J. 1976. Aquaculture techniques for creating and restoring beds of giant kelp, *Macrocystis* spp. J. Fish. Res. Board Can. 33: 1015-1023.

Smith, B. M. and F. L. Harrison. 1978. Sensitivity of *Macrocystis* gametophytes to copper. U.S. Nuclear Regulatory Commission, NUREG/CR-0694; UCRL-52481. 1-34.

Sokal, R. R. and F. J. Rohlf. 1969. Biometry. W.H. Freeman and Company, San Francisco.

Thompson, R. S. and E. M. Burrows. 1984. The toxicity of copper, zinc, and mercury to the brown macroalga *Laminaria saccharina*. In: *Ecotoxicological Testing For The Marine Environment*. G Persoone, E. Jaspers, and C Claus (Eds). State Univ. Ghent and Inst. Mar. Scient. Res. Brendene, Belgium 2: 588pp.

Zar, J. H. 1974. Biostatistical Analysis. Prentice-Hall, Inc., Inglewood Cliff, N.J.

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. 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).

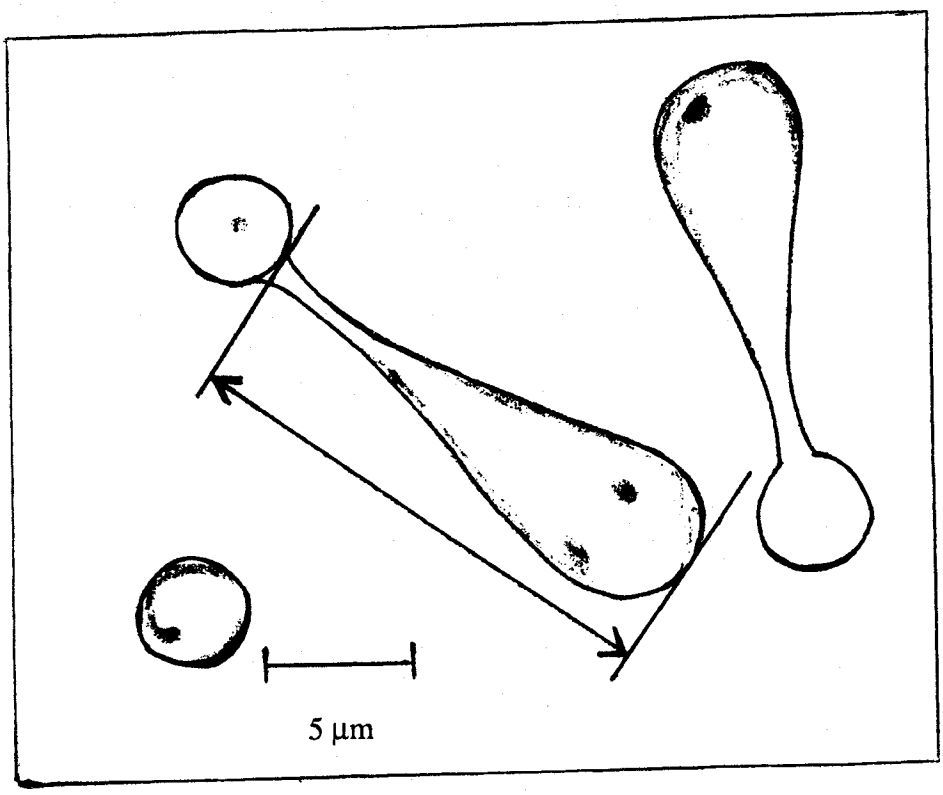

Figure 1. Microscopic view of non-germinated *Macrocystis* zoospore (bottom left) and two germinated spores. Arrow indicates length measured for the germ-tube growth endpoint.

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 blades are placed in release beaker: _____

Time blades are removed from release beaker: _____

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: _____

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	Date	Organism	Investigator
→	0.0 ml in 1000 ml flask →	Control			
→	0.0 ml in 1000 ml flask →	Brine Control	Notes		
→	_____ →	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

Kelp Toxicity Test Data Sheet

Test Start Date: | **Test End Date:** | NOEC:

Start Time: | **End Time:** | **Analyzed by:**

Microscope: | **Micrometer Conversion Factor:**

Toxicant: | **Ref. Tox. Chem. Sample Vial No.'s:**

Effluent Source: | **Collection Date:**

Test Container Number	Nominal Conc. $\mu g\ l^{-1}$ or %	Number Spores Germ.	Number Spores Not Germ.	Percent Not Germ.	Length Measurements (in ocular micrometer units)											Mean Length (in µm)
					L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	Mean	

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 II

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
  - ice chest and blue ice
- 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 flask.)
- 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° 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 and does not use harmful chemicals.

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. 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 the first 100 larvae encountered from each vial under 100x magnification. 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. 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 or effluent 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

American Society for Testing and Materials, 1987. Annual book of ASTM standards; water and environmental technology. Vol 11.04: 382 - 388.

Anderson, B.S., Hunt, J.W., Turpen, S.L., Palmer, F.H., and M. Martin. 1990. Marine Bioassay Project fifth report: interlaboratory toxicity testing and further protocol development with giant kelp, red abalone, and topsmelt. State Water Resources Control Board. Sacramento, California.

Anderson, B.S., J.W. Hunt, M. Martin, S.L. Turpen and F.H. Palmer. 1988. Marine Bioassay Project third report; protocol development: reference toxicant and initial complex effluent testing. DWQ report No. 88-7, State Water Resources Control Board, Sacramento, California.

Ault, J. 1985. Some quantitative aspects of reproduction and growth of the red abalone , *Haliotis rufescens* Swainson. J. World Maricult. Soc. 16: 398.

Bruland, K.W., R.P. Franks, G.A. Knauer, and J.H. Martin. 1979. Sampling and analytical methods for the determination of copper, cadmium, zinc, and nickel at the nannogram per liter level in seawater. Anal. Chem. Acta 150: 233 - 245.

Ebert, E. E. and J. L. Houk. 1984. Elements and innovations in the cultivation of red abalone *Haliotis rufescens*. Aquaculture. 39: 375-392.

Grigg, R.W. and R.S. Kiwala. 1970. Some ecological effects of discharged wastes on marine life. Calif. Fish Game 56: 145 - 155.

Hahn, K.O. 1989. *Handbook of culture of abalone and other marine gastropods*. CRC Press, Inc. Boca Raton, Florida. 348 pp.

Hines, A.H. and J.H. Pearse. 1982. Abalones, shells, and sea otters: dynamics of prey populations in central California. Ecology 63: 1547 - 1560.

Hunt, J.H. and B.S. Anderson. 1989. Sublethal effects of zinc and municipal effluents on larvae of the red abalone Haliotis rufescens. Mar. Biol. 101: 545 - 552.

Hunt, J.W., B.S. Anderson, S.L. Turpen, A.R. Coulon, M. Martin, F.H. Palmer, and J.J. Janik. 1989. Marine Bioassay Project fourth report: Experimental evaluation of effluent toxicity testing protocols with giant kelp, mysids, red abalone, and topsmelt. DWQ report No. 89-5WQ, State Water Resources Control Board, Sacramento, California.

Kikuchi, S. and N. Uki. 1974. Technical study on artificial spawning of abalone, genus *Haliotis* II. Effect of irradiated seawater with ultraviolet rays on inducing to spawn. Bull. Tohoku Reg. Fish. Res. Lab., 33: 79-86.

Kikuchi, S. and N. Uki. 1974. Technical study on artificial spawning of abalone, genus *Haliotis* IV. Duration of fertility related to temperature. Bull. Tohoku Reg. Fish. Res. Lab., 34: 73.

Leighton, D.L. 1974. The influence of temperature on larval and juvenile growth in three species of southern California abalones. Fishery Bulletin 72: 1137 - 1145.

Linfield, J.L.T., M. Martin and J. Norton. 1985. Marine Bioassay Project first progress report: bioassay species selection and recommended protocols. California State Water Resources Control Board, Sacramento, California.

Morris, R., D. Abbott, and E. Haderlie. 1980. Intertidal invertebrates of California. Stanford Univ. Press. 690pp.

Morse, D. E., H. Duncan, N. Hooker, and A. Morse. 1977. Hydrogen peroxide induces spawning in molluscs, with activation of prostaglandin endoperoxide synthetase. Science 196: 298-300.

Owen, B., J. McLean, and B. Meyer. 1971. Hybridization in the eastern Pacific abalone (*Haliotis*). Bull. Los Angeles County Museum Nat. Hist. Sci. (9).

Sokal, R. R. and F. J. Rohlf. 1969. Biometry. W.H. Freeman and Company, San Francisco.

Zar, J. H. 1974. Biostatistical Analysis. Prentice-Hall, Inc., Inglewood Cliff, N.J.

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.

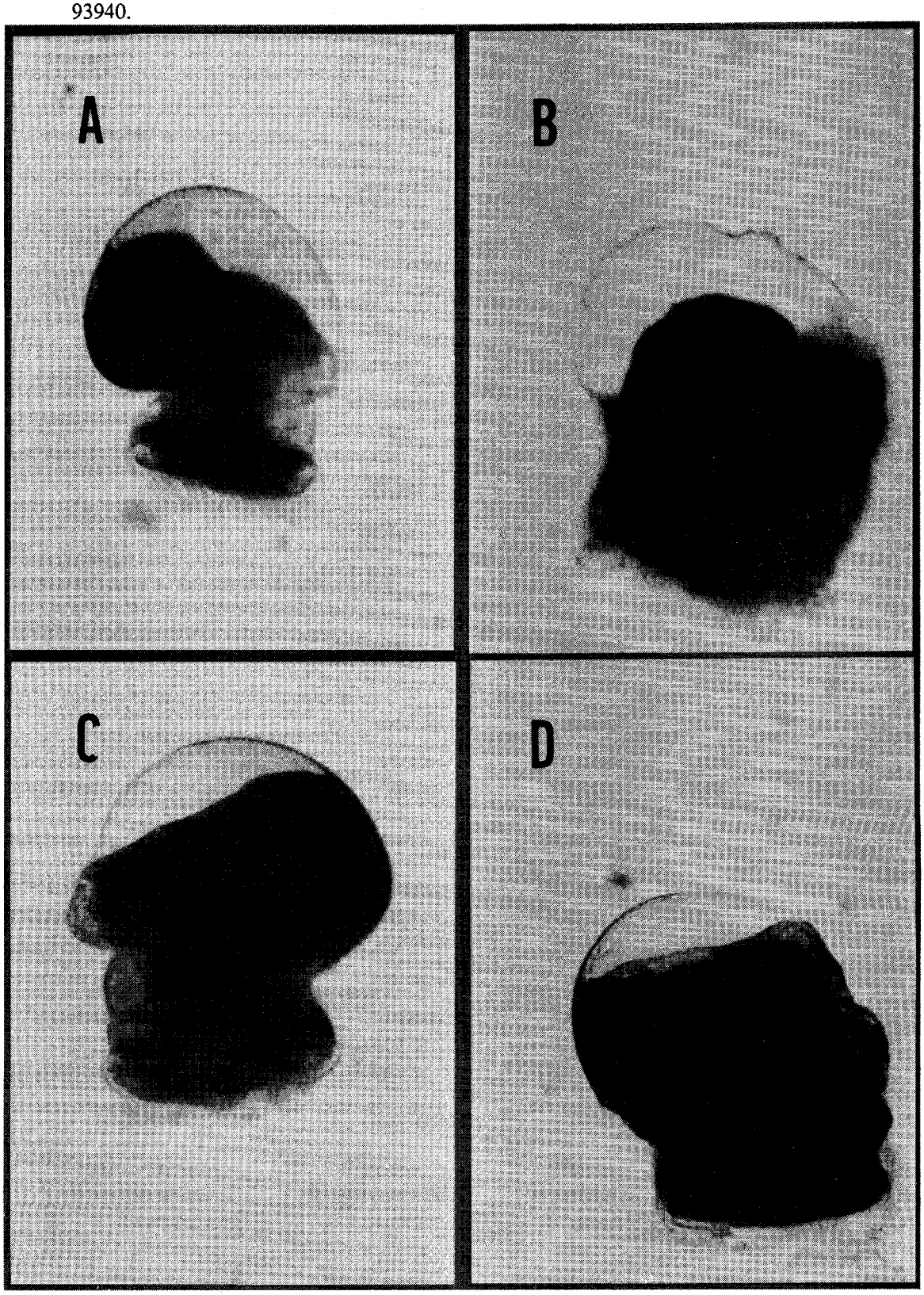
I. Normal larva with well calcified shell and one small indentation at leading edge.

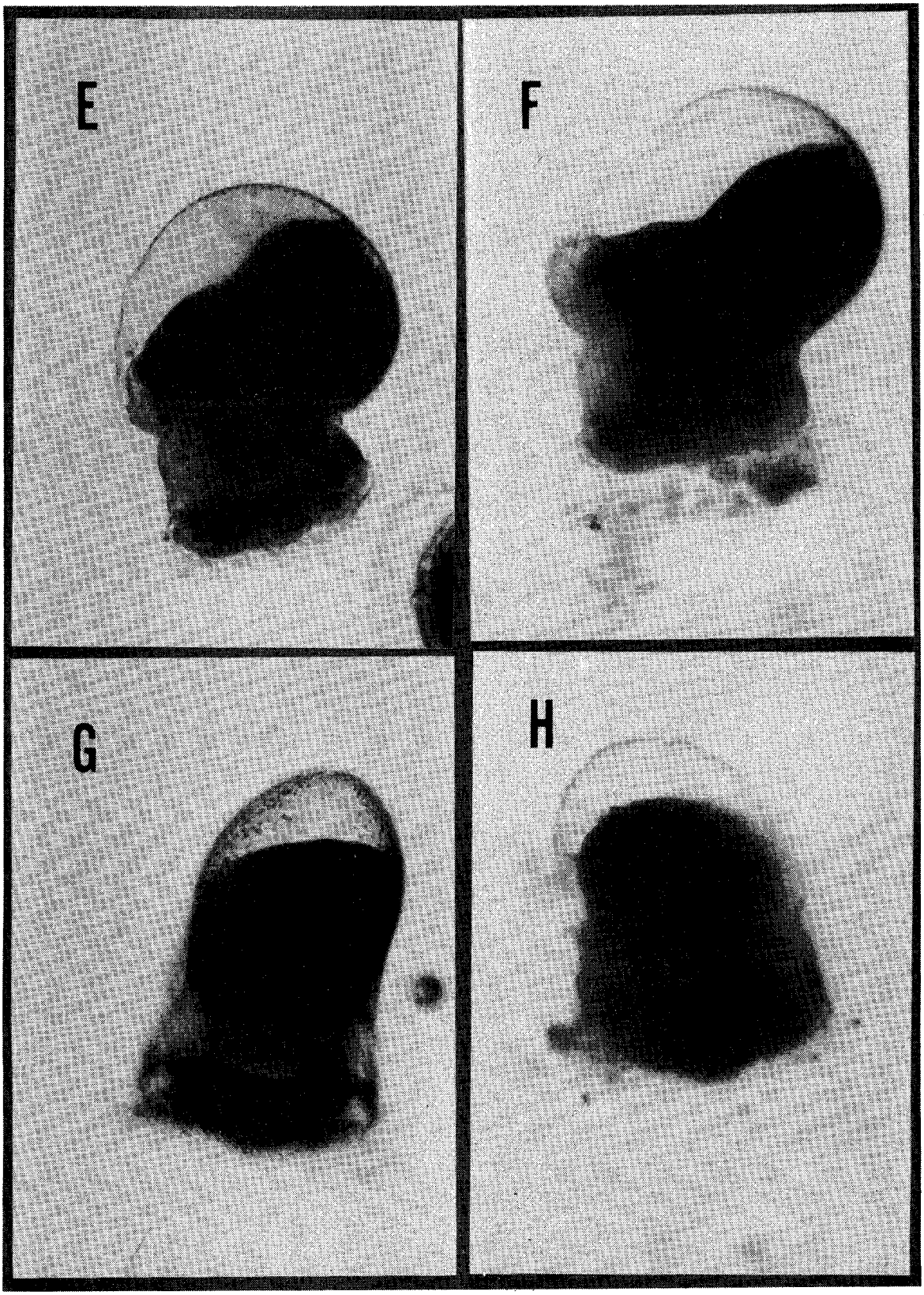
J. Abnormal larva with shell transparencies, indentations and irregular shape.

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.

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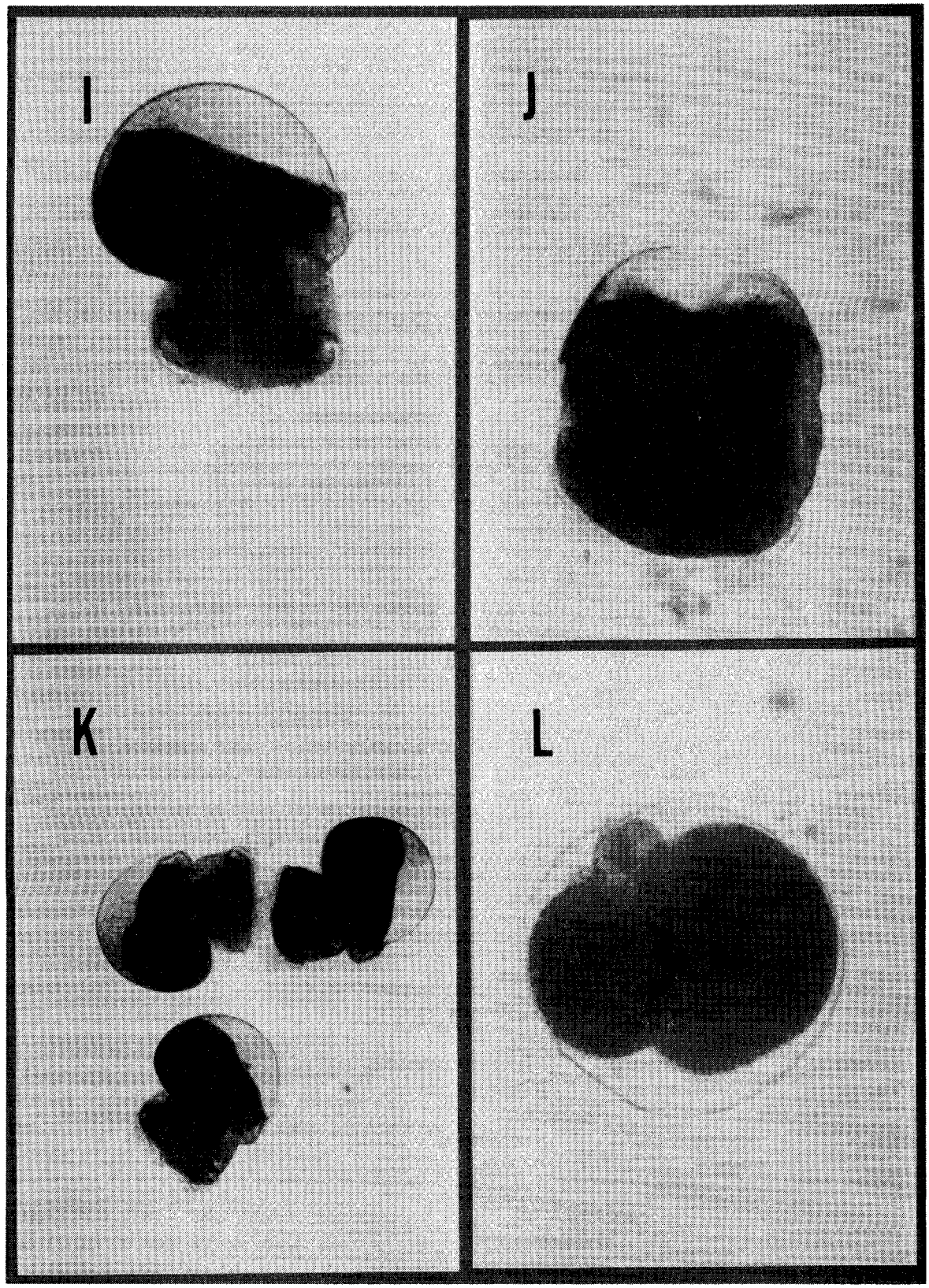
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Abalone Spawning Worksheet

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. _____

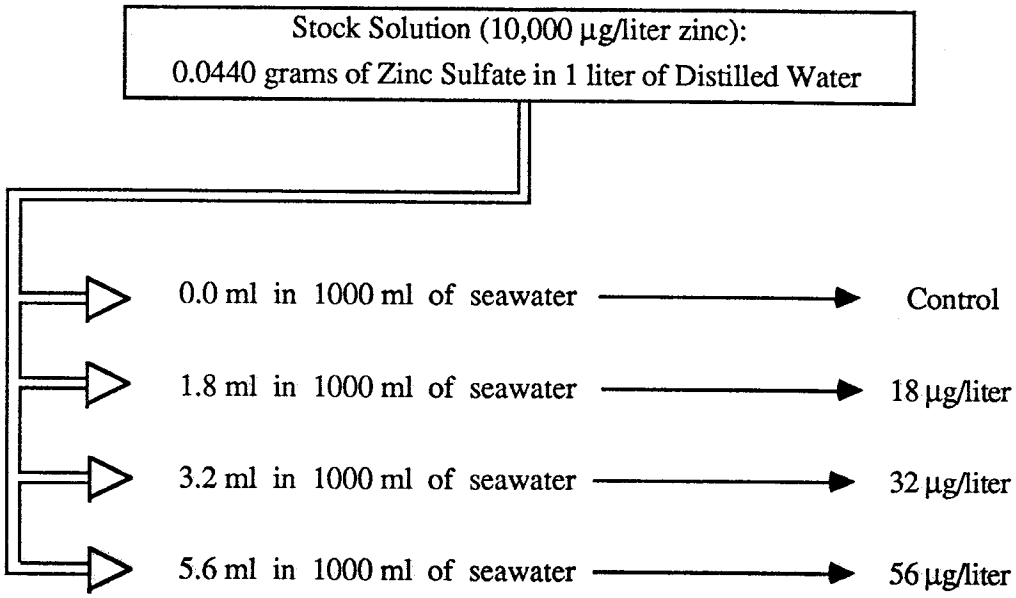
Add 1000 embryos to each test container by pipetting the volume calculated below from the well-mixed beaker sampled above.

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

Abalone Toxicity Test Data Sheet

Test Container Number	Corresponding Toxicant Concentration

Test Start Date: Test End Date:

Start Time: End Time:

Date Test Analyzed: Analyzed by:

Toxicant: Ref. Tox. Chem. Sample Vial No.'s:

Effluent Source: Collection Date:

Vial Number *	Test Container Number	Nominal Concentration ($\mu g\ l^{-1}$ or %)	Number of Abnormal Larvae	Number of Normal Larvae	Percentage of Abnormal Larvae	Arcsin $\sqrt{x}$ Percent Abnormal	Mean % Abnormal ± s.d. for each Concentration

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 III

MYSID, *HOLMESIMYSIS COSTATA*, TOXICITY TEST PROTOCOL

HOLMESIMYSIS COSTATA, MYSID EARLY LIFE-STAGE LETHALITY TOXICITY TEST PROTOCOL

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1.0 Introduction

This protocol gives step-by-step instructions for performing a 96-hour static renewel 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 96 hours, after which the percentage mortality is determined in each toxicant concentration. These data are used to derive No Observed Effect Concentrations (NOECs) or median lethal concentrations (LC_{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/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* 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
- glass tubes [5-mm-bore, 15-cm length, with suction bulbs (for handling adults)]
- wide-bore 10-ml pipet (for handling juveniles)
- 1000-ml glass beaker
- plastic 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, dissolved oxygen, and ammonia
- refractometer
- thermometer and thermograph (for continuous measurement)
- water bath or environmental chamber
- test containers (see Section 5.0)
- sample vials for trace metal 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 test (see Quality Assurance Section 2.1.2)
- pesticide-free 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 store in a dark refrigerator 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 96 hours. 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 half of the original test solution from each container, taking care to avoid losing 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.

Refill the half-empty containers to the 200-ml mark by carefully pouring new test solution down the side of the test container.

5.0 Test Containers

Test containers must hold 200 ml of test solution and should provide ample flat surface area to separate individual mysids. Polystyrene tissue culture flasks (250-ml capacity) are tentatively recommended for all toxicants. Testing with these containers has been limited, but the containers have a large flat surface area and are sealed to prevent evaporation and volatilization. Tissue culture test containers should be soaked over-night in dilution seawater prior to testing. Other 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.

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 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. 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 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* (= *Acanthomysis*) *costata* (Holmes 1900). 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), but *H. costata* is numerically abundant in kelp forest habitats and is considered 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. In *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 span.

A number of toxicity studies have used *H. costata* (Tatem and Portzer, 1985; Davidson et al., 1986; Machuzac and Mikel, 1987; Reish and Lemay, 1988; Martin et al., 1989; Asato, 1988). 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

Mysids must be identified to species. Use Holmquist (1979, 1981) as a guide for identification. Recently, there have been changes in the taxonomy of *H. costata*. Most previous authors have used the name *Acanthomysis sculpta*. However, Holmquist (1979) reviewed the taxonomy of several mysid genera and considers previous references to *Acanthomysis sculpta* in California to be synonymous with *Holmesimysis costata*. We have considered 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 10 to 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 (1° C per 2 hours). 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 constant gentle aeration and fronds or blades of Macrocystis, which serve as 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 20 *Artemia* nauplii per mysid per day and 100 mg of ground Tetramin® flake food per 100 adults. 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 reuse.

8.4 Isolation of Test Animals

To provide 500 to 800 juveniles for each set of toxicity tests (effluent and reference toxicant), approximately 150 gravid female mysids must be isolated. Gravid females can be identified by their large, extended marsupia filled with (visible) eyed juveniles. The marsupia of females that are close to releasing are grey in color.

Gravid females are most easily isolated from other mysids by using the following procedure: 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 dry screen, 4) gently draw the gravid females off the screen with a suction bulb and smooth glass tube (5-mm bore), 5) collect the gravid females in a separate screen tube, or place them directly into the release aquarium (see below). 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. Provide 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; it is important to turn all lights off at night to facilitate release. In the morning, the screened compartment containing the females should be removed and placed in a separate clean aquarium. Juveniles should be slowly siphoned into a 150-µm-mesh screen-bottom tube (25 cm diam.) immersed in a bucket filled with clean seawater. Once the releasing aquarium is emptied, it should be washed with hot fresh water to eliminate the possibility of mixing cohorts.

Juvenile test organisms concentrated in the screen-tube can be counted by subsampling 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 the next day's release. Initial experiments indicate that there may be differences in toxicant sensitivity among different aged mysids (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 20-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 are not forced down onto the screen. The height of the bucket determines the level of water in the screen tube. About 100 to 200 juveniles should be held in each screen-tube (100 juveniles per static 4-liter beaker). Juveniles should be fed 5 to 10 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 (13° ± 1° C if collected north of Pt. Conception). Half 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, place 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 2 juveniles, repeat the process, adding two mysids at a time until each cup contains 8 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 in a few cups. If more than 5 mls of water are added to the test solution with the juveniles, report the amount on the data sheet. Carefully pour the juveniles into the test containers immediately after reducing the water volume. Juveniles can become trapped in drops; have a squirt bottle ready to gently rinse down any trapped mysids. 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 eight animals are transferred 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 96 hours. Fifty percent of the test solution must be renewed after 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). Do not aerate the test containers. 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 10 newly released *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. (Rinsing and concentrating is most easily done using a 100-μm-mesh screen tube). Nothing else should be added to the test containers.

9.3 Endpoint Determination

Mysid death is the toxicity test endpoint. Immobile mysids that do not respond to a stimulus are counted as 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 counted as alive. 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.

Count and record the number of live and dead mysids at the end of the 96-hour period. Missing mysids are assumed to be dead.

10.0 Data Analysis

Use probit analysis to determine the LC_{50} and 95% confidence intervals. 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.

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

Divide the number of dead mysids in each replicate by the total (eight) 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, include all data in the ANOVA, and use the appropriate control for the Dunnett's comparison. 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).

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 20% 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 median lethal concentration (LC_{50}) must fall between 60 and 100 µg/liter zinc in the reference toxicant test.

4) 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).

5) 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).

12.0 References

Anderson, B.S., J. W. Hunt, M. Martin, S. L. Turpen, and F. H. Palmer. 1988. Marine Bioassay Project third report; protocol development: reference toxicant and initial complex effluent testing. Division of Water Quality report no. 88-7WQ, California State Water Resources Control Board, Sacramento, California.

Anderson, B.S., J.W. Hunt, S.L. Turpen, F.H. Palmer, and M. Martin. 1990. Marine Bioassay Project fifth report: interlaboratory toxicity testing and further protocol development with giant kelp, red abalone, and topsmelt. State Water Resources Control Board. Sacramento, California.

Asato, S.L. 1988. The effects of heavy metals on survival and feeding of *Holmesimysis costata* (Mysidacea). Master's Thesis, California State University at Long Beach.

ASTM. 1987. Annual book of standards, water and environmental technology. American Society for Testing and Materials, Philadelphia, Pennsylvania Vol. 11.04; 375.

Clark, W. 1971. Mysids of the southern kelp region. In W. North, ed., Biology of Giant Kelp Beds *(Macrocystis)* in California. J. Cramer Publisher, Lehre, Germany. Pages 369 - 380.

Clutter, R. I. 1967. Zonation of nearshore mysids. Ecology. 48(2):200-208.

Clutter, R. I. 1969. The microdistribution and social behavior of some pelagic mysids. J. exp.mar Biol. Ecol. 3: 125-155.

Davidson, B. M., A. O. Valkirs and P. F. Seligman. 1986. Acute and chronic effects of tributyltin on the mysid *Acanthomysis sculpta* (Crustacea, Mysidacea). Proceedings, Oceans 86, Washington, D.C., Sept 23-24, 1986, Pages 1219-1225.

DeLisle, P.F. and M.H. Roberts. 1988. The effect of salinity on cadmium toxicity to the estuarine mysid *Mysidopsis bahia*: role of chemical speciation. Aquatic Toxicology 12: 357 - 370.

Gaetz, C.T. 1986. Toxicity of used drilling fluids to mysids (Mysidopsis bahia). Environ. Toxicol. Chem. 5: 813 - 821.

Gentile, S. M., J. H., Gentile, J. Walker, and J. F. Heltshe. 1982. Chronic effects of cadmium on two species of mysid shrimp: *Mysidopsis bahia* and *Mysidopsis bigelowi.* Hydrobiologia. 93: 195-204.

Green, J. M. 1970. Observations on the behavior and larval development of *Acanthomysis sculpta* (Tattersall), (Mysidacea). Can. J. Zool. 48:289-292.

Holmquist, C. 1979. *Mysis costata* and its relations (Crustacea, Mysidacea). Zool. Jb. Syst. 106: 471-499.

Holmquist, C. 1981. The genus *Acanthomysis* Czerniavsky, 1882 (Crustacea, Mysidacea) Zool. Jb. Syst. 108: 386-415.

Hunt, J.W., B.S. Anderson, S.L. Turpen, A.R. Coulon, M. Martin, F.H. Palmer, and J.J. Janik. 1989. Marine Bioassay Project, fourth report: experimental evaluation of effluent toxicity testing protocols with giant kelp, mysids, red abalone, and topsmelt. Division of Water Quality report no. 89-5WQ, California State Water Resources Control Board, Sacramento, California.

Linfield, J.L.T., M. Martin and J. Norton. 1985. Marine Bioassay Project first progress report: bioassay species selection and recommended protocols. California State Water resources Control Board, Sacramento, California.

Lussier, S. M., J. H. Gentile and J. Walker. 1985. Acute and chronic effects of heavy metals and cyanide on *Mysidopsis bahia* (Crustacea: Mysidacea). Aquatic Toxicology. 7: 25-35.

Machuzak, M. J. and T. K. Mikel, Jr. 1987. Drilling fluid bioassays using Pacific ocean mysid shrimp, *Acanthomysis sculpta*, a preliminary introduction. In W.J. Adams, G.A. Chapman, and W.G. Landis, eds., Aquatic Toxicology and Hazard Assessment: 10, ASTM STP 971. American Society for Testing and Materials, Philadelphia, Pennsylvania, Pages. 447-453.

Martin, M., J. W. Hunt, B. S. Anderson, S. L. Turpen, and F. H. Palmer. 1989. Experimental evaluation of the mysid *Holmesimysis costata* as a test organism for effluent toxicity testing. Environ. Toxicol. Chem. 8: 1003 - 1012.

Mauchline, J. 1980. The Biology of Mysids and Euphausids. Advances in Marine Biology. 18: 681.

McLuscky, D.S. and L. Hagerman. 1987. The toxicity of chromium, nickel, and zinc: effects of salinity and temperature, and the osmoregulatory consequences in the mysid *Praunus flexuosus*. Aquatic Toxicology 10: 225 - 238.

Nimmo, D. R., L. H. Bahner, R. A. Rigby, J. M. Sheppard, and A. J. Wilson, Jr. 1977. *Mysidopsis bahia*: an estuarine species suitable for life-cycle tests to determine the effects of a pollutant. ASTM STP 634, American Society for Testing and Materials, Philadelphia, PA. Pages 109-116.

Reish, D.J., and J.A. Lemay. 1988. Bioassay manual for dredged sediments: U.S. Army Corps of Engineers. Research report, U.S. ACE Environmental Resources Branch, Contract No. DACW-09--83R-005: 34 pp.

Sokal, R. R. and F. J. Rohlf. 1969. Biometry. W.H. Freeman and Company, San Francisco.

Tatem, H.E. and A.S. Portzer. 1985. Culture and toxicity tests using Los Angeles district bioassay animals, *Acanthomysis* and *Neanthes*. U.S. Army Corps of Engineers, Misc. Paper EL-85-6, Waterways Experiment Station, Vicksburg, Mississippi.

Weber, C.I., W.B. Horning, D.J. Klemm, T.W. Neiheisel, P.A. Lewis, E.L. Robinson, J. Menkedick, and F. Kessler. 1988. Short-term methods for estimating chronic toxicity of effluents and receiving waters to marine and estuarine organisms. EPA/600/4-87/028. U.S. Environmental Protection Agency, Cincinnati, Ohio.

Zar, J. H. 1974. Biostatistical Analysis. Prentice-Hall, Inc., Inglewood Cliffs, N.J.

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 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 < 25 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 two mysids at a time into as many plastic cups as there are test containers. Repeat the process until each cup has exactly eight juvenile mysids (Section 9.1).

D. Eliminate excess water from the cups (no more than five mls should remain) and pour the mysids into the test containers Make sure no mysids are left in the randomization cups. Count the number of juveniles in each test container to verify that each has eight (Section 9.1).

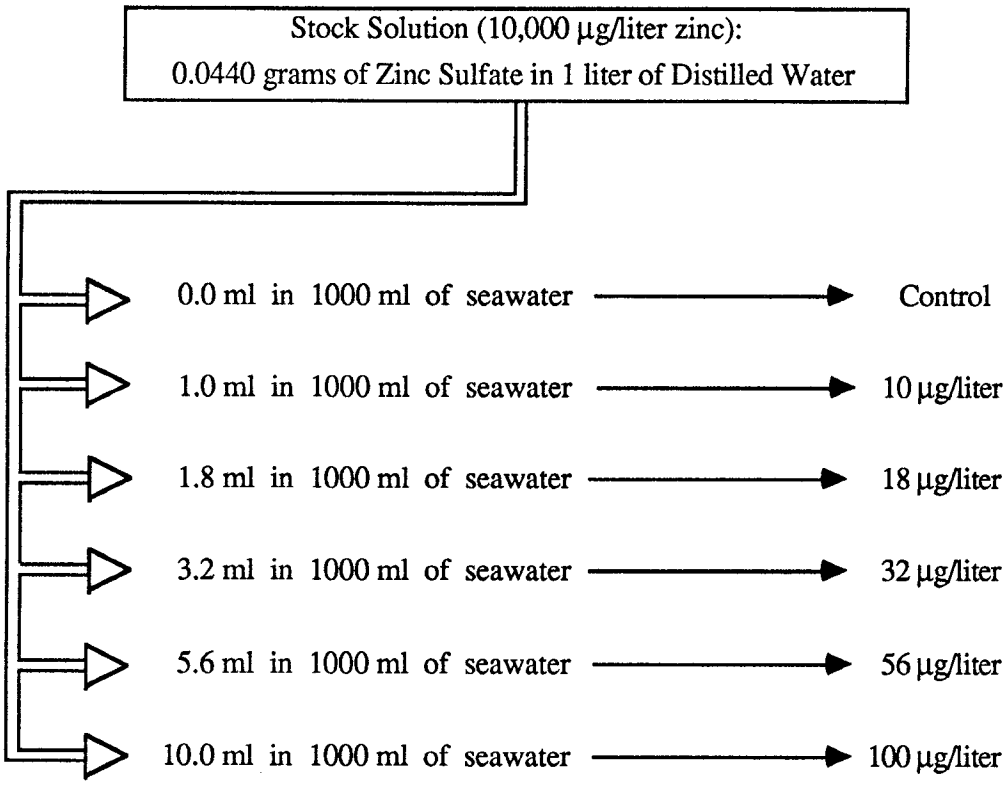
E. Remove all dead mysids daily, and add ten newly hatched *Artemia* nauplii/mysid/day; Section 9.2).

F. After 48 hours, renew 50% of the test solution in each container (Section 4.4).

G. After 96 hours, 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).

H. Calculate LC_{50}'s and 95% confidence intervals using probit analysis. Calculate NOECs using Analysis of Variance and Dunnett's test (Section 10.0).

Zinc Dilution Schedule for Mysid 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

Mysid Toxicity Test Data Sheet

Test Start Date: Test End Date: NOEC:

Start Time: End Time:

Mysid Age at Start:

Toxicant: Ref. Tox. Chem. Sample Vial No.'s:

Effluent Source: Collection Date:

Test Container Number	Nominal Concentration ($\mu g\ l^{-1}$ or %)	Number Dead				Total Number Dead	Total No. of Mysids at Start	Percent Dead	Notes	
		Day 1	Day 2	Day 3	Day 4					

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 ($^{o}/_{oo}$)	Ammonia ($mg\ l^{-1}$)

Notes

APPENDIX IV

TOPSMELT TOXICITY TEST PROTOCOL

TOPSMELT 12-DAY EMBRYO DEVELOPMENT TOXICITY TEST PROTOCOL (Tentative)

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1.0 Introduction

This protocol gives step-by-step instructions for performing a 12-day static renewal test using larval hatching success and developmental abnormalities to determine the toxicity of chemical compounds, complex effluents, or impacted marine waters. In this procedure, early-blastula topsmelt embryos are exposed to test solutions for 12 days. The test is terminated when all of the embryos have either hatched as larvae, or died. Hatched larvae are then examined microscopically to determine the percentage of abnormal larvae. These data are used to derive No Observed Effect Concentrations (NOECs) which give a numerical indication of toxicity. Because the test measures effects on developmental 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

- beach seine w/ one-cm-mesh
- 100-liter plastic trash cans with lids
- 5-liter plastic buckets
- compressed oxygen or air with air stones

•recirculating pump

•cool-white lights

•filter system

•600-watt immersion heater

•(2) 1000-liter tanks

•fish food (Tetramin™ flake food, freeze dried krill)

•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 10-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

•inverted microscope

•dissecting microscope

•environmental chamber

•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)

•9-ml glass tissue culture tubes with teflon-lined caps (240)

•stainless steel culture tube racks (4)

•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 twenty times. Every test must contain twenty 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 twenty 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. Reagent grade copper chloride ($CuCl_2 \cdot 2H_2O$) 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 twenty replicates of the following copper concentrations: 0, 32, 56, 100, and 180 µg/liter. Prepare one liter of each concentration by adding 0, 3.2, 5.6, 10.0, and 18.0 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 embryos from the same spawn as those used in the effluent test.

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 topsmelt embryos. 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 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

Seven milliliters of test solution are needed for each test container. Although more than 20 replicate test solutions can be mixed in a 1-liter volumetric flask, this volume is used as an example. 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 20 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 20 replicate containers.

4.4 Test Solution Renewals

All effluent test solutions are renewed every 48 hours; copper solutions are not renewed. Assuming the test starts on day 0, this requires effluent solution renewals on days 2, 4, 6, 8, and 10. 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 pipetted out, leaving 0.5 ml covering the embryo. New solutions are carefully poured down the side of the tissue culture tube to minimize disturbance to the embryo. **Note:** We have found that this protocol performs well under static conditions using copper chloride. Other researchers have successfully tested complex effluents under completely static conditions. In situations where toxicity information is required on a single sample where no renewals are possible, a static test can be conducted provided that the water quality measurements at the beginning and end of the test adhere to the quality assurance specifications for this protocol.

5.0 Test Containers

Use glass tissue culture tubes for all toxicants (94mm x 16mm; Bellco Glass, Trenton NJ). Each tube holds 7 ml of test solution and has 7 mm of head space. Tubes are capped with a

teflon-lined cap, and stored horizontally in stainless steel racks to increase the volume of test media exposed to the airspace, and facilitate the exchange of oxygen. Each rack holds 36 tubes.

Note: Other test containers have been used successfully with this protocol. For example, polystyrene tissue culture containers may be substituted for the above containers as long as the reference toxicant test results using alternative containers meet test acceptability requirements (Section 11).

5.1 Randomization

To randomize placement of the early-blastula embryos, order the test containers from 1 to 100 (for reference toxicant tests, or 1 to N for effluent tests, with N being the total number of test containers) and add 7 mls of the appropriate toxicant concentration to each. Next, arrange the numbered test containers randomly using a random numbers table, and place one embryo in each test container (see section 9.1). When all of the embryos are in, reorder the containers to facilitate measuring water quality.

To prevent bias in the analysis of the results, the test container numbers are taped over after the final water quality samples have been taken, and before the endpoints are quantified (see section 9.2).

6.0 Physical/Chemical Measurements of Test Solutions

One sample from each test concentration should be measured as follows: measure test solution temperature daily; measure water bath or environmental chamber temperature continuously; measure salinity, pH, and dissolved oxygen at the beginning and end of the test, and before every solution renewal. It may be necessary to combine solution from several replicates in order to measure dissolved oxygen concentrations at the end of the test. 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 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 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.

Note: Tissue culture tube caps are cleaned as follows: 1) rinse three times in deionized water, 2) rinse briefly three times with acetone, 3) rinse three times with deionized water, 4) rinse briefly three times with 2N HCL, 5) rinse three times with deionized water, 6) soak 24 hours in deionized water, 7) rinse three times with deionized water, 8) dry in a clean area.

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 either 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. Caps are cleaned as above.

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 embryos of the topsmelt, *Atherinops affinis*. Topsmelt occur from the Gulf of California to Vancouver Island, British Columbia (Miller and Lea, 1972). They are ubiquitous in coastal waters and, in terms of biomass and numbers, rank 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). 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.*, in press). The topsmelt is being considered for use in routine effluent toxicity testing by State Water Resources Control Board because of its ecological importance and potential susceptibility to coastal pollution (eg., Hose *et al.*, 1983).

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 dorsal 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 are 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 enough 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 pre-soaked 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 20 ° C during transport. See Section 8.6 for transport of embryos.

8.4 Broodstock Culture

Once in the laboratory the fish should be treated for 2 days with a general antibiotic (eg., Prefuran®as per label instructions) in a separate tank, 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 should be recirculated using the system 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 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 daily (at 1100 to 1200 hrs) approximately 0.3g of freeze dried krill (eg., Superba®, First Choice) crumpled into 0.1 - 0.5 mm^2 pieces. Fish also receive approximately 0.3g of Tetramin ™ flake food (1300-1500 hrs) daily. Tanks are siphoned clean once weekly.

Polyester fiber 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 should be 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 20 °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.

8.6 Embryo Transport

Newly-fertilized embryos can be transported in a wide-mouth, one-liter thermos bottle. Strands of embryos should first be wrapped diagonally around stainless steel screens (12 cm x 3 cm with 8-mm mesh) to form a spiral configuration of embryos. No more than approximately 300 embryos should be transported in any one thermos bottle, and the embryos should be wrapped to minimize overlapping. Each screen should be placed in a one-liter, light-tight plastic thermos filled with 34 ± 2 ‰ seawater at 10 °C. The seawater in the thermos should be aerated with pure oxygen for 30 seconds prior to introduction of the embryos. The thermos should be packed in an ice chest with blue ice for transport. Embryos should be shipped via air-express overnight couriers. Because most transport services cannot deliver parcels within 8 hours, it is probably not possible for laboratories relying on air-freight-transported-embryos to use early-blastula embryos. Research is currently being conducted to investigate the possibility of retarding development so that embryos can be shipped to arrive in the early-blastula stage, and to determine whether there are differences in sensitivity between early-blastula and later developmental stages.

9.0 Toxicity Test Procedure

9.1 Exposure of Test Organism

9.1.1 Embryo Preparation

Eggs are collected from the polyester fiber spawning substrates and their developmental stage determined; refer to Lagler *et al.* (1962) for staging. The eggs can be stripped from the substrates using tweezers. Clamp the tweezers around the clumps of fiber with attached eggs, and pull the fiber through the clamped tweezers, leaving the eggs on the edge of the tweezers. It is convenient to remove eggs from small sections of the substrate, rather than the whole substrate at once. Place the eggs in a watch glass for staging and sorting using a dissecting microscope (36x). Use only synchronous, early blastula embryos for this protocol (approximately 8 hours old, see Figure 1.). The eggs should be of uniform shape and size; 240 embryos are needed for a typical effluent test (140 embryos for the effluent test and 100 embryos for the copper reference test).

9.1.2 Incubation

After the test solutions have been prepared and the test containers are randomized (Section 5.1), the embryos can be transferred to the containers using a wide bore, fire-polished pipet. The containers are then re-ordered from #1 to 140 (or #1 to 100) in the racks and 7 ml of the appropriate test solution are pipetted into each. The racks can then be placed into the incubator on their sides so that the tubes are horizontal. The racks should be rotated, end over end, four times daily to mix the solutions.

9.2 Endpoint

After all of the embryos have either hatched or died (indicated by the absence of a heartbeat), the test is terminated (usually within 12 days). At the end of the test the final water quality samples are taken and the number of each test container is taped over to prevent reader bias in the analysis of the results. After each container is read, the tape is removed and the container number recorded. The number of embryos that have hatched is tallied for each concentration. Each hatched larvae is observed (live) and those with obvious terata are noted using the methods described by Weiss and Weiss (1982); see, also, Anderson *et al.* (in review), and Middaugh *et al.* (1988). Hatched live larvae can be placed in a petri dish or depression slide and anesthetized with MS-222 for observation. Table 1 (attached) lists the possible terata to be quantified. Terata are combined for the statistical analyses. Any larvae with any combination of terata listed in Table1 is counted as abnormal. Hatched dead larvae are also considered to be abnormal. The percentage of abnormal larvae is the number of abnormal larvae with terata listed in Table 1, plus the number of hatched dead larvae, plus the number of dead embryos, divided by twenty (the number of replicates).

10.0 Data Analysis

A row by column test is employed to compare the data. This test of independence uses the G statistic and is based on the chi-squared distribution. Pairwise comparisons with an adjusted alpha (alpha' = 0.05) are used to compare test concentrations to controls to calculate No Observed Effect Concentrations (NOEC's) for each endpoint (refer to Sokal and Rohlf 1969).

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 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 probit analysis to calculate EC_{50}'s and 95 % confidence intervals.

11.0 Test Acceptability

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

1) The mean percentage of abnormal larvae must not exceed 20% in the reference toxicant or effluent test brine controls if the tests used embryos spawned in house. If the tests used embryos shipped from outside the testing laboratory the mean percentage of abnormal larvae must not exceed 30% in either the reference toxicant or effluent test brine controls.

2) The EC_{50}'s for percentage of abnormal larvae in the copper reference test must be below 231 µg/liter (chemically verified copper concentrations).

12.0 References

Allen, L.G. and M.H. Horn. 1975. Abundance, diversity and seasonality of fishes in Colorado Lagoon, Alamitos Bay, California. Estuar. Coastal Mar. Sci. 3: 371- 380.

Allen, L.G. 1982. Seasonal abundance, composition, and productivity of the littoral fish assemblage in upper Newport Bay, California. Fish. Bull., U.S. 80 (4): 769-790.

Anderson, B.S., Middaugh, D.P., Hunt, J. W., and S. L. Turpen. In Press. Copper toxicity to sperm, embryos, and larvae of topsmelt *Atherinops affinis*, with notes on induced spawning. Mar. Environ. Res.

American Society for Testing Materials (ASTM). 1987. Annual book of ASTM standards; water and environmental technology, 11.04: 382-388.

Borthwick, P.W., Patrick, J.M. Jr., and D.P. Middaugh. 1985. Comparative acute sensitivities of early life stages of atherinid fishes to chlorpyrifos and thiobencarb. Arch. Environ. Contam. Toxicol. 14: 465-473.

Croaker, R. S. 1934. The spawning of *Atherinops insularum insularum.* Copeia 1934: 43.

Fronk, R.H. 1969. Biology of *Atherinops affinis* Hubbs in Newport Bay. M.S. Thesis, Univ. of Calif., Irvine. 106p.

Horn, M.H. 1979. Diel and seasonal variation in abundance and diversity of shallow-water fish populations in Morro Bay, California. Fish. Bull.. 78: 759-770.

Hose, J. E., Stoffel, R. J., and K. E. Zerba. 1983. Behavioural responses of selected marine fishes to chlorinated seawater. Mar. Env. Res. 9: 37-59.

Lagler, K.F., Bardach, J.E., and R.R. Miller. 1962. *Ichthyology*. John Wiley and Sons, New York. pp 545.

Middaugh, D.P., Hemmer, M.J., and L.R. Goodman. 1987. Methods for spawning, culturing and conducting toxicity tests with early life stages of four atherinid fishes: The inland silverside, *Menidia beryllina*, Atlantic silverside, *M. menidia*, tidewater silverside, *M. peninsulae* and California grunion, *Leuresthes tenuis*. EPA 600 8-87 004. pp 56 .

Middaugh, D.P. and J.M. Shenker. 1988. Salinity tolerance of young topsmelt, *Atherinops affinis*, cultured in the laboratory. Calif. Fish Game, 74 (4): 232-235

Middaugh, D.P., and M.J. Hemmer. 1984. Spawning of the tidewater silverside *Menidia peninsulae* (Goode and Bean), in response to tidal and lighting schedules in the laboratory. Estuaries 7(2): 139 - 148.

Middaugh, D.P., Hemmer, M.J., and E.M. Lores. 1988. Teratological effects of 2,4-dinitrophenol 'produced water' and naphthalene on embryos of the inland silverside *Menidia beryllina*. Dis. Aquat. Org. 4 : 53-65.

Miller, D.J. and R.N. Lea. 1972. Guide to the coastal marine fishes of California. Calif. Dep. Fish Game Bull. 157. 235 p.

Sokal, R.R. and F.J. Rohlf. 1969. *Biometry*. W.H. Freeman and Company, San Francisco. pp 859.

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), 18, 32, 56, 100, and 180 µg/liter by adding 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 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 - 100 and pour 7 mls of the appropriate test solution into each container. Randomize the test containers, and place one early-blastula embryo in 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) Collect early-blastula embryos from polyester fiber spawning substrates. Place them on a watch glass for sorting under a dissecting microscope (Section 9.1.1).

B) Sort out 240 synchronous early-blastula embryos (Section 9.1.1).

C) Pour 7 ml of the appropriate test solution into each test container (Section 4.0).

D) Randomize test containers and place one embryo 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, 8, and 10 (Section 4.4).

F) After all of the embryos have either died or hatched (on approximately day 12), determine the number of hatched live larvae with terata. The number of dead embryos, dead larvae, and abnormal larvae are totaled and divided by the number of replicates (20) to give the proportion of abnormal larvae at each test concentration. Read the test blind by taping over the container numbers before quantifying the endpoints (Section 9.2).

G) Use a row by column test followed by a multiple comparison of each test concentration to the control 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 probit analysis.

Table 1. List of observable terata for topsmelt embryos and larvae.

Craniofacial Index, CFI

Progressive eye convergence

0. Normal
1. Slight convergence
2. Convergence to touching
3. Synophthalmia (two separate lenses have formed)
4. Fusion of optic cups but two lenses formed
5. Cyclopodia
6. Anacephaly

Cardiovascular Index, CVI

Heart development

0. Normal
1. Slight defect in structure or function, including hemostasis somewhere in body
2. Tube heart beating
3. Tube heart not beating
4. Beating tissue but no heart structure
5. No discernible heart tissue

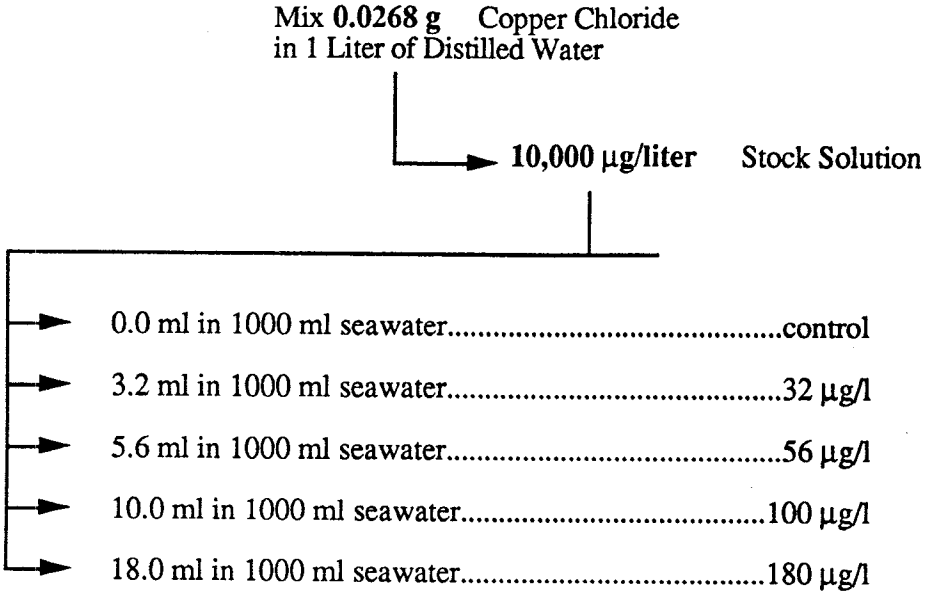
Skeletal Index, SKI

Skeletal deformities

0. Normal
1. Slight bend or kink
2. Major bend or kink (>90°) or more than one bend or kink
3. Stunted
4. Very stunted but axis discernible
5. No axis discernible

Reprinted from: Weiss, P. and J.S. Weiss. 1982. Toxicity of methylmercury, mercuric chloride, and lead in killifish (*Fundulus heteroclitus*) from Southhampton, New York. Env. Res. Vol. 28, pp. 364-374.

Copper Dilutions
for Topsmelt 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

APPENDIX V

QUALITY ASSURANCE / 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. 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

APHA. 1985. Standard methods for the examination of water and wastewater. American Public Health Association, Washington, D.C.

ASTM. 1987. Annual book of ASTM standards. Section 11: Water and environmental technology, Vol. 11.04. American Society for Testing and Materials, Philadelphia, Pennsylvania, 01–110487–48 (see Designation D 4447 - 84 for information on disposal of laboratory and toxic materials).

Gaston, P.J. The care, handling, and disposal of dangerous chemicals. Inst. of Science Technology, Northern Publishers (Aberdeen) Ltd., Aberdeen, Scotland.

Holmquist, C. 1979. *Mysis costata* Holmes, 1900, and its relations (Crustacea, Mysidacea). Zool. Jb. Syst. 106: 471 - 499.

Holmquist, C. 1981. The genus *Acanthomysis* Czerniavsky, 1882 (Crustacea, Mysidacea). Zool. Jb. Syst. 108: 386 - 415.

Huguenin, J.E. and J. Colt. 1989. Design and operating guide for aquaculture seawater systems. Elsevier Science Publ., pp. 263.

ITII. 1977. Toxic materials and hazardous industrial chemical safety. International Technical Information Institute, Tokyo, Japan. pp. 591.

Miller, D.J. and R.N. Lea. 1972. Guide to the coastal marine fishes of California. Calif. Dept. Fish Game. Fish Bull. 157: 82-83.

US EPA. 1977. Occupational health and safety manual. Office of Planning and Management, U. S. Environmental Protection Agency, Washington, D. C.

US EPA. 1979. Handbook for analytical quality control in water and wastewater laboratories. U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio, EPA/600/4-79/019.

US EPA. 1988. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to marine and estuarine organisms. U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio, EPA/600/4-87/028.

Walters, D.B. and C.W. Jameson. 1984. Health and safety for toxicity testing. Butterworth Publ. Co.Woburn, Massachusetts.

THE MARINE BIOASSAY PROJECT

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