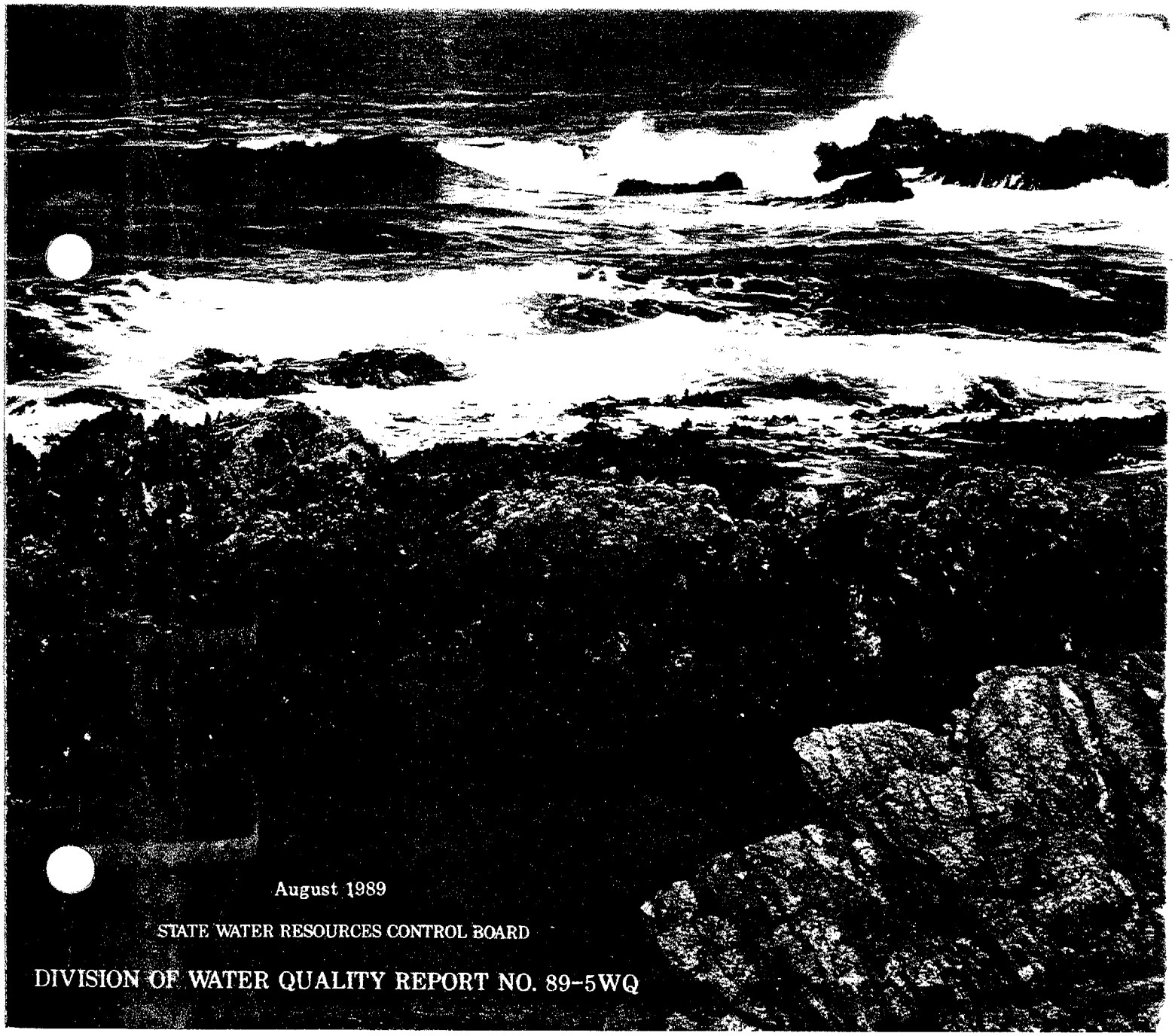
MARINE BIOASSAY PROJECT

FOURTH REPORT

EXPERIMENTAL EVALUATION OF EFFLUENT TOXICITY TESTING PROTOCOLS WITH GIANT KELP, MYSIDS, RED ABALONE, AND TOPSMELT



August 1989

STATE WATER RESOURCES CONTROL BOARD

DIVISION OF WATER QUALITY REPORT NO. 89-5WQ

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DIVISION OF WATER QUALITY REPORT NO. 89-5WQ

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SUMMARY AND OVERVIEW OF THE MARINE BIOASSAY PROJECT

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 to marine waters. The Project's primary objective is development of short-term tests to measure the toxicity of these discharges. Emphasis has been placed on developing tests that are sensitive to toxicants present in effluents. 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 tests are designed to estimate more subtle long-term adverse effects that ultimately may damage populations of aquatic species. The new toxicity tests generally use early life stages of sensitive aquatic organisms and measure sub-lethal responses such as abnormal development or decreased germination. 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.

Toxicity Testing

Aquatic toxicology is a relatively new and evolving area of study that uses 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. In the current terminology of aquatic toxicology, the "Marine Bioassay Project" would be more accurately described as the "Marine Toxicity Testing Project".

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 definition is less clear when referring to tests of either other organisms with shorter reproductive cycles such as invertebrates and algae or to early life stages of fish.

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, 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 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 represent 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. Whole effluent toxicity tests measure the bioavailability of toxicants in a complex mixture and integrate the adverse effects of the mixture. However, some important toxic properties, such as biomagnification, bioaccumulation, and carcinogenicity, will be missed by short-term toxicity tests. The MBP is focusing its efforts on developing short-term, critical life stage toxicity tests for estimating long term toxic effects of complex effluents.

The development of toxicity test protocols for whole effluents has several major requirements in addition to conducting a series of laboratory experiments. First, the necessary facilities must be modified or constructed to provide a laboratory for rearing and spawning test organisms. For marine organisms, a flowing seawater system is preferable. Other laboratory needs include a separate area for conducting tests with toxicants, constant temperature water baths and instruments to monitor water quality, washing facilities for special laboratory glassware, an area provided with compound and dissecting microscopes and other equipment to analyze test results, and a treatment system to remove toxicants before discharge of laboratory wastewater.

Toxicity test development involves conducting a test several times to refine and simplify methods to be standardized into a formal protocol. 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 four years of intensive effort. For the most part, this has included the sustained work of two principal investigators and two laboratory technicians.

Regulatory Framework

The development of these toxicity test protocols to estimate long-term effects of wastewater discharges is consistent with both federal and state requirements. In 1984, the United States Environmental Protection Agency (EPA) issued a national "Policy for 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 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 by January 1, 1990. Ocean dischargers of 100 million gallons per day or more will be required in their permits to use these 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 for 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 Board shall use the multispecies toxicity testing program with representative marine species in adopting the bioassay protocols specified in Section 13170.2 of the Water Code."

On March 19, 1987 the State Board adopted a workplan for triennial review of the California Ocean Plan, based on public hearings held in October 1986. The workplan listed 26 issues raised during hearings and identified 7 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.

These marine toxicity tests will be implemented in regulatory programs of the State Board and six coastal Regional Water Quality Control Boards. For example, ocean discharge permits may incorporate "no observed effect concentrations" (NOEC's) that are derived from tests of sensitive life stages of native marine species. Thus a given discharger with an outfall design providing for 100:1 dilution might have a requirement that a one percent dilution of effluent show no observed effect in toxicity tests.

Alternatively, a discharge permit could be expressed in toxicity units (TU). A TU is defined as 100 divided by the no observed effect concentration:

$$TU = 100 / NOEC$$

The NOEC is defined as the maximum percent effluent, or any water being tested, that does not result in any observed effect on test organisms. In the example of a requirement that no toxicity be observed in effluent diluted to one percent, the discharge permit would require that the effluent toxicity limit be 100 toxicity units or less.

$$TU = 100 / 1 = 100$$

Phase One (November 1984 - February 1986):

The Marine Bioassay Project is designed as a four 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 Marine Pollution Studies Laboratory (MPSL) located south of Monterey.

During this first phase, efforts were made to obtain wide-spread participation in developing the scope of the project. Initially a draft report (Linfield et al., 1985a) was released for review in March 1985. This draft described a number of potential species for marine toxicity tests, recommended twelve of these as most suitable, and presented the appropriate protocols for each of the twelve. The report was sent to a number of interested parties, including representatives of Federal and State regulatory agencies (EPA, SWRCB, RWQCB's), the Southern California Coastal Water Research Project, and a number of individual scientists. A workshop was planned to discuss the draft report and propose development of the project.

The workshop 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 (Linfield et al., 1985b) was released in June and 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 review project 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 MBP staff. Overall, a major

accomplishment of 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.

Phase I of the project accomplished three main tasks. First, the Department of Fish and Game laboratory was extensively refurbished for animal culture and rearing of marine species, and methods were developed for maintaining and spawning selected marine species. Second, a mobile laboratory was purchased and used to conduct aquatic toxicity tests. Finally, 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 (Martin et al., 1986).

Phase Two (March 1986 - October 1987):

In Phase 2, three new short-term protocols were developed after repeated testing using zinc as a reference toxicant. All three protocols are static tests; that is, the test solutions are not changed or renewed during testing. Each protocol measures a different effect or endpoint. These protocols, designed to estimate chronic toxicity of discharges 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 two representative complex effluents from municipal wastewater plants; one primary and one secondary. In addition, longer term reference toxicant tests were used with each species to calibrate the relative sensitivity of the short-term protocols.

The short-term larval abalone protocol is a 48-hour test in which abnormal shell development is the endpoint used as the measured effect of toxicity. The average no observed effect concentration for zinc based on three repetitive tests was 39 parts per billion (ppb). In preliminary tests on two municipal wastewater treatment plants, a NOEC of 10 percent was determined for the secondary treatment plant and 3.2 percent for the primary treatment plant.

The short-term giant kelp procedure is a 48-hour test that measures two different endpoints: zoospore germination and growth of the germination tube. These two endpoints were found to be relatively insensitive to zinc. With preliminary wastewater tests, the NOEC was 0.56 percent for the primary effluent and 18 percent for the secondary treatment effluent.

The short-term mysid protocol is a 96-hour test with an endpoint of lethality in juvenile mysids. Primary and secondary effluents had NOECs of 1 percent and 32 percent, respectively in preliminary testing. The Project's third report (Anderson et al., 1988) provides detailed descriptions of the developmental work completed in Phase 2.

Phase Three (November 1987 - December 1988):

This report, the fourth in a series, describes the work performed from November 1987 to December 1988. During this phase, the abalone, kelp, and mysid shrimp toxicity tests developed during Phase 2 were further refined by examining complex effluent from two large municipal ocean dischargers. In addition, preliminary tests were conducted using a fish species, the topsmelt (Atherinops affinis). A summary of the test results from this phase of the MBP is presented in the accompanying tables and following discussion:

Toxicity testing with the kelp produced a number of important findings. The germ-tube growth endpoint was more sensitive than germination in tests using complex effluent. Seasonal variability was found for both endpoints (germ-tube growth and germination) using copper as a reference toxicant. The reasons for these patterns are unclear. A likely cause is seasonal fluctuations in the health of adult plants from which the sporophylls were collected. Toxicity

tests were conducted jointly by the MBP and Coastal Resources Associates (CRA) as an "interlaboratory comparison". The results demonstrated that the kelp test can be performed by a qualified laboratory having no previous experience with the protocol. A long-term test (20-day) showed that sporophyte production (resulting from sexual reproduction between male and female gametophytes) was more sensitive to copper that sporophyte growth, spore germination, or germ-tube growth.

The 96-hour mysid shrimp (Holmesimysis) toxicity tests were further evaluated and the protocols developed in Phase 2 were modified. Juvenile mysid survival was inhibited at 1.0 and 5.6 percent effluent from two municipal wastewater dischargers (Los Angeles County Joint Pollution Control Plant and Los Angeles Hyperion Treatment Plant). Response of mysids to the zinc reference toxicant was variable throughout the year. This variability does not appear to be related to oceanographic or climatic patterns. Interlaboratory comparisons conducted at MPSL and the Hyperion Plant, using complex effluent, produced identical NOECs. An interlaboratory comparison between MPSL and CRA using zinc as a reference toxicant produced some variability due either to the handling of mysids or different dilution water used in the tests. In a longer 7-day test using zinc as the reference toxicant, gravid (egg-bearing) females were more tolerant to the toxicant than juveniles. In the same tests, the growth endpoint was less sensitive than, or equal in sensitivity to mortality.

A number of experiments were carried out with the red abalone. In toxicity tests from two large municipal discharges, NOEC's ranged from 1.0 to 3.8 percent. The reference toxicant tests using zinc produced similar response curves. These reference toxicant tests are conducted to provide a standard response to indicate the general health of test organisms and the quality of the testing environment. The variability in the interlaboratory comparison (MPSL and CRA) with zinc as a reference toxicant may have been due to high test solution salinities and different water sources used by each laboratory. Based on these test results, a number of refinements were made to improve the protocol for the 48-hour abalone larval test.

Preliminary fish toxicity tests were conducted with Atherinops affinis, a common nearshore coastal and estuarine fish species. In one experiment, larval survival was significantly inhibited by copper at 96 ppb (NOEC = 53 ppb). Topsmelt were found to be a good choice for use in these toxicity tests based on their suitability in laboratory culture, ease in obtaining fertilized eggs, and their ecological importance in coastal marine waters.

Phase Four (January 1989 - December 1989):

Phase 4 is the final and implementation phase of the MBP. Implementation of biological testing of complex effluents discharged to marine waters includes the following activities:

1. Develop a short-term protocol for a fish species native to California.

2. Conduct interlaboratory verification of test protocols using complex effluent.

3. Develop a procedures manual containing the necessary protocols to conduct toxicity tests on marine organisms.

4. Train Regional Board and discharger staff in the use of the protocols to conduct toxicity tests.

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SUMMARY OF NO OBSERVED EFFECT CONCENTRATION (NOEC) STUDIES PERFORMED WITH COMPLEX EFFLUENT AND REFERENCE TOXICANTS DURING PHASE 3 OF THE MARINE BIOASSAY PROJECT

	Effluent A[1]		Effluent B	
	Test Number			
	1	2	1	2
Complex Effluent Tests (percent effluent)				
Kelp 48-Hour Test				
Germination	≥10.0%	1.0%	≥10.0%	≥10.0%
Germ-tube Growth	3.2	<0.56	5.6	5.6
Mysid 96-Hour Test	5.6	<1.0	3.2	3.2
Abalone 48-Hour Test	1.0	1.0	3.2	1.8
Reference Toxicant (units = ppb)				
Kelp 48-Hour Test with Copper				
Germination	<41	19	99	54
Germ-tube Growth	<41	<10	<31	19
Mysid 96-Hour Test with Zinc	<18	18	56	56
Abalone 48-Hour Test with Zinc	18	32	32	18

[1] Effluents were each sampled and tested twice. Effluent A tests were conducted in February and August 1988. Effluent B was tested in May and October.

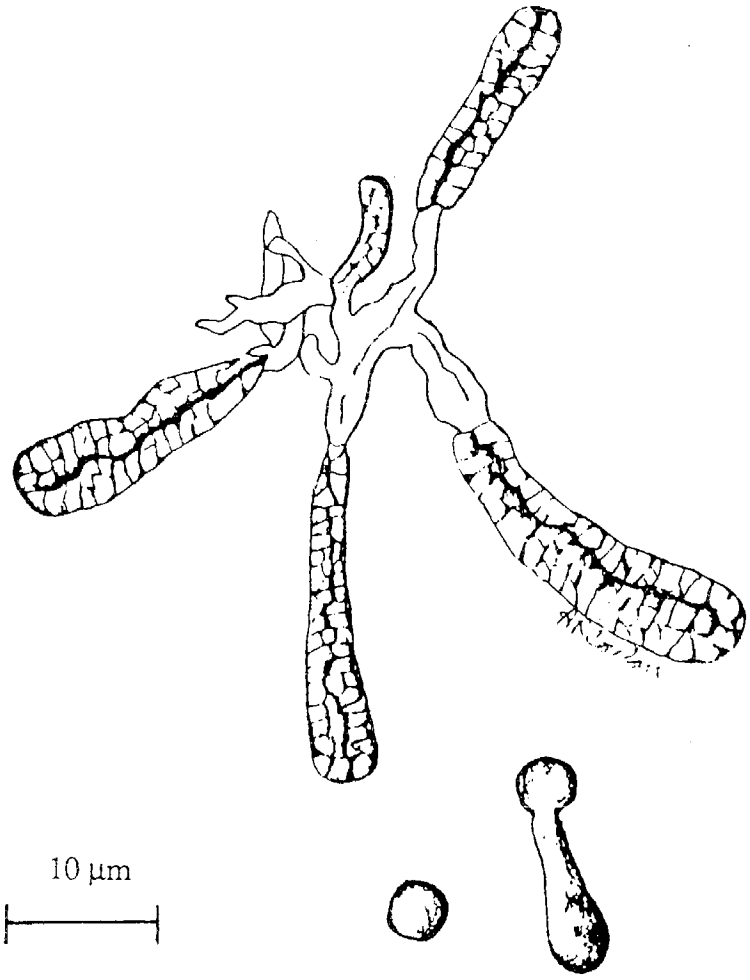
SUMMARY OF NO OBSERVED EFFECT CONCENTRATIONS (NOEC)
FOR INTERLABORATORY TESTS CONDUCTED DURING PHASE 3
OF THE MARINE BIOASSAY PROJECT
(Concentrations in ppb)

	MPSL[1]	Verification Laboratory
Kelp 48-Hour Test with Copper		
Germination	<41	56 [2]
Germ-Tube Growth	<41	56 [2]
Abalone 96-Hour Test with Zinc	18	7 [2]
Mysid 96-Hour Test with Zinc	56	18 [2]
Mysid 96-Hour Test with Effluent	3.2%	3.2% [3]

[1] MPSL = Marine Pollution Studies Laboratory
[2] Coastal Resources Associates, Encinitas, California
[3] Los Angeles Hyperion Treatment Plant

Section 1
Giant Kelp Experiments

Brian S. Anderson
John W. Hunt



Macrocystis pyrifera - sporophyte
non-germinated spore
germinated spore

Introduction

Giant kelp *Macrocystis pyrifera* is the dominant canopy-forming alga in southern and central California, and an important primary producer in near-shore marine communities. *Macrocystis* forests are complex ecosystems which provide a valuable economic resource to California. There is increasing concern over the effects of waste discharge on kelp forest ecosystems because of their proximity to coastal pollution sources.

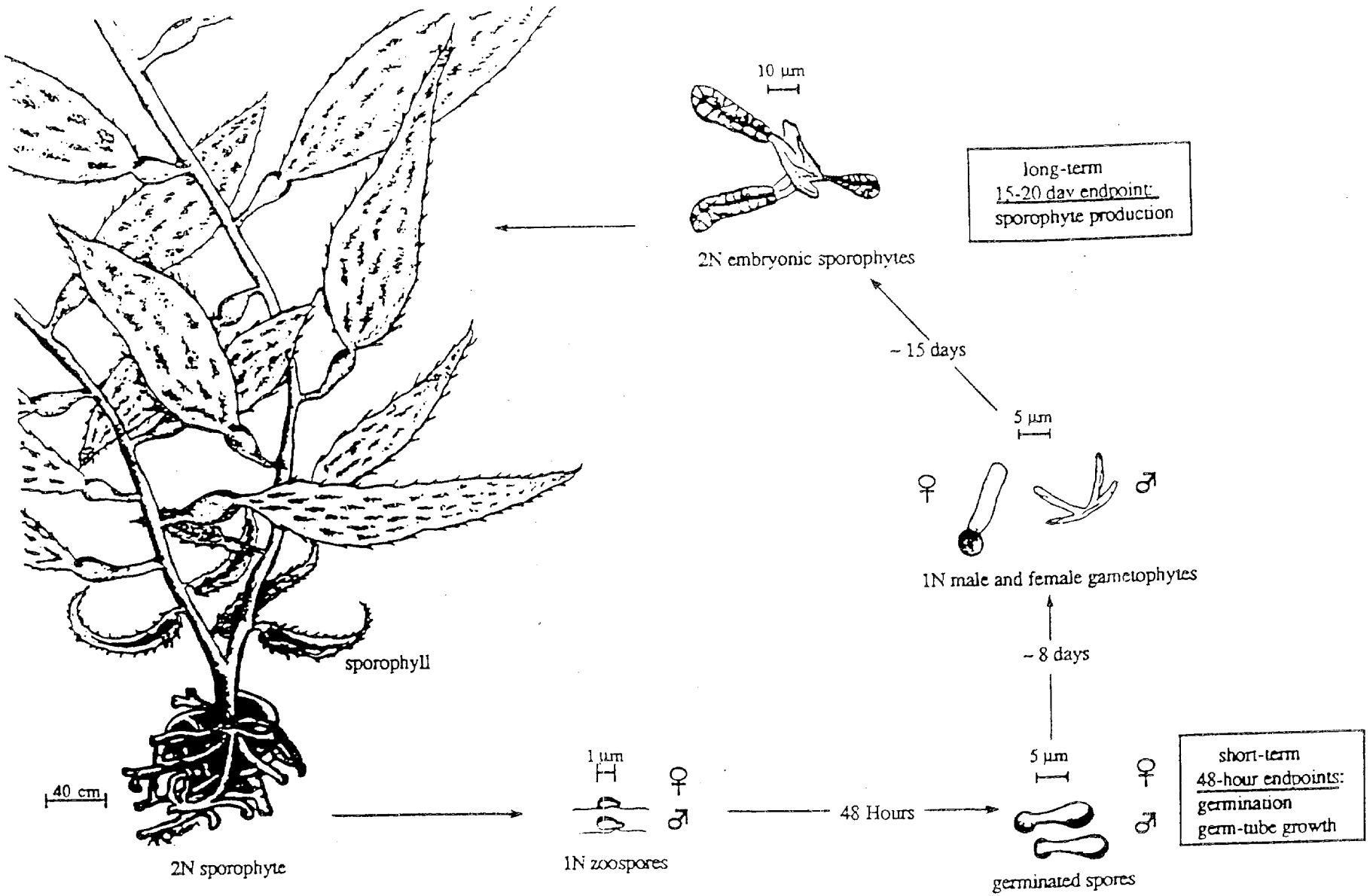
Macrocystis was chosen as a macroalga toxicity test species for the Marine Bioassay Project because of its economic and ecological importance, and amenability to laboratory culture (Linfield *et al.*, 1985). Giant kelp has been cultured extensively in laboratory studies and its life history has been well described (North, 1971, and 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 and 1981) are applicable to the interpretation of toxicity test results. James *et al.* (1987) used the microscopic stages of several laminarian species, including *Macrocystis*, for evaluating 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 15-20 day test. The 48-h 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 (see Figure 1).

The results of initial experiments comparing the short- and long-term tests indicated that the 48-h toxicity test was more appropriate for use in routine effluent testing. The long-term test was time consuming and cultures were subject to contamination during the 15-20 day test. Emphasis was placed on the continued development of the 48-h test using reference toxicants and complex effluents. The results of these studies demonstrated that this test was adaptable for evaluating effluent toxicity, provided that problems concerning test replicability were resolved (Anderson and Hunt, 1988).

The Phase 3 *Macrocystis* experiments focused on the continued testing of complex effluents and replicate reference toxicant testing. Effluent testing of two large southern California dischargers (Los Angeles City's Hyperion Waste Treatment Plant and L.A. County's JWPCP WTP) was performed quarterly. Each effluent was tested twice over a one year period. Reference toxicant tests were run concurrently with the effluent tests to determine whether variability was a function of the effluent, the test, or seasonal variations in kelp sensitivity. Copper chloride was substituted for zinc sulfate as a reference toxicant to determine if a more toxic metal would reduce between-test variability.

Figure 1. Life cycle diagram for giant kelp *Macrocystis pyrifera*. Short-term and long-term endpoints indicated in boxes.



In addition, three 48-h copper tests were performed within the same month to compare between-test variability with seasonal variability in the quarterly copper reference toxicant tests.

An interlaboratory test was conducted between the Department of Fish and Game's Marine Pollution Studies Laboratory (MPSL) and a second laboratory, Coastal Resources Associates (CRA), to refine the test protocol and ensure that the other laboratories could successfully conduct the test.

A long-term (20 day) experiment compared the sensitivities of 4 different endpoints to copper. Sporophyte production and growth after a 20 day copper exposure were compared to spore germination and gametophyte growth after 48 hours.

An experiment comparing the relative sensitivities of kelp obtained from different locations, and two experiments investigating the mechanism of germ-tube growth were also completed.

Methods

Detailed methods for the kelp experiments discussed in this section are given in the *Macrocystis* protocol (Appendix I). Protocol modifications for some of the experiments are given below. Unless otherwise stated, the methods were the same as those given in Appendix I.

Facilities

All of the experiments were done between November, 1987 and December, 1988 at MPSL. Located on the Big Sur coast in Monterey County, the MPSL seawater intake is at least 4 km from any known pollution source. Background concentrations of copper in the laboratory seawater reported by Martin *et al.* (1981) were 0.057 ± 0.030 µg/l.

Quarterly Effluent Tests

Four short-term tests of complex effluent were conducted, one during each quarter of the year. The effluents were obtained from two different waste treatment plants and each plant's effluent was tested twice. Effluents from the Los Angeles County Joint Water Pollution Control Plant (JWPCP) were tested in February and August 1988. The L.A. City Hyperion Treatment Plant effluents were tested in May and October. All experiments used 24-hour composite samples collected the day before the test and shipped to MPSL in cooled plastic containers via overnight courier. The JWPCP effluent was a mixture of 45% secondary and 55% primary treated wastewater that had been chlorinated and then dechlorinated. The Hyperion effluent was a mixture of 33% secondary and 67% primary treated wastewater and was not chlorinated. A series of effluent dilutions were tested with natural seawater used as the diluent. The effluent dilutions for the first JWPCP test were 0, 1.0, 3.2, 5.6, and 10.0%. The dilutions for the first Hyperion test were 0, 0.56, 1.0, 3.2, 5.6, and 10%. The effluent dilutions for the second JWPCP and Hyperion tests were 0, 0.56, 1.0, 1.8, 3.2, 5.6, and 10.0%. The zoospores used in the quarterly effluent and reference toxicant tests came from sporophylls collected at either Monastery Beach or Granite Canyon, Monterey County.

Quarterly Reference Toxicant Tests

Reference toxicant tests were done concurrently with the effluent tests using copper chloride as the toxicant. These tests served as reference to validate the procedures followed in the effluent tests, and gave an indication of any seasonal differences in kelp sensitivity to toxicants. The effluent tests and concurrent reference toxicant tests used spores released from the same mixture of sporophyll, with equal densities of spores in each test container.

The copper reference toxicant tests were scheduled quarterly in February, May, August, and October to evaluate seasonality. The copper concentrations tested were as follows: Test #1 = 0, 56, 100, 180, 320, 560 µg/l; Test #2 = 0, 32, 56, 100, 180, 320; Tests #3 and #4 = 0, 10, 18, 32, 56, 100, 180 µg/l.

Replicate Reference Toxicant Experiments

To compare the seasonal variability observed in the quarterly tests with short-term variability, we conducted three 48-h tests in October. The methods for the replicate copper tests followed the 48-h protocol except some modifications were made to minimize variability. Sporophylls were collected from one plant instead of several plants for all tests; sporophyll weight was standardized prior to spore release for each test.

An adult plant was marked in the kelp bed at Granite Canyon and used as the sporophyll source for all tests. Equal weights (116 g) of sporophylls were placed in 2 liters of 0.2 µm-filtered sea water at the start of each test. After 1.5 hours the spore density was calculated and adjusted so that approximately 3×10^6 spores were pipeted in 0.5 ml water into each test container. After 48 hours the test slides were removed and the endpoints quantified. Copper concentrations were 0, 10, 32, 56, 100, and 180 µg/l. An 18 µg/l test concentration was added to the last test.

Interlaboratory Testing

One interlaboratory test of the 48-h standard protocol was conducted. Methods followed the procedures for interlaboratory tests given in Anderson *et al.* (1988). Experiments were run concurrently at MPSL and the CRA laboratory in Encinitas. The staff at CRA were experienced in culturing kelp and familiar with the kelp protocol but had no prior experience conducting the 48-h test.

Macrocystis sporophylls were collected in the kelp beds at Monastery Beach, Monterey County and San Onofre, San Diego County one day before the test. Half of the sporophylls collected at each site were retained in coolers overnight for use by the respective labs. The other half was shipped on ice via overnight courier to the other participating lab. Dilution water collected from the MPSL seawater system was shipped to the CRA lab and water collected by CRA at Scripps pier in La Jolla was shipped to MPSL. Spores from the same collection site were used to produce spores to eliminate variability from interpopulation differences. Sporophyll from San Onofre were used because they released more spores. The copper concentrations tested were 0, 56, 100, 180, 320, 560 µg/l, with 5 replicates per concentration. The control solutions were accidentally not inoculated in the CRA copper test. The control values for germination and germ-tube

growth given for the CRA data are those from the CRA duplicate controls using San Onofre sporophyll in MPSL seawater. Except for the control, the dilution water in the CRA test was from Scripps Pier in La Jolla (see discussion).

In addition to the standard 48-h test design, triplicate controls using combinations of sporophyll and dilution water from two different sites (San Diego County and Monterey County) were compared. Three control combinations were tested at MPSL: Monastery sporophyll and MPSL water; Monastery sporophyll and La Jolla water; and San Onofre sporophyll and MPSL water. The control combinations tested at CRA were San Onofre sporophyll and La Jolla water; San Onofre sporophyll and MPSL water; and Monastery sporophyll and La Jolla water.

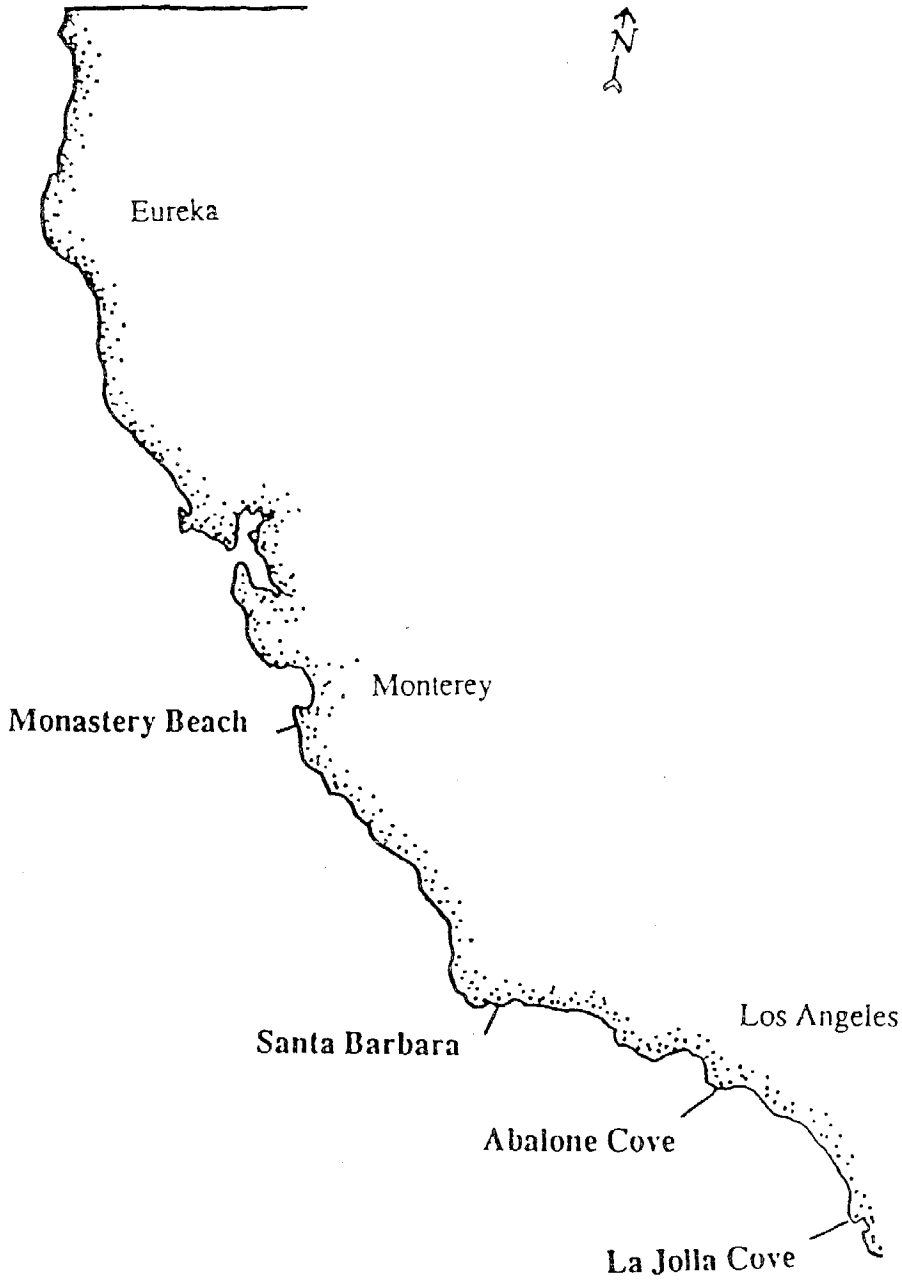
Sensitivity of Different Kelp Populations

To compare the sensitivity of kelp zoospores from geographically isolated *Macrocystis* populations, sporophylls were collected from 4 different kelp beds: La Jolla Cove, San Diego County; Abalone Cove, Los Angeles County; Campus Point, Santa Barbara County; and Monastery Beach, Monterey County (Figure 2). Three groups of sporophylls were collected on December 8, 1988, blotted and placed in coolers at 9 °C, and transported overnight to MPSL. The sporophylls from Campus Point were collected on December 1, placed in flowing seawater (at ambient temperature ~16 °C) in a greenhouse, then blotted and placed in a cooler on December 8, and transported overnight to MPSL. The fourth group of sporophylls were collected at Monastery Beach and stored in a refrigerator at 5 °C instead of a cooler for the night before the test.

On December 9 all of the sporophylls were rinsed in 0.2 µm-filtered seawater then left to release zoospores in 1 liter of water for 1 hour. Equal weights of sporophylls were used in the spore release process and equal spore densities distributed to each test container. After the zoospores were pipetted into the test containers, the standard 48-h protocol was followed, except that two microscope slides were placed into each container. The first microscope slide was removed to quantify germination and germ-tube growth after 48 hours. At this time the lights were changed from cool-white fluorescent to full-spectrum Vitalights®(see next section for detailed methods). Nutrients were added (PES, minus EDTA-chelated Iron, plus germanium dioxide). From this point on the test solutions were changed on days 4 and 8. The second slide was removed after 16 days and quantified. Sporophyte numbers were counted at 100x magnification in three 0.2 x 18.0 mm transects on each replicate slide with a compound microscope. Transect counts were used to quantify sporophyte production rather than estimating the percentage of female gametophytes producing sporophytes, because the number of female gametophytes was too dense to allow accurate counting (see below). Five concentrations were tested: 0, 18, 32, 56, and 100 µg/l copper.

Germination and germ-tube growth data were calculated as a percentage of control means. A two-way ANOVA was used to determine differences between the four spore sources. No Observed Effect Concentrations (NOEC's) were determined for each spore source using ANOVA followed by Dunnett's test as described in Appendix 1.

Figure 2. Sporophyll collection sites for experiment comparing sensitivities of different kelp populations to copper.



To analyze the sporophyte production data, the mean of the three transects on each test slide was calculated to give a mean number of sporophytes per transect for each replicate slide. These data were then analyzed with ANOVA followed by Dunnett's to compare the different spore sources and derive NOEC's for each.

Long-term Reference Toxicant Experiment

The methods for the long-term kelp test were similar to those given by Anderson and Hunt (1988). As in the short-term protocol (Appendix I) this experiment started with motile zoospores. The initial procedures were the same as the 48-h experiments, with the following modifications: nutrients were added to the tests solutions to induce gametogenesis (PES without EDTA-chelated Iron); germanium dioxide was added to prevent fouling by diatoms; full-spectrum Vitalights® were adjusted to give 50 μ E $m^{-2}sec^{-1}$ on a 24 hour photoperiod; test solutions were changed every 96 hours; and the test was conducted in an incubator at 13°C in clear-plastic-covered 250 ml polyethylene containers. The copper concentrations tested were 0, 10, 32, 56, 100, and 180 µg/l.

Three microscope slides were placed in each test container at the start of the experiment. After 48 hours one slide was removed and germination and germ-tube growth were quantified following our standard 48 hour protocol.

After 19 days a second slide was removed and the effect of copper on sporophyte production was evaluated. Sporophyte production is a measure of kelp sexual reproduction. For this endpoint we quantified the number of sporophytes as a percentage of female gametophytes. One-hundred female gametophytes were counted and the number with sporophytes attached was noted. Only sporophytes with at least one cell division were counted. Sporophyte production was quantified this way to eliminate bias from unequal distribution of female gametophytes on replicate slides.

On day 20, the final slide was removed and the effect of copper on sporophyte growth was quantified. Sporophyte length was measured in microns with an ocular micrometer at 100x magnification. The first 10 sporophytes encountered in a 0.2 x 18.0 mm transect across the slide were measured in each replicate.

Copper Toxicity and Lighting Experiments

The effect of different light levels on germination and growth was evaluated using the standard 48-h protocol. Motile spores were settled and grown at five different light levels for 48 hours. The light levels tested were: 0, 10, 30, 50, and 70 µE m^{-2} sec^{-1}. Light was measured with a flat plate, cosine corrected light sensor (Licor® model 185-B, Licor Inc., Lincoln, Nebraska). Each treatment was replicated five times.

In a second experiment, the standard 48-h protocol using copper was conducted in complete darkness.

Reference Toxicant Verification and Water Chemistry

As in our previous work, samples for copper analyses were taken at the beginning and end of each test. One replicate from each concentration was sampled and analyzed using a Perkin Elmer Model 603 atomic absorption spectrophotometer . Unless otherwise noted, all reported copper values are analytically verified concentrations. Those not analytically verified are referred to as nominal concentrations. No chemical analysis was done on the solutions used in the interpopulation experiment.

Water quality was monitored by measuring dissolved oxygen, pH, salinity, and temperature at the beginning and end of each experiment.

Results and Discussion

The results of the kelp experiments performed during Phase 3 of the Marine Bioassay Project are summarized in Table 1.

Table 1. Summary of Phase 3 kelp test data. † = concentrations are nominal values. * = NOEC for sporophyte growth. § = median and range of copper NOEC's for germination and germ-tube growth for all tests except Copper No-Light Test.

Toxicant	Date	No Observed Effect Concentration		
		Germination	Growth	Sporophyte Prod.
Complex Effluent (Quarterly Tests)				
JWPCP	February	≥10.0 %	3.20 %	- - -
Hyperion	May	≥10.0 %	5.60 %	- - -
JWPCP	August	1.0 %	<0.56 %	- - -
Hyperion	October	≥10.0 %	5.60 %	- - -
Copper Reference Toxicant (Quarterly Tests)				
Q #1	February	<40.8 µg/l	<40.8 µg/l	- - -
Q #2	May	99.1 µg/l	<31.1 µg/l	- - -
Q #3	August	19.4 µg/l	<10.1 µg/l	- - -
Q #4	October	54.1 µg/l	18.8 µg/l	- - -

(Continued on next page)

Table 1. Summary of Phase 3 kelp test data (cont.)

Toxicant	Date	No Observed Effect Concentration		
		Germination	Growth	Sporophyte Prod.
Short-Term Variability				
Copper Replicate Tests				
Test #1	October	55.8 µg/l	8.8 µg/l	- - -
Test #2	October	94.5 µg/l	9.3 µg/l	- - -
Test #3	October	54.1 µg/l	18.8 µg/l	- - -
Copper Interlaboratory Tests				
CRA	February	56.0 µg/l†	<56.0 µg/l†	- - -
MPSL		<40.8 µg/l	<40.8 µg/l	- - -
Copper Long-Term Test				
	October	50.1 µg/l	31.1 µg/l	<10.2 µg/l 10.2 µg/l *
Copper Interpopulation Test†				
Monastery	December	56.0 µg/l	<18.0 µg/l	<18.0 µg/l
Santa Barbara	December	≥100.0 µg/l	<18.0 µg/l	<18.0 µg/l
La Jolla	December	≥100.0 µg/l	<18.0 µg/l	<18.0 µg/l
Abalone Cove	December	≥100.0 µg/l	18.0 µg/l	<18.0 µg/l
Copper No-Light Test	October	10.0 µg/l	32.5 µg/l	- - -
Median Copper NOEC §		56.0 µg/l	<18.0	
(Range)		(19.4 - ≥100)	(8.8 - <56)	

Quarterly Effluent and Reference Toxicant Tests

The results of the two Los Angeles County Joint Water Pollution Control Plant (JWPCP) effluent tests were variable (Figures 3a, 4a). In the February effluent test (Figure 3a) germination was not significantly inhibited at any test concentration, but in August germination was significantly inhibited at effluent concentrations of 3.2 % and greater (NOEC = 1.0%). The germ-tube growth endpoint was more sensitive in both tests (Figure 4a). The JWPCP effluent significantly inhibited germ-tube growth at concentrations of 5.6 % and greater (NOEC = 3.2%) in the February test, and at the lowest test concentration in the August test (NOEC < 0.56%).

The results of the reference toxicant tests indicate that there is seasonal variability in kelp sensitivity to toxicants and suggest that the difference observed in the two JWPCP effluent tests was not due solely to variability in effluent toxicity. It is more likely that both effluent toxicity and kelp sensitivity varied between the two tests. The greater variability in the effluent germination and germ-tube growth response curves relative to the copper response curves suggest that effluent toxicity varied.

The results of the Hyperion effluent tests are given in Figures 5a and 6a. Unlike the JWPCP test results, the two Hyperion effluent tests were similar. There were no effects on germination in either the May or October effluent tests. The NOEC's for germ-tube growth were the same for both tests (NOEC = 5.6% effluent in May and October). The germination and germ-tube growth endpoints were variable in the May and October copper tests (Figures 5b and 6b). The NOEC's for germination were 99.1 µg/l in May and and 54.1 µg/l in October (Figure 5b), while the NOEC's for germ-tube growth were < 31.1 µg/l in May and 18.8 µg/l in October (Figure 6b). The greater sensitivity in the October tests again suggest seasonal variability in kelp sensitivity to the copper reference toxicant. Although the percentage of secondary treated wastewater was greater in the JWPCP effluent, it was consistently more toxic than the Hyperion effluent.

Figure 3a. JWPCP Complex Effluent Tests
(Germination)

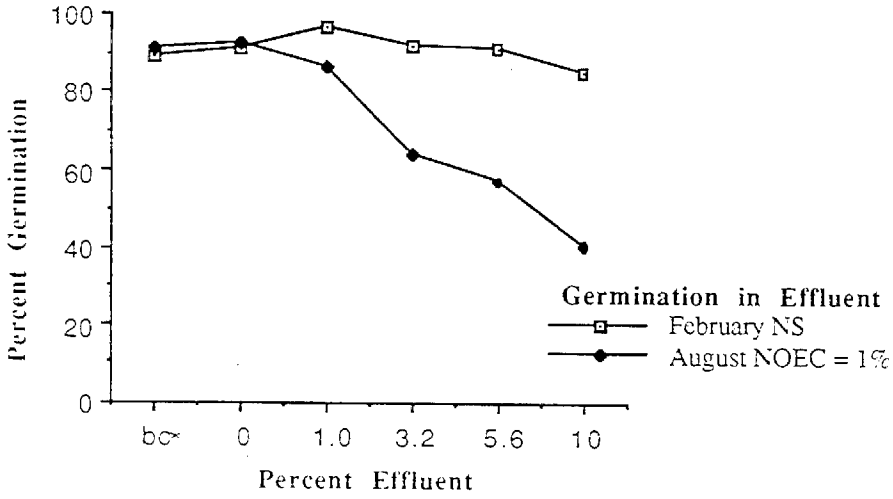
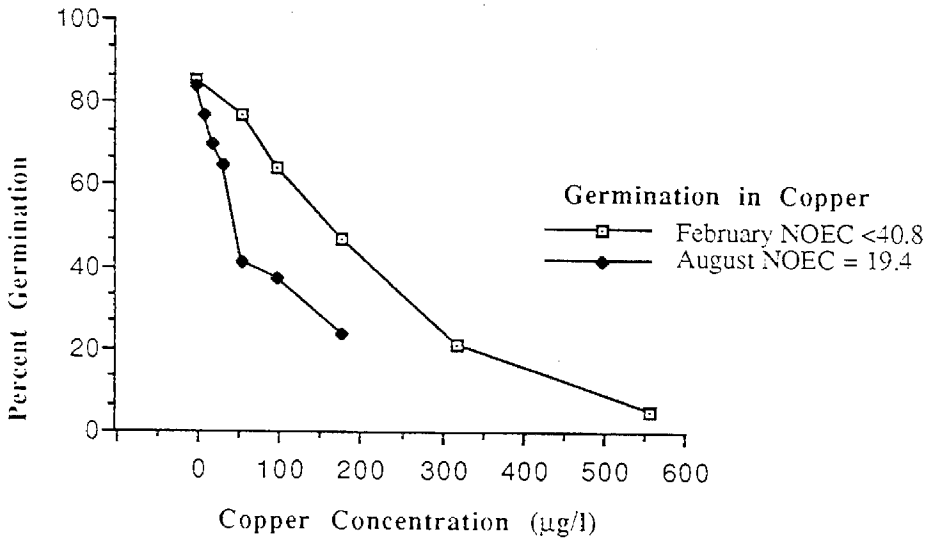


Figure 3b. JWPCP Concurrent Copper Reference Toxicant Tests
(Germination)



* bc = brine control
NS = No significant difference

Figure 4a. JWPCP Complex Effluent Tests
(Germ-Tube Growth)

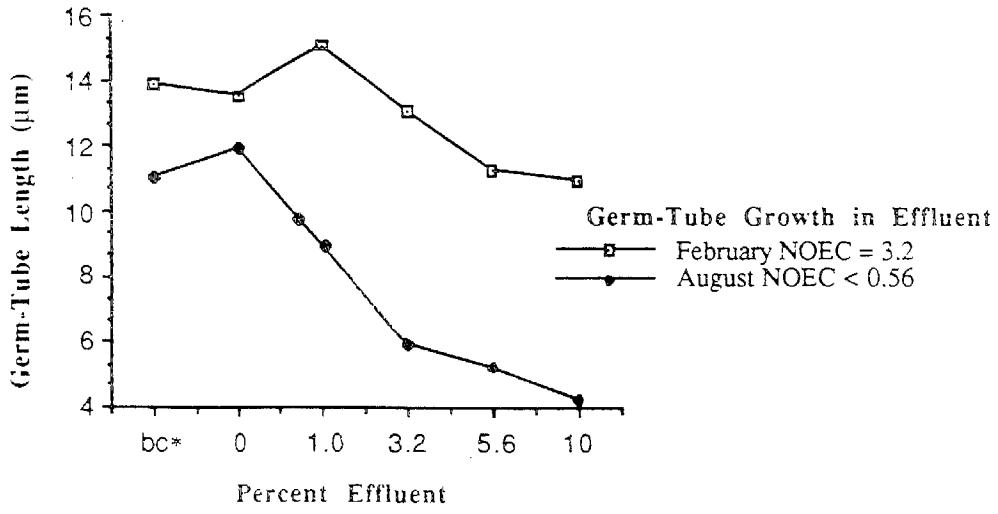
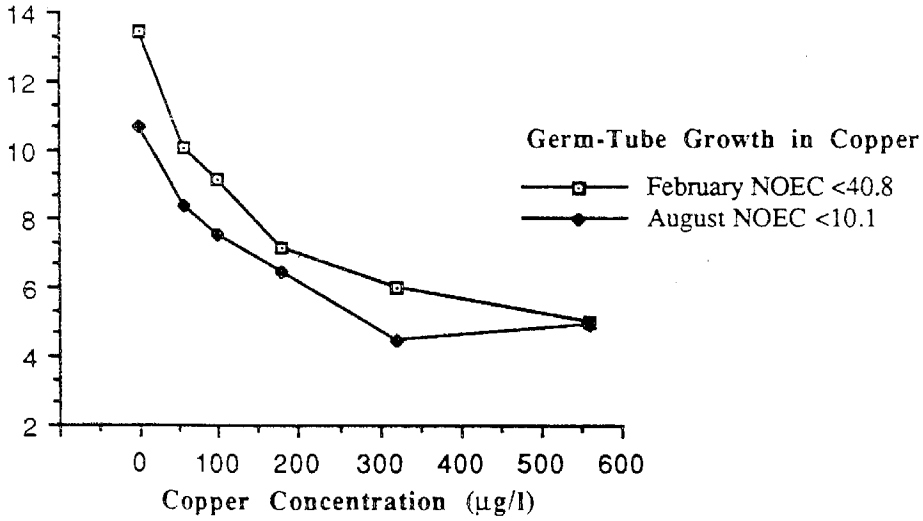


Figure 4b. JWPCP Concurrent Copper Reference Toxicant Tests
(Germ-Tube Growth)



*bc = brine control

Figure 5a. Hyperion Complex Effluent Tests
(Germination)

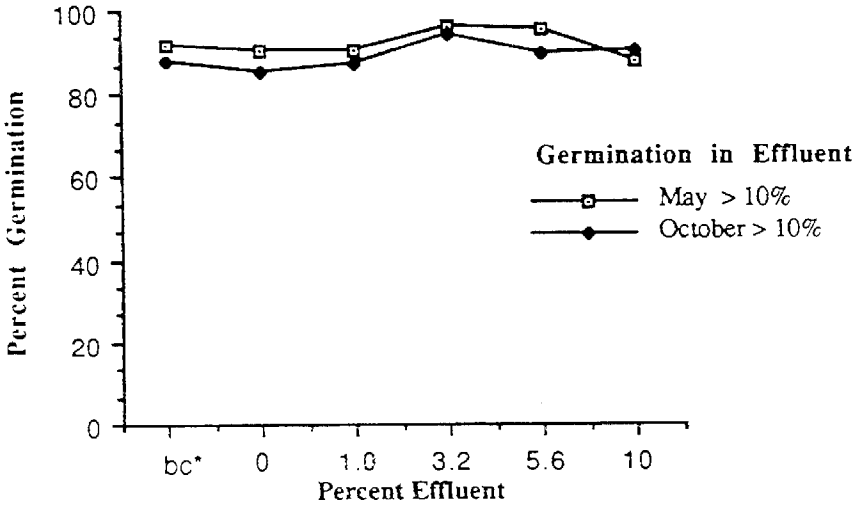
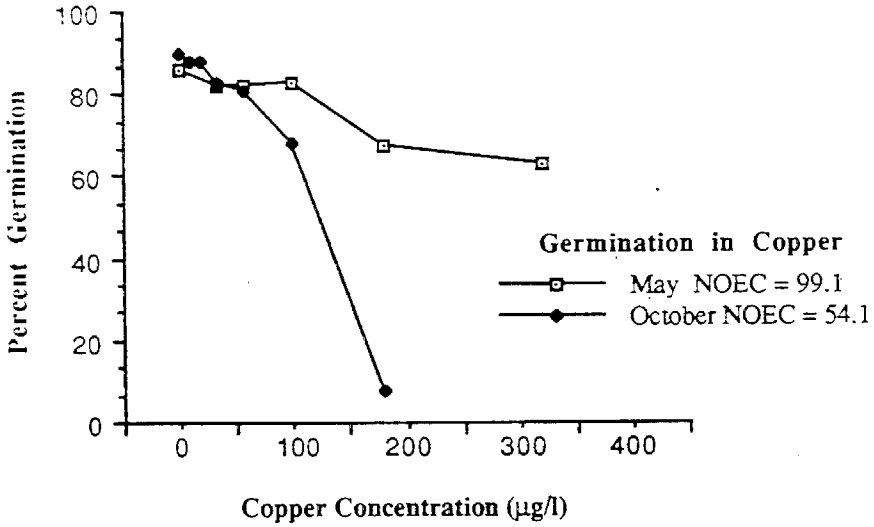


Figure 5b. Hyperion Concurrent Copper Reference Toxicant Tests
(Germination)



* bc = brine control

Figure 6a. Hyperion Complex Effluent Tests
(Germ-Tube Growth)

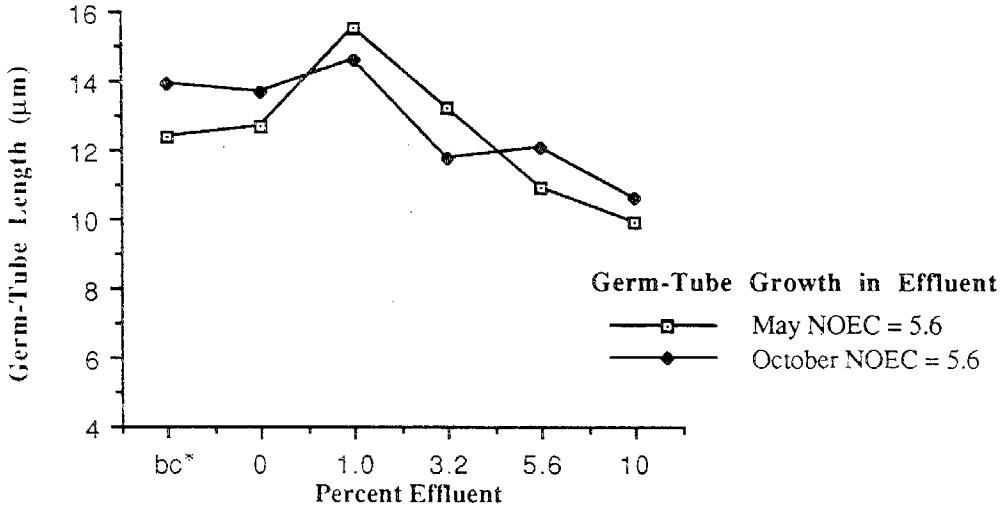
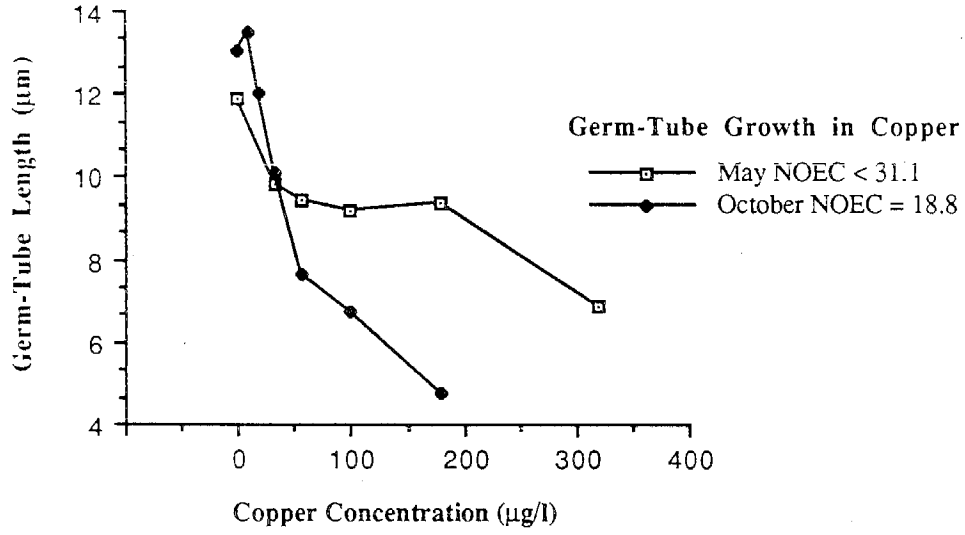


Figure 6b. Hyperion Concurrent Copper Reference Toxicant Tests
(Germ-Tube Growth)



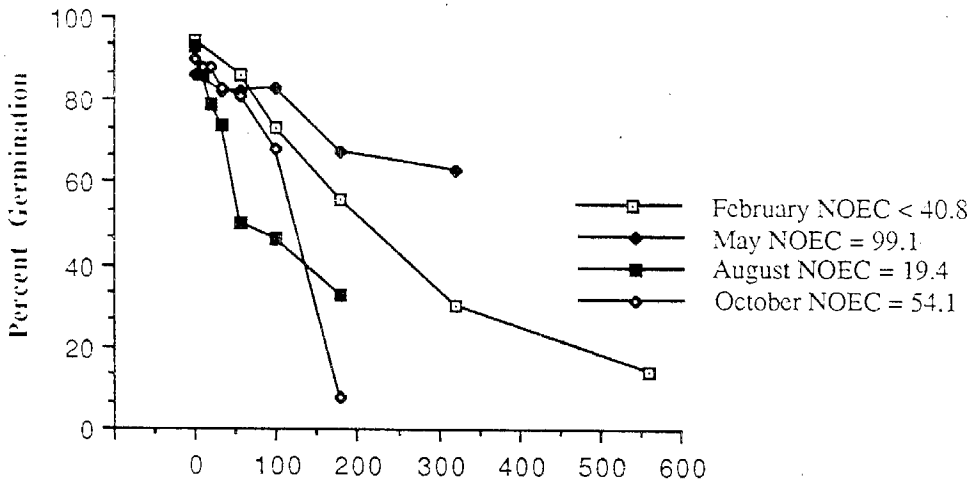
* bc = brine control

Seasonal variability in copper sensitivity of *Macrocystis* can be seen more clearly in the combined quarterly test data (Figure 7a & b). Germination and germ-tube growth were variable in the four quarterly tests. Both endpoints in the February and May tests were apparently less sensitive than in August and October. The reason for this seasonal variability is unknown, but may be due to seasonal fluctuations in the general health of adult plants from which the sporophylls were collected. Marine algae are dependant on ambient nutrient supplies which fluctuate seasonally. Nitrogen (as NO_3^- and NH_4^+) in particular, may be limiting during certain times of the year (Jackson, 1977; Wheeler and North, 1981; Gerard, 1982). In southern California, nitrogen limiting conditions generally occur in late summer and fall during times of no upwelling (North *et al.*, 1982). Although nitrogen limiting conditions occur less frequently in central California where the water is cooler and and upwelling is more consistent (Gerard, 1982 ZZ; Broenkow and Smethie, 1978), it is possible that the differences in sensitivity of *Macrocystis* zoospores and gametophytes observed in the quarterly experiments were related to seasonal fluctuations in the availability of nutrients to the plants at our collection site. If the plants were nutrient depleted, it is reasonable to infer that their spores were likewise affected, perhaps increasing their sensitivity to toxicants. No nutrients were added to the test containers in the quarterly 48-h experiments.

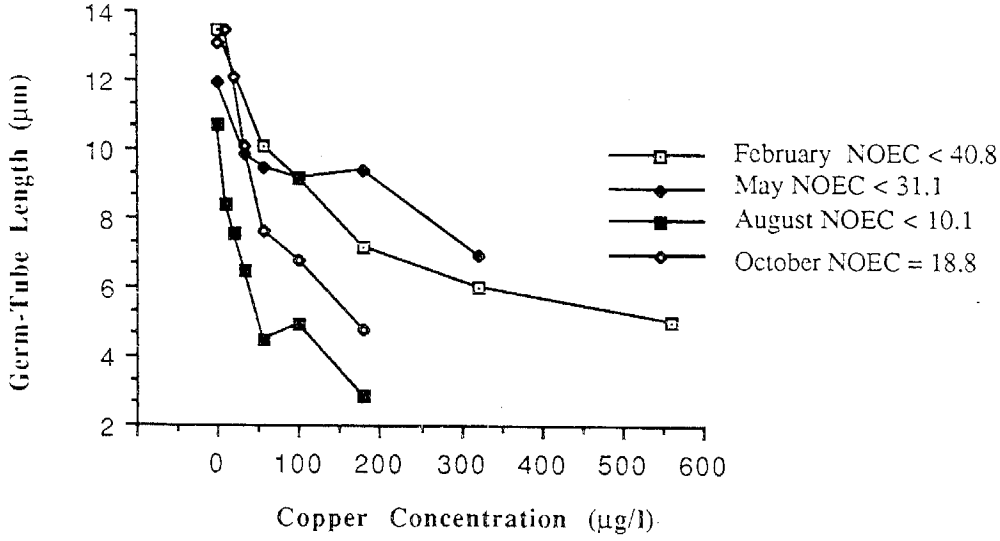
Another possible explanation for the variability observed in the quarterly tests is seasonal differences in the chelating capacity of the dilution water used in the experiments. Chelation of divalent cations (e.g. Cu^{2+}) occurs on dissolved organic compounds and particles in the dilution water (Kuwabara, 1983). The concentration of Cu^{2+} ions in the test containers could have been affected by fluctuations of chelators in the dilution water, which may vary seasonally. Because the cationic form of copper is most likely the form toxic to plants (Sunda and Guilliard, 1976), a fluctuation in chelating capacity could have affected the results. Analysis of divalent ions rather than total copper concentration would help resolve if chelation affects the results. Divalent ion analysis is relatively difficult and has not been the emphasis of MBP. An alternative approach would be to use an organic toxicant which would eliminate chelation problems.

Figure 7a-b. Kelp Quarterly Copper Reference Toxicant Tests

a) Germination



b) Germ-Tube Growth



Replicate Reference Toxicant Experiments

Results of the three replicate copper experiments, conducted sequentially in October, were less variable than the four quarterly tests (Figure 8a-b). The NOEC's for inhibition of germination were 55.8, 94.5, and 54.1 µg/l for tests 1-3, respectively. The response curve in test #3 had a more severe decrease in germination at higher concentrations than the first 2 tests. The NOEC's for inhibition of germ-tube growth were 8.8, 9.3, and 18.8 µg/l for the 3 tests.

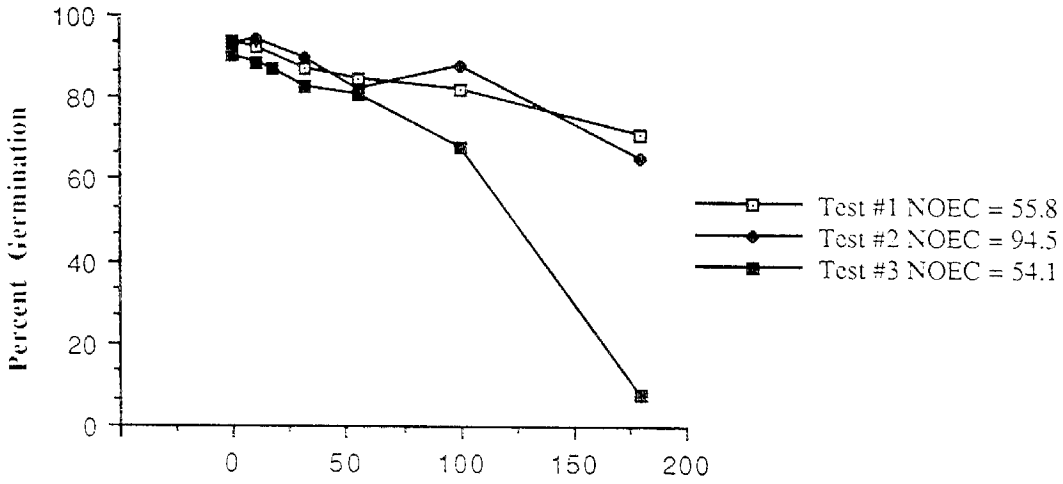
The difference in NOEC's in the growth data are probably due to the range of copper concentrations used in the three experiments. The first two tests used a 10 µg/l but not an 18 µg/l (nominal) concentration.

Results of the three replicate tests indicate that seasonality contributes to variability in *Macrocystis* sensitivity to toxicants. The replicate copper tests were less variable than the quarterly copper tests. Although the quarterly tests used spores obtained from a pooled sample of sporophylls from several adult plants, this probably did not contribute to the variability between the tests. For example, the results of the three zinc sulfate tests from 1987 are included for comparison (Figure 9a&b). These tests were all done in December - January with spores obtained from a mixture of sporophylls. As with the copper results, the zinc germination NOEC's were more variable than the growth NOEC's, but the between-test variability for both endpoints was less than in the quarterly copper tests. The shape of the copper response curves for both endpoints are similar to those for zinc, even though the copper tests used sporophylls from the same plant while the zinc tests used pooled sporophylls. It is not clear why the length data are more consistent than the germination data but this has been the trend for all of the replicate experiments conducted to date.

An effort was made in these replicate experiments to standardize the wet-weight of sporophyll used in the release process of the 3 replicate tests. This was done to minimize differences in the amount of natural chelators (eg., surface polysaccharides and polyphenols) released by the sporophylls. It has been shown that these chemicals are capable of chelating divalent ions (Haug and Smidsrod, 1965; Ragan *et al.*, 1979), and variation in the amount of chelators present could contribute to between-test variability. The initial spore density was calculated and diluted accordingly with 0.2 µm-filtered seawater, so that 0.5 ml was added to each container to provide approximately 3×10^6 spores. More work is necessary to determine whether this type of standardized procedure reduces variability. The Phase II zinc tests (Figure 9a-b) had similar variability without standardizing sporophyll weight, which suggests that chelation effects from algal exudates are negligible if the spore densities used in replicate tests are the same.

Figure 8a-b. Kelp Replicate Copper Reference Toxicant Tests

a) Germination



b) Germ-Tube Growth

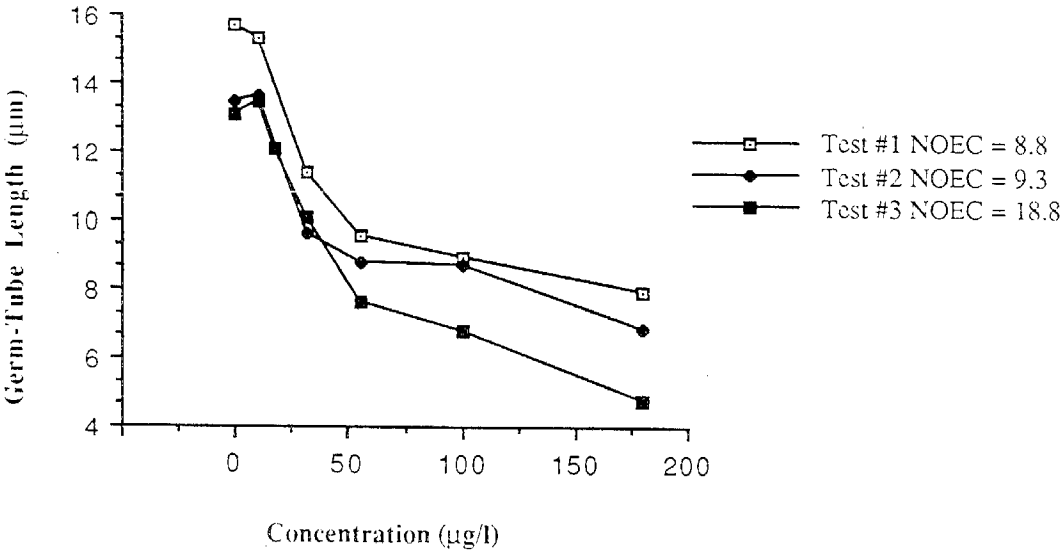
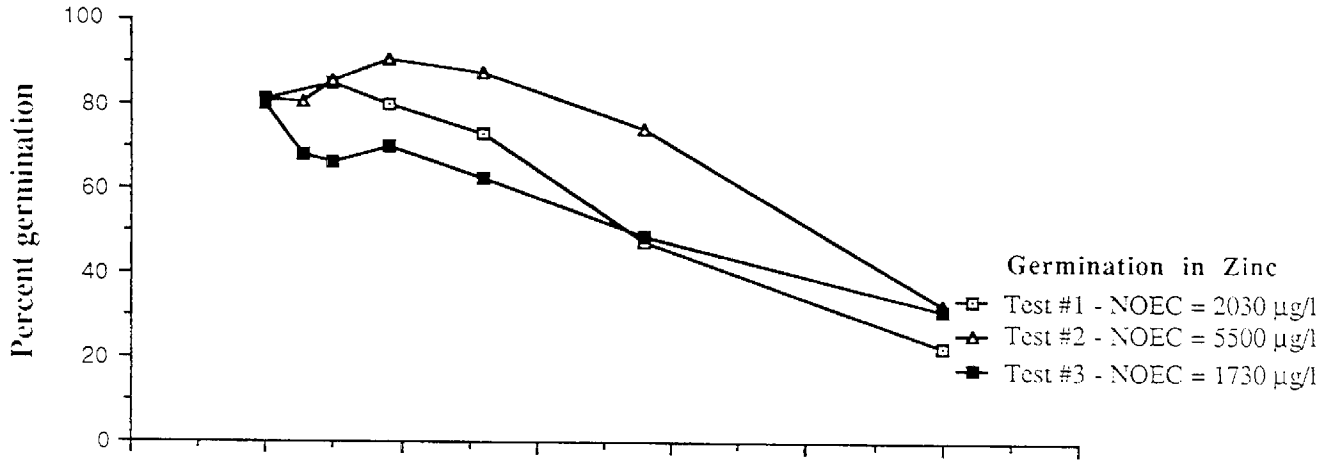
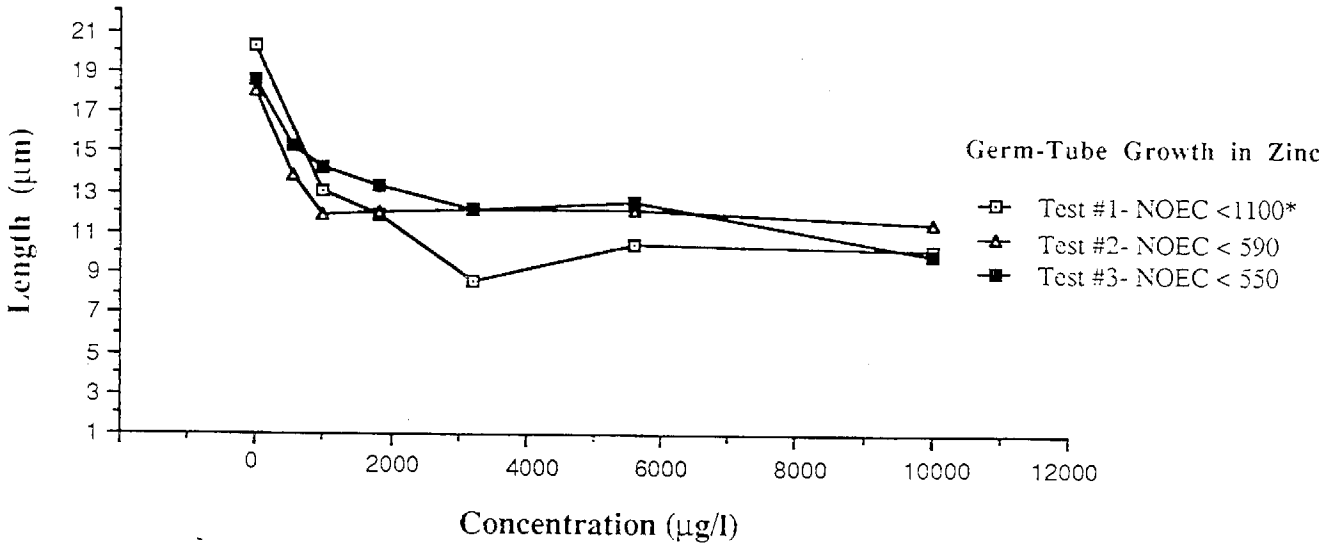


Figure 9 a-b. Kelp Replicate Zinc Reference Toxicant Tests
(From Anderson and Hunt, 1988)

(a) Germination



(b) Germ-Tube Growth



Interlaboratory Testing

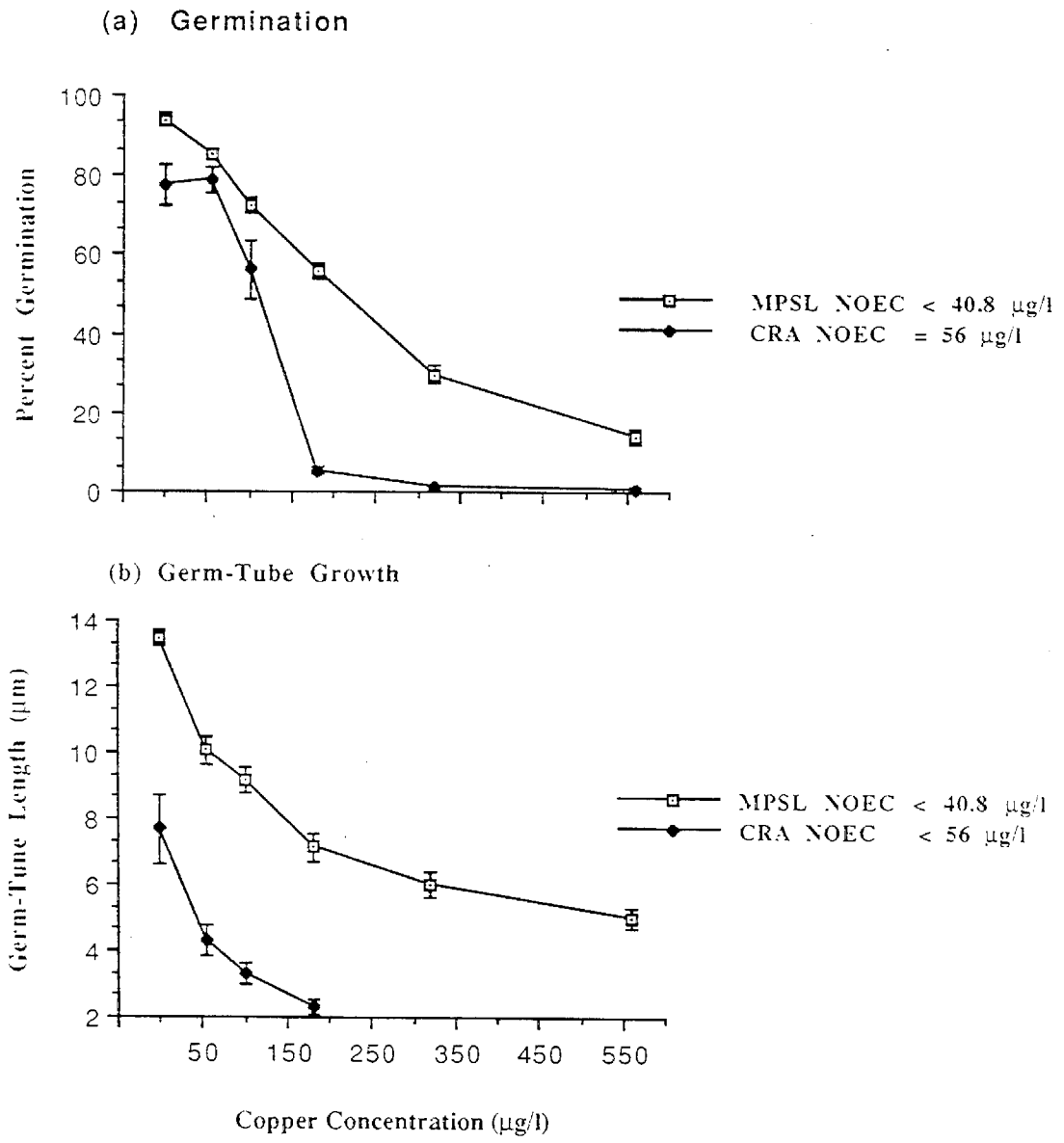
The results of the interlaboratory tests between MPSL and CRA were relatively uniform (Figure 10a-b). As mentioned earlier, because the CRA control replicates were inadvertently not inoculated, it is difficult to make direct comparisons. The control numbers for germination and germ-tube growth given for the CRA response curves (Figure 10) are from the duplicate controls using San Onofre sporophylls in MPSL seawater. It is probably reasonable to substitute this control because Analysis of Variance indicated that the dilution water source had no effect on toxicity (Table 2).

The germination data were slightly more variable than the germ-tube growth data (Figure 10a). The NOEC for germination in the MPSL test was less than the lowest test concentration (<40.8 µg/l). The NOEC for germination in the CRA test was 56 µg/l (nominal concentration). Germ-tube growth results were consistent (Figure 10b); both labs derived NOEC's less than the lowest (nominal) concentration, 56 µg/l. The shapes of the germ-tube growth response curves were similar, except that germination tubes were consistently shorter at all test concentrations in the CRA test. It is not clear what caused this difference. The measured light levels were the same for both labs, but different light meters were used and these may have been calibrated differently. It is also not clear why the germination rate for the MPSL control group was higher than the CRA control group (Figure 10a). Both used the same water and sporophyll source (Table 2).

Table 2. Percent germination and germ-tube growth in duplicate controls from the CRA and MPSL interlaboratory tests (Mean and S.D.). * = used as the control in the 48-h interlaboratory copper toxicity test. N.D. = no data.

Laboratory	Water Source	Spore Source	Mean % Germination	Mean Germ-Tube Length
CRA	MPSL	San Onofre	77.4 (11.6)	7.7 (2.3)*
CRA	La Jolla	Monastery	70.2 (13.6)	7.5 (0.5)
CRA	La Jolla	San Onofre	N.D.	N.D.
MPSL	MPSL	Monastery	70.7 (3.6)	11.2 (0.8)
MPSL	MPSL	San Onofre	93.9 (3.1)	13.5 (0.4)*
MPSL	La Jolla	Monastery	74.5 (4.4)	12.1 (0.9)

Figure 10 a-b. Kelp Copper Interlaboratory Tests: MPSL-CRA



Sensitivity of Different Kelp Populations

The results of the interpopulation experiment suggest that there is little difference in response to copper between isolated populations of *Macrocystis* (Figure 11a-c, Table 3). While there was slight variability in the germination data, it was not statistically significant (ANOVA $p = 0.05$). All of the spores showed slight germination inhibition in response to copper, but the only statistically significant decrease occurred with the Monastery Beach sporophyll (NOEC = 56 µg/l, Figure 11a). The germination rates of spores from the Santa Barbara sporophylls were significantly less than for spores from the three other sporophyll sources (Figure 11a), probably because they were handled differently. They were collected 8 days before the test and held in seawater, while the others were collected the day before the test. The low germination rate was probably not a result of copper toxicity, because control germination was also low, and the effect on germ-tube growth was no different than for the other spore sources.

The germ-tube growth data showed no significant difference between the different spore sources. The NOEC for germ-tube growth was less than the lowest test concentration (18 µg/l) at Monastery, Santa Barbara, and La Jolla while Abalone Cove had a NOEC of 18 µg/l. The response curves were similar for the four sporophyll sources (Figure 11b).

Although the relative numbers of sporophytes produced varied between the different spore sources, the inhibition of sporophyte production by copper was the same. Sporophyte production was completely inhibited in all of the cultures at the lowest test concentration, 18 µg/l (Figure 11c). Because we evaluated the effect of copper on three different endpoints, each having varying degrees of sensitivity, a wide range of copper concentrations were tested. Including more concentrations between 0 and 18 µg/l would have given more information on sporophyte production.

While the results of this experiment indicate little difference in copper sensitivity between different populations of *Macrocystis*, it should be noted that this experiment was conducted at only one time period and with sporophylls collected on one day from only four locations. It is possible that a more extensive experiment with more locations tested over the course of a year might reveal greater differences in kelp sensitivity. For example, if the seasonal effect observed in the quarterly tests was related to physiological differences coupled to nutrient availability, one might expect to see differences between central and southern California kelp populations at times when significant upwelling is occurring in central California but not in southern California. Geographical differences in physiology have been demonstrated for temperature tolerance by gametophytes (Deysher, unpublished data) and sporophytes (North, 1971), and for photosynthesis in sporophytes (Zimmerman, 1988). Ecotypic differences in physiology have been reported for *Laminaria saccharina* (Gerard ,1988), and genetically-based variability in tolerance to copper have been reported for other macroalga species (Russell and Morris, 1970; Hall *et al.*, 1981; Reed and Moffat, 1983).

Figure 11 a-c. Kelp Interpopulation Copper Experiment

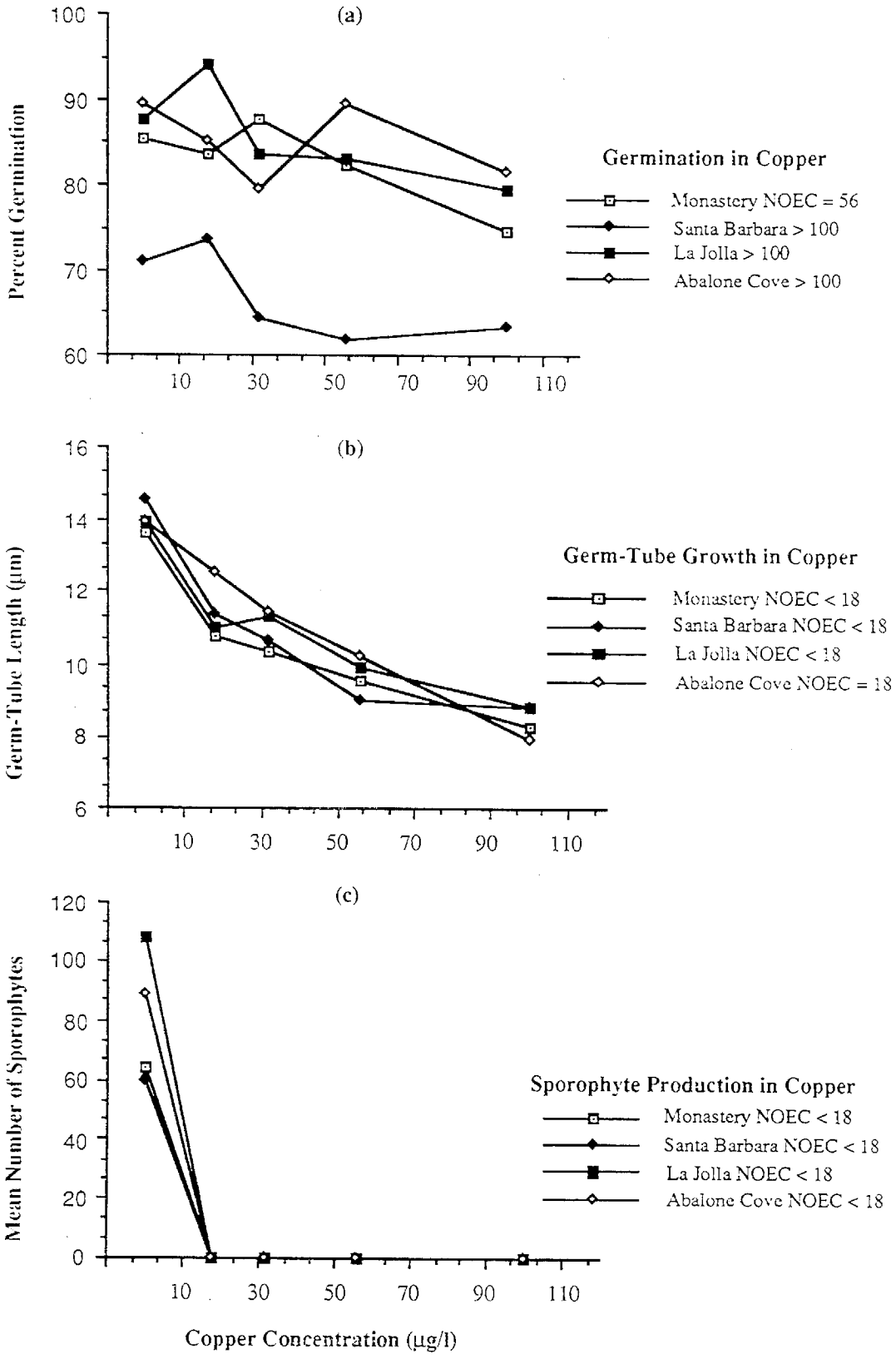


Table 3. Percent germination and germ-tube growth of spores from four different kelp populations exposed to copper chloride (Mean and S.D.).

Copper Conc. (µg/l)	Sporophyll Sources: Monastery Beach	Santa Barbara	La Jolla	Abalone Cove
	Germination (%)			
0	84.4 (3.7)	70.9 (6.5)	87.6 (6.9)	89.6 (5.8)
18	83.5 (6.7)	73.7 (5.1)	84.1 (6.1)	85.1 (6.0)
32	87.8 (4.3)	64.4 (4.7)	83.5 (3.9)	79.4 (7.0)
56	82.3 (7.6)	61.7 (3.7)	83.2 (3.7)	89.4 (5.3)
100	74.7 (6.5)	63.3 (8.7)	79.5 (3.7)	81.6 (6.1)
	Germ-Tube Growth (µm)			
0	13.6 (0.5)	14.6 (0.7)	14.0 (0.4)	14.0 (0.9)
18	10.7 (0.5)	11.4 (0.5)	11.0 (1.1)	12.6 (1.2)
32	10.4 (0.8)	10.6 (0.7)	11.3 (0.6)	11.5 (0.9)
56	9.6 (0.7)	9.0 (0.4)	9.9 (0.8)	10.2 (1.3)
100	8.2 (1.2)	8.8 (0.9)	8.8 (0.7)	7.9 (0.8)

Long-Term Reference Toxicant Experiment

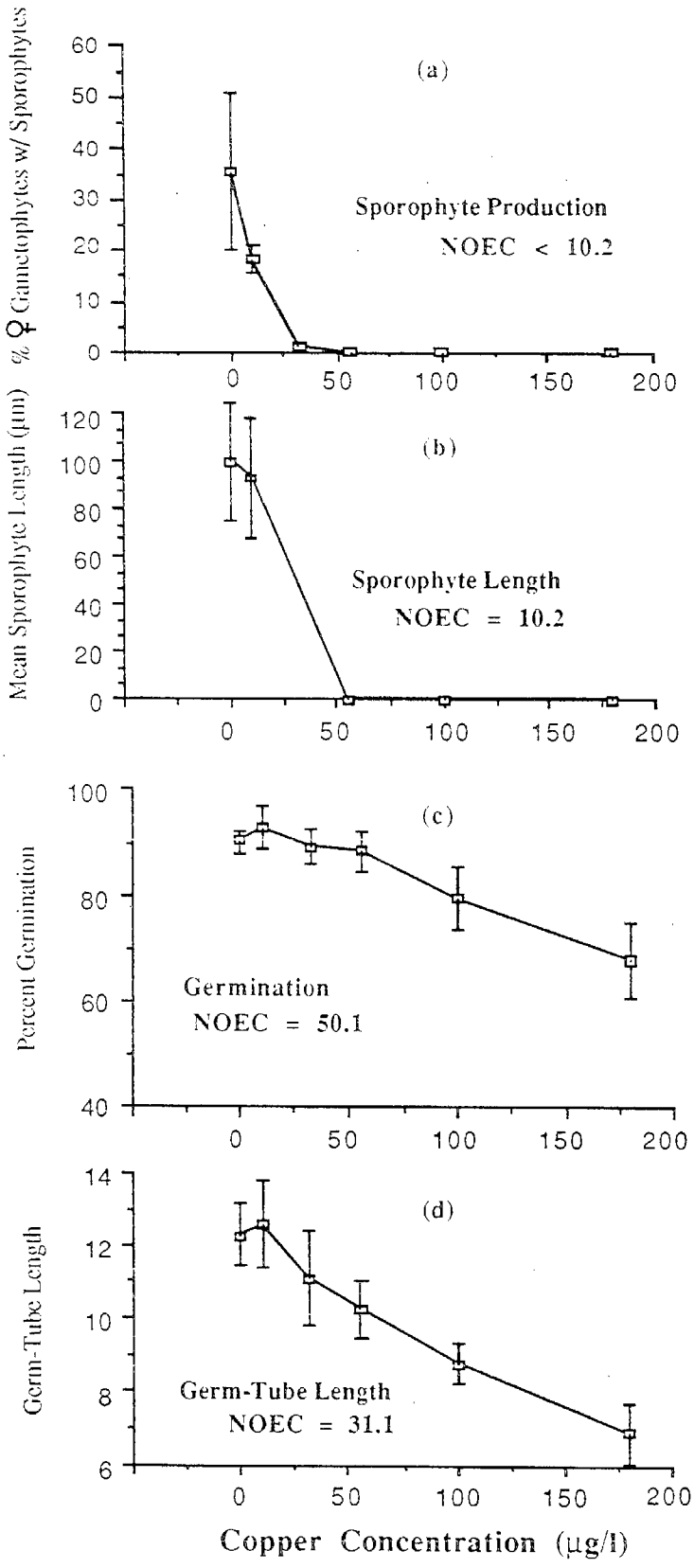
Results of the 20-day long-term experiment comparing 4 different endpoints showed that sporophyte production was more sensitive to copper than sporophyte growth, spore germination or germ-tube growth (Figure 12a-d). The percentage of female gametophytes producing sporophytes declined at the lowest test concentration, 10.2 µg/l (NOEC < 10.2 µg/l, Figure 12a). Once produced, the sporophytes appeared to grow in the lower copper concentration (NOEC = 10.2 µg/l, Figure 12b), although the sizes of the sporophytes present were variable . There were so few sporophytes present in the 32 µg/l concentration that it was impossible to measure an adequate number for a statistical comparison. Germination was inhibited at 90.6 µg/l copper (NOEC = 50.1 µg/l, Figure 12c) and germ-tube growth was inhibited at 50.1 µg/l (NOEC = 31.1 µg/l, Figure 12d).

Although the results indicate that sporophyte production is about three times as sensitive as germ-tube growth, the NOEC for germ-tube growth derived in this experiment was greater than the value typically calculated for copper in our previous experiments. For example, the October quarterly copper test conducted concurrently with this long-term test, had a NOEC of 18.8 µg/l. The difference between the quarterly test and the long-term test was light and nutrients. For the quarterly test we used cool-white fluorescent lights instead of the daylight spectra Vitalights® used in the long-term test. In addition, no nutrients were added to the quarterly test while PES was added to the long-term test. It is possible that the lights or nutrients affected the comparisons within the long-term test by decreasing the sensitivity of germ-tube growth. In the interpopulation test, the first 48 hours of the test were conducted using cool-white lights. After the germination and germ-tube growth endpoints were quantified, the cool-white lights were replaced with full-spectra Vitalights®. The NOEC's for germ-tube growth and sporophyte production were both less than the lowest test concentration (< 18 µg/l) in the interpopulation test. The entire long-term test was conducted using Vitalights® and nutrients. The NOEC for germ-tube growth in this test was 31.1 µg/l and the NOEC for sporophyte production was < 10.2 µg/l (Table 1).

Other researchers have found inhibition of reproduction to be a more sensitive indicator of toxicity than vegetative growth in early life stage toxicity tests using laminarian algae. Smith and Harrison (1978) found that copper inhibited the vegetative growth of *Macrocystis* gametophytes at 50 µg/l, while production of eggs by female gametophytes was inhibited at 30 µg/l. Thompson and Burrows (1984) found that vegetative growth of *Laminaria saccharina* sporophytes was inhibited at 50 µg/l, and reproduction (sporophyte production) was inhibited at 10 µg/l copper. Chung and Brinkhuis (1986) found that the release of *Laminaria saccharina* zoospores, gametophyte growth and gametogenesis were inhibited at copper concentrations greater than 50 µg/l.

Although less sensitive than the reproductive endpoint, inhibition of germ-tube growth is a consistent indicator of toxicity. Germ-tube growth is preferable to the reproductive endpoint for routine testing because it requires considerably less technical expertise, is relatively consistent in repetitive tests, and takes only 48 hours to conduct. The drawbacks are that germ-tube growth is somewhat less sensitive than the reproductive endpoint, and the ecological relevance is less easily defended because it is not clear how declines in germ-tube growth affect field populations. Comparison with the reproductive endpoint indicate that factors inhibiting germ-tube growth also inhibit reproduction. Germination is a less sensitive and more variable indicator of toxicity, but its ecological relevance is more obvious since spores that do not germinate will not develop.

Figure 12 a-d. Kelp Long-Term Copper Reference Toxicant Test



Lighting Experiments

Different light levels did not affect germination rates (Figure 13a), but did affect germ-tube growth (Figure 13b). Germ-tube lengths were significantly longer in the no-light and 10 $\mu E\ m^{-2}\ sec^{-1}$ light levels. The increased germ-tube length observed in the low- and no-light treatments probably resulted from etiolation, a process of seedling elongation which commonly occurs in plants grown in the dark (Raven *et al.*, 1981). The ability to grow in the absence of light suggests that the spores are not relying on active photosynthesis for germination and initial growth. Amsler (1988) has shown that *Macrocystis* zoospores photosynthesize at a rate that just offsets the cost of respiration and net photosynthesis is negligible. Zoospores probably use stored photosynthate for germination and initial growth (C. Amsler, UCSB, personal communication). Luning (1980) found that germination and production of the first primary cell in *Laminaria* species can occur in complete darkness and suggested that the subsequent vegetative growth of the gametophyte is dependent on the production of new photosynthate.

The results of the 48-h copper experiment in total darkness indicate that light may influence inhibition of germination or germ-tube growth by copper (Figure 14a-b). The NOEC for germination was 10 µg/l and the NOEC for germ-tube growth was 32.5 µg/l. These results were not consistent with previous copper experiments in the light. Compared to our previous tests, there was greater within-replicate variability in the length data, and the NOEC for germination was considerably lower (Table 1).

These experiments indicate that light does not affect germination and initial growth, but may affect the inhibition of these processes by copper. In an earlier attempt to explain the mechanism of toxicity we hypothesized that inhibition of germination and germ-tube growth might be caused by inhibition of photosynthesis (Anderson and Hunt, 1988). This does not appear to be correct based on the results of this experiment; it appears that some other process is involved. It is not clear why germination was more sensitive to copper than germ-tube growth in the dark; elucidation will require further research.

Figure 13. The Effects of Light Level on Kelp Germination and Germ-Tube Growth (μm).

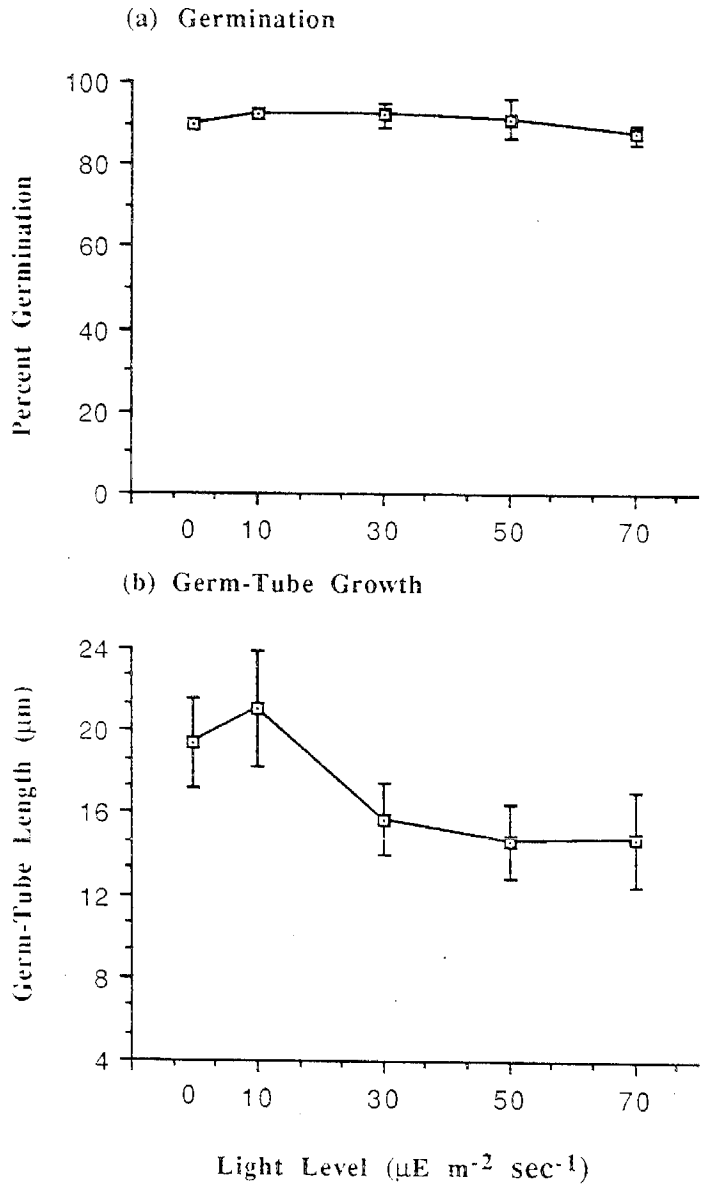
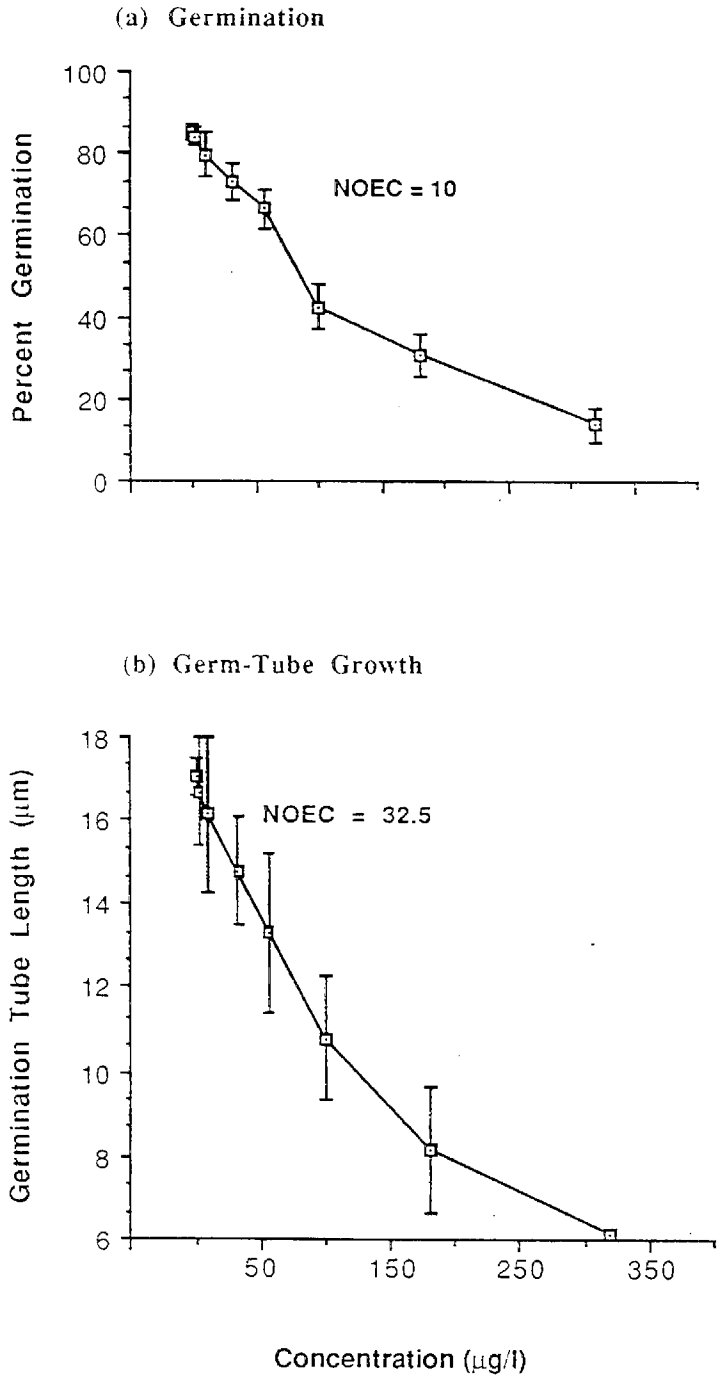


Figure 14. The Effects of Copper on Kelp Spores in Total Darkness



Physical and Chemical Measurements and Reference Toxicant Verification

In general the physical and chemical values for all of the tests were within normal ranges (Table 4). Salinity values in the first quarterly copper test (which was also the MPSL copper interlab test) were abnormally high, ranging from 35 ppt at the start of the test to 47 ppt at the end. The increased salinity in this test resulted from evaporation from the open containers, but this did not appear to affect the results. The salinity also increased in the CRA interlab test but the results were consistent with past copper tests. Subsequent tests used covered containers. Temperature during the kelp interpopulation experiment increased to 19 °C for approximately 8 hours due to a malfunctioning temperature control unit. Although high temperature can inhibit kelp, it did not appear to affect the results of this experiment.

Table 4. Ranges of physical and chemical data for Phase 3 kelp experiments.

Test	DO ($mg\ l^{-1}$)	pH	Salinity (ppt)	Temp (°C)
JWPCP Effluent #1	7.59-8.15	7.85-7.95	34-36	15.5-16.0
Hyperion Effluent #1	7.59-8.87	7.65-7.85	33-36	15.0-16.0
JWPCP Effluent #2	7.19-7.95	7.80-8.00	35-36	15.0-15.5
Hyperion Effluent #2	6.19-7.22	7.86-8.24	35-36	13.5-14.5
Copper Quarterly #1	7.70-8.05	7.85-7.95	35-47	15.2-16.6
Copper Quarterly #2	7.70-8.00	7.75-7.90	36-37	15.0-15.5
Copper Quarterly #3	7.54-8.00	7.85-8.00	35-37	14.5-15.5
Copper Quarterly #4	6.65-7.76	7.96-8.27	35-36	13.5-16.0
Copper Replicate Test #1	6.00-7.06	7.91-8.26	34-36	13.0-13.5
Copper Replicate Test #2	6.40-6.88	7.96-8.27	35-36	13.0-13.5
Copper Replicate Test #3	6.65-7.76	7.96-8.27	35-36	13.5-16.0
Copper Interlab CRA	No Data	7.90-7.90	33-44	13.7-14.0
Copper Interlab MPSL	7.70-8.05	7.85-7.95	35-47	15.2-16.0
Copper Interpopulation	6.96-7.10	8.14-8.29	35-36	15.0-19.0
Copper No-Light	7.41-7.85	7.79-8.16	35-35	15.0-15.0
Copper Long-Term	6.14-7.02	7.72-8.04	34-35	13.0-14.5

The chemical analyses of the copper concentrations in the Phase 3 experiments showed that the nominal and analytically verified concentrations were in relatively close agreement in all experiments (Table 5).

Table 5. Chemical verification of copper concentrations used in kelp tests. Concentrations are given as nominal, analyzed at the start, and analyzed at the end of the test. ND = not detected

		Test Concentrations ($\mu g\ l^{-1}$)						
Quarterly Copper Tests								
Q #1	Nominal	0	56.0	100.0	180.0	320.0	560.0	
	Analyzed start	2.4	62.5	111.0	193.0	339.0	608.0	
	Analyzed end	4.5	19.0	74.0	103.0	218.0	314.0	
Q #2	Nominal	0	32.0	56.0	100.0	180.0	320.0	
	Analyzed start	1.2	30.3	57.3	99.0	163.0	285.0	
	Analyzed end	2.3	31.9	57.3	99.2	185.0	306.0	
Q #3	Nominal	0	10.0	18.0	32.0	56.0	100.0	180.0
	Analyzed start	ND	9.4	21.4	37.5	52.6	102.8	176.7
	Analyzed end	ND	10.8	17.3	27.7	38.5	89.4	137.3
Q #4	Nominal	0	10.0	18.0	32.0	56.0	100.0	180.0
	Analyzed start	ND	10.2	18.7	32.5	55.5	94.2	175.8
	Analyzed end	ND	9.9	18.9	32.1	52.6	91.4	164.7
Replicate Copper Tests								
Rep. #1	Nominal	0	10.0	32.0	56.0	100.0	180.0	
	Analyzed start	ND	9.4	32.6	58.6	41.7	168.2	
	Analyzed end	ND	8.1	30.2	52.9	45.2	163.1	
Rep. #2	Nominal	0	10.0	32.0	56.0	100.0	180.0	
	Analyzed start	ND	8.3	33.4	56.4	89.1	160.9	
	Analyzed end	ND	10.3	25.5	61.3	99.9	176.9	
Rep. #3	Nominal	0	10.0	18.0	32.0	56.0	100.0	180.0
	Analyzed start	ND	10.2	18.7	32.5	55.5	94.2	175.8
	Analyzed end	ND	9.9	18.9	32.1	52.6	91.4	164.7
Interlaboratory Copper Test								
MPSL	Nominal	0	56.0	100.0	180.0	320.0	560.0	
	Analyzed start	2.4	62.5	111.0	193.0	339.0	608.0	
	Analyzed end	4.5	19.0	74.0	103.0	218.0	314.0	
No-Light Copper Test								
	Nominal	0	10.0	32.0	56.0	100.0	180.0	320.0
	Analyzed start	ND	11.7	35.4	54.1	101.6	181.5	339.9
	Analyzed end	ND	8.2	29.5	47.0	93.0	152.8	293.7
Long-Term Copper Test								
	Nominal	0	10.0	32.0	56.0	100.0	180.0	
	Analyzed start	ND	11.0	30.9	46.0	91.8	147.7	
	Analyzed end	ND	9.3	31.2	54.9	89.3	140.3	

Summary

The following is a brief summary of the results of research conducted with *Macrocystis* as part of Phase 3 of the Marine Bioassay Project:

1. The 48-h protocol was easy to perform and relatively reliable; of 17 experiments, the results of only one were not used. There were no problems conducting the effluent tests ; *Macrocystis* was reasonably sensitive to the effluents tested. There was high salinity due to evaporation in the first quarterly copper test, but all other copper tests were problem free.
2. Seasonal (quarterly) variability in the sensitivity of kelp spores to copper was greater than variability over a one month time span.
3. Germ-tube growth was more sensitive and less variable to copper and effluents than germination.
4. The 48-h *Macrocystis* protocol was successfully conducted by an outside laboratory having no previous experience using the protocol.
5. No significant differences in sensitivity to copper were found among spores from four different geographical areas in central and southern California.
6. The long-term effect on reproduction (sporophyte production) was more sensitive to copper than the short-term effects on germination and germ-tube growth.
7. Germination was not affected by variations in light. Germ-tube growth was greater at lower light levels. The affect of light on toxicity is unknown.
8. Germination of spores was more sensitive to copper in the dark than in the light, while germ-tube growth was similar.
9. Minor changes to the *Macrocystis* 48-h protocol are recommended before implementation. We are exploring the possibility of using smaller, disposable test containers (eg. tissue culture containers) that can be used in conjunction with an inverted microscope. Instead of microscope slides, the spores are settled directly onto the bottom of the test container. After 48 hours the container is placed on the stage of an inverted microscope and the gametophytes are observed without being removed. Early trials of this technique have been successful. We are also investigating the possibility of using a preservative to fix the kelp cultures at the end of the test. This allows the investigators to quantify the endpoints at their convenience.

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Section 2
Mysid Experiments

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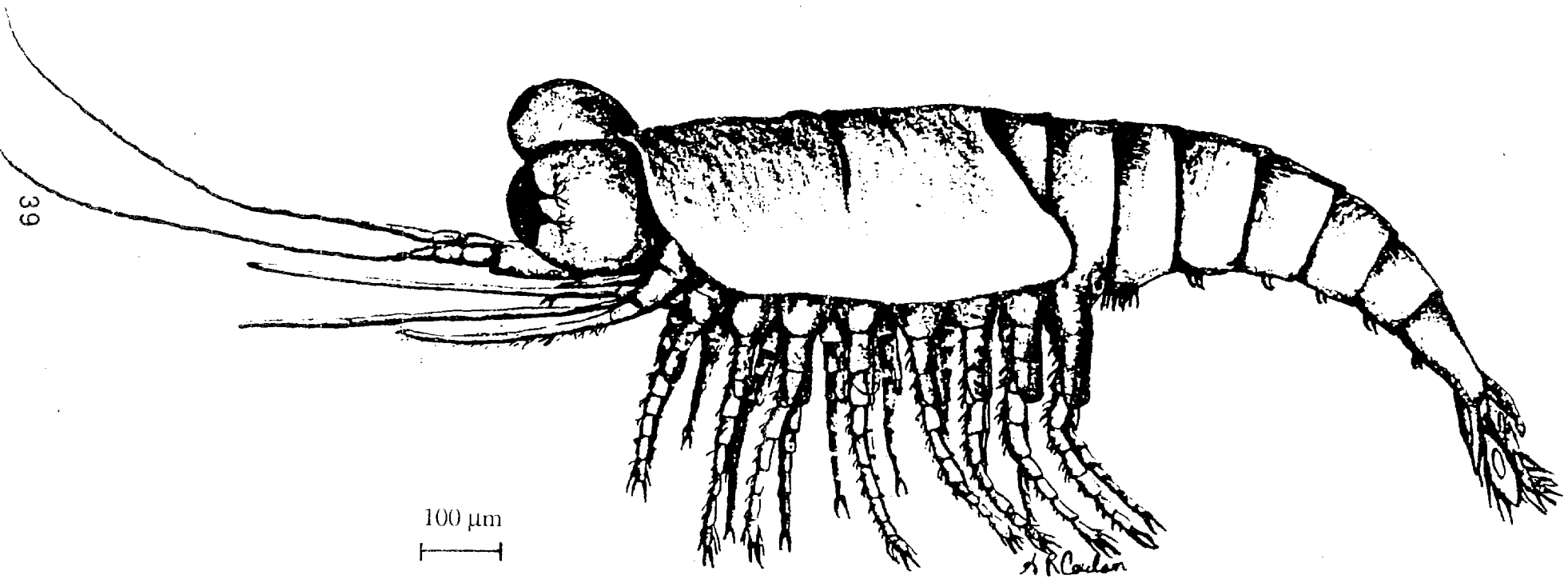


Holmesimysis costata - juvenile

Introduction

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

Holmesimysis (=Acanthomysis) costata* (Holmes 1900) is a common near-shore mysid that ranges the length of California (Clutter, 1967; Clark, 1971; Hobson and Chess, 1976). *Holmesimysis* occurs in the surface canopy of the giant kelp, *Macrocystis pyrifera*, where it is ecologically important as a food source for fishes, particularly juvenile kelp canopy species (Mauchline, 1980; Clark, 1971; Hobsen and Chess, 1976). There are few published references on the biology of this species (Clutter, 1967; Clutter, 1969; Green, 1970). Adult field-captured *Holmesimysis** have been used previously in mortality bioassays (Machuzak and Mikel, 1987), and the acute and chronic toxicity of tributyltin to this species has been evaluated (Davidson *et al.*, 1986). Little is known about pollution effects on *Holmesimysis* or on mysid populations in general, but because *Holmesimysis* occurs near shore, populations are likely to come into contact with discharged effluents. Effluent constituents have been shown to adversely affect other mysid species in laboratory experiments (Nimmo *et al.*, 1977). Discharged sewage effluents have probably affected mysid populations indirectly through observed declines in kelp forest habitat (Grigg, 1978; Wilson, 1982).

In Phase 2 of the Marine Bioassay Project, juvenile *Holmesimysis* were exposed to complex effluent samples and a reference toxicant, zinc sulfate, in multiple 48- and 96-hour mortality tests (Anderson *et al.*, 1988). This report describes experiments on *Holmesimysis* designed to further evaluate the 96-hour toxicity test protocol. Several aspects of test performance were investigated, including: 1) feasibility for use with effluents from large municipal waste treatment plants, 2) variation in effects of effluents and reference toxicants, 3) seasonality of the mortality response to a reference toxicant, 4) variation in results obtained at different laboratories, 5) sensitivity of mysids of different ages, and 6) sensitivity to copper, a widely used reference toxicant. Research was initiated on growth inhibition in juvenile *Holmesimysis* as a sublethal toxicity test endpoint. Results from 22 experiments were used to evaluate and modify the *Holmesimysis* toxicity test protocol given in Appendix II.

* Note: There are questions concerning the taxonomy of *Holmesimysis/Acanthomysis* species. The most current taxonomic interpretation (Holmquist, 1981) considers previous references to *Acanthomysis sculpta* in California to be synonymous with *Holmesimysis costata*, and we are considering this version to be definitive (see also Mauchline, 1980).

Methods

Facilities

The experiments were conducted between January and December 1988 at the California Department of Fish and Game Marine Pollution Studies Laboratory (MPSL) at Granite Canyon. The laboratory is located on the Big Sur Coast in Monterey County, California. The MPSL seawater intake is at least 15 km from any known source of pollution. The site is 0.5 km from a California State Mussel Watch station at Soberanes Point that has low concentrations of trace elements, pesticides, and petroleum hydrocarbons (Stephenson *et al.*, 1979; Martin and Castle, 1984).

Holmesimysis Toxicity Test Protocol

Methods for conducting 96-hour mortality tests are described in the *Holmesimysis* toxicity test protocol given in Appendix II. A number of experiments required additional steps or modifications, and these are presented below.

Effluent Tests

Effluent samples were collected from two large southern California waste treatment plants that discharge to marine waters: the Los Angeles County Joint Water Pollution Control Plant (JWPCP) and the Los Angeles City Hyperion waste treatment plant. Twenty-four-hour composite samples were shipped overnight to MPSL in 1-liter polyethylene bottles packed in blue ice. Hyperion effluent was not chlorinated. JWPCP samples were collected after chlorination and dechlorination. Before diluting samples for test solutions, effluents were agitated to evenly distribute particles. Test concentrations were 0, 0.56%, 1.0%, 1.8%, 3.2%, 5.6%, and 10.0% effluent in seawater. MPSL natural seawater (0.2 µm-filtered) was used as the dilutant. Test solutions were adjusted with calculated volumes of hypersaline brine to compensate for low effluent salinity relative to natural seawater. Commercially available sea salts were used to make hypersaline brines. Sea salt brines were not always satisfactory; some brine mixtures were measurably toxic, as discussed below. All effluent tests used brine controls containing seawater, the same volume of hypersaline brine used in the highest effluent concentration, and distilled water in place of effluent to adjust salinity to that of the dilution seawater. Toxicant concentrations were compared against brine controls to derive No Observed Effect Concentrations (NOECs) in effluent tests. (See protocol in Appendix II for details of statistical analysis)

Effluents from each treatment plant were tested twice at approximately five month intervals. Each effluent test was accompanied by a concurrent test using a reference toxicant.

Reference Toxicant Tests

Zinc (zinc sulfate, $ZnSO_4 \cdot 7H_2O$) was used as a reference toxicant in the following tests: reference toxicant tests concurrent with effluent tests, interlaboratory tests, age sensitivity tests, and longer-term sublethal tests using growth as an endpoint. Zinc is a suitable reference toxicant because it is stable in solution, easy to analyze chemically, relatively non-hazardous to laboratory personnel, and found in high concentrations in the target effluents (Anderson *et al.*, 1988).

Six 96-hour zinc tests were conducted throughout the year to investigate seasonal variability. Tests were done in February, March, June, July, September, and November. These tests were compared statistically using a 2-way Analysis of Variance to detect differences between toxicant concentrations and between tests. Student-Newman-Keuls (SNK) multiple comparisons were used to make distinctions among the individual tests.

One *Holmesimysis* experiment used copper (cupric chloride, $CuCl_2 \cdot 2H_2O$) as the toxicant, so that results could be compared to numerous published copper toxicity studies with a wide range of organisms.

Interlaboratory Testing

In May 1988, recently collected gravid female mysids were sent by overnight air delivery from MPSL to Coastal Resources Associates (CRA) laboratories in San Diego County, California. Juvenile mysids were hatched from the gravid females at the CRA facility. Three-day-old juveniles were then used in the 96-h *Holmesimysis* test with zinc as the toxicant. Four weeks later, mysids treated identically were held at MPSL and tested for comparison with the CRA results. Both laboratories used zinc sulfate supplied by MPSL, but seawater from different sources was used. All toxicant concentrations were verified by chemical analysis (see below), and NOEC's and LC_{50}'s for all tests were derived using identical statistical procedures (see below).

In November, juvenile mysids were sent by overnight air delivery to the Hyperion waste treatment plant in Los Angeles, California. There they were held in an aquarium until they were 3 days old, then transferred to test containers and exposed to effluent for 96 hours. A split sample of the same effluent was packed with ice and sent by overnight air delivery to MPSL, where 3-day-old mysids from the same hatch were also exposed to the effluent for 96 hours. Brine solutions used to adjusting salinity were prepared at MPSL and shipped to Hyperion for the test. Each laboratory used a different dilution water.

Age-Dependent Sensitivity Experiments

Mysids of different ages were tested to investigate age-specific toxicant sensitivity. Several distinct mysid cohorts were isolated as described in the protocol. To prevent intermixing of cohorts, the hatching aquarium was drained and rinsed with hot freshwater after each cohort was isolated. Isolated juveniles were maintained, prior to testing, in flowing filtered seawater and fed to excess with newly hatched *Artemia* nauplii and Tetramin® flake food. In the first age sensitivity test, 1, 3, 5, 7, and 9-day old juveniles and gravid females were exposed simultaneously to zinc concentrations of 0, 100, 180, and 320 µg/l using the 96-hour protocol. Each treatment was replicated 3 times. The second test compared 3, 5, 7, and 9-day old juveniles and a mixture (two 3-day, three 5-day, three 7-day, and two

9-day olds). All mysids were exposed simultaneously to zinc concentrations of 0, 56, and 100 µg/l using the 96-hour protocol. Each treatment was replicated 5 times. Data from each treatment were compared statistically using a two-way ANOVA and Student-Neuman-Keuls multiple comparison to determine significant differences between concentrations and age groups (Sokal and Rohlf, 1969; Zar, 1974).

Growth Experiments

Because *Holmesimysis* has a 70-day life cycle, a reproductive endpoint was considered impractical for routine effluent testing. Instead, growth was evaluated as a sublethal endpoint. Juvenile mysids were cultured and delivered to test containers for growth experiments in the same manner as for 96-hour mortality tests. In separate tests, 3-day old mysids were exposed to zinc and effluents for 4 and 7 days. In addition, a study was undertaken to determine the most appropriate mysid age for testing growth effects. Cohorts of newly hatched mysids were followed throughout their 70-day life cycle, and growth measurements were taken on daily samples of 10 mysids. Marked increases in length over time were used to estimate moulting frequencies. Based on those results, a test was designed to incorporate 2 moults within a 7 day span. Because moulting appeared to occur on days 9 and 13 (see Results), tests were designed to begin with 8-day old mysids. Unfortunately, two separate 8-day exposures of 8-day-old mysids were unsuccessful because of high test solution salinity. A 7-day growth experiment using 9-day old mysids was successfully completed, however. At the end of each growth experiment, all surviving mysids were fixed in 5% buffered formalin. Carapace lengths were measured from the base of the eyestock to the posterior mid-dorsal edge of the carapace at 40x magnification within 1 week after preservation. Following length measurements, mysids from each replicate were wrapped together in foil, dried at 55° C for 48 hours, and weighed. The mean weight per mysid was determined for each replicate by division. All growth tests were static renewal tests in which 50% of the toxicant solution was replaced every 48 hours.

Physical/Chemical Measurements of Test Solutions

Physical/chemical parameters were measured in test solutions from one random replicate of each toxicant concentration at the beginning and end of each test and before each renewal. Dissolved oxygen and pH were measured with an Orion EA 940 Ion Analyzer. Salinity was measured with an Atago refractometer and temperature was measured using a standard thermometer.

Reference Toxicant Chemical Verification

As in our previous work, reference toxicant concentrations were sampled for chemical analysis at the beginning and end of all tests. Chemical analyses based on the methods of Bruland *et al.* (1979) were performed on Perkin Elmer model 603 or 5000 atomic absorption spectrometers at the Moss Landing Marine Laboratories. Although measured concentrations from previous tests have been similar to nominal concentrations (Anderson *et al.*, 1988), measured concentrations this year varied substantially from nominal concentrations. We suspect that contamination of sample vials caused the variability between nominal and measured concentrations, although attempts

to document this were unsuccessful. Due to the uncertainty regarding chemical analysis, all zinc concentrations are reported as nominal concentrations. Comparisons of nominal and measured zinc concentrations are given in Table 6.

Measured copper concentrations were similar to nominal values. There was no evidence of copper contamination of test solutions or sample vials. All copper values are reported as measured concentrations.

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

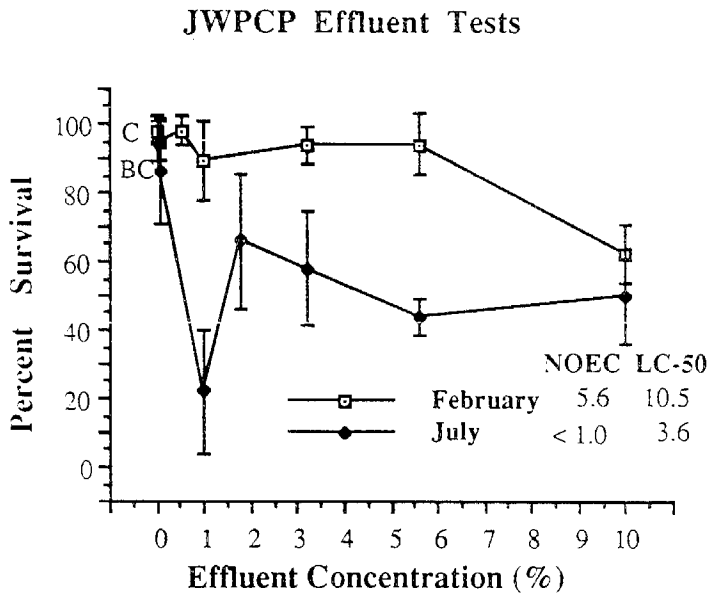
Toxicant & Test	Date	Test Solution Concentrations						
Nominal Concentrations		0	10	18	32	56	100	180
Zinc (Concurrent)	2/23-27	22.2	-	27.6	47.2	139.0	114.0	205.0
Zinc (repeat)	3/7-11	3.7	-	25.9	21.2	127.8	99.5	190.5
Zinc (Concurrent)	6/10-14	10.6	-	25.9	21.2	234.4	99.5	190.5
Zinc (Concurrent)	7/29-8/2	N.D.	-	24.8	38.2	56.6	85.0	102.4
Zinc (Concurrent)	11/1-5	5.9	17.2	30.7	42.8	58.3	97.7	-
Zinc (CRA Interlab)	5/12-16	9.8	-	33.2	45.5	71.4	120.0	200.0
Zinc (MPSL Interlab)	6/10-14	10.6	-	25.8	21.2	234.4	99.5	190.5
Zinc Age #2	3/7-11	3.7	-	25.8	21.2	127.8	99.5	190.5
Zinc Grow 9d-old	6/16-23	4.1	18.8	23.4	37.1	59.5	105.3	-
Zinc Grow 3-4d-old	7/29-8/5	30.1	31.9	28.5	38.6	52.2	108.0	-
Zinc Grow 3d-old	9/22-29	2.0	13.8	27.1	34.7	54.2	103.2	-
Mean for all Zinc Tests		3.0	17.7	27.0	36.9	55.3	99.6	202.7
Copper	7/29-8/2	0.5	11.1	21.6	29.8	53.3	100.8	-

Results and Discussion

Effluent Tests

Juvenile mysids responded differently to the two samples of JWPCP effluent (Figure 15a). In the February test, survival was significantly inhibited only in the highest effluent concentration (10%); while in the July test, survival was significantly inhibited in 1% effluent (Table 7). It is not clear why the July effluent sample was more toxic. Effluent toxicity may have been affected by the season or day of the week of collection (the February sample was collected on the weekend, the July sample during the week). There was an unusually strong response in the 1% effluent treatment in the July test. All five replicates of the 1% dilution had poor survival, though there was nothing unusual in the physical/chemical measurements from this treatment. All higher concentrations were also significantly lower than controls, however, so the July NOEC was calculated as <1%.

Figure 15a.



C = control; BC = brine control

Survival in brine controls was lower than in dilution water controls in all tests, indicating varying degrees of brine toxicity. Brine controls were lower by: 2% in the February JWPCP test, 8% in the June Hyperion test, 10% in the July JWPCP test, and 22% in the November Hyperion test. Brines were made from Instant Ocean® artificial sea salt. The suitability of this and other brands of artificial sea salt is now being evaluated. In all effluent tests, effluent treatments were compared to the brine controls to determine NOEC's. Salinity adjustment is recommended because decreased salinities have been shown to increase toxicant uptake in crustaceans (O'Hara, 1973; Vernberg *et al.*, 1977), and thus affect toxicity test results.

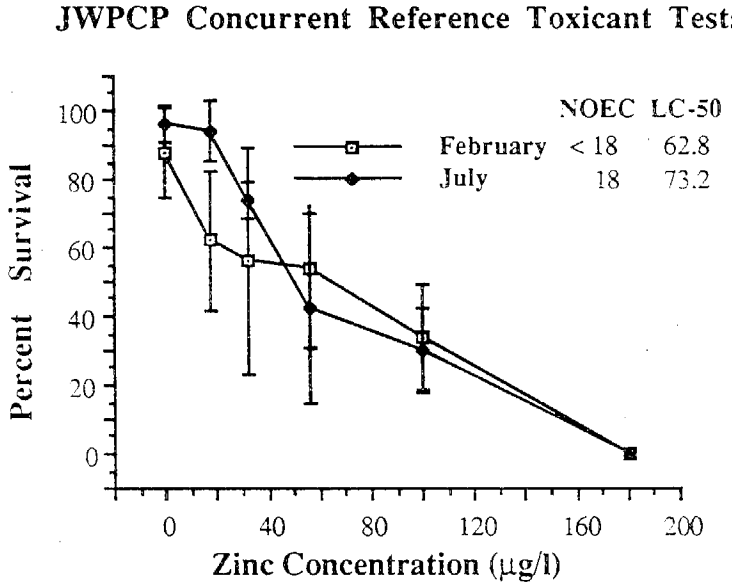
Table 7. Summary of results of mysid experiments. NOEC is the No Observed Effect Concentration. LC_{50} is the median lethal concentration calculated using linear regression. JWPCP = Los Angeles County Joint Water Pollution Control Plant. Hyp.= Los Angeles City Hyperion plant. Interlab signifies paired tests used for interlaboratory comparison. na = not applicable, NSD = no significant difference. Growth is growth in carapace length.

Toxicant	Date	NOEC	LC_{50}
JWPCP Effluent	2/23-27	5.6 %	10.5 %
Zinc (Concurrent)	2/23-27	<18 µg/l	62.8 µg/l
Zinc (Repeat)	3/7-11	56 µg/l	80.7 µg/
Hyp. Effluent	6/10-14	3.2 %	4.3 %
Zinc (Concurrent)	6/10-14	56 µg/l	88.4 µg/l
JWPCP Effluent	7/29-8/2	<1.0 %	3.6 %
Zinc (Concurrent)	7/29-8/2	18 µg/l	73.2 µg/l
Hyp. Effluent	11/1-5	3.2 %	3.7 %
Zinc (Concurrent)	11/1-5	56 µg/l	70.2 µg/l
Zinc 96 hour	9/22-25	32 µg/l	93.6 µg/l
Zinc (CRA Interlab)	5/12-16	18 µg/l	78.8 µg/l
Zinc (MPSL Interlab)	6/10-14	56 µg/l	88.4 µg/l
Hyp. Effluent (Hyp Interlab)	11/1-5	3.2 %	2.9 %
Hyp. Effluent (MPSL Interlab)	11/1-5	3.2 %	3.7 %
Copper	7/29-8/2	<11 µg/l	27.2 µg/l
Age Sensitivity Test #1			
1-day-olds	1/16-20	na	na
3-day-olds	1/16-20	<100 µg/l	82 µg/l
5-day-olds	1/16-20	<100 µg/l	85 µg/l
7-day-olds	1/16-20	<100 µg/l	74 µg/l
9-day-olds	1/16-20	<100 µg/l	76 µg/l
Gravid Females	1/16-20	>320 µg/l	>320 µg/l
Age Sensitivity Test #2			
3-day-olds	3/7-11	56 µg/l	85 µg/l
5-day-olds	3/7-11	>100 µg/l	165 µg/l
7-day-olds	3/7-11	56 µg/l	60 µg/l
9-day-olds	3/7-11	56 µg/l	na
mixed (3, 5, 7, and 9)	3/7-11	56 µg/l	61 µg/l

Growth Tests	Date	Mortality NOEC	Growth NOEC
Zinc 9d-old/7d test	6/16-23	32 µg/l	10 µg/l
Zinc 3-4d-old/7d test	7/29-8/5	18 µg/l	NSD
Zinc 3d-old/7d test	9/22-29	32 µg/l	NSD
Effluent 3d-old/4d test	2/23-27	5.6 %	NSD

Though less variable than the JWPCP effluent tests, concurrent reference toxicant tests produced different NOECs (Figure 15b). Zinc NOEC and LC_{50} values were lower in February than in July. Elevated test solution salinities (Table 8) may have increased the overall toxicity of the February test solutions (Table 7). Elevated salinities have been shown to increase zinc toxicity in estuarine mysids and other crustaceans (McLusky and Hagerman, 1987; McKenney and Neff, 1979).

Figure 15b.



Test results from the Hyperion effluent samples were similar. Response curves from the two tests corresponded closely, with identical NOEC's and similar LC_{50}'s (Figure 16a, Table 7). The June effluent was from a weekday sample, the November effluent from a weekend sample; this difference apparently did not affect test results. Mysid survival in brine controls was poor: 80% in June and 50% in November. This did not affect the calculation of NOECs in either test, because there were sharp breaks between affected and unaffected treatments (Figure 16a).

There was little difference between the two reference toxicant tests run concurrently with the Hyperion effluent tests. Both had similar curves, identical NOEC's and similar LC_{50}'s (Figure 16b; Table 7). Although the two curves had similar shapes, survival in the November test was about 10% less at all concentrations. The reason for this is not clear, though variation in the condition of test organisms or physical/chemical characteristics of test solutions may have been responsible (Table 8).

Figure 16a. Results of tests with Hyperion waste treatment plant effluent. C = control, BC = brine control.

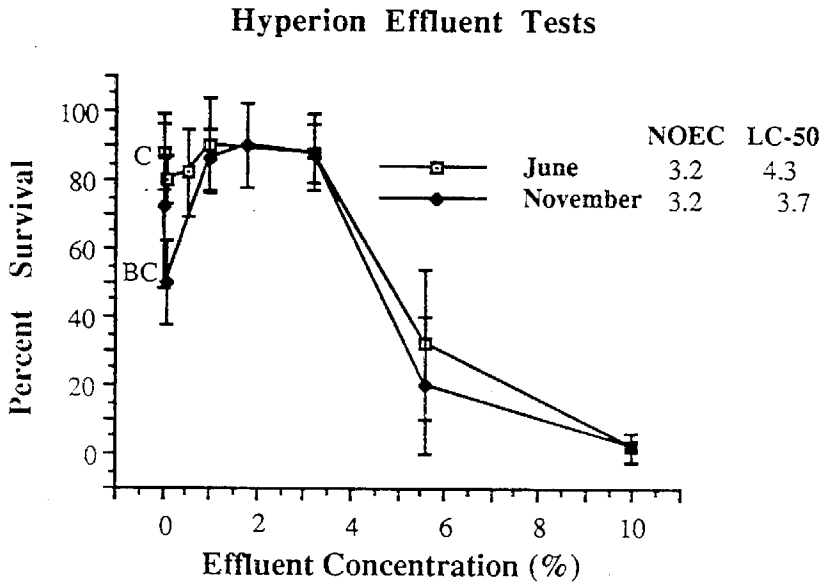


Figure 16b.

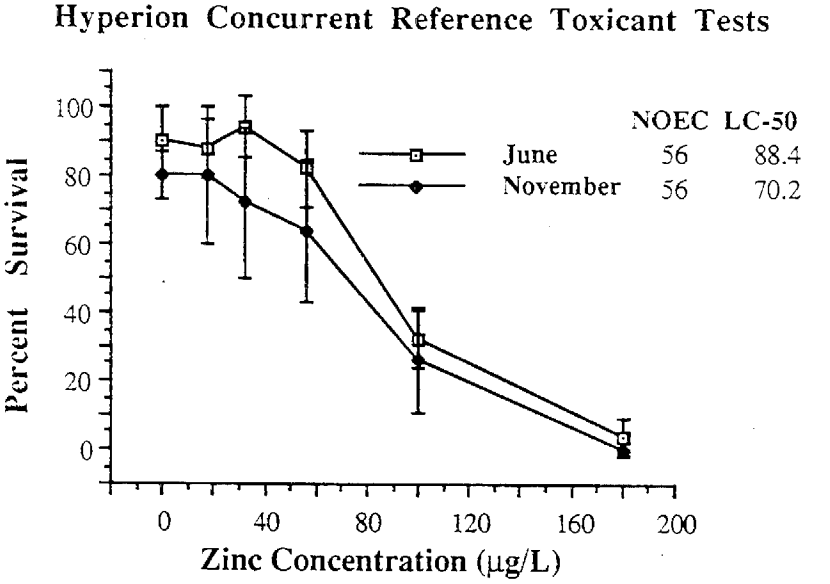


Table 8. Ranges of physical/chemical data for mysid experiments. Measurements were taken from one randomly selected replicate of each test concentration at the beginning and end of each test and at each water change. Each reported range is for all measurements from a given test. JWPCP = Los Angeles County Joint Water Pollution Control Plant. Hyp.= Los Angeles City Hyperion plant. Interlab signifies paired tests used for interlaboratory comparison.

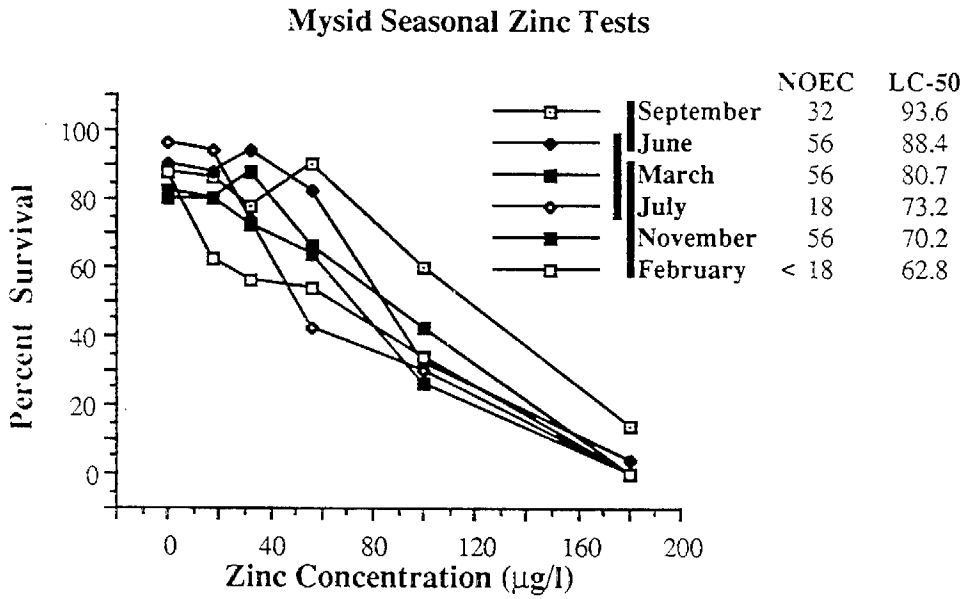
Toxicant	Date	Dissolved O_2 (mg/l)	pH	Salinity (°/oo)	Temperature (°C)
JWPCP Effluent	2/23-27	7.03 - 7.95	7.70 - 7.95	34 - 39	12.6 - 16.5
Zinc (Concurrent)	2/23-27	7.08 - 7.90	7.70 - 7.90	35 - 42	13.5 - 16.5
Zinc (repeat)	3/7-11	7.34 - 7.90	7.70 - 7.95	34 - 42	13.1 - 15.3
HYP Effluent	6/10-14	6.90 - 8.21	7.60 - 7.95	34 - 37	14.0 - 17.0
Zinc (Concurrent)	6/10-14	6.73 - 8.15	7.70 - 7.90	35 - 38	14.0 - 15.0
JWPCP Effluent	7/29-8/2	5.96 - 7.85	7.46 - 7.90	35	14.5 - 16.0
Zinc (Concurrent)	7/29-8/2	7.03 - 7.85	7.75 - 7.90	34 - 36	13.0 - 16.0
HYP Effluent	11/1-5	6.87 - 7.12	7.90 - 8.03	35	13.0
Zinc (Concurrent)	11/1-5	6.38 - 7.18	7.86 - 8.10	34 - 35	12.0 - 13.0
Zinc 96 hour	9/22-25	5.79 - 7.10	7.98 - 8.15	34 - 35	13.0 - 13.5
Zinc (CRA Interlab)	5/12-16	7.60 - 8.40	7.50 - 7.80	34 - 36	12.9 - 14.3
Zinc (MPSL Interlab)	6/10-14	6.73 - 8.15	7.70 - 7.90	35 - 38	14.0 - 15.0
HYP Effluent (Interlab)	11/1-5	9.20 - 10.40	8.06 - 8.20	27 - 35	13.2 - 13.5
HYP Effluent (MPSL)	11/1-5	6.87 - 7.12	7.90 - 8.03	35	13.0
Copper	7/29-8/2	7.54 - 8.21	7.75 - 7.85	36 - 38	13.0 - 15.0
Zinc Age #1	1/16-19	6.78 - 7.90	7.00 - 7.95	34 - 39	14.0 - 17.0
Zinc Age #2	3/7-11	7.34 - 7.90	7.70 - 7.95	34 - 42	13.1 - 15.3
Zinc Grow: 9d-old	6/16-23	6.83 - 8.15	7.75 - 7.95	35 - 36	15.0 - 16.0
Zinc Grow: 3-4d-old	7/29-8/5	7.03 - 7.85	7.75 - 7.90	34 - 36	13.0 - 16.0
Zinc Grow: 3d-old	9/22-29	5.79 - 7.10	7.98 - 8.15	34 - 35	13.0 - 13.5
Effluent Grow: 3d-old	2/23-27	7.03 - 7.95	7.70 - 7.95	34 - 39	12.6 - 16.5

Seasonal Variability

The responses of mysids to zinc were variable over time. The six 96-hour zinc tests produced different response curves, NOEC's and LC_{50}'s (Figure 17). Statistically significant differences broke the tests into overlapping groups (ANOVA, SNK p =.05). There were no differences between data from September and June (the group with the highest overall survival), between June, March and July (the intermediate group), or between March, July, November and February (the group with the lowest overall survival; see overlapping bars on Figure 17). These groupings do not correspond well with recognized oceanographic or climatic seasonal patterns: December through

February (winter Davidson current), March through July (spring/summer upwelling), and August through November (summer/fall oceanic season; Reid, 1960). Although seasonal factors may affect the reproductive condition, habitat, or food supply of mysid populations in the field, they do not appear to be responsible for the observed variation between toxicity test results in our study. Other factors may have a greater effect on between-test variability. For example, high salinity was a probable cause for higher toxicity in the February test. Another potential factor affecting test variability was variation in the concentration of the organic chelating agents that reduce trace metal toxicity by sequestering free ions. Quantitative analysis of free ions (Zn^{2+}) in test solutions was beyond the scope of our study.

Figure 17.



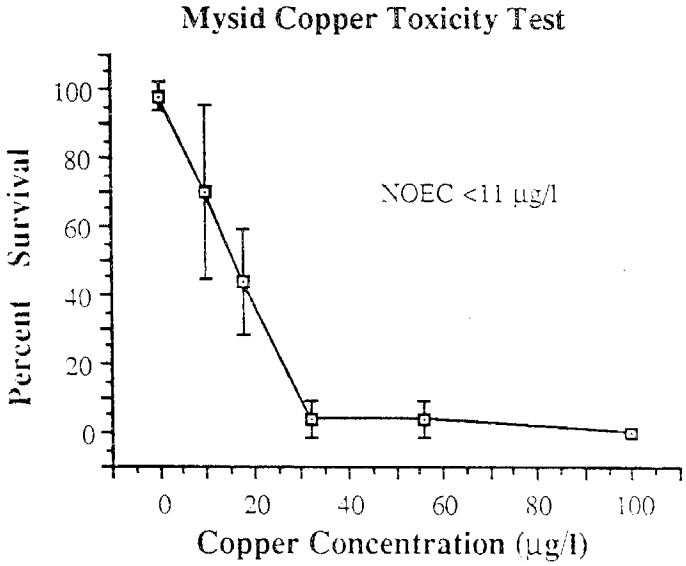
Zinc Toxicity

Holmesimysis was sensitive to zinc. The NOEC's for the six *Holmesimysis* zinc tests ranged from <18 to 56 µg/l (Table 7). The mean LC_{50} for the six zinc tests was 78.2 µg/l. For comparison, Lussier *et al.* (1985) reported a 96-hour zinc NOEC of 120 µg/l and a 96-hour zinc LC50 of 499 µg/l for the mysid, *Mysidopsis bahia.* Other reported zinc LC_{50} values for marine Crustacea were: 456 µg/l for zoea of the Dungeness crab, *Cancer magister* (Martin *et al.*, 1981); 713 µg/l for the copepod, *Tisbe holothuridae* (48-hour; Verriopoulos and Hardouvelis, 1988); and 10,200 µg/l for adult marine shrimp, *Callianassa australiensis* (Ahsanullah *et al.*, 1981).

Copper Toxicity

As with kelp, abalone, and topsmelt, *Holmesimysis* was more sensitive to copper than to zinc. Copper LC_{50} and NOEC values were 27 and <11 µg/l, respectively (Figure 18). For comparison, Lussier *et al.*, (1985) reported 96-hour copper LC_{50} and NOEC values of 181 and 77 µg/l, respectively, for *Mysidopsis bahia*.

Figure 18.



Interlaboratory Testing

The two sets of interlaboratory tests produced contrasting results. In the MPSL/CRA zinc tests, the response curves and LC_{50}'s from the two laboratories were roughly similar, but the NOEC's were different (Figure 19a). In the MPSL/Hyperion effluent tests with, the shapes of the response curves were different, but the NOEC's were the same (Figure 19b).

In the MPSL/CRA study, survival of CRA mysids was significantly lower in 32 µg/l zinc than in controls (CRA NOEC = 18 µg/l), while survival of MPSL mysids was higher in 32 µg/l than in controls (MPSL NOEC = 56 µg/l). Differences in zinc concentration between laboratories may have caused the difference: the nominal 32 µg/l concentration was measured as 21 µg/l for MPSL and 45 µg/l for CRA. The accuracy of chemical analysis is uncertain, however, because of sample contamination. Differences in mysid handling or differences in the composition of dilution waters are other possible sources of interlaboratory variability.

The MPSL/Hyperion interlaboratory test using effluent was adversely affected by the brine mixture. Brine controls had unacceptably low survival values (Figure 19b). Despite large differences between laboratories for most effluent concentrations, the NOEC's, derived by comparison with brine controls, were the same for both laboratories. Effluent interlaboratory testing will be repeated using a different brand of sea salt to adjust salinity.

Figure 19a.

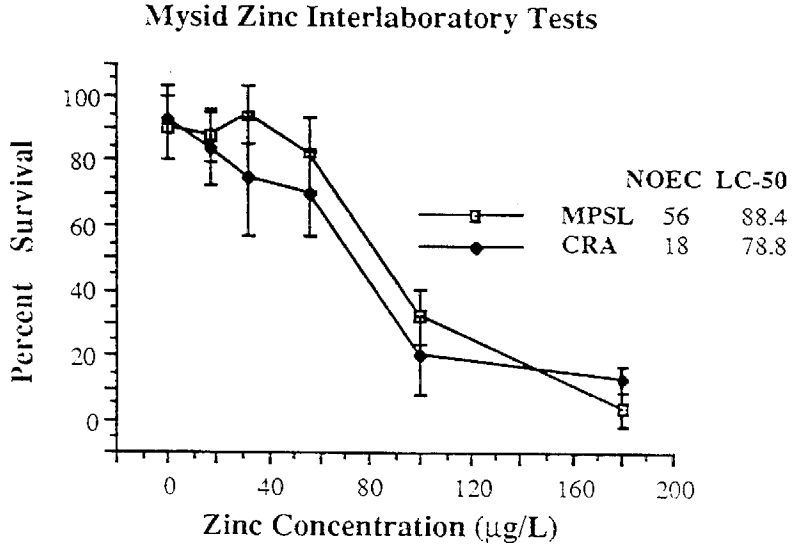
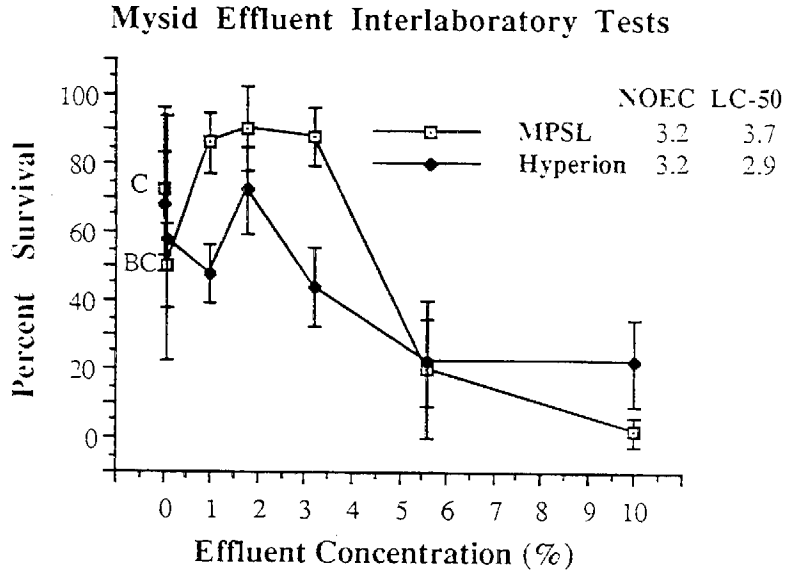


Figure 19b.



C = control; BC = brine control

Age-Dependent Sensitivity

In the first comparison of age-dependent toxicant sensitivity, juvenile mysids aged 3, 5, 7, and 9 days responded similarly. Gravid females were significantly less sensitive, and 1-day-old juveniles had unacceptably low control survival. The LC_{50} values for the 3- through 9-day-olds were within a narrow range from 74 to 85 µg/l, and there was no significant difference between the data from these age groups (ANOVA, p =.82; Figure 20a). The

relative sensitivity of 3- to 9-day-olds was examined at lower toxicant concentrations in a second experiment. Of the juvenile mysid groups, survival was highest among 5-day-olds and lowest among 9-day-olds (ANOVA, SNK $p < .05$). Survival was not significantly different among 3-day-olds, 7-day-olds, and the mixed group (Figure 20b; ANOVA, SNK $p > .05$). High test solution salinities may have affected test results (Table 8), and testing with more zinc concentrations is recommended before the three mysid age classes are used interchangeably in toxicity tests. Nevertheless, the two experiments suggest that juveniles in the 3- to 9-day-old age range represent a sensitive and appropriate life stage for toxicity testing, while gravid females and 1-day-olds are unsuitable because of low sensitivity and low control survival, respectively.

Figure 20a. Mysid Age-Specific Sensitivity to Zinc

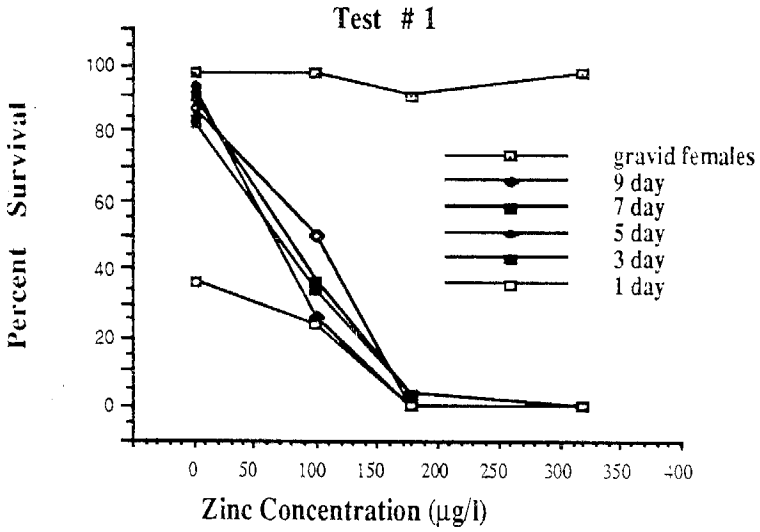
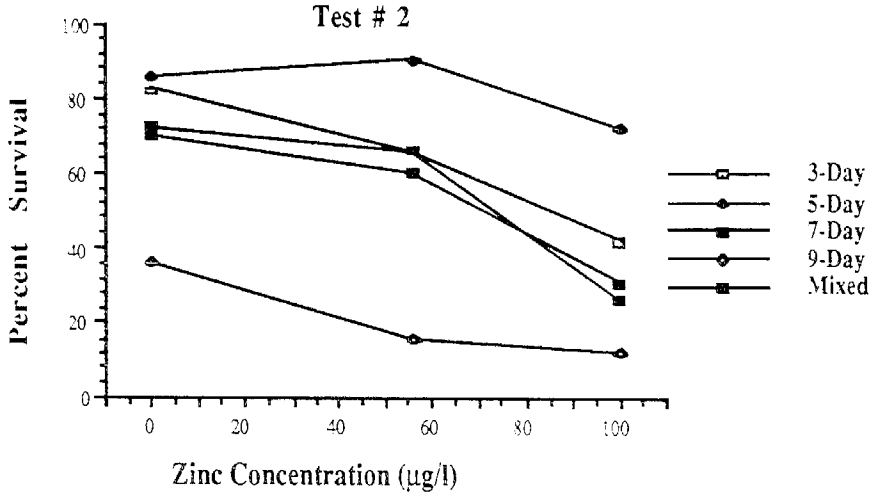


Figure 20b. Mysid Age-Specific Sensitivity to Zinc



Growth Experiments

In tests with 3-day-old mysids, growth was a less sensitive endpoint than mortality. Zinc and effluent concentrations causing significant decreases in survival did not significantly inhibit growth in length or weight. In a 4-day effluent test, 3-day-old mysids showed a gradual decline in carapace length with increasing effluent concentration up to 10% (Figure 21a); but this decrease was not significant, while survival declined significantly above the NOEC of 5.6%. In a 7-day zinc test, 3-day-olds showed no decrease in growth in concentrations up to 100 µg/l, while survival decreased significantly above the NOEC of 32 µg/l (Figure 21b). In another 7-day test, 3- and 4-day-old mysids showed a marked increase in growth above control levels in intermediate concentrations, and growth never declined below control values, while survival produced a NOEC of 18 µg/l (Figure 21c). Weight measurements never changed significantly with toxicant concentration, partially because of the very low dry weights of the juveniles (0.4 mg mean per test container; 0.05 mg mean per mysid) relative to the sensitivity of the scale (0.1 mg).

To better distinguish growth effects, older mysids that moulted more often were used in 7-day growth experiments. Measurements of mysids in laboratory culture produced a growth curve that was punctuated with apparent jumps in growth over time (Figure 22). These increases were assumed to indicate moulting. Two jumps occurred between the ages of 8 and 15 days, so 7-day-long exposures beginning with 8-day-old mysids were initiated. These tests were unsuccessful because of poor control water quality, but a 7-day exposure with 9-day-olds was completed (Figure 23). Growth in length of the 9-day-old mysids was significantly reduced in zinc concentrations above the NOEC of 18 µg/l, the same NOEC produced by the survival data. The response curves for the two effects were similar (Figure 23). The survival data was more variable than the growth data (average between-replicate coefficient of variation for survival data = 57%, average CV for growth data = 17%). Weight measurements in this test produced no statistically significant differences between exposed mysids and controls.

Previous investigators have studied the relative sensitivity of lethal and sublethal effects on *Holmesimysis* and other marine Crustaceans. Sublethal effects on growth and reproduction in *Holmesimysis* have been shown to occur at lower tributyltin concentrations than those causing death (Davidson *et al.*, 1986). Zinc concentrations affecting reproduction in the copepod, *Tisbe holothuridae*, were lower than those causing death (Verriopoulos and Hardouvelis, 1988). The relative sensitivity of lethal and sublethal effects may depend on the toxicant studied (Lussier *et al.*, 1985). Measuring both lethal and sublethal effects may be the most effective way to determine the toxicity of complex effluents that contain many potentially toxic constituents. Because 70-day whole lifecycle or reproductive tests are not practical for routine testing with *Holmesimysis*, 7-day growth and survival tests might provide the most sensitive alternative.

Figure 21a.

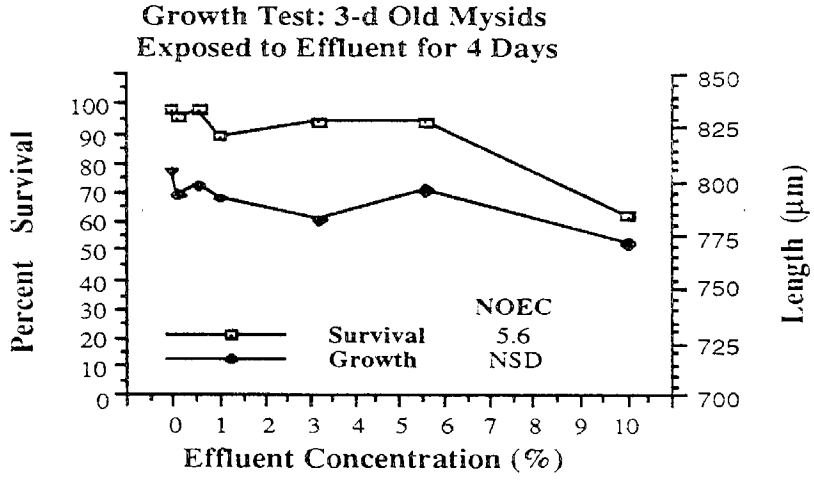


Figure 21b.

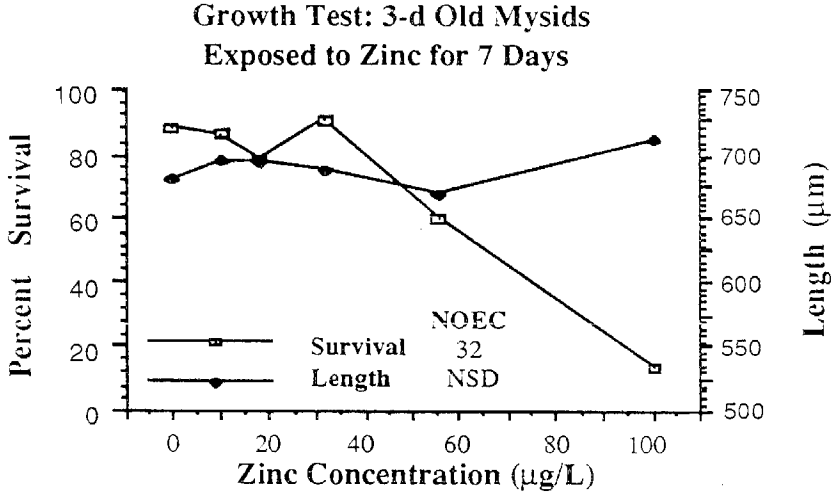


Figure 21c.

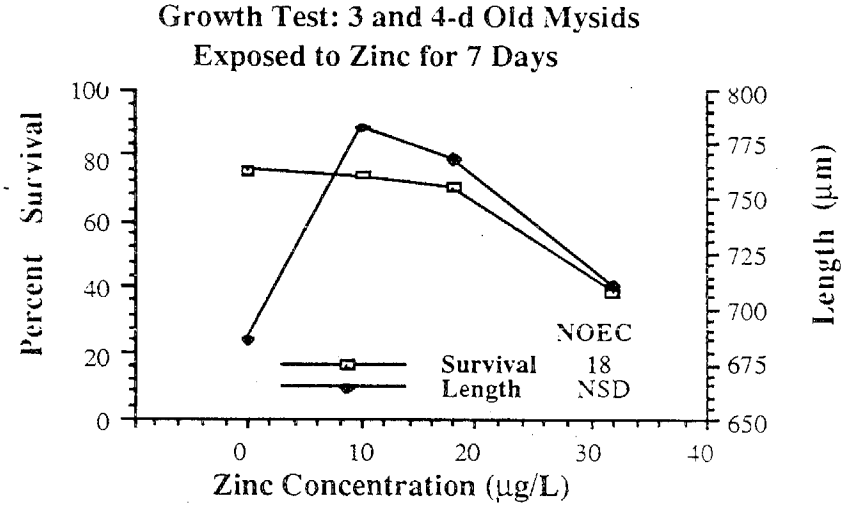
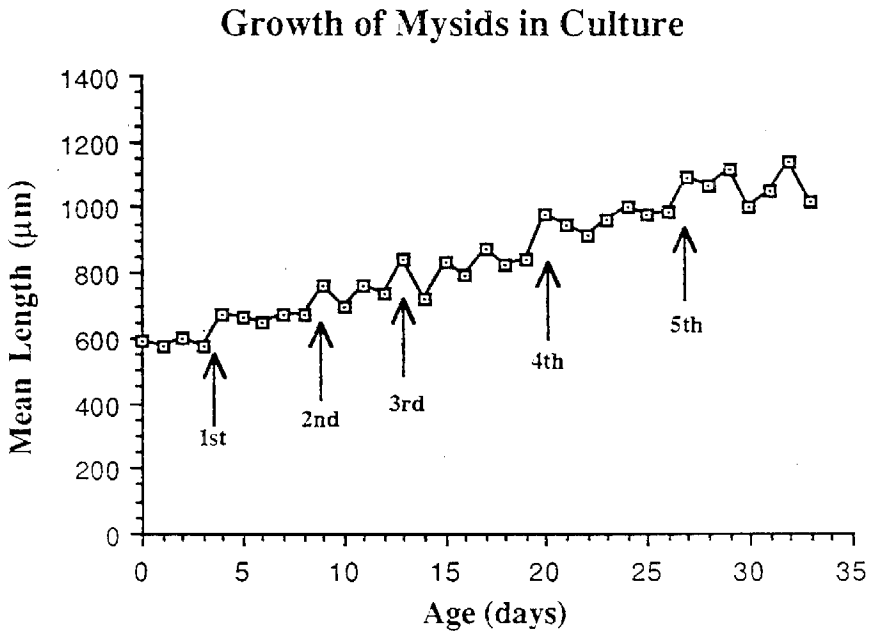
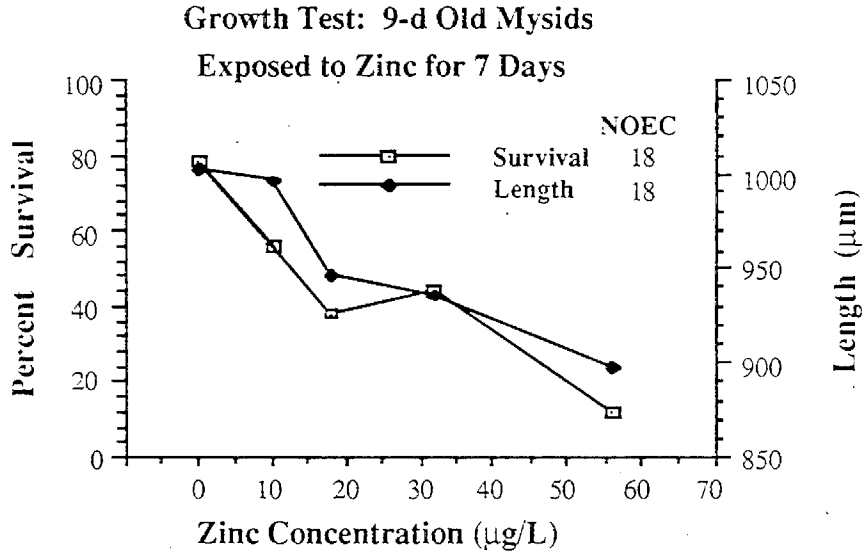


Figure 22.



Arrows indicate jumps in growth assumed to indicate moulting.

Figure 23.



Summary

1. *Holmesimysis costata* is an ecologically important Pacific coast mysid crustacean that inhabits kelp canopies. *Holmesimysis* is easily collected and gravid females can be maintained in the laboratory for extended periods, producing juvenile mysids suitable for toxicity testing. Gravid females and newly hatched juveniles can be shipped by overnight courier to testing laboratories.

2. A 96-hour toxicity test protocol has been developed and tested with reference toxicants and complex effluents. The 96-hour mortality test has been a sensitive indicator of effluent toxicity, with effluent NOEC values comparable to those produced by the sublethal abalone and kelp tests described in this report.

3. *Holmesimysis* juveniles were sensitive to toxicity from hypersaline brines used to adjust effluent salinity. Further research is needed to determine suitable sea salts and methods for producing brine mixtures suitable for effluent testing with *Holmesimysis*.

4. NOEC values from 96-hour reference toxicant tests varied between 18 and 56 µg/l. Variations in test results were not correlated with seasonal changes.

5. Interlaboratory variation was similar to variation between tests conducted at MPSL, with zinc NOECs of 18 and 56 µg/l. Interlaboratory tests with effluent produced the same NOEC, 3.2%, although these tests were affected by poor brine control survival.

6. Gravid female adult *Holmesimysis* were insensitive to zinc (relative to juveniles), and one-day-old mysids had low control survival. Neither of these age groups was suitable for toxicity testing. Juvenile *Holmesimysis* aged 3, 5, 7, and 9 days responded similarly to zinc in one experiment. A second experiment using lower toxicant concentrations showed that 3-day-olds, 7-day-olds, and a mixed group (3-, 5-, 7-, and 9-days-old) responded similarly, while 5-day-olds were significantly more tolerant and 9-day-olds were significantly more sensitive to zinc. Further research is recommended before juvenile mysids of varying age are used interchangeably in toxicity tests.

7. Although the 96-hour mortality test is a simple and sensitive indicator of effluent toxicity, it is currently unsuitable for use because of regulatory preferences for sublethal tests. Reproductive endpoints are not feasible with *Holmesimysis* because of the animal's 70-day lifecycle. Growth effects were studied as an alternative sublethal endpoint. In tests using zinc and effluents, growth was equally or less sensitive than mortality. Growth may be more sensitive to other toxicants.

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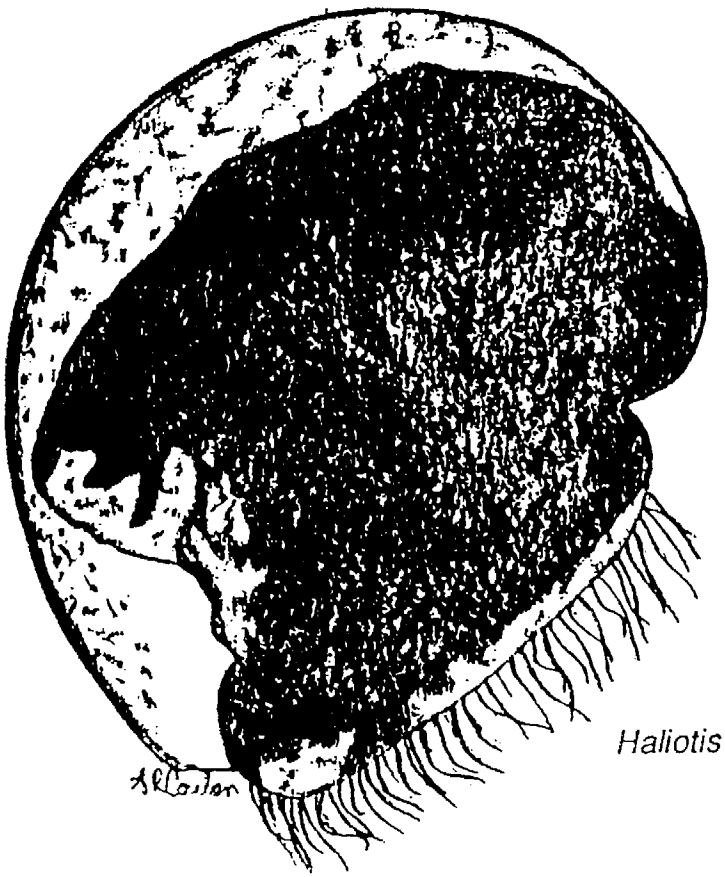
Section 3
Red Abalone Experiments

John W. Hunt
Brian S. Anderson



Haliotis rufescens - veliger

Introduction

The red abalone is the molluscan species developed for toxicity testing by the Marine Bioassay Project. The abalone is widely recognized in California as a seafood delicacy. It supports a popular recreational fishery throughout the state and is harvested commercially from Southern California waters. The largest of the California gastropods, it consumes a variety of seaweeds and small incidental organisms, and is an important food source for sea otters, lobsters and octopods. Abalone inhabit nearshore rocky subtidal and intertidal areas. Declines in some populations near ocean outfalls have been associated with past effluent discharges (Grigg and Kiwala, 1970).

Several attributes make the red abalone an appropriate organism for routine effluent toxicity testing. Culture techniques have been well developed by researchers interested in its potential for commercial aquaculture, and larval test organisms can be easily obtained throughout most of the year. Previous toxicity studies have shown abalone larvae to be sensitive to trace metals and a variety of organic toxicants (Martin *et al.*, 1977; Morse *et al.* 1979). An effluent toxicity test protocol for red abalone was developed in Phase 2 of this project, and the protocol was tested with reference toxicants and primary and secondary complex effluents (Anderson *et al.*, 1988). The test protocol provides step-by-step instructions for a 48-hour static bioassay in which newly fertilized abalone eggs develop into veliger larvae while exposed to a series of effluent dilutions. Abnormal development of the larval shell is used to indicate toxicity. Also developed during Phase 2 was a 9-day flow-through test. This longer test was conducted to verify whether zinc concentrations causing abnormal shell development also affected the larvae's ability to metamorphose into juvenile abalone (Hunt and Anderson, in press).

This report describes a number of experiments designed to refine the red abalone test for routine detection of effluent toxicity. Experiments were conducted to analyze the following aspects of test performance: 1) feasibility for use with complex effluents from large municipal waste treatment plants; 2) variation in the effects of effluents and reference toxicants; 3) seasonality of response to a reference toxicant (zinc); 4) variation in the interpretation of the shell abnormality endpoint by different investigators; 5) sensitivity of the shell abnormality endpoint relative to a sperm viability endpoint; 6) variation in results obtained at different laboratories; and 7) sensitivity to copper, a widely used toxicant. The present revision of the test protocol given in this report (Appendix III) reflects information gained in conducting 17 tests this year. The following sections describe the methods and results of these experiments, and these are discussed in terms of refining the protocol for routine effluent testing.

Methods

Red Abalone Short-term Toxicity Test Protocol

Methods for conducting the 48-h abalone larval development toxicity test are described in the protocol given in Appendix III. The protocol provides a set of step-by-step instructions for conducting the test, and includes techniques for culture, spawning and fertilization, preparation of test solutions, and preservation and analysis of test organisms. All of the tests described in this report used 5 replicates of each toxicant concentration. No Observed Effect Concentrations (NOEC's) were calculated for all tests using analysis of variance (ANOVA) followed by Dunnett's multiple comparison as described in the protocol. The experiments were conducted at the Marine Pollution Studies Laboratory (MPSL) at Granite Canyon, Monterey County, California, except for one interlaboratory experiment conducted with Coastal Resources Associates (CRA) in Encinitas, California.

Effluent Tests

Effluents were collected from two large Southern California waste treatment plants that discharge complex effluents into marine waters: the Los Angeles County Joint Water Pollution Control Plant (JWPCP) and the Los Angeles City Hyperion waste treatment plant. Twenty four-hour composite samples were collected and shipped overnight to MPSL in 1-liter polyethylene bottles packed with blue ice. Hyperion effluent was 67% primary and 33% secondary, and was not chlorinated. JWPCP effluent was 45% primary and 55% secondary, and was collected after chlorination and dechlorination. Samples were agitated to evenly distribute effluent particles before each test dilution. Test concentrations were 0 (control), 0.56%, 1.0%, 1.8%, 3.2%, 5.6%, and 10.0% effluent in seawater. MPSL natural seawater (0.2 µm-filtered) was used as the dilutant. Calculated volumes of hypersaline brine were added to test solutions to compensate for the low salinity of effluents relative to natural seawater. Commercially available sea salts were used to make hypersaline brines. Brine pH was adjusted to that of the dilution water by addition of 2N hydrochloric acid. All effluent tests used brine controls containing seawater, the same volume of hypersaline brine used in the highest effluent concentration, and distilled water in place of effluent to adjust salinity to that of the dilution water. Brine controls were compared against effluent treatments to determine NOECs (see Appendix III for details).

Brine solutions made with commercial salts were satisfactory with one exception, when brine was inadvertantly mixed using twice the normal amount of salt (70 vs. 35 g/l of seawater). This caused 50% abnormality in brine controls, and the test was discarded. Experiments are planned to compare the suitability of brines made with different brands of commercial sea salts.

Effluents from each treatment plant were tested twice, with the tests approximately six months apart. Each effluent test was accompanied by a concurrent test using a reference toxicant.

Reference Toxicant Tests

Zinc (zinc sulfate, $ZnSO_4 \cdot 7H_2O$) was used as a reference toxicant in seven trials of the protocol. Zinc is a suitable reference toxicant because it is stable in solution, easy to analyze chemically, relatively non-hazardous to laboratory personnel, and found in high concentrations in the target effluents (Anderson *et al.*, 1988).

Four of the zinc tests were run concurrently with effluent tests and were timed on a quarterly basis to investigate seasonal variability. Test dates were February 2, April 26, August 23, and November 15, 1988. These, and a fifth test begun August 17, were compared statistically using a 2-way analysis of variance. A Student-Newman-Keuls (SNK) multiple comparison test was used to find statistical differences between the individual tests.

One experiment used copper (cupric chloride $CuCl_2 \cdot 2H_2O$) as the toxicant, so that results could be compared to numerous published copper toxicity studies with a variety of organisms.

Comparison of Endpoint Interpretation Among Different Investigators

A number of steps have been taken to minimize variation in the interpretation of larval shell abnormality. The protocol contains a number of photographs that illustrate shell development. These are used to clarify the verbal definition of an abnormal shell as "a thin, non-calcified shell with visible indentations." In the past, two investigators have separately analyzed every replicate of three zinc tests and compared the results with paired sample t-tests. No significant differences between investigators were found in these comparisons (Hunt and Anderson, in press). In the present study, our counts were compared to those made by investigators analyzing the abalone test for the first time. Two samples from each concentration of a zinc test were analyzed by two combinations of one new and one experienced investigator. An effluent test was similarly analyzed by two other combinations of investigators. The results were compared using paired sample t-tests. Graphical comparisons were made of results from zinc and effluent tests in which four different investigators analyzed duplicate samples from each test concentration.

Comparison of Toxicant Sensitivity: Larval Shell Development vs. Sperm Cell Viability

To better protect marine populations, toxicity tests focus on the most sensitive life stage of the test organism. Sperm cells of some marine echinoderms are sensitive to effluents in rapid tests (Dinnel and Stober, 1987). An experiment was conducted to test whether abalone sperm cells were more sensitive than developing abalone veliger larvae when exposed to zinc.

Several modifications were made to the protocol to expose sperm cells prior to fertilization. Abalone broodstock were spawned sequentially so that males spawned about 1.5 hours before females. Concentrated sperm suspension was pipetted from just above the respiratory pore of a spawning male abalone, and placed in a 250-ml flask with fresh 0.2-µm filtered seawater (14° C). Sperm density was calculated and the sperm stock diluted so that 1 ml of sperm suspension was added to each test container to provide a test density of 4,250 sperm/ml or 850 sperm per egg. The sperm cells were exposed to the zinc toxicant solutions for 1 hour. One thousand unfertilized eggs

were then pipetted to each container. No-sperm controls were used to verify that eggs were unfertilized prior to contact with the test sperm cells. After an 18-hour exposure, half of the eggs in the test containers were pipetted into Sedgewick-Rafter cells and viewed at a 100x magnification. Unfertilized eggs were visible as single cells. Fertilized embryos had developed into trochophore larvae. Percent fertilization data were calculated for each replicate and analyzed with the same statistical methods outlined in the protocol.

The remaining embryos continued to incubate in each test container for a total of 48 hours to allow development to the veliger larval stage. These were then sampled, counted, and analyzed as described in the protocol.

Interlaboratory Testing

Male and female abalone broodstock were shipped overnight from MPSL to the Coastal Resources Associates (CRA) laboratory in San Diego County, California, using the transportation techniques described in the protocol. Broodstock were acclimated overnight in seawater collected from La Jolla, California. The following day, investigators from CRA, who had not previously conducted the test, initiated a 48-h abalone test using methods described in the protocol. Test solutions were inadvertantly made up to yield prescribed concentrations of zinc sulfate (compound weight = 287.56) as opposed to zinc (atomic weight = 65.38). Therefore, nominal zinc concentrations in CRA test solutions were 4.4 times lower than those used at MPSL. Chemical verification of test solutions was unsuccessful, as described below. Dilution water in the CRA test was collected from La Jolla.

Abalone were packed for transportation but retained at MPSL for use in the MPSL 48-h test. The MPSL dilution water was collected at Granite Canyon. All methods for this test were as described in the protocol.

Reference Toxicant Chemical Verification

As in previous work on this project, all reference toxicant concentrations were sampled at the beginning and end of each test. These were analyzed for zinc or copper concentration on a Perkin Elmer model 5000 atomic absorbtion spectrometer following the methods of Bruland *et al.* (1979). Previously, nominal concentrations have been similar to measured concentrations (see Table 9). This year, however, measured concentrations from many experiments varied widely from nominal concentrations. These discrepancies were probably caused by sample vial contamination, rather than contamination of test containers or solutions. For example, control solutions in which abalone developed normally had measured zinc concentrations well above those causing 100% abnormality in previous (chemically verified) tests. Variability between measured concentrations was high, both between tests and between samples from the same test (Table 9). Attempts to document sample vial contamination were unsuccessful because most used vials had been acid cleaned before the problem was discovered. Because sampling problems compromised chemical analysis, all zinc concentrations in this report are given as nominal concentrations.

Measured copper concentrations were close to nominal concentrations (Table 9), and there was no evidence of sample contamination. All copper values are reported as measured concentrations.

Table 9. Comparison of nominal and chemically measured zinc concentrations for abalone tests from this report and from previous work on the Marine Bioassay Project. ND = not detectable: concentrations below the detection limit (2.6 µg/l) of the AA mass spectrometer.

Nominal Zinc Concentration (µg/l)	Chemically Measured Zinc Concentrations (mean ± s.d.; µg/l)	
	Abalone Tests (this report) (n = 6)	Previous MBP Abalone Tests (n = 7)
0	33.22 ± 37.43	All ND (< 2.6)
10	20.75 ± 14.11	13.2 ± 3.6
18	42.33 ± 31.47	22.1 ± 3.7
32	43.46 ± 21.70	39.1 ± 2.4
56	84.38 ± 44.03	69.1 ± 5.0
100	110.55 ± 33.70	126.8 ± 7.9

Results and Discussion

Effluent Tests

Dilutions of effluent from JWPCP were toxic to abalone larvae at concentrations greater than 1%. Tests done in February and August produced a NOEC of 1% (Figure 24a, Table 10). The response curves for the two effluent samples were not identical. Ten percent of the abalone larvae in 3.2% effluent were normally developed in the February test, while 61% were normally developed in the same concentration in the August test. Both of these effects were significantly different from controls, however, and did not affect the NOEC value. The 1.8% effluent concentration in the August test produced 95% normal larvae, but because of the low variability between replicates, this value was significantly less than the control. There was no 1.8% concentration in the February test.

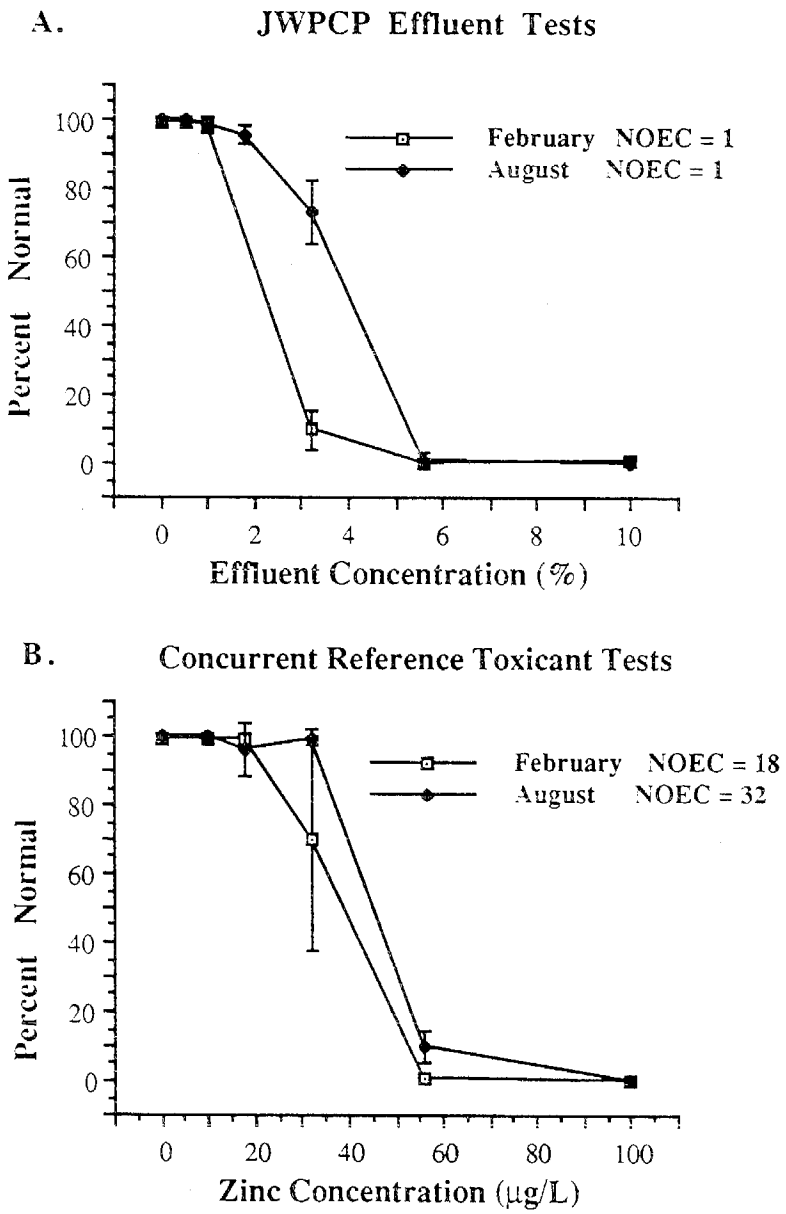
Brine controls from all effluent tests (n = 4) produced a mean of 99.3 ± 0.5% (s.d.) normal larvae, compared to 99.0 ± 0.8% normal larvae for dilution water controls from the same tests.

Table 10. Summary of abalone test data. NOEC = No Observed Effect Concentration. JWPCP = Los Angeles County Joint Water Pollution Control Plant. Hyperion = Los Angeles City Hyperion waste treatment plant. "Concurrent" signifies reference toxicant tests run concurrently with effluent tests. "Interlab" signifies paired tests done at different laboratories to investigate interlaboratory variability.

Toxicant	Date	NOEC
Effluent Tests		
JWPCP	February	1.0 %
JWPCP	August	1.0 %
Hyperion	June	1.8 %
Hyperion	November	3.2 %
Zinc Tests		
Concurrent	February	18.0 µg/l
Zinc	April	32.0 µg/l
Concurrent	June	18.0 µg/l
Sperm exposed	August	32.0 µg/l
Concurrent	August	32.0 µg/l
Concurrent	November	32.0 µg/l
CRA Interlab	February	7.0 µg/l
MPSL Interlab	February	18.0 µg/l
Copper Test	June	< 6.0 µg/l

Although the two JWPCP effluent tests produced the same NOEC, there were differences between results of concurrent reference toxicant tests (Figure 24b, Table 10). The February zinc NOEC was 18 µg/l, while the August zinc NOEC was 32 µg/l. Only 70% of the larvae developed normally in 32 µg/l in the February test, while 99% developed normally in the 32 µg/l concentration in the August test. High test solution salinities due to evaporation may have affected zinc toxicity in the February test (Table 11), although this was not directly tested.

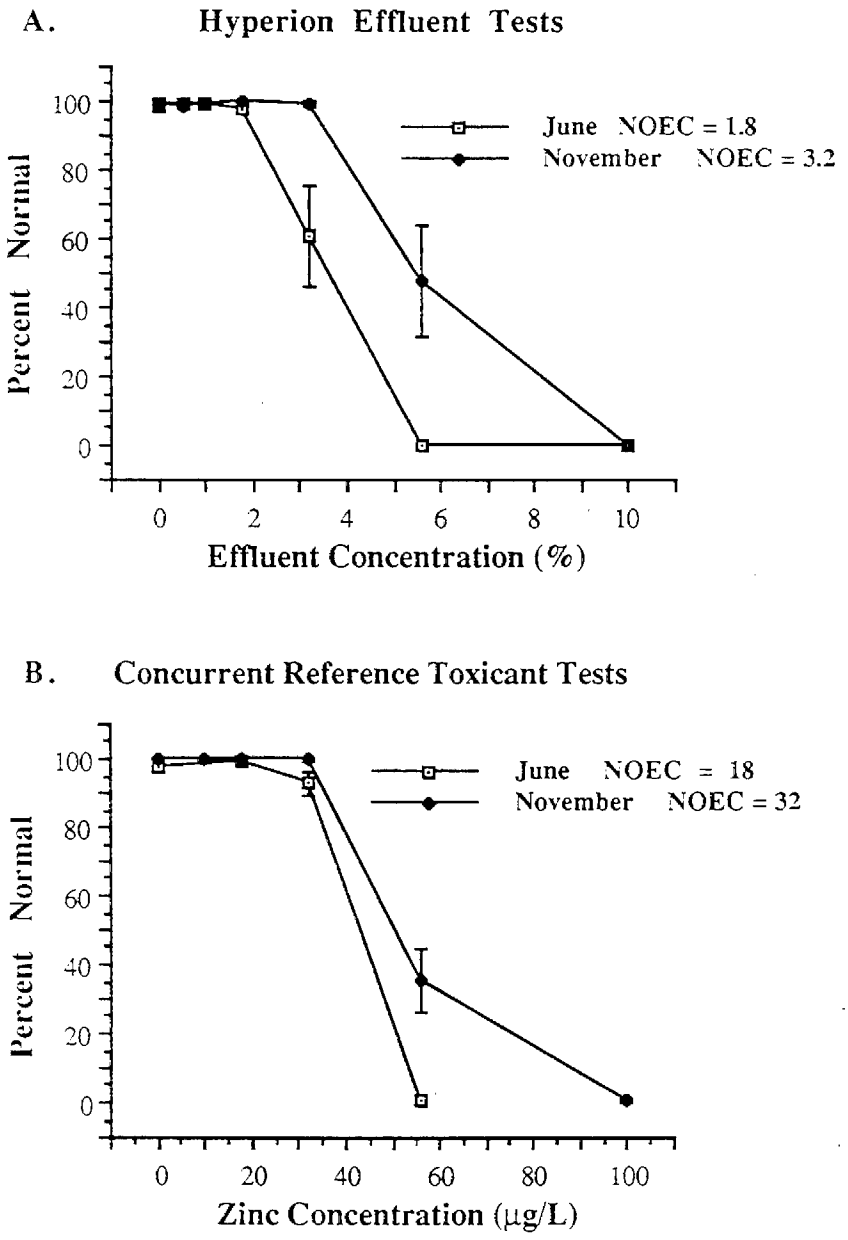
Figure 24.



Responses to Hyperion effluent varied, with NOEC values of 1.8% in June and 3.2% in November (Table 10). The difference in response was most significant in the 3.2% effluent treatments, where 99% of the larvae developed normally in November while only 61% developed normally in June (Figure 25a).

The concurrent zinc tests produced different NOEC's (Table 10), although the differences in the response curves were slight (Figure 25b). The June test had a lower NOEC (18 µg/l) primarily because low between-replicate variability resulted in a statistically significant difference between the control response (98% normal) and the response at 32 µg/l (93%).

Figure 25.

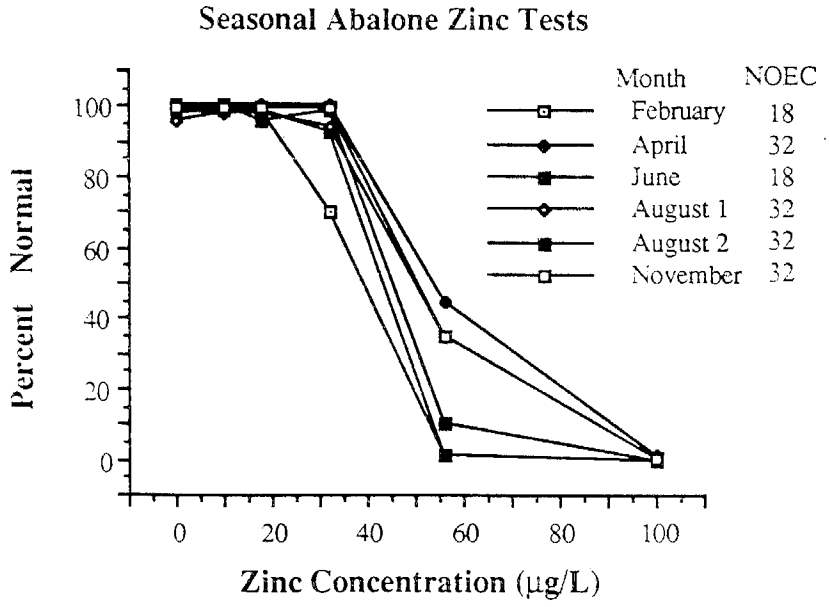


The primary reason for conducting reference toxicant tests concurrently with effluent tests is to provide some type of standard response to indicate the general health of the test organisms and the quality of the testing environment. Using the February concurrent zinc test as an example, lower test values may have been indicative of poor quality control with regard to test solution salinity. In 10 abalone zinc tests conducted at MPSL, all NOEC's have been either 18 or 32 µg/l nominal (22 to 43 µg/l measured) concentrations. Further testing with other laboratories, different seawaters, and different broodstocks will be necessary to define the criteria for test acceptability in a regulatory setting.

Seasonal Effects on Reference Toxicant Tests

Of six zinc tests run this year, two (February and June) had NOEC's of 18 µg/l and four had NOEC's of 32 µg/l (Table 10, Figure 26). Analysis of variance testing showed significant differences between the zinc tests. Multiple comparisons (SNK) broke the test results into three statistically distinct groups. The February test was the lowest, the two tests in August were similar and intermediate, and the November and April tests were similar and had the highest mean percentages of normal larvae. The June test was not compared because of differences in the number of concentrations used.

Figure 26.



It is difficult to say whether variability between tests is the result of seasonal factors. April and November coincide with distinct oceanographic periods off California. April is characterized by intense upwelling with associated high levels of dissolved oxygen, inorganic nutrients and phytoplankton (Reid, 1960). November surface waters originate offshore and are warmer and poorer in oxygen and nutrients; yet there was no statistical difference between tests conducted in these months. Likewise, environmental conditions in February and June are quite different, but tests from these months produced the same NOEC. Seasonal factors appeared to affect test results

less than uncontrolled factors (e.g. test solution salinity in the February and CRA tests; Table 11). As stated above, there are uncertainties regarding measurement of zinc concentrations, and this may be an important source of variation. Measurement of zinc free ion concentration, which is more directly indicative of zinc toxicity (Sunda and Guillard, 1976; Overnell, 1975), is beyond the scope of this project. Although sources of variation are unclear, this series of tests helped to define the range of results that might be expected throughout the year.

Table 11. Ranges of physical/chemical data for abalone experiments. One randomly chosen replicate was measured from each toxicant concentration. Ranges include all measurements from a given test. JWPCP = Los Angeles County Joint Water Pollution Control Plant. Hyperion = Los Angeles City Hyperion waste treatment plant. "Concurrent" signifies reference toxicant tests run concurrently with effluent tests. "Interlab" signifies paired tests done at different laboratories to investigate interlaboratory variability.

Toxicant	Date	Dissolved O_2 (mg/l)	pH	Salinity ($^{O}/oo$)	Temperature (°C)
Effluent					
JWPCP	February	7.70 - 7.95	7.85 - 7.95	35 - 36	13.5 - 14.2
JWPCP	August	7.08 - 7.85	7.80 - 8.12	33 - 35	13.0 - 15.0
Hyperion	June	7.44 - 8.00	7.85 - 8.05	34 - 36	16.0
Hyperion	November	6.43 - 6.92	7.97 - 8.18	34 - 35	13.0
Zinc					
Concurrent	February	7.59 - 8.00	7.90 - 8.00	35 - 38	13.6 - 14.0
Zinc	April	7.70 - 8.10	7.70 - 7.85	34 - 36	16.0 - 16.5
Concurrent	June	7.14 - 8.10	7.85 - 7.90	35 - 36	16.0
Sperm exposed	August	7.39 - 8.05	7.80 - 8.20	35 - 36	14.0 - 15.0
Concurrent	August	7.29 - 7.90	7.92 - 8.14	35 - 36	13.0 - 14.5
Concurrent	November	6.89 - 7.46	7.92 - 8.27	34 - 35	13.0
MPSL Interlab	February	7.59 - 8.00	7.90 - 8.00	35 - 38	13.6 - 14.0
CRA Interlab	February	na	7.90	33 - 38	13.7 - 14.4
Copper	June	7.54 - 8.00	7.85 - 7.90	34 - 35	14.5 - 15.0

Zinc Toxicity

The mean zinc EC_{50} for the abalone tests reported here was 50 µg/l. The mean EC_{50} from 3 previous zinc tests using chemically verified zinc concentrations was 68 µg/l (Hunt and Anderson, in press). A comparison of these values with those for other molluscs and echinoderms indicates that abalone larvae are sensitive indicators of zinc toxicity (Table 12).

Table 12. Zinc and copper toxicity values from literature sources (µg/l).

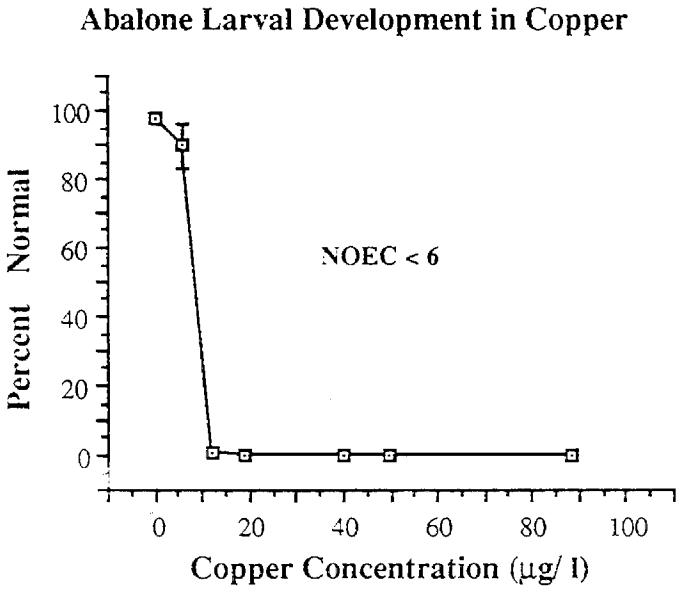
Organism	Effect Studied	Zn EC_{50} (LC_{50})	Cu EC_{50} (LC_{50})	Source
Echinoderms				
Arbacia punctulata	Sperm cell; fertilization	121	12	Nacci *et al.*, 1986
Strongylocentrotus purpuratus	Sperm cell; fertilization	262	24	Dinnel *et al.*, 1983
S. droebachiensis	Sperm cell; fertilization	383	58	Dinnel *et al.*, 1983
Molluscs				
Mytilus edulis	48-h larval development	175	5.8	Martin *et al.*, 1981
Crassostrea gigas	48-h larval development	119	5.3	Martin *et al.*, 1981
C. virginica	48-h larval development	206	15	MacInnes and Calabrese, 1978
M. mercenaria	10-day larval mortality	195	16	Calabrese et al 1977
Haliotis rufescens	48-h larval development	68	-	Hunt and Anderson, in press
Haliotis rufescens	48-h larval development	50*	8.8	Hunt and Anderson, this report

* nominal concentration

Copper Toxicity

Copper was more toxic than zinc to abalone larvae. The NOEC was less than 6 µg/l , the lowest concentration tested (Figure 27). The EC_{50} for copper was 8.8 µg/l, based on linear regression using chemically measured concentrations. The EC_{50} of 8.8 µg/l is among the lowest published values for copper (Table 12).

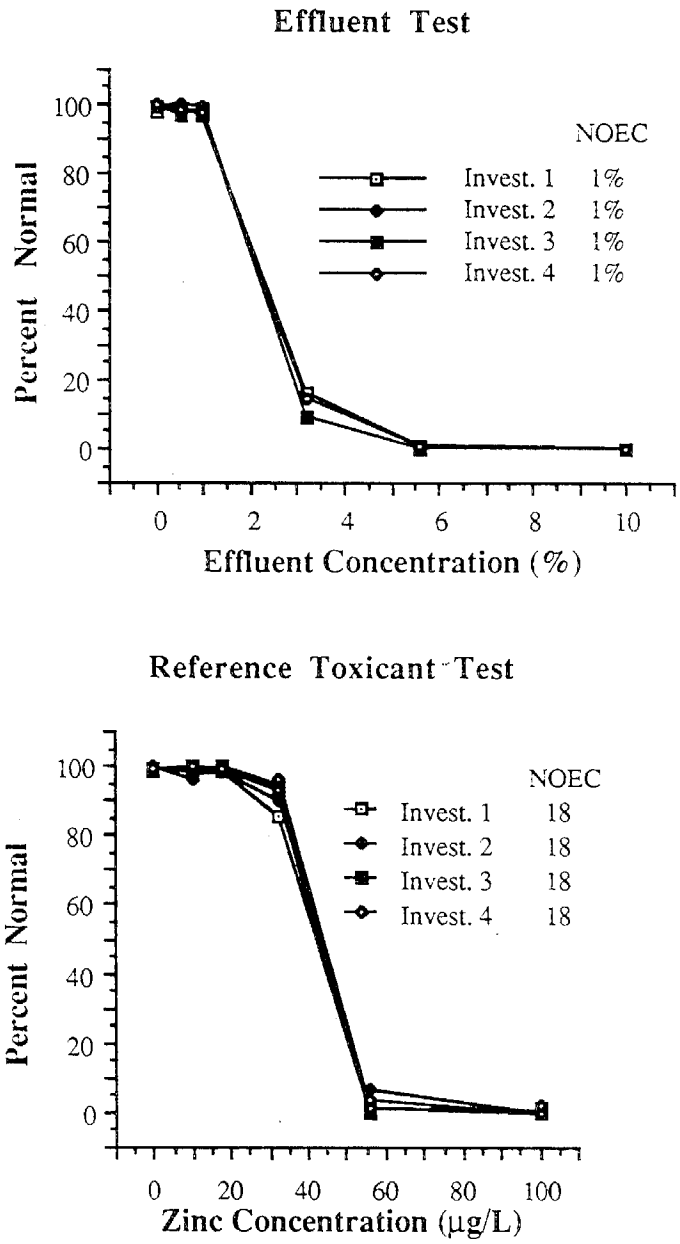
Figure 27.



Endpoint Interpretation

Four paired comparisons (two for a zinc test, two for an effluent test, each with a different combination of an experienced and inexperienced investigator) showed no statistically significant differences between analyses of larval abalone samples (p = .69, .26, .19, and .49 for paired sample t-tests). Graphs of zinc and effluent tests read by four different investigators show that the counts were all close, and that the greatest differences were between counts of samples from intermediate concentrations (32 and 56 µg/l in the zinc test, and 3.2 % in the effluent test; Figure 28). Intermediate concentrations also have the largest between replicate variability (see Figures 24, 25, 27, 29 and 30). The data suggest that variability in investigator interpretation of larval development does not significantly affect the results or NOEC's from the abalone tests. One further comparison is planned in which each of a number of investigators will analyze five replicates from the most variable intermediate concentration.

Figure 28. Endpoint Comparisons Between Different Investigators

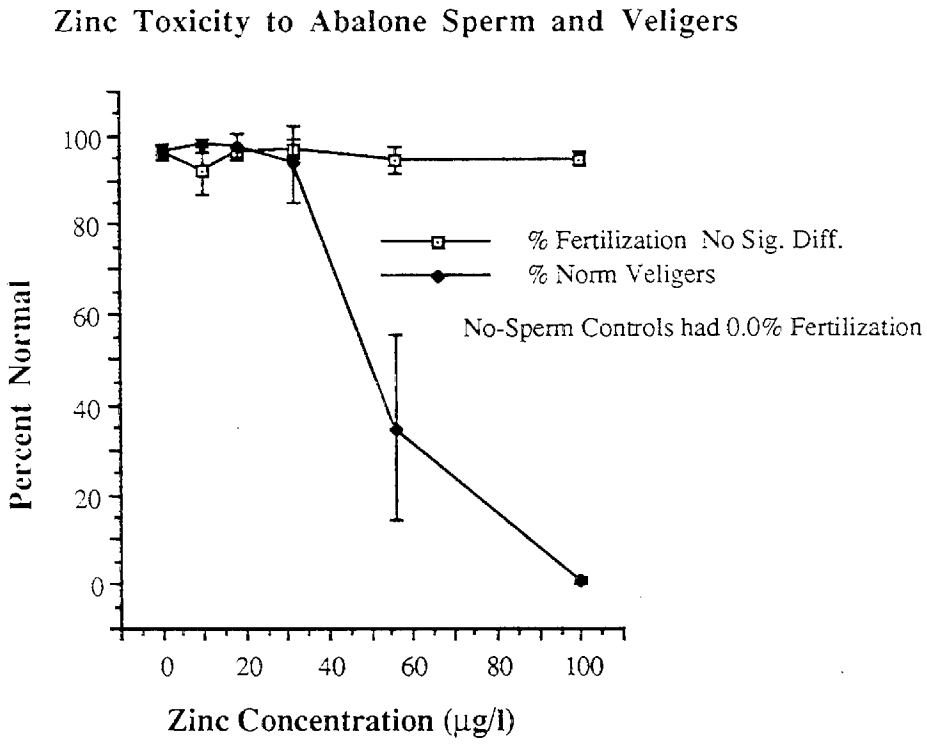


Abalone Sperm Test

Sperm viability and fertilization success were unaffected at zinc concentrations causing 100% abnormal development in veliger larvae that developed in the same test solutions (Figure 29). Nearly 100% fertilization occurred in all zinc concentrations. No fertilization occurred in separate sperm-free controls, indicating that eggs were not fertilized prior to exposure. Veliger larval results from this test were similar to those from other 48-h tests, even though in this test, sperm were exposed and fertilization occurred in zinc solutions. Analysis of variance detected no significant difference between the larval development data from the sperm cell test and that from a standard 48-hour test conducted the next week (August tests in Figure 26).

The sperm to egg ratio has been shown to affect the sensitivity of echinoderm sperm cell tests (Dinnel *et al.*, 1987). It may be that lower sperm to egg ratios would increase the sensitivity of an abalone sperm cell test. Exposure of eggs prior to fertilization might also prove to be a more sensitive indicator of toxicity. Even if exposure of abalone gametes proves sensitive, the lack of a fertilization membrane in the organism makes analysis of resulting embryos somewhat more difficult than with echinoderms.

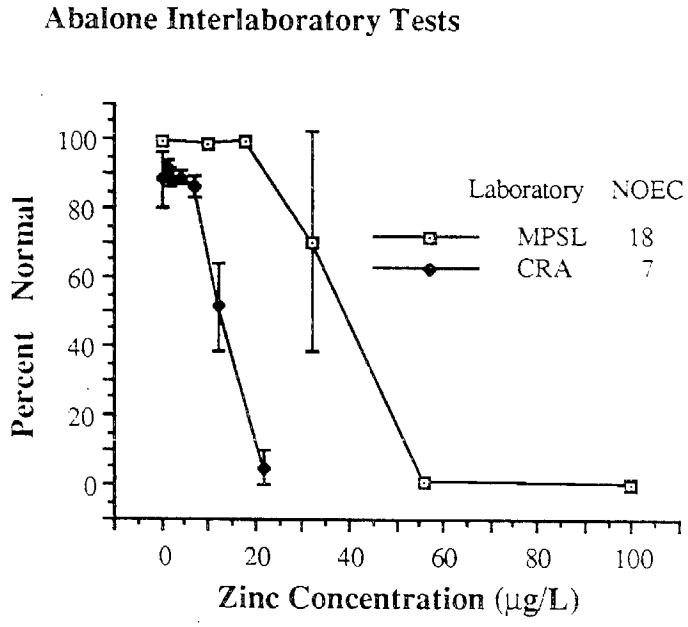
Figure 29.



Interlaboratory Tests

The two laboratories participating in the interlaboratory tests produced different results (Figure 30). The test conducted at the Marine Pollution Studies Laboratory (MPSL) doubled as the February reference toxicant test discussed above. The NOEC for this test was 18 µg/l, a low value compared to other MPSL tests. Seventy percent of the larvae in 32 µg/l zinc developed normally, while 99% developed normally in 18 µg/l. In contrast, 5% developed normally in 22 µg/l in the test at Coastal Resources Associates (CRA), and only 52% developed normally in 12.7 µg/l. The NOEC at CRA was 7 µg/l zinc. Lower zinc concentrations were used at CRA (see Methods), and different water sources were used at each lab. Test salinities were high in both tests, ranging up to 38 °/oo at both labs.

Figure 30.



Physical/Chemical Measurements of Test Solutions

With the exception of high salinities in the February tests, all measurements of salinity, temperature, pH, and dissolved oxygen were within normal ranges (Table 11).

Summary

1. Abalone were easy to culture and spawn. Spawning was successful in 15 of 18 trials this year, with two unsuccessful spawning attempts in December and one in February.
2. The abalone 48-hour toxicity test worked well with effluents. Brine control results were similar to control results in four tests. Effluent NOEC's ranged from 1% to 3.2%.
3. Variation among reference toxicant tests was low, with zinc NOEC's ranging from 18 to 32 µg/l in six tests over the course of the year. Test variation did not correspond to season.
4. Abalone larvae were sensitive to copper, with a NOEC of <6 µg/l and an EC_{50} of 8.8 µg/l.
5. Differences in interpretation of the larval shell abnormality endpoint were minimal among new and experienced investigators. There were no significant differences in four paired comparisons, and NOEC's derived by all investigators were identical in zinc and effluent tests.
6. The larval shell development endpoint was more sensitive than a sperm viability endpoint in one test. Fertilization success was unaffected in concentrations that produced 100% abnormal larval shell development.
7. Differences between two laboratories conducting the abalone test were significant. High test solution salinity and uncertainty about zinc test concentrations may have been responsible.
8. The following specific recommendations have been added to the protocol based on Phase 3 experimentation with the 48-h abalone larval development test:
   a. Spawning induction should include four ripe adult abalone of each sex (rather than three) to increase the probability of spawning success.
   b. Initiate fertilization within one hour of female spawning. Eggs should not be left to aggregate on the bottom of spawning buckets prior to fertilization. Instead, slowly siphon them into a clean bucket at a low enough density that they form a monolayer on the bottom without contacting each other.
   c. Deliver eggs to test containers within one hour after fertilization. Immediately after delivery, examine remaining eggs microscopically to verify they have not progressed past the one-cell stage. Handling two-celled embryos can damaged them and affect test results.
   d. Begin spawning induction when effluent is delivered so that spawning occurs 2 to 3 hours later. This will allow time for making effluent dilutions and allowing test solutions to achieve the correct temperature prior to the addition of embryos.
   e. Decant hypersaline brines to remove precipitates, and adjust brines to the pH of the dilution water.
   f. Design testing facilities to minimize evaporation of test solutions. Test containers should be covered with clear acrylic sheets to minimize airflow across the surface of test solutions. Tissue culture flasks eliminate evaporation problems.

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Section 4

Topsmelt Experiments

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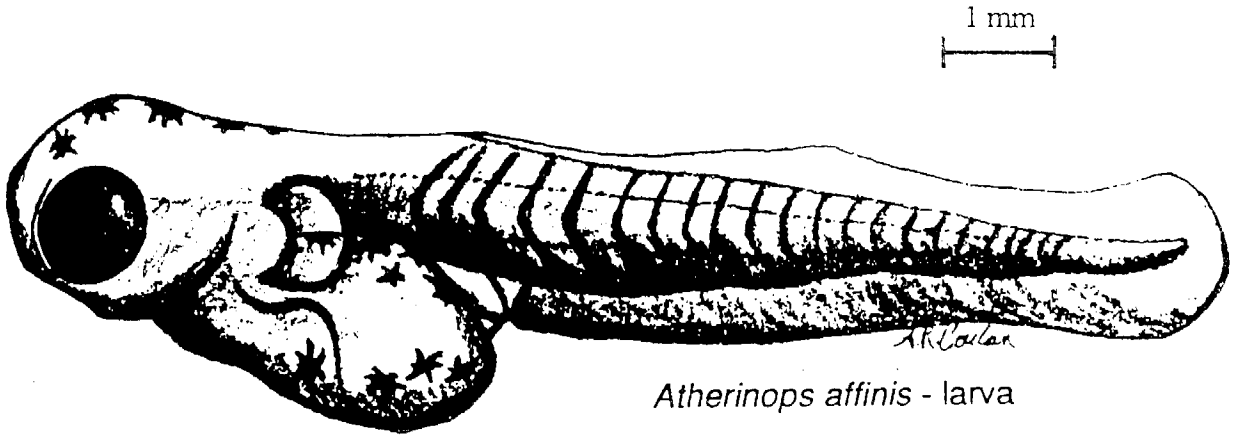


Atherinops affinis - larva

Introduction

One objective of the Marine Bioassay Project is to develop a toxicity test protocol using a fish species indigenous to California's marine waters. To date we have conducted preliminary investigations with two species, northern anchovy, *Engraulis mordax*, and kelp bass, *Paralabrax clathratus*. Adult anchovy were obtained from a local bait dealer and maintained at MPSL for two years. Anchovy are a relatively delicate pelagic species and susceptible to trauma. Broodstock mortality rates in our culture tank were approximately 2.5 % per week. Several attempts to spawn them using synthetic hormone treatments described by Leong (1971) were unsuccessful. Because of difficulty in maintaining broodstock and obtaining fertilized eggs, anchovy were considered unsuitable for use in routine toxicity testing.

Adult kelp bass were obtained from the Occidental College Marine Laboratory at Redondo Beach, and were maintained on warm seawater (18 °C) for six months to facilitate gametogenesis. Attempts to spawn these fish with hormone treatments were unsuccessful. Because development of a toxicity test protocol for kelp bass is near completion by Hose and Parker (in review), further work with this species was considered to be a duplication of effort. Evaluation of a fish species for effluent toxicity testing has continued in Phase 3 with research on topsmelt, *Atherinops affinis*.

Topsmelt range from the Gulf of California to British Columbia and are numerically abundant in nearshore coastal and estuarine habitats (Miller and Lea, 1972; Quast, 1971; Allen, 1982). Like many atherinidae, topsmelt are demersal spawners and generally spawn in estuarine habitats from May to August in California (Hubbs, 1918).

Topsmelt have little economic value, but are ecologically important in estuarine and nearshore marine habitats (Allen and Horn, 1975; Horn, 1979; Allen, 1982). Little toxicological research has been conducted with this species, but there is increasing interest in using them in bioassays (Middaugh *et al.* in press; Reisch and Lemay, 1988). Preliminary culture techniques have been completed by Middaugh *et al.* (in press) and Middaugh and Shenker (1988), suggesting that topsmelt may be utilized for toxicity testing, particularly in situations where waste is discharged into estuarine habitats. Researchers with the U.S. EPA have successfully developed bioassay protocols for other atherinids (Middaugh *et al.*, 1987), including silversides *Menidia* sp. We used techniques similar to Middaugh *et al.* (1987) culture and test *Atherinops* larvae in preliminary toxicity tests. The results of this work are presented here.

Methods

The culture and toxicity test methods for the topsmelt experiments followed the basic procedures developed for silversides (*Menidia* sp.) by Middaugh and Hemmer (1984), and Middaugh *et al.* (1987). The protocol is a static renewal toxicity test that measures larval survival and growth after a 10 day toxicant exposure. Growth is measured as dry weight.

Culture Methods

Approximately 50 adult topsmelt were collected at Moss Landing Harbor (Monterey County) in June 1988. The fish were collected on an incoming tide with a seine and placed in a 250 liter plastic tank, supplied with air, and transported to MPSL. Once at MPSL, the fish were transferred to a 3,800 liter circular tank equipped with flowing

seawater. Fish were treated 3 times with a general antibiotic (Prefuran®) to reduce infections that might arise from capture and transport trauma. After treatment, the tank temperature was slowly increased at a rate of 1 °C per day from the ambient temperature of 15 °C to 20 °C using heated seawater supplied by a heat exchanger. The tank's inflow pipe was positioned on the surface at an angle so that the water in the tank flowed in a constant circular motion at approximately 5 cm sec^{-1}. The fish were fed twice daily with ground squid, supplemented with Purina® trout chow and Tetramin® flake food.

Atherinops affinis is a demersal spawner. To cue spawning, the inflow water to the tank was turned off each day for 1 hour in the morning and evening. Plastic aquarium algae was attached to the bottom to provide artificial spawning substrate. The algae were inspected daily, and when new eggs were present, the algae and eggs were transferred to a 15-liter aquarium equipped with flowing seawater adjusted to 21 °C with a 100 Watt immersion heater. The eggs hatched after nine days incubation. larvae were then isolated in screen tubes equipped with flowing seawater at 15-16 °C. Topsmelt larvae were able to eat newly hatched *Artemia* nauplii within 48 hours of hatching. The larvae were held in the screen tubes for 7 days and fed *Artemia* nauplii in excess. Seven day-old *Atherinops* larvae were used in all tests because work by Heber *et al.* (1987) comparing newly-hatched *Menidia beryllina* to 4-6 day, and 7-9 day-old larvae suggested that, of these three groups, the 7-9 day old larvae were the most sensitive to copper chloride.

Toxicity Test Methods

At the start of each test, 7-day-old larvae were transferred to the test containers. As with mysids (Appendix II), the larvae were pre-randomized by pipeting them 2 at a time into a cup until a total of 10 were transferred. These were then added to 650 ml borosilicate glass beakers filled with 400 mls of test solution. The experiments used 5 replicates each of 1 control and 4 toxicant concentrations. All tests were conducted at 17 °C in a water bath. Larvae were fed newly hatched *Artemia* nauplii in excess during all experiments. *Artemia* densities were not quantified.

The larvae were exposed to the toxicant for 10 days. The two endpoints evaluated were survival and growth. The number of dead larvae were tallied daily and removed from the test containers; on day 10 the percentage survival was calculated. To measure growth, all of the live larvae in each replicate on day 10 were dried at 55 °C for 24 hours. All dried fish were pooled together and weighed, and the total weight was divided by the total number of fish to give a mean weight per fish for each replicate. These data were analyzed following the procedures described in the previous protocols. The percentage data were first transformed to the arcsine of the square root and analyzed with ANOVA followed by Dunnett's test. Untransformed weight data were analyzed with ANOVA followed by Dunnett's test. The No Observed Effect Concentration (NOEC) was derived for all tests as the highest toxicant concentration that was not statistically different from the control at $p \leq 0.05$.

Three reference toxicant experiments were conducted: two with zinc sulfate, and one with copper chloride. The concentrations used in the first zinc test were: 0, 560, 1,000, 1,800, 3,200, and 5,600 µg/l. The test solutions were renewed (90% renewal) every 2 days. The concentrations used in the second zinc test were: 0, 180, 320, 560, 1,000, and 1,800 µg/l, and were renewed (90%) every 4 days. The test concentrations used in the copper test were: 0, 56, 100, and 180 µg/l, and were renewed (90%) once on day five. The two zinc tests used 10 animals per replicate. There were 6 fish in each replicate in the copper test.

All toxicant concentrations were chemically verified with a Perkin Elmer model 603 or 5000 atomic absorption spectrometer. One randomly selected replicate from each test concentration was sampled for chemical analysis at the beginning of every experiment and prior to each water change. The reported concentrations and NOEC's are the means of these measured test concentrations.

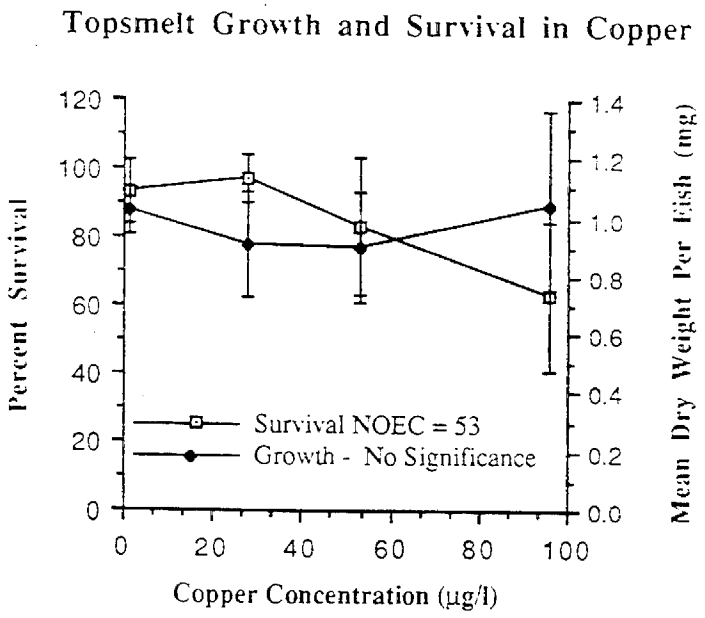
Chemical and physical measurements of the experimental solutions were taken at the beginning and end of each test. Samples were randomly selected from each of the test concentrations and analyzed for dissolved oxygen, pH, salinity, and temperature.

Results and Discussion

Survival of topsmelt larvae was significantly inhibited at 96 µg/l after a 10 day exposure to copper chloride (NOEC = 53 µg/l). Heber *et al.*, (1987) reported a copper NOEC of 102 µg/l for *Menidia beryllina* larvae. Rice and Harrison (1978) found significant mortality with *Engraulis mordax* larvae at 300 µg/l copper. The measured copper concentrations in our test were variable; the NOEC reported was the mean measured value for the 100 µg/l nominal concentration (53 ± 50 µg/l; see Table 1). After the initial dilution, old copper stock solutions were inadvertently used and this could have resulted in lower measured values.

There was no significant larval growth inhibition in this experiment (Figure 31). The mean weight per fish was not significantly different between controls and the highest concentration (96 µg/l). We observed significant weight increases in all of the fish regardless of exposure concentration. Growth measured as standard length was not significantly inhibited by copper either (data not shown).

Figure 31.

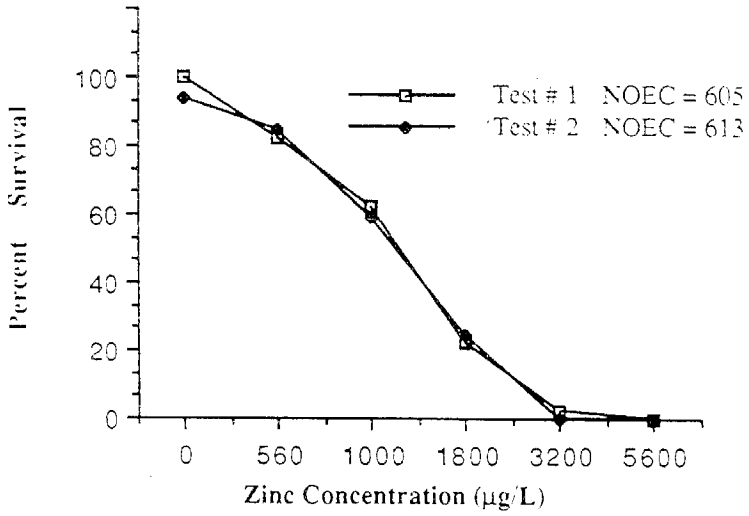


Zinc sulfate inhibited larval survival at concentrations greater than 605 and 613 µg/l in zinc tests #1 and #2 respectively (Figure 32a). Significant growth inhibition occurred at concentrations greater than 605 µg/l in test #1, but no significant growth inhibition was found in test #2 (Figure 32b).

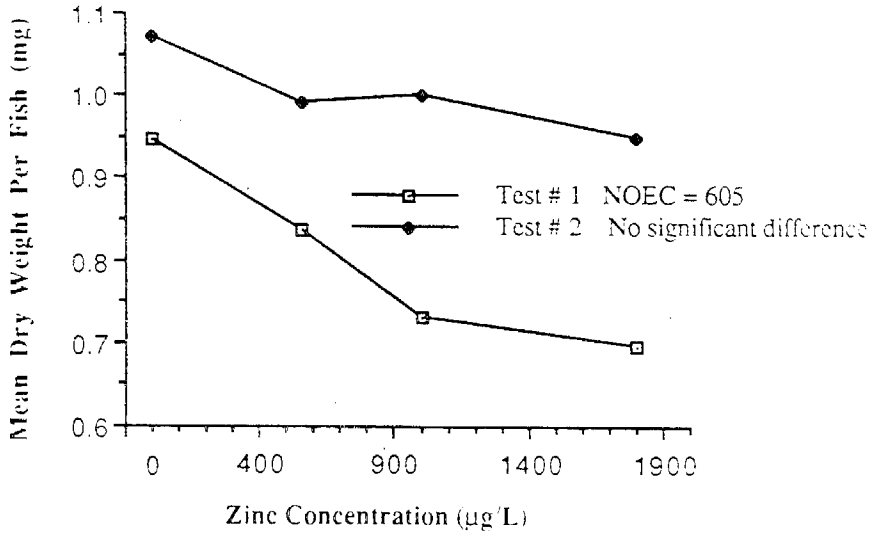
It is possible that growth effects were masked in the copper and zinc experiments because *Artemia* nauplii concentrations were not adjusted as mortality increased. All replicates were fed *Artemia* in excess regardless of the number of surviving larvae, and the *Artemia* concentrations were not quantified. As larvae died in the higher concentrations, the number of *Artemia* per larvae increased relative to the lower concentrations. The greater relative food density may have mitigated the toxic effect of the metals.

Figure 32a-b.

a. **Topsmelt Survival in Zinc**



b. **Topsmelt Growth in Zinc**



The measured copper and zinc concentrations are given in Table 31. As discussed above, the copper concentrations were variable probably due to the use of old stock solutions. The measured zinc concentrations were close to nominal values (Table 13).

Table 13. Chemical verification of toxicants concentrations used in Phase 3 topsmelt tests. Measured concentrations are means of initial concentrations and samples before renewals (± s.d.). All values are in µg/l.

Copper Test						
Nominal	0	56	100	180		
Measured	1 (1)	28 (28)	53 (50)	96 (85)		
Zinc Tests						
# 1						
Nominal	0	560	1000	1800	3200	5600
Measured	4 (5)	605 (98)	877 (69)	1637 (145.0)	2903 (319)	5110 (386)
# 2						
Nominal	0	180	320	560	1000	1800
Measured	5 (3)	224 (12)	372 (26)	613 (47)	1010 (69)	1872 (75)

Physical and chemical measurements of test solutions in the copper and zinc tests were within normal ranges (Table 14).

Table 14. Physical and chemical ranges for Phase 3 topsmelt tests.

Toxicant	D.O. (mg/l)	pH	Salinity (ppt)	Temp. (°C)
Copper	7.08 - 8.41	8.35 - 8.55	34 - 36	17.0 - 18.0
Zinc #1	6.88 - 8.26	8.20 - 8.65	35 - 36	14.0 - 17.0
Zinc #2	6.88 - 7.80	8.35 - 8.70	34 - 36	15.0 - 17.0

Although the sensitivity of the 7-day old *Atherinops* larvae appeared to be comparable to that of *Menidia beryllina*, other fish species appear to be more sensitive to zinc and copper. For example, Hose and Parker (in review) reported a copper NOEC of 18 µg/l using embryonic kelp bass *Paralabrax clathratus*, a pelagic spawning species. Pelagic spawners are generally thought to be more sensitive to toxicants than demersal spawners like *Atherinops* (J.E. Hose, personal communication).

Embryonic stages of *Atherinops* are probably more sensitive to toxicants than larval stages. Rice *et al.* (1978 and 1980) suggest that embryonic stages of marine fish are generally more sensitive to metals than larval stages. Engel and Sunda (1979) found that the embryonic Atlantic siversides (*Menidia menidia*) were most sensitive to cupric ions ($CuSO_4$) at or about the time of hatching. Middaugh *et al.* (1988) used 2- to 4-cell embryos of *Menidia beryllina* in 8-day experiments. Various teratological endpoints were used to evaluate toxicity including cranialfacial, cardiovascular, and skeletal defects. Development or teratological effects might be more sensitive indicators of toxicity than larval growth and survival with *Atherinops*. Experiments are now being planned to compare the relative sensitivities of larval growth and survival to various developmental endpoints using topsmelt embryos.

Summary

1. Topsmelt were amenable to laboratory culture and fertilized eggs were obtained with relative ease.
2. Growth was equally or less sensitive than survival in tests using zinc sulfate and copper chloride. The lack of quantification or adjustment of *Artemia* concentrations may have masked growth effects.
3. *Atherinops affinis* and *Menidia beryllina* larvae had similar sensitivities to copper chloride.
4. Larval topsmelt were relatively insensitive to zinc and copper. Future research will focus on effects of toxicants on embryonic stages.

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APPENDIX I

GIANT KELP TOXICITY TEST PROTOCOL

GIANT KELP GERMINATION AND GROWTH SHORT-TERM TOXICITY TEST PROTOCOL

B.S. Anderson

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1.0 Equipment

(35) 350 mL polyethylene plastic food containers

or

(35) 600 mL borosilicate glass beakers

(35) microscope slides and cover slips

(1) hemacytometer

(1) 2 L plastic or glass beaker

(2) 1 L volumetric flasks

pH meter

DO meter (NH_3 electrode)

Thermometer

Refractometer

Microscope (w/ocular micrometer)

Waterbath (or) Incubator

Cleaning liquids

Pipets: (vol. 1 ea. 1- 100 mL)

(grad. 1 ea. 10 mL)

Cool white fluorescent lights

Analytical balance

Light meter

Hand Counters

2.0 Test Organism

2.1 Species

The test organisms for this protocol are the zoospores of the giant kelp *Macrocystis pyrifera*. *Macrocystis* is the dominant canopy forming 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 test species because of its availability, economic and ecological importance, history of

successful laboratory culture (North, 1976; Luning, 1980; Kuwabara, 1981; Deysher and Dean, 1984), and previous use in toxicity testing (Clendenning, 1958; Smith and Harrison, 1978; James *et al.*, 1987; Anderson and Hunt, 1988). Like all kelps, *Macrocystis* has an alternation of generations 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 may settle onto the bottom substrate and germinate. The spores are either male or female; the male spores develop into male gametophytes and the females develop into female gametophytes. The male gametophytes produce flagellated gametes which may fertilize eggs produced by the female gametophytes. Fertilized eggs develop into sporophytes, completing the lifecycle. The entire process from zoospore release to sporophyte production can be completed in the laboratory in approximately 12-16 days.

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. Once settled, the spores germinate by producing a germ tube through which the cytoplasm of the spore is extruded 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 elongation of the germination tube (Figure 1).

2.2 Collection

Macrocystis zoospores are obtained from the reproductive blades of the adult plant. The reproductive blades, sporophylls, are located near the base of the plant just above it's conical holdfast. Sporophylls must be collected subtidally and should be collected from several different plants in one location to give a good genetic representation of the population (at least 5). 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 and basal location on the adult plant, and general lack of pneumatocysts (air bladders).*

(*For information regarding sporophyll collection, contact: The Marine Pollution Studies Laboratory, oast Route 1, Granite Canyon, Monterey CA, 93940. (408) 624-0864 or 624-0947).

2.3 Preparation

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 in moist paper towels at approximately 9-12 °C until needed (note: the zoospores must be released within 24 hours of collection to insure their viability).

2.4 Test Conditions

2.4.1 Lighting

This test must be done under controlled temperature and lighting in either an environmental chamber or water bath. Whatever is used, the test chamber should be designed to provide adequate uniform lighting and cooling and allow easy access to all test containers. The lights used in this protocol are simple cool white fluorescent lights.

The lights should be adjusted to give 50 µE $m^{-2}s^{-1}$ at the top of each test container. It is important that each test container receive the same quanta of light (± 5 µE 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.

2.4.2 Temperature

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. If excessive evaporation occurs, clear plastic food storage wrap can be used to cover the containers. The plastic can be attached to the individual containers with a rubber band.

3.0 Toxicity Test Procedure

3.1 Experimental Design

This protocol is based on the use of 5 effluent concentrations and 2 controls. Each of these is replicated 5 times, thus 35 test containers are needed for each test. Effluent concentrations are usually assigned in a logarithmic sequence as 0% (control), 0% b.c. (brine control), 0.10%, 0.18%, 0.32%, 0.56%, and 1.00% effluent; or, as an example of a wider range, 0% , 0% b.c., 1.0%, 1.8%, 3.2%, 5.6%, and 10.0% effluent. The range and number of concentrations is based on the toxicity of the effluent being tested.

A preliminary range finding test using concentrations from 0 to 100% effluent may be necessary when nothing is known about the toxicity of the target effluent. Because a hypersaline brine is used to adjust the salinity of the effluent dilutions (see section 3.3.2), a brine control is needed in addition to a dilution water control.

3.2 Test Containers

Standard Microscope Method

For tests using complex effluents or organic toxicants, use 600mL borosilicate glass beakers as the test containers. For tests involving trace metal toxicants, use 350 mL polypropylene or polyethylene food storage containers. If new food storage containers are being used, they should be cleaned using the instructions given in section 5.0. With both container types, place one standard microscope slide in each test container to serve as substratum for the zoospores to settle upon. The microscope slide is removed at the end of the experiment.

3.3 Test Solutions

It is convenient to prepare the effluent dilutions for the 48-h kelp protocol concurrently with the zoospore release process (Section 3.6). The test solutions can then be equilibrating in the constant temperature room or water bath while the zoospores are being isolated. Prepare test solutions by diluting the effluent with an approved dilution water using volumetric flasks and pipets. Mix test solutions by combining effluent, hypersaline brine (if necessary, see below), and dilution water in a 1 L volumetric flask. The solutions should be mixed from the lowest concentration (i.e., control) to the highest concentration to avoid contamination. After the test containers have been filled, they should be covered with clear plastic or acrylic sheets to prevent contamination and evaporation.

3.3.1 Dilution Seawater

Unless the actual receiving water is specified as the dilution water, obtain dilution water from clean reference areas that are not contaminated by toxic substances. The minimum requirement for acceptable dilution seawater is that the test organisms survive, grow, and reproduce normally in it. Filter the dilution water to exclude particles greater than 0.2 μm in diameter, unless the effects of dilution water particulates are being specifically addressed in the study. The dilution water salinity should be $34 \pm 2\ ^{o}/_{oo}$.

3.3.2 Salinity Adjustment

Sewage effluents generally have a salinity of 0-2 ppt. In order to conduct toxicity tests with marine organisms it is therefore necessary to adjust the salinity of the effluent dilutions to 34 °/oo with a hypersaline brine. We recommend using a mixture of synthetic seasalts and seawater to make the brine.* It is important to use synthetic salts that do not contain chelators and are free of metal contaminants. Two brands that meet these criteria are Wimex™ and 40 Fathoms™. To make a brine dissolve 35 grams of synthetic seasalt in 1 liter of clean, filtered seawater. This mixture gives a brine with a final salinity of approximately 68-70 °/oo, and is sufficient for adjusting the salinity of most effluents, assuming the highest effluent concentration tested is no greater than approximately 50 %. The pH of the brine should be 7.9, but we have found that brines mixed with synthetic salts are often slightly alkaline. If this occurs, the pH of the brine should be adjusted with a 5 % solution of 6N HCl prior to using the brine for salinity adjustments. As with any titration, the brine solution should be adjusted 1 drop at a time with the dilute acid, and it's pH should be monitored frequently. In the event the pH of the test solution is acidic, adjust it using NaOH as above.

To calculate the amount of brine to add to each test solution, determine the salinity of the brine (SB, in ppt), the salinity of the effluent (SE, in ppt), and the 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:

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

Use brine controls in all tests where brine is used. Make brine control solutions by adding as much brine as is used in the highest effluent concentration (usually 10%). Add distilled water to adjust the salinity to 34 °/oo, then fill the remainder of the mixing flask with dilution water. To determine the amount of distilled water to add, use the above equation, setting SE equal to zero, and solving for VE. Mix the brine control solutions thoroughly.

3.3.3 Example Test Dilution Calculation

An example of a typical dilution calculation is given in the back of this protocol.

* We are currently researching new methods for making hypersaline brines. This is the method that has given us the best results to date.

3.4 Randomization

Standard method

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 35 (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 test organisms have been examined at the end of the test. Arrange the test containers randomly in the water bath or controlled temperature room.

3.5 Physical/Chemical Measurements

Measure the temperature, salinity, pH, dissolved oxygen, and ammonia concentration in one randomly selected replicate of each test concentration at the beginning and end of the experiment. Samples at the beginning of the test can be taken directly from the volumetric mixing flask. Samples at the end of the test can be taken directly from the test containers and should be taken and analyzed before reading the test results. Be careful not to disturb the growing gametophytes on the bottom of the test containers. Measure temperature using a thermometer accurate to at least 1 °C. Measure salinity with a refractometer accurate to 1 °/₀₀. Measure oxygen in mg/L using an oxygen probe accurate to 0.5 mg/l. Use a pH probe accurate to 0.1 pH units. Determine ammonia concentration to the nearest 0.1 mg/l.

3.6 Zoospore Release

Zoospore release is induced by slightly desiccating the sporophyll blades then placing them in filtered seawater. To desiccate the sporophyll, blot the blades with paper towels and let them sit exposed to the air for 1 hour. The number of sporophyll blades needed depends upon their maturity; usually 10-15 blades are sufficient. After 1 hour the blades can be placed in a 2 L glass or plastic beaker filled with 0.2 µm filtered seawater at ambient (15-16°C) temperature: the release water should not exceed 18°C. After 1 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 (400x). 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 2 hours. If it takes longer than 2 hours to get an adequate density of zoospores (~7,500 zoospores / ml of water), repeat the release process with new sporophylls.

3.7 Zoospore Density

After the zoospores are released, determine their density using a hemacytometer. Take a 9 ml sample of swimming spores from the top of the release beaker to avoid sampling the dead zoospores and kelp exudate that have settled to the bottom. To obtain an accurate count, fix the spores in 37 % buffered formalin and shake the sample well before placing it on the hemacytometer. We fix the sample by mixing 9 mls of spore solution with 1 ml of formalin. After counting, the density is multiplied by 1.111 to correct for the dilution caused by adding 1 ml of formalin to the sample. Use at least 3 replicate counts. The standard deviation of these counts should be less than 10% of the mean; if it is not, 3 more replicate counts should be made. 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.

3.8 Exposure of Test Organisms

After the zoospore density has been determined and the volume needed to give 7,500 zoospores / ml test solution is calculated, add this volume to each test container. Again, take only the viable zoospores which are swimming at the top of the release beaker. Because this protocol is designed to expose the zoospores before the germination process has begun, observe a sample under magnification to insure that the spores are swimming before adding them to the test containers.

3.9 Endpoint Determination

After 48 hours the test is terminated. Decant the test solution out of the test container being careful not to tip the microscope slide over. Take the slide out and blot the bottom on a paper towel then place a cover slip on the slide. We usually blot the excess water around the edge of the cover slip to eliminate the flow of water under the cover slip. The slide is then ready for reading under the microscope. The spores are observed under 400x magnification.

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 and determine whether

they are circular (non-germinated) or have a protuberance that extends at least 1 spore diameter (about 3.0 µm) from the edge of the spore (germinated). Spores with a germination tube less than 1 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.

The growth endpoint is the measurement of the total length of the germination tube from the edge of the original spore membrane (Figure 1). For this endpoint only germinated spores with straight germination tubes are 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, and measuring 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.

4.0 Data Analysis

After a 48 hour exposure period, add the number of germinated and non-germinated spores to get 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 percentage data to the arcsine of their square root. 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 a 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 ($p \leq 0.05$).

No data transformation is necessary for the length data. Analyze the data using ANOVA followed by Dunnett's multiple comparison test and derive the NOEC as above.

5.0 Suggested Criteria for Acceptability

The following are the biological and physical/chemical criteria for a successful 48-hour toxicity test using *Macrocystis* spores and gametophytes. At least 80% of the control spores must germinate. The temperature of the test solutions must be less than 17 °C. The salinity of the test solutions must be 34 ± 3 ‰. The dissolved oxygen in the test solutions must be greater than 60% saturation. The pH of the test solutions must be 7.9 ± 0.3. If any of these criteria are not met, the test should be considered invalid, and be repeated.

6.0 Cleaning Procedure

Test Containers (glass): All glass test chambers used in organics and complex effluent bioassays should be cleaned as follows: 1) rinse 3 times with hot tap water, 2) rinse 3 times with acetone, 3) rinse 3 times with deionized water, 4) soak 24 hours in 3N HCL, 5) rinse 3 times with deionized water, 6) soak 24 hours in deionized water, 7) rinse 3 times in deionized water, 7) dry in clean oven.

Test Containers (plastic): All plastic test chambers used in metals bioassays should be cleaned as follows: 1) rinse 3 times with deionized water, 2) soak for 24 hours in a mild detergent, 3) rinse 3 times with deionized water, 4) soak for 24 hours in 3N HCL, 5) rinse 3 times with deionized water, 6) soak 24 hours in 3N HNO_3, 7) rinse 3 times with deionized water, 8) soak 24 hours in deionized water, 9) dry in a clean oven.

.0 Macrocystis Toxicity Test Protocol Summary

1) Collect sporophyll and rinse in 0.2 µm filtered seawater. Store at 9-12 °C for no more than 24 hours before zoospore release.

2) Blot sporophyll dry and leave exposed to air for 1 hour.

3) Place 10-15 sporophyll blades in 2 L of 0.2 µm filtered seawater for no more than 2 hours. The presence of zoospores is indicated by a slight cloudiness in the water.

4) Take a sample of the zoospore solution from the top layer 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 1 % of the test solution volume.

5) Check to make sure that the zoospores are swimming, then pipet the volume of water necessary to give 7,500 spores / ml into each of the test containers. Remember to take the water from the top of the release beaker so that only swimming zoospores are used.

6) 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.

7) Calculate the percentage of germinated spores for each replicate of each concentration. Transform this percentage value 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 a 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.

8.0 References

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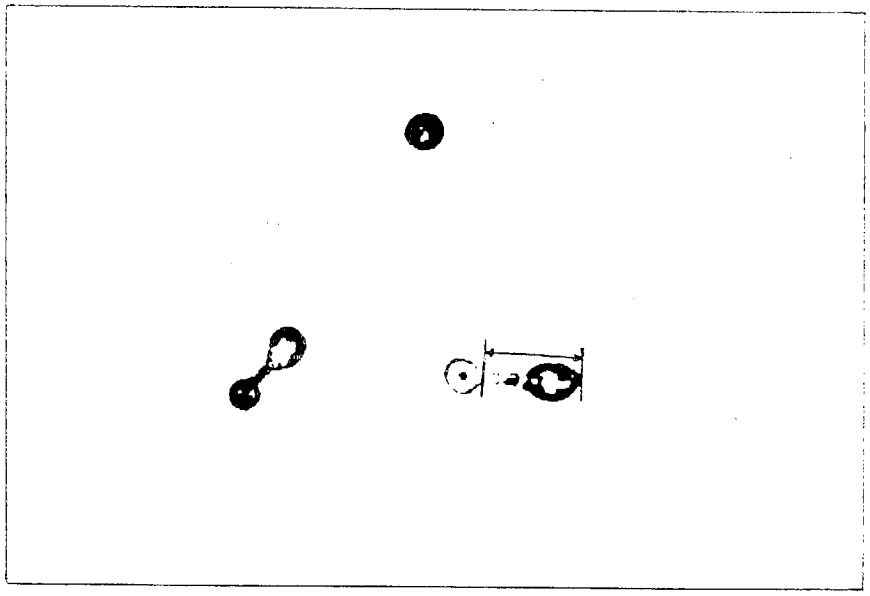

Figure 1. Germinated (lower two) and non-germinated spores of *Macrocystis pyrifera*. Arrows mark germ-tube length measurement (400x).

Effluent Dilution Sheet

100% Effluent is the Stock Solution

		Corresponding Beaker Numbers
0.0 ml in 1000 ml flask →	Control	3, 7, 12, 27, 28
0.0 ml in 1000 ml flask →	Brine Control	33, 18, 40, 34, 22
5.6 ml in 1000 ml flask →	0.56%	35, 17, 2, 8, 13
10.0 ml in 1000 ml flask →	1.0%	36, 23, 24, 29, 37
18.0 ml in 1000 ml flask →	1.8%	25, 30, 31, 38, 4
32.0 ml in 1000 ml flask →	3.2%	9, 14, 19, 26, 39
56.0 ml in 1000 ml flask →	5.6%	32, 20, 15, 28, 10
100.0 ml in 1000 ml flask →	10.0%	11, 16, 1, 21, 6

Salinity Adjustment Using Hypersaline Brine

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

Quantities to be measured : SB = Salinity of the Brine (°/oo)
SE = Salinity of the Effluent (°/oo)

Quantities to be calculated: VB = Volume of the Brine to be added (ml)

SB = 74 °/oo SE = 2 °/oo

Brine Salinity should be 60 to 80 °/oo

Note: Always adjust the pH of the brine solution to that of the dilution water.

The equation to calculate the volume of brine to add for each concentration is: $VB = VE\left(\frac{34 - SE}{SB - 34}\right)$

For the test effluent and the test brine calculate: $\frac{34 - SE}{SB - 34} = 0.80$

Then multiply this number by the volume of effluent for each test concentration.

*Note: Distilled water is used instead of effluent in brine controls.

Concentration	VE		VB	Final Test Solution Mixture
Control	0.0 ml	x (.8) =	0.0 ml Brine	+ 0.0 ml Effluent in a 1000 ml flask; fill with seawater
Brine Control*	100.0 ml	x (.8) =	80.0 ml Brine	+ 100.0 ml Dist. Water* in 1000 ml flask; fill with seawater
0.56%	5.6 ml	x (.8) =	4.5 ml Brine	+ 5.6 ml Effluent in a 1000 ml flask; fill with seawater
1.0%	10.0 ml	x (.8) =	8.0 ml Brine	+ 10.0 ml Effluent in a 1000 ml flask; fill with seawater
1.8%	18.0 ml	x (.8) =	14.4 ml Brine	+ 18.0 ml Effluent in a 1000 ml flask; fill with seawater
3.2%	32.0 ml	x (.8) =	25.6 ml Brine	+ 32.0 ml Effluent in a 1000 ml flask; fill with seawater
5.6%	56.0 ml	x (.8) =	44.8 ml Brine	+ 56.0 ml Effluent in a 1000 ml flask; fill with seawater
10.0%	100.0 ml	x (.8) =	80.0 ml Brine	+ 100.0 ml Effluent in a 1000 ml flask; fill with seawater

Date 1/1/89 Test Organism White Rhino Investigator's initials JH

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
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%

Salinity Adjustment Using Hypersaline Brine

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

Quantities to be measured : SB = Salinity of the Brine ($^{o}/_{oo}$)
SE = Salinity of the Effluent ($^{o}/_{oo}$)

SB = _____ $^{o}/_{oo}$ SE = _____ $^{o}/_{oo}$

Quantities to be calculated: VB = Volume of the Brine to be added (ml)

Brine Salinity should be 60 to 80 $^{o}/_{oo}$

Note: Always adjust the pH of the brine solution to that of the dilution water.

The equation to calculate the volume of brine to add for each concentration is: $VB = VE\left(\frac{34 - SE}{SB - 34}\right)$

For the test effluent and the test brine calculate: $\frac{34 - SE}{SB - 34}$ = _____

Then multiply this number by the volume of effluent for each test concentration.

*Note: Distilled water is used instead of effluent in brine controls.

Concentration	VE		VB	Final Test Solution Mixture
Control	0.0 ml	x ( ) =	_____ ml Brine	+ 0.0 ml Effluent in a 1000 ml flask; fill with seawater
Brine Control*	100.0 ml	x ( ) =	_____ ml Brine	+ 100.0 ml Dist. Water* in 1000 ml flask; fill with seawater
0.56%	5.6 ml	x ( ) =	_____ ml Brine	+ 5.6 ml Effluent in a 1000 ml flask; fill with seawater
1.0%	10.0 ml	x ( ) =	_____ ml Brine	+ 10.0 ml Effluent in a 1000 ml flask; fill with seawater
1.8%	18.0 ml	x ( ) =	_____ ml Brine	+ 18.0 ml Effluent in a 1000 ml flask; fill with seawater
3.2%	32.0 ml	x ( ) =	_____ ml Brine	+ 32.0 ml Effluent in a 1000 ml flask; fill with seawater
5.6%	56.0 ml	x ( ) =	_____ ml Brine	+ 56.0 ml Effluent in a 1000 ml flask; fill with seawater
10.0%	100.0 ml	x ( ) =	_____ ml Brine	+ 100.0 ml Effluent in a 1000 ml flask; fill with seawater

Date _____ Test Organism _____ Investigator's initials _____

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

te	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

MYSID, *HOLMESIMYSIS COSTATA*, TOXICITY TEST PROTOCOL

MYSID, *HOLMESIMYSIS COSTATA*, SHORT-TERM MORTALITY BIOASSAY PROTOCOL

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1.0 Equipment†

(1) 1000 ml, tall glass beaker
Magnifying lens
(35) 350 ml glass stacking dishes
(or)
(35) 250 ml polyethylene food storage containers
(35) 50 ml polyethylene beakers
Graduated pipets: 1 & 10 ml
Cleaning chemicals (Acetone, 3N HCL)
Artemia nauplii
10 liters of 1 µm-filtered dilution seawater
20 liter, aquaria (static or flow through)
800 liter circular tank (static or flow through)
pH meter
DO meter (w/ NH_3 electrode)
Refractometer
Thermometer
15 °C water bath (or) Temp. control room
Wide-bore 10 ml pipet
(4) Polyethylene 1000 ml volumetric flasks Volumetric pipets: 1, 5, 10, 25, & 100 ml
Plastic spray bottles
140 µm Nitex screen tube (10" dia.)
5 liter plastic bucket
100 µm-mesh hand nets

† This is a list of suggested equipment and is given only as a guideline for the culturing and toxicity testing procedures described in this protocol. Some of the items are optional, depending upon the needs of individual laboratories. Because there is some flexibility in the procedures given below, this list should not be considered complete.

2.0 Test organism

2.1 Species

The test organisms for this protocol are juvenile mysid shrimp *Holmesimysis* (= *Acanthomysis**) *costata* (Holmes 1900). *H. costata* occurs in the surface canopy of the giant kelp *Macrocystis pyrifera*. The ecology of this mysid species is poorly described (Holmquist 1979, Clutter, 1967 & 1969, Green 1970), but *H. costata* are numerically abundant in kelp forest habitats and are considered to be an important food source for kelp forest fish (Clark 1971, Mauchline 1980). Although there has been limited use of *H. costata* in toxicity testing (Davidson *et al.* 1986, Machuzac and Mikel 1987), mysids in general are considered to be excellent 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.* 1984 & 1985).

Adult female *H. costata* brood between thirty and forty embryos in an abdominal pouch, the marsupium, and these develop *in vivo* and hatch as juveniles. The generation time for *H. costata* is approximately 60 - 70 days, making lifecycle tests with this species impractical for a routine toxicity testing program (Anderson and Hunt unpublished data). Consequently, the initial development of a toxicity test protocol with *Holmesimysis* has focused on a 96-hour mortality toxicity test. For background information on the development of this protocol see Anderson *et al.* (1988) and Martin *et al.* (In Press).

2.2 Collection

Holmesimysis can usually be obtained by pulling a small mesh net through the water just under the surface canopy blades of giant kelp *Macrocystis pyrifera*. The mesh size should be approximately 300 µm to retain the adults but not juveniles. The juvenile mysids used in this protocol are hatched in the laboratory from the gravid female adults captured in the field. For any given series of tests, all juveniles should be descendants from brooding adult mysids collected from the same location and having the same conditioning and handling. Broodstock should be collected from waters free of point and non-point source pollution to minimize the possibility of physiological adaptation to toxicants. Mysids can be transported for a short time (< 3 hours) in lidded 12 L plastic buckets. The buckets should be gently aerated to reduce transport mortality; we use a battery powered portable air pump.

* There are questions concerning the taxonomy of *Holmesimysis*/*Acanthomysis* species. We are considering Holmquists (1981) interpretation to be definitive and are considering previous references to *A. sculpta* in California to be synonymous with *H. costata* (see also Mauchline, 1980).

For longer transport times the mysids can be shipped in sea water in sealed plastic bags. The following transport procedure has proven to be successful: 1) fill the plastic bag with 1 L of sea water, 2) saturate the seawater by bubbling in pure oxygen, 3) place 25-30 (adult) mysids in each bag, 4) top off the water to eliminate air space, 5) seal the bag securely then place it in an ice chest. The ice chest should be cooled to approximately 15 °C with Blue Ice (a range of 12 -17 °C is tolerable). Use a layer of newspapers to insulate the plastic bags from the ice.

Specimens should be positively identified prior to testing; for a review of the taxonomy of this genus consult Holmquist (1979, 1981).

2.3 Broodstock Culturing

After collection the mysids should be transported quickly to the laboratory and placed in aquaria or tanks equipped with flowing seawater at a flow rate of approximately 1 L per minute. The density of animals depends upon the size of the tank; no more than approximately 20 animals per liter of water is recommended for extended lengths of time. The water temperature should be held below 17 °C.

We culture mysids in 3 types of containers at MPSL: an 800 L circular tank, a 20 L aquarium, and a 25 cm diameter screen-tube in a 5 L bucket. Newly caught adult mysids are held in the 800 L circular tank. This tank is equipped with flowing seawater, and serves as the broodstock culture tank. The brooding females produce the juveniles that are used in the test protocol. Brooding female mysids can be easily identified by their large, extended marsupia filled with (visible) eyed juveniles. The marsupia of females that are close to hatching are grey in color. For each toxicity test 350 juveniles are needed; approximately 60 brooding adults should be isolated to provide enough juveniles for one test. The gravid female mysids are transferred to a 20 liter aquarium when juveniles are needed for a test. This "hatching" aquarium has a removable, 240 µm mesh cradle. The adults are placed within the cradle so that as the juveniles hatch, they swim through the mesh into the bottom of the aquarium. This provides the juveniles a refuge from the adults, which can be cannibalistic. The cradle and gravid females are removed and placed into a separate aquarium, leaving the juveniles behind, and these can be siphoned into a separate container. We generally hold the juveniles in a 25 cm diameter, 140 µm mesh screen- tube. The screen-tube is suspended in a 5 liter bucket so that seawater (0.5 l / minute) flows into the top and out through the bottom of the tube, then over the side of the bucket. The height of the bucket determines the level of water in the screen tube. Juveniles can be held indefinitely in this type of container provided adequate food is supplied. All of the culture containers described above are fitted with drip feeders that supply newly hatched *Artemia* nauplii to the mysids (see below).

2.3.1 Static Culturing

All of the containers described above can be used as static culture systems provided adequate aeration is supplied and the water is changed periodically. At least 50 % of the water in the 800 L tank should be replaced once

each week. All of the water in the 20 L aquarium should be replaced every 3 days, and every 2 days in the screen-tube / 5 L bucket.

2.4 Isolation of Test Animals

Hatchings generally occur at night, so the hatching aquarium should be checked each morning for the presence of newly hatched juvenile mysids. This aquarium should be kept free of incidental hatches (less than 350 juveniles), until a sufficiently large enough hatch (greater than 350 juveniles) occurs. This will insure that all of the juveniles used in the test are the same age. The hatching aquarium is siphoned to remove incidental hatches at the end of the day, and checked the next morning for juveniles. When at least 350 juveniles are present, they can be slow-siphoned into the screen tube arrangement described above. This allows the juvenile mysids to be held in a relatively clean environment, and makes it easy to consolidate them into a smaller container when it is time to deliver them to the test chambers. This protocol requires the use of three day old juvenile mysids, thus the newly hatched mysids are held in the screen tube for three days.

2.5 Feeding

All broodstock mysids should be fed a diet of newly hatched *Artemia* nauplii in excess (approximately 1000 nauplii per liter of culture water). Because newly-hatched mysids are not affective predators, we supplement their diet with flake food for the first 2 days after hatching (e.g.. Tetramin™, or any pet store flake fish food with > 5% lipid content). A small pinch of finely ground flake food twice a day is sufficient for young juveniles. Gentle aeration in the culture chambers keep the food suspended which facilitates feeding. It is possible to increase the nutritive value of the nauplii by pretreating the *Artemia* cultures with unialgal food, for example *Isochrysis galbana* (reference).

The feeding rates in the test beakers should be closely controlled. A feeding rate of twenty nauplii per test animal every 24 hours is sufficient. To reduce the accumulation of debris in the test containers, test animals should be fed *Artemia* only; it is not necessary to aerate the test containers. The *Artemia* can be delivered to the test containers with a pipet. To avoid test water dilution, the food animals should be concentrated and delivered to test containers with a minimum volume of water (1 mL). The *Artemia* should be rinsed to remove excess waste products prior to pipetting them into the test containers.

3.0 Toxicity Test Procedure

3.1 Experimental Design

This protocol is based on the use of 5 effluent concentrations and 2 controls. Each of these is replicated 5 times, thus 35 test containers are needed for each test. Effluent concentrations are usually assigned in a logarithmic sequence as 0% (control), 0% b.c. (brine control), 0.10%, 0.18%, 0.32%, 0.56%, and 1.00% effluent; or, as an example of a wider range, 0% , 0% b.c., 0.10%, 0.32%, 1.00%, 3.2%, and 10% effluent. The range and number of concentrations is based on the toxicity of the effluent being tested, and the requirements of the agency responsible for monitoring effluent toxicity. A preliminary range finding test using concentrations from 0 to 100% effluent may be necessary when the toxicity of the target effluent is unknown. Because a hypersaline brine is used to adjust the salinity of the effluent dilutions, a brine control is needed in addition to a dilution water control (see section 3.3.2).

3.2 Test Containers

For tests using complex effluents or organic toxicants, use 350 mL borosilicate glass stacking dishes as the test containers. For tests using trace metal toxicants, use 350 mL polypropylene or polyethylene food storage containers.

3.3 Test Solutions

It is convenient to prepare the effluent dilutions for the 96-h mysid protocol before isolating the juvenile mysids for the test. The test solutions can then be equilibrating in the constant temperature room or water bath while the test animals are being isolated. Prepare test solutions by diluting the effluent with an approved dilution water using volumetric flasks and pipets. Mix test solutions by combining effluent, hypersaline brine (if necessary, see below), and dilution water in a 1 L volumetric flask. The solutions should be mixed from the lowest concentration (i.e.., control) to the highest concentration to avoid contamination. After the test containers have been filled, they should be covered with clear plastic or acrylic sheets to prevent contamination and evaporation.

3.3.1 Dilution Seawater

Unless the actual receiving water is specified as the dilution water, obtain dilution water from clean reference areas that are not contaminated by toxic substances. The minimum requirement for acceptable dilution seawater is that the test organisms survive, grow, and reproduce normally in it. Filter the dilution water to exclude particles greater than 1.0 µm in diameter, unless the effects of dilution water particulates are being specifically addressed in the study. The dilution water salinity should be 34 ppt ± 2 $^{o}/_{oo}$.

3.3.2 Salinity Adjustment

Sewage effluents generally have a salinity of 0-2 $^{o}/_{oo}$. In order to conduct toxicity tests with marine organisms it is necessary to adjust the salinity of the effluent dilutions to 34 $^{o}/_{oo}$ with a hypersaline brine. We recommend using a mixture of synthetic sea salts and seawater to make the brine. We are currently evaluating brands of sea salt to find the most suitable for making hypersaline brines. It is important to use synthetic salts that do not contain chelators and are free of metal contaminants. Two brands that meet these criteria are Wimex™ and 40 Fathoms™. To make brine we dissolve 35 grams of synthetic seasalt in 1 liter of clean, filtered seawater. This mixture results in a brine with a final salinity of approximately 68-70 ppt, and is sufficient for adjusting the salinity of most effluents assuming the highest effluent concentration tested is no greater than approximately 50 %. The pH of the brine should be 7.9, but we have found that brines mixed with synthetic salts are often slightly alkaline. If this occurs, the pH of the brine should be adjusted with a 5 % solution of 6N HCl prior to using the brine for salinity adjustments. As with any titration, the brine solution should be adjusted by adding the dilute acid 1 drop at a time while frequently monitoring the pH . In the event the brine is acidic, use NaOH to adjust the pH.

To calculate the amount of brine to add to each test solution, determine the salinity of the brine (SB, in ppt), the salinity of the effluent (SE, in ppt), and the 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:

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

Use brine controls in all tests where brine is used. Make the brine control solutions by adding as much brine as is used in the highest effluent concentration (usually 10%). Add distilled water to adjust the salinity to 34 ppt, then fill the remainder of the mixing flask with dilution water. To determine the amount of distilled water to add, use the above equation, setting SE equal to zero, and solving for VE. Mix the brine control solutions thoroughly.

3.3.3 Example Test Dilution Calculation

An example of a typical dilution calculation is given in the Effluent Dilution Sheet at the end of this protocol.

3.4 Randomization

3.4.1 Test Containers

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 35 (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 test organisms have been examined at the end of the test. Arrange the test containers randomly in the water bath or controlled temperature room.

3.4.2 Test Animals

We have found that juvenile *Holmesimysis* less than 3 days old have poor survival (Martin *et al.* In Press). We therefore recommended that 3 day old juvenile mysids be used for this protocol. Although not always possible, it is convenient to isolate a cohort of newly hatched juveniles on Friday of the week before a given test so that a test can be started on Monday with 3 day old animals. This allows time to conduct the 96-hour test during the work week.

The juvenile mysids should be randomized before placing them into the test containers. We do this by first transferring them from the screen-tube culture container (described in section 2.3) into a 1 liter beaker. From the beaker we pipet them 2 at a time into 35 randomization cups using a 10 ml, wide-bore pipet. When each of the 35 cups contains 2 juveniles, the process is repeated, by adding 2 mysids at a time until each cup contains 10 animals. The juveniles are then poured into the test containers. Decant off the excess water in the cups before pouring the animals into the test containers; no more than 5 mls should be added with the juveniles. Also, the mysids are easily trapped in water drops, so care should be taken to make sure all 10 animals are transferred into each test container. We verify our counts after delivering the mysids into the test containers.

3.5 Physical/Chemical Measurements of Test Solutions

Measure the temperature, salinity, pH, dissolved oxygen, and ammonia concentration in one randomly selected replicate of each test concentration at the beginning and end of the experiment. Samples at the beginning of the test can be taken directly from the volumetric mixing flask. Samples at the end of the test can be taken directly from the test containers and should be taken and analyzed before reading the test results. Be careful not to disturb the mysids in the test containers. Measure temperature using a thermometer accurate to at least 1 °C. Measure salinity with a refractometer accurate to 1 ppt. Measure oxygen in mg/L using an oxygen probe accurate to 0.5 mg/L. Use a pH probe accurate to 0.1 pH units. Determine ammonia concentration to the nearest 0.1 mg/L.

3.6 Endpoint Determination

The endpoint for the 96 hour mysid toxicity test is animal death. Because the juvenile mysids are often immobile on the bottom of the test container, it is difficult to determine whether or not they are dead. Death is defined here as the lack of response when gently probed. If an individual lying on the bottom moves even slightly when touched with a probe, it is considered to be alive; if it does not respond, it is considered to be dead. The dead mysids are removed after each counting to prevent fouling of the test containers. Care should be taken when removing the dead mysids to insure that cross contamination of the test containers does not occur. A separate disposable pipet should be used to remove mysids from each container. The number of live mysids are counted at 96 hours.

A sublethal endpoint based on growth is being evaluated for this protocol. Until this endpoint is developed, mortality will be used as an indication of toxic response.

4.0 Data Analysis

Add the number of dead and live mysids at 96 hours to get the total number of mysids counted for each replicate. Calculate the number of living mysids as a percentage of this total for each replicate. Transform the percentage data to the arcsine of their square root. 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 a Dunnett's multiple comparison test to compare each concentration against the control (Zar, 1974; Sokal and Rohlf, 1969). Derive the No Observed Effect Level (NOEL) as the highest concentration that is not significantly different from the control. Use an alpha level of $p = .05$ to determine statistical significance.

5.0 Suggested Criteria for Acceptability

The following are the biological and physical/chemical criteria for a successful 96-hour toxicity test using juvenile *Holmesimysis*. At least 80% of the control mysids must survive. The temperature of the test solutions must be less than 17 °C. The salinity of the test solutions must be 34 ± 2 °/oo. The dissolved oxygen in the test solutions must be greater than 60% saturation. The pH of the test solutions must be 7.9 ± 0.3. If any of these criteria are not met, the test should be considered invalid, and be repeated.

6.0 Cleaning Procedure

Test Containers (glass): All glass test chambers used in organics and complex effluent bioassays should be cleaned as follows: 1) rinse 3 times with hot tap water, 2) rinse 3 times with acetone, 3) rinse 3 times with deionized water, 4) soak 24 hours in 3N HCL, 5) rinse 3 times with deionized water, 6) soak 24 hours in deionized water, 7) rinse 3 times in deionized water, 7) dry in clean oven.

Test Containers (plastic): All plastic test chambers used in metals bioassays should be cleaned as follows: 1) rinse 3 times with deionized water, 2) soak for 24 hours in a mild detergent, 3) rinse 3 times with deionized water, 4) soak for 24 hours in 3N HCL, 5) rinse 3 times with deionized water, 6) soak 24 hours in 3N HNO_3, 7) rinse 3 times with deionized water, 8) soak 24 hours in deionized water, 9) dry in a clean oven.

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8.0 96-h Juvenile Mysid Toxicity Testing Protocol Summary

1. Isolate approximately 60 gravid female mysids into a static sea water hatching aquarium (15 °C).

2. Siphon clean the hatching aquarium to remove incidental hatches. When a significant (>350) hatch occurs, isolate the newly hatched individuals into their own container.

3. Maintain the juveniles for 3 days on finely ground flake fish food (> 5% lipid) and *Artemia* nauplii.

4. Fill 35 test containers with 200 mL of test solution and arrange them randomly in a constant temperature room or water bath at 15 °C. The solutions tested should include 5 effluent concentrations and 2 controls, one for dilution water and one for the brine, all replicated 5 times. Have a coded number on each replicate test container that corresponds to the correct replicate number and concentration.

5. Randomize the 3 day old juvenile mysids into 35 randomization cups. Place 2 mysids at a time into each of the 35 cups until each cup has exactly 10 juvenile mysids in it (e.g.. 2 animals in cup 1, then 2 animals in cup 2 and so on until each of the 35 cups has 2 animals, then start the whole process again and proceed until all have 10 animals in them). Use the minimum amount of water for this process.

6. After all cups have exactly 10 mysids in them, pour the mysids into the test containers. Make sure no mysids are left in the randomization cups. Count the number of mysids in each test container to verify that each has 10 juveniles.

7. Feed the test mysids 24-hour post hatch *Artemia* nauplii (20 nauplii/mysid/day).

8. Remove all dead mysids daily and record.

9. At 96 hours count the number of live mysids in each container and record. Use the code, after counting, to get the correct concentration for each count (see #4).

10. Calculate the percentage of living and dead mysids for each replicate, transform this percentage value to arcsine of the square root, and conduct an analysis of variance (ANOVA) to discern differences between concentrations. Compare each concentration to the control using a Dunnett's multiple comparison test. Determine the NOEC value as the highest concentration that is not significantly different from the control ($p < 0.05$).

Effluent Dilution Sheet

100% Effluent is the Stock Solution			Corresponding Beaker Numbers
→ 0.0 ml in 1000 ml flask	→	Control	3, 7, 12, 27, 28
→ 0.0 ml in 1000 ml flask	→	Brine Control	33, 18, 40, 34, 22
→ 5.6 ml in 1000 ml flask	→	0.56%	35, 17, 2, 8, 13
→ 10.0 ml in 1000 ml flask	→	1.0%	36, 23, 24, 29, 37
→ 18.0 ml in 1000 ml flask	→	1.8%	25, 30, 31, 38, 4
→ 32.0 ml in 1000 ml flask	→	3.2%	9, 14, 19, 26, 39
→ 56.0 ml in 1000 ml flask	→	5.6%	32, 20, 15, 28, 10
→ 100.0 ml in 1000 ml flask	→	10.0%	11, 16, 1, 21, 6

Salinity Adjustment Using Hypersaline Brine

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

Quantities to be measured : SB = Salinity of the Brine (°/oo)
SE = Salinity of the Effluent (°/oo)

SB = 74 °/oo SE = 2 °/oo

Quantities to be calculated: VB = Volume of the Brine to be added (ml)

Brine Salinity should be 60 to 80 °/oo

Note: Always adjust the pH of the brine solution to that of the dilution water.

The equation to calculate the volume of brine to add for each concentration is: $VB = VE\left(\frac{34 - SE}{SB - 34}\right)$

For the test effluent and the test brine calculate: $\frac{34 - SE}{SB - 34} = 0.80$

Then multiply this number by the volume of effluent for each test concentration.

*Note: Distilled water is used instead of effluent in brine controls.

Concentration	VE		VB	Final Test Solution Mixture
Control	0.0 ml x (.8) =		0.0 ml Brine	+ 0.0 ml Effluent in a 1000 ml flask; fill with seawater
Brine Control*	100.0 ml x (.8) =		80.0 ml Brine	+ 100.0 ml Dist. Water* in 1000 ml flask; fill with seawater
0.56%	5.6 ml x (.8) =		4.5 ml Brine	+ 5.6 ml Effluent in a 1000 ml flask; fill with seawater
1.0%	10.0 ml x (.8) =		8.0 ml Brine	+ 10.0 ml Effluent in a 1000 ml flask; fill with seawater
1.8%	18.0 ml x (.8) =		14.4 ml Brine	+ 18.0 ml Effluent in a 1000 ml flask; fill with seawater
3.2%	32.0 ml x (.8) =		25.6 ml Brine	+ 32.0 ml Effluent in a 1000 ml flask; fill with seawater
5.6%	56.0 ml x (.8) =		44.8 ml Brine	+ 56.0 ml Effluent in a 1000 ml flask; fill with seawater
10.0%	100.0 ml x (.8) =		80.0 ml Brine	+ 100.0 ml Effluent in a 1000 ml flask; fill with seawater

Date 1/1/89 Test Organism White Rhino Investigator's initials JH

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
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%

Salinity Adjustment Using Hypersaline Brine

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

Quantities to be measured : SB = Salinity of the Brine ($^{o}/_{oo}$)
SE = Salinity of the Effluent ($^{o}/_{oo}$)

SB = _____ $^{o}/_{oo}$ SE = _____ $^{o}/_{oo}$

Quantities to be calculated: VB = Volume of the Brine to be added (ml)

Brine Salinity should be 60 to 80 $^{o}/_{oo}$

Note: Always adjust the pH of the brine solution to that of the dilution water.

The equation to calculate the volume of brine to add for each concentration is: $VB = VE\left(\frac{34 - SE}{SB - 34}\right)$

For the test effluent and the test brine calculate: $\frac{34 - SE}{SB - 34} =$ _____

Then multiply this number by the volume of effluent for each test concentration.

*Note: Distilled water is used instead of effluent in brine controls.

Concentration	VE		VB	Final Test Solution Mixture
Control	0.0 ml	x ( ) =	_____ ml Brine	+ 0.0 ml Effluent in a 1000 ml flask; fill with seawater
Brine Control*	100.0 ml	x ( ) =	_____ ml Brine	+ 100.0 ml Dist. Water* in 1000 ml flask; fill with seawater
0.56%	5.6 ml	x ( ) =	_____ ml Brine	+ 5.6 ml Effluent in a 1000 ml flask; fill with seawater
1.0%	10.0 ml	x ( ) =	_____ ml Brine	+ 10.0 ml Effluent in a 1000 ml flask; fill with seawater
1.8%	18.0 ml	x ( ) =	_____ ml Brine	+ 18.0 ml Effluent in a 1000 ml flask; fill with seawater
3.2%	32.0 ml	x ( ) =	_____ ml Brine	+ 32.0 ml Effluent in a 1000 ml flask; fill with seawater
5.6%	56.0 ml	x ( ) =	_____ ml Brine	+ 56.0 ml Effluent in a 1000 ml flask; fill with seawater
10.0%	100.0 ml	x ( ) =	_____ ml Brine	+ 100.0 ml Effluent in a 1000 ml flask; fill with seawater

Date _____ Test Organism _____ Investigator's initials _____

Mysid Toxicity Test Data Sheet

T art 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:

est ontainer umber	Nominal Concentration (µg l^{-1} or %)	Number Dead Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Total Number Dead	Total No. of Mysids at Start	Percent Dead	Arcsin $\sqrt{x}$ Percent Dead	Mean ± s.d. Percent Dead for each Conc

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

RED ABALONE TOXICITY TEST PROTOCOL

ABALONE LARVAL DEVELOPMENT
SHORT TERM TOXICITY TEST PROTOCOL

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This 48 hour toxicity test uses early development of abalone larvae to test wastewater toxicity. Abalone develop from fertilized eggs into veliger larvae in the test solution, and are then examined microscopically. Abnormal larval shell development is the indicator of toxic effect.

1.0 Necessary Equipment

constant temperature room

or constant temperature water bath (15 ± 1°C)

ultraviolet water sterilization unit (4 to 5 foot UV bulb) for UV spawning method

or hydrogen peroxide (reagent grade H_2O_2, 30%, refrigerated)

and Tris biological buffer [Tris (hydroxymethyl) aminomethane] for H_2O_2 spawning method

compound light microscope (100x)[a]

or inverted microscope (100x)[b]

meter and probes for dissolved oxygen, pH, and ammonia

salinity refractometer

thermometer

analytical balance

compressed oxygen, polyfoam sponges, large plastic bags, blue ice, ice chest (for transporting abalone spawners)

2 or more darkened aquaria for broodstock

supply of *Macrocystis* or other macroalgae (if broodstock are to be held at the lab longer than 5 days)

stainless steel butter knife, with a rounded smooth-edged blade (for handling adult abalone)

flowing 20 µm-filtered seawater (2 L per minute, for maintaining broodstock prior to testing; if flowing seawater is not available, recirculating aquaria equipped with aeration, temperature control, and oyster shell and activated charcoal filters can be used)

60 L of 1 µm-filtered UV-sterilized seawater (for spawning)

15 L polyethylene buckets (3)

1000 ml beaker (tall form)

perforated plunger (for stirring eggs in the 1000 ml beaker, see Section 2.6)

1.0 Necessary Equipment (continued)

1 L volumetric flasks (2)

volumetric pipettes: 1 ml, 5 ml, 10 ml, 25 ml, 50 ml, 100 ml (1 each)

graduated pipettes: 1 ml, 10 ml (1 each)

wide-bore pipettes: 1 ml, 10 ml (1 each)

10 L of 1 µm-filtered seawater (for dilution)

10 L polyethylene water bottle

250 ml beakers (borosilicate glass for effluents and organics, polypropylene for metals, 35 per test)[a]

or 250 ml (or 70 ml) tissue culture flasks[b]

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]

perforated plunger (for stirring eggs in the 1000 ml beaker, see Section 2.6)

Sedgewick-Rafter counting cell microscope slide[a]

hand counters (2)

buffered formalin [formaldehyde 37% (1 L), sodium borate (3 g), and glycerin (50 ml)]

200 ml per test[a]

or 300 to 1050 ml per test (depending on tissue culture flask size)[b]

reagent grade acetone (1 L per test)[a]

3N hydrochloric acid (15 L per test; can be reused 3 times)[a]

data sheets

[a,b] Two techniques can be used to incubate and analyze larvae in this toxicity test. The first technique has been used in the experiments described in this report and in all previous work by the Marine Bioassay Project. In this first technique, abalone are exposed to test solutions in open 250 ml beakers. Larvae are then screened, concentrated into vials, and analyzed in Sedgewick Rafter slides under a standard compound microscope. Any equipment used only in this technique is marked with a bold superscript [a]. An alternative technique is based on the use of an inverted microscope to analyze larval samples. Larvae are exposed to toxicants and analyzed in closed disposable tissue culture flasks. This modification is designed to make the test quicker and more cost effective, while reducing opportunities for contamination and volatilization of toxicant solutions. Equipment used only in this alternative technique is marked with a bold superscript[b]. Where appropriate, instructions for conducting both techniques are included in the following text. It is important to note that the inverted microscope technique is in development and has not been fully tested.

2.0 Test Organism

2.1 Species Identification

The species used in this test is *Haliotis rufescens*, the red abalone. The red abalone is recommended for use in California because it is naturally distributed along the entire California coast (including areas impacted by effluents), it is important economically, and it is amenable to laboratory culture.

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).

2.2 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. 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.

2.3 Broodstock Transport

Abalone broodstock can transported for short time periods from the field or supply facility in clean covered plastic buckets filled with seawater. Supply aeration using small battery powered aerators, and maintain water temperatures between 8° and 18° C. Four abalone in a 15 L bucket should remain healthy for up to 4 hours under these conditions.

Abalone can be transported up to 30 hours in sealed, oxygen-filled plastic bags containing moist (seawater) polyfoam sponges. Wrap 2 small blue ice blocks in sections of newspaper (about 15 pages thick) for insulation, and place the wrapped blue ice in the bottom of a small (10 - 15 L) ice chest. Cut the polyfoam into sections (about 20 x 40 cm), and allow them to soak in seawater for 24 hours. Rinse the sponges in fresh seawater and wring them out well so they are just moist. 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 in the ice chest on the wrapped blue ice. Fill the bags with pure oxygen, squeeze the bags to purge all the air, then refill with oxygen.

Inflate the bags until they fill the ice chest, seal the bags (air-tight) and close the chest. Avoid transporting the ice chest in temperatures below freezing or above 80° F.

2.4 Broodstock Conditioning

At the testing facility, place the abalone in aerated aquaria with flowing seawater (1-2 L/min). Ideal maintenance temperature is 15° C. 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, Pelagophycus*) or any fleshy red algae can be substituted. Keep a tight fitting lid on the aquaria to prevent escape and desiccation. Empty and rinse aquaria twice weekly to prevent build-up of detritus. Remove any dead abalone immediately, and drain and scrub their aquarium.

Broodstock can be held in recirculating aquaria if necessary. Supply constant aeration and temperature control. Add only a few blades of algal food at each cleaning to prevent its accumulation and decay. Use oyster shell and charcoal filters, and monitor the ammonia or nitrite-N content of recirculating seawater to check that filters are removing metabolites.

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. Ripe spawners have a distinct color difference between the gray digestive gland and the green or cream-colored gonad. Less developed gonads appear grayer (in females) or brown (in spent males). Large (20 cm) abalone ripen at least once a year and provide up to 10 million viable eggs per spawning. Smaller abalone (7 to 10 cm) can be spawned three or four times annually, producing 100,000 to 1,000,000 eggs per spawning. For further information on red abalone culture, see Ebert and Houk (1984).

2.5 Spawning Induction

Ripe abalone can be induced to spawn by stimulating the synthesis of prostoglandin-endoperoxide in the reproductive tissues. 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).

2.5.1 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 L/min) 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 L) 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 L 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 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 hydrogen peroxide 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 and 5 mM H_2O_2. Allow the abalone to remain in contact with the chemicals for 2.5 hours at 15° C. Maintain constant aeration.

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 3 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.

2.5.2 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 L/min) 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 L) 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/min) 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.

2.6 Fertilization

As the females spawn, allow the eggs to settle to the bottom. 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. Do not allow eggs to gather into clumps in either bucket; each egg should be individually distinguishable, and not touching other eggs. 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. Leave some eggs in the spawning bucket. If they are too densely settled, siphon them to another bucket and keep them in reserve.

As the males spawn, siphon sperm from directly above the respiratory pore and collect this in a 500 ml flask. Keep the flask at 15°C, and use it as a back-up in case the males stop spawning. Usually the males will continue to spawn, turning the water in the bucket milky white. As long as the males continue spawning, partially drain and refill the bucket every so often to replace old sperm-laden water with fresh seawater (15°C).

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). 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 L/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 min). Fertilization is then complete. Carefully pour or siphon off the water from above the settled eggs to remove as much of the sperm laden water as possible without losing substantial numbers of eggs. Slowly refill the bucket with 1 µm-filtered seawater (15°C). Allow the eggs to settle, decant again, and slowly add enough seawater to resuspend the eggs in water about 10 cm deep. When the eggs have settled, 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 eggs in the short amount of time it takes to fill the beaker. Examine a sample

of the eggs at 100x magnification. One to one hundred sperm should be visible attached to each egg. If sperm are so dense they appear fuzzy, the abalone will develop abnormally and cannot be used.

2.7 Estimation of Embryo Density

Evenly mix the eggs in the 1000 ml beaker by gentle vertical stirring with a clean perforated plunger. The plunger is a plastic disk with numerous holes, slightly smaller in diameter than the beaker. A plastic rod is fixed vertically to the center of the disk as a handle. Never allow eggs to settle densely in the bottom of the beaker, and take care not to crush the eggs while stirring. Take 5 samples of the evenly suspended eggs using a 1 ml wide bore graduated pipet. Hold the pipet up to the light and count the individual eggs using a hand counter. Discard the sampled eggs after counting. Take the mean of five samples to estimate the number of eggs per ml. The standard deviation for five counts should be no more than 10% of the mean. If it is, count five more samples and take the mean of the 10 samples to obtain a density estimate. Density of eggs in the beaker should be between 200 and 300 eggs/ml. Fewer eggs/ml will cause excessive dilution of the test solutions; more eggs/ml may degrade water quality in the beaker.

3.0 Toxicity Test Procedure

The steps described in the following section must be done concurrently with those described above. Test containers and solutions must be ready to accept the embryos as soon as they are fertilized. Culture work (spawning, etc.) and toxicant work should be done in separate laboratory rooms, and care should be taken to avoid contaminating the organisms prior to testing.

3.1 Experimental Design

This protocol is based on the use of 5 effluent concentrations, a dilution water control, and a brine control. Each of these is replicated 5 times, so there are 35 test containers needed for each test. Effluent concentrations are usually assigned in a logarithmic sequence as 0% (control), 0% (brine control) 0.56%, 1.0% 1.8%, 3.2%, 5.6%, and 10% effluent. Set the range and number of concentrations to be used by consulting the responsible monitoring agency. A preliminary range finding test using a wider range of concentrations may be necessary if nothing is known about the toxicity of the target effluent.

3.2 Test Containers

Two types of containers can be used, depending on whether a standard[a] or inverted[b] microscope is to be used to analyze the samples at the end of the test. For tests using a standard scope, 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. Tissue culture flasks[b] are used for all tests with an inverted microscope. The tissue culture flasks have not been thoroughly tested, and different sizes are being considered. Initially, 250 ml flasks are being tested. Smaller, 70 or 50 ml flasks have the advantage of requiring less formalin for sample preservation (see Section 3.6.3), but larval density and water quality factors need to be examined.

3.3 Test Solutions

Prepare test solutions by combining effluent, hypersaline brine (Section 3.3.2), and dilution water using volumetric flasks and pipets. Clean all glassware prior to use (see Section 4.0). Mix test solutions from the lowest concentration (control) to the highest concentration to avoid contamination.

3.3.1 Dilution Water

Obtain dilution water from clean reference areas that are not contaminated by toxic substances, unless the actual receiving water is specified as the dilution water. The source for dilution water should be consistent and specified for any toxicity testing program. The minimum requirement for acceptable dilution water is that the test organisms survive, grow, and reproduce normally in it. Filter the dilution water to exclude particles greater than 1 µm, unless the effects of dilution water particulates are being specifically addressed in the study. Dilution water salinity should be 34 ‰ ± 2 ‰ .

3.3.2 Salinity Adjustment

Adjust the salinity of effluent dilutions to 34 ‰ by adding hypersaline brine. Brines can be made in two ways: by evaporating natural seawater or by adding artificial sea salts to natural seawater.

Evaporation is a slow process and must be initiated at least one week in advance of the test date. Heat and aerate dilution water until about half has been evaporated away. Do not use temperatures in excess of 40° C, or concentrate the brine to salinities greater than 100 ‰ .

To make brines with artificial sea salts, add approximately 35 g of sea salt to each liter of natural seawater. Stir for at least one hour, let any precipitates settle, and decant the supernatent brine mixture. Make sea salt brines on the day of the test. Wimex Marine Mix® and Forty Fathoms® brand sea salts are recommended based on limited testing.

Check the pH of all brine mixtures and adjust to that of the dilution water by adding, dropwise, hydrochloric acid (2 N) or sodium hydroxide (2 N).

3.6.3 Sample Preservation

After the 48 hour exposure, the abalone larvae are fixed in formalin. Two methods for sample preservation are described.

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 with the broad side down for counting on the inverted microscope.

3.6.4 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 200 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.

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 200 larvae encountered in each flask under 100x magnification. Count the number of normal and abnormal larvae using hand counters.

3.6.5 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. Count larvae as abnormal if they have thin clear shells with multiple dents. Severe deformations of the larval shell are also counted as abnormal. Refer to the accompanying photographs (**Figure 1**) for classification of marginally deformed larvae.

Larvae that have arrested development at the two-cell stage or beyond are also counted as abnormal. One celled eggs are not counted because arrested development may have occurred before exposure. Some larvae are found remaining in the egg membrane as veligers after 48 hours. These are not counted. Shells separated from rest of the

animal are not counted, nor are larvae with broken shells, since these may have been the result of handling rather than toxicity.

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

4.0 Cleaning Procedure

4.1 Glass Test Containers

All glass test chambers used in organics and complex effluent bioassays should be cleaned as follows: 1) rinse 3 times with hot tap water, 2) rinse 3 times with new reagent grade acetone, 3) rinse 3 times with deionized water, 4) soak 24 hours in 3N HCL, 5) rinse 3 times with deionized water, 6) soak 24 hours in deionized water, 7) rinse 3 times with deionized water, 8) dry in a clean oven at 50°C.

4.2 Plastic Test Containers

All plastic test chambers used in metals bioassays should be cleaned as follows: 1) rinse 3 times with deionized water, 2) soak for 24 hours in a mild detergent (Alconox®), 3) rinse 3 times with deionized water, 4) soak for 24 hours in 3N HCL, 5) rinse 3 times with deionized water, 6) soak 24 hours in HNO_3, 7) rinse 3 times with deionized water, 8) soak 24 hours in deionized water; 9) rinse 3 times with deionized water, 10) dry in a clean oven at 50° C.

NOTE: All volumetric flasks and pipets used for diluting test solutions must be cleaned regardless of which type of test containers are used. In method [a] using a standard compound microscope and beakers, all reusable beakers must be washed as above. In method [b] using an inverted microscope and tissue culture flasks, the flasks are disposable and need not be washed, but can be recycled.

5.0 Data Analysis

Determine the percentage of normal larvae in each replicate container. Transform the percentage data to the arcsine of their square root. Check the original test container randomization sheet (see Section 3.4), 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 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.

6.0 References

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To calculate the amount of brine to add to each effluent dilution, determine the salinity of the brine (SB, in ‰), the salinity of the effluent (SE, in ‰), and the 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 ‰.

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, and solving for VE.

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

3.3.3 Example Test Solution

Two hundred milliliters of test solution are needed for each test container. Five replicates can be mixed in a 1 L volumetric flask. To make a test solution at a concentration of 1% effluent, add 10 ml of effluent to the 1 L volumetric flask using a volumetric pipet. Assuming an effluent salinity of 0 ‰ and a brine salinity of 68 ‰, add 10 ml of brine using another 10 ml volumetric pipet. Fill the volumetric flask to the 1 L mark with dilution water, stopper it, and shake to mix. Pour equal volumes into the 5 replicate containers.

3.4 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 35 (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. 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.

3.5 Physical/Chemical Measurements of Test Solutions

Measure the temperature, salinity, pH, dissolved oxygen, and ammonia concentration in one randomly chosen replicate of each test concentration at the beginning and end of the 48 hour test. Prior to testing, compile a list of containers (which are numbered randomly) for measurement, so that each day one container from each concentration is measured. Measure temperature using a thermometer accurate to at least 1° C. Measure salinity with a refractometer accurate to 1 ‰. Measure oxygen in mg/L using an oxygen probe accurate to at least 0.5 mg/L. Use a pH probe accurate to 0.1 pH units. Determine total ammonia concentration to the nearest 0.1 mg/L.

3.6 Exposure of Test Organisms

3.6.1 Delivery of Fertilized Eggs

Using the estimate of egg density in the 1000 ml beaker, calculate the volume of water that contains 1000 eggs. (This is the number used in 250 ml beakers[a] and 250 ml tissue culture flasks[b]. For smaller flasks use 4 eggs per milliliter of flask volume[b]). Remove 1000 eggs by drawing the appropriate volume of water from the well mixed beaker using a 10 ml wide bore pipet. Deliver the eggs into the test containers directly from the pipet making sure not to touch the pipet to the test solution. Stir the egg beaker with the plunger between taking aliquots. The temperature of the egg suspension must be within 1° C of the temperature of the test solution (As above, all solutions are kept at 15°C).

Eggs must be delivered into the test solutions within one hour of fertilization. Immediately after the eggs have been delivered, take a sample from the egg beaker and examine it under 100x magnification. All eggs should still be in the one-cell stage. Two-celled eggs are more easily damaged, and transferring the eggs at this stage can result in abnormally developed larvae.

3.6.2 Incubation

Incubate the test organisms for 48 hours in the test containers at 15° C under constant low lighting. Fertilized eggs become trochophore larvae, hatch, and develop into veliger larvae in the test solutions during the exposure period.

7.0 Abalone Toxicity Test Protocol Summary

1. Induce 4 male and 4 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.

2. Siphon eggs to 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.

3. Suspend the eggs evenly in a 1000 ml beaker and count 5 samples in a 1 ml pipet to estimate egg density.

4. Fill 35 test containers (5 effluent concentrations, 1 control, and 1 brine control, all replicated 5 times) with 200 ml of test solution and arrange them randomly in a constant temperature room or water bath at 15° C.

5. Pipet 1000 fertilized eggs into each test container. Incubate for 48 hours.

6. 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 and top off with dilution water.

7. 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.

8. Count the number of normal and abnormal larvae in each replicate container. Use larval shell development as the test endpoint.

9. Calculate the percentage of normal larvae for each replicate, transform this percentage value to the arcsine of the square root, and do an analysis of variance (ANOVA) to indicate differences between concentrations.

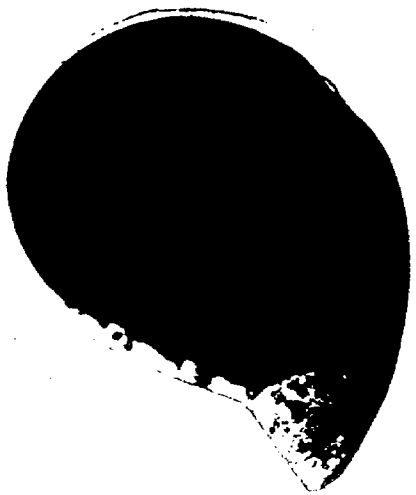
10. Compare each concentration to the control group using a Dunnett's multiple comparison test. Determine the NOEC value as the highest concentration that is not significantly different from the control.

[a] Techniques and equipment for use with a compound microscope and open beakers as test containers.

[b] Techniques and equipment for use with an inverted microscope and tissue culture flasks as test containers. (See Section 1.0)

A. Normal veliger. Note smooth striated shell.

B. Borderline normal veliger (larva withdrawn into shell). Shell striated with one small dent.



C. Borderline abnormal. Thin clear shell with multiple indentations.

D. Abnormal veliger. Severly deformed shell.

Effluent Dilution Sheet

100% Effluent is the Stock Solution			Corresponding Beaker Numbers
→ 0.0 ml in 1000 ml flask	→	Control	3, 7, 12, 27, 28
→ 0.0 ml in 1000 ml flask	→	Brine Control	33, 18, 40, 34, 22
→ 5.6 ml in 1000 ml flask	→	0.56%	35, 17, 2, 8, 13
→ 10.0 ml in 1000 ml flask	→	1.0%	36, 23, 24, 29, 37
→ 18.0 ml in 1000 ml flask	→	1.8%	25, 30, 31, 38, 4
→ 32.0 ml in 1000 ml flask	→	3.2%	9, 14, 19, 26, 39
→ 56.0 ml in 1000 ml flask	→	5.6%	32, 20, 15, 28, 10
→ 100.0 ml in 1000 ml flask	→	10.0%	11, 16, 1, 21, 6

Salinity Adjustment Using Hypersaline Brine

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

Quantities to be measured: SB = Salinity of the Brine (°/oo)
SE = Salinity of the Effluent (°/oo)

SB = 74 °/oo SE = 2 °/oo

Quantities to be calculated: VB = Volume of the Brine to be added (ml)

Brine Salinity should be 60 to 80 °/oo

Note: Always adjust the pH of the brine solution to that of the dilution water.

The equation to calculate the volume of brine to add for each concentration is: $VB = VE\left(\frac{34 - SE}{SB - 34}\right)$

For the test effluent and the test brine calculate: $\frac{34 - SE}{SB - 34} = 0.80$

Then multiply this number by the volume of effluent for each test concentration.

*Note: Distilled water is used instead of effluent in brine controls.

Concentration	VE		VB	Final Test Solution Mixture
Control	0.0 ml	x (.8) =	0.0 ml Brine	+ 0.0 ml Effluent in a 1000 ml flask; fill with seawater
Brine Control*	100.0 ml	x (.8) =	80.0 ml Brine	+ 100.0 ml Dist. Water* in 1000 ml flask; fill with seawater
0.56%	5.6 ml	x (.8) =	4.5 ml Brine	+ 5.6 ml Effluent in a 1000 ml flask; fill with seawater
1.0%	10.0 ml	x (.8) =	8.0 ml Brine	+ 10.0 ml Effluent in a 1000 ml flask; fill with seawater
1.8%	18.0 ml	x (.8) =	14.4 ml Brine	+ 18.0 ml Effluent in a 1000 ml flask; fill with seawater
3.2%	32.0 ml	x (.8) =	25.6 ml Brine	+ 32.0 ml Effluent in a 1000 ml flask; fill with seawater
5.6%	56.0 ml	x (.8) =	44.8 ml Brine	+ 56.0 ml Effluent in a 1000 ml flask; fill with seawater
10.0%	100.0 ml	x (.8) =	80.0 ml Brine	+ 100.0 ml Effluent in a 1000 ml flask; fill with seawater

Date 1/1/89 Test Organism White Rhino Investigator's initials JH

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
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%

Salinity Adjustment Using Hypersaline Brine

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

Quantities to be measured : SB = Salinity of the Brine ($^{o}/_{oo}$)
SE = Salinity of the Effluent ($^{o}/_{oo}$)

SB = _____ $^{o}/_{oo}$ SE = _____ $^{o}/_{oo}$

Quantities to be calculated: VB = Volume of the Brine to be added (ml)

Brine Salinity should be 60 to 80 $^{o}/_{oo}$

Note: Always adjust the pH of the brine solution to that of the dilution water.

The equation to calculate the volume of brine to add for each concentration is: $VB = VE\left(\frac{34 - SE}{SB - 34}\right)$

For the test effluent and the test brine calculate: $\frac{34 - SE}{SB - 34}$ = _____

Then multiply this number by the volume of effluent for each test concentration.

*Note: Distilled water is used instead of effluent in brine controls.

Concentration	VE		VB	Final Test Solution Mixture
Control	0.0 ml	x ( ) =	_____ ml Brine	+ 0.0 ml Effluent in a 1000 ml flask; fill with seawater
Brine Control*	100.0 ml	x ( ) =	_____ ml Brine	+ 100.0 ml Dist. Water* in 1000 ml flask; fill with seawater
0.56%	5.6 ml	x ( ) =	_____ ml Brine	+ 5.6 ml Effluent in a 1000 ml flask; fill with seawater
1.0%	10.0 ml	x ( ) =	_____ ml Brine	+ 10.0 ml Effluent in a 1000 ml flask; fill with seawater
1.8%	18.0 ml	x ( ) =	_____ ml Brine	+ 18.0 ml Effluent in a 1000 ml flask; fill with seawater
3.2%	32.0 ml	x ( ) =	_____ ml Brine	+ 32.0 ml Effluent in a 1000 ml flask; fill with seawater
5.6%	56.0 ml	x ( ) =	_____ ml Brine	+ 56.0 ml Effluent in a 1000 ml flask; fill with seawater
10.0%	100.0 ml	x ( ) =	_____ ml Brine	+ 100.0 ml Effluent in a 1000 ml flask; fill with seawater

Date _____ Test Organism _____ Investigator's initials _____

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 $\mu g\ l^{-1}$	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

THE MARINE BIOASSAY PROJECT

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