MARINE BIOASSAY PROJECT

THIRD REPORT

PROTOCOL DEVELOPMENT:

REFERENCE TOXICANT AND INITIAL COMPLEX EFFLUENT TESTING

DIVISION OF WATER QUALITY REPORT NO. 88-7

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April 1988

STATE WATER RESOURCES CONTROL BOARD

ACKNOWLEDGEMENTS

We would like to express our thanks to the members of our Scientific Review Committee: Howard Bailey, UC Davis; Steven Bay, Southern California Coastal Water Research Project (SCCWRP); Tom Dean, UC Santa Barbara, Kelp Ecology Project; and Phil Oshida, U.S. EPA Region 9, San Francisco. Their technical advice and overall guidance is greatly appreciated. Dan Otis, Craig Wilson, and the State Water Resources Control Board staff contributed to the planning and review process, and we appreciate their participation in the project. We gratefully acknowledge Earl Elbert of the California Department of Fish and Game Marine Culture Laboratory for sharing his experience with abalone and for contributing broodstock, Jo Ellen Hose of Vantuna Research Group for assisting with the kelp bass bioassays, Don Kent of Hubbs Research for providing sea bass larvae, and Michael Newshall of Newshall Mariculture for providing cultures of vegetative Macrocystis gametophytes. We would also like to thank Gary Chapman, U.S. EPA Newport, for contributing data from cooperative secondary effluent tests. Interlaboratory tests at SCCWRP were performed by Steven Bay and Darrin Greenstein, and Don Smith of the California Mussel Watch program performed the chemical analysis of reference toxicant test solutions; their cooperation is appreciated.

This report was prepared through agreement with the California State Water Resources Control Board (Contract No. 5-222-250, California Department of Fish and Game, and Subcontract C-1728 between California Department of Fish and Game and University of California, Santa Cruz).

Partial funding for phase 2 of this study has been provided by the California State Water Resources Control Board using Section 205(j) grant funds made available by the United States Environmental Protection Agency.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

SUMMARY AND OVERVIEW OF THE MARINE BIOASSAY PROJECT

The goal of the Marine Bioassay Project, authorized by the State Water Resources Control Board in 1985, is to protect California's ocean resources by determining the impacts of toxic waste discharges on marine waters. The Project's primary objective is development of sensitive short-term bioassay protocols for use in performing toxicity tests on these discharges. Most bioassay tests currently used are relatively insensitive because they measure lethal effects on adult organisms. In colloquial terms, those are referred to as the "Kill them and count them" variety of bioassay test. In contrast, a newer generation of tests is being developed by the Marine Bioassay Project and other groups. These are designed to estimate more subtle, chronic (long-term) effects of waste discharges. The new tests generally make use of sensitive, early life stages of aquatic organisms and measure sub-lethal effects such as abnormal development or reproductive failure. In addition, because the tests will measure discharges to ocean waters, the bioassay protocols being developed by the Marine Bioassay Project have emphasized the use of marine species native to California.

Regulatory Framework:

The development of these bioassay protocols to measure the long-term effects of waste discharges is consistent with both federal and state requirements. In 1984, the United States Environmental Protection Agency (EPA) issued a national "Policy for the Development of Water Quality-based Limitations for Toxic Pollutants" (49 CFR, No.48, March 9, 1984). This policy outlined a technical approach for controlling discharge of toxic substances through the federal system of discharge permits. In addition to meeting numerical standards for individual chemicals, the policy requires EPA and the States to use biological testing to complement chemical testing. Biological testing is especially useful for assessing the toxicity of complex discharges where it may be virtually impossible to characterize toxicity solely by chemical analysis. Biological testing also provides information not available from chemical testing. For example, it incorporates measurement of 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 bioassay protocols by January 1, 1990. Ocean dischargers of over 100 million gallons per day will be required in their permits to use these bioassay protocols for monitoring complex effluents by January 1, 1991. The same requirement will be applied to smaller dischargers effective January 1, 1992. Section 3 of AB 3500 expressed legislative intent that the organisms used in testing be representative marine species:

> "If the State Water Resources Control Board determines through its Marine Bioassay Project that a multispecies toxicity testing program with representative marine species for monitoring complex ocean effluent discharges is appropriate, the 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 the hearings and identified seven as being high priority

for Ocean Plan review. Refinement of bioassay protocols and implementation of their use was one of the high priority issues.

Implementation of these marine bioassay techniques will be used in regulatory programs of the State Water Resources Control Board and six coastal Regional Water Quality Control Boards. For example, ocean discharge permits may incorporate biological and chemical "no observable effect concentrations" (NOEC's) that are derived from tests on sensitive life stages of native marine species. Thus a given discharger might have a requirement that a one percent dilution of effluent show no observable effect in the bioassay 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:

$$TU = \frac{100}{NOEC}$$

The NOEC is defined as the maximum percent effluent, or any water being tested, that does not result in any observable 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.

$$TU = \frac{100}{1} = 100$$

Phase One (Previous Work):

The Marine Bioassay Project has been designed as a four phase program to develop and implement use of short-term bioassay tests for toxicity testing of complex effluents discharged to the ocean. Actual laboratory work has been conducted at the California Department of Fish and Game's (DFG) Marine Pollution Laboratory located south of Monterey.

The first two reports issued by the Marine Bioassay Project described the Project's first phase and provided recommendations for continuing phases. Phase I involved three major preparatory tasks:

1. Extensive refurbishment of a Department of Fish and Game laboratory for animal culture and rearing of marine species, and development of methods for maintaining and spawning selected species.

2. Purchase and initial use of mobile laboratory specifically designed to conduct aquatic toxicity bioassays.

3. Development of range-finding and definitive acute bioassays on three indigenous marine species. Performing and validating acute tests is a preliminary, but necessary, step in development of short-term tests to estimate chronic toxicity.

Phase Two (Current Work):

This report, the third in a series, describes work performed during the period from March 1986 to October 1987. Three new short-term bioassay protocols were developed after repeated testing using zinc as a reference toxicant. 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 (= Acanthomysis) costata, and the giant kelp (Macrocystis pyrifera). After some refinement, preliminary testing with the three protocols was performed on two representative complex effluents, a primary and a secondary municipal wastewater. In addition, longer term reference toxicant tests were used with each species to calibrate the relative sensitivity of the short-term test protocols.

All three short-term protocols are static tests; that is, the test solutions are not changed or renewed during the bioassay. Each protocol measures a different effect, or endpoint. A summary of test results is given in the accompanying table.

The short-term larval abalone bioassay 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, an NOEC of 10 percent was determined for the secondary treatment plant and 3.2 percent for the primary treatment system.

The short-term giant kelp bioassay is a 48-hour test that measures two different endpoints: zoospore germination and growth of the germination tube. Because kelp are relatively insensitive to zinc, the tests also were conducted with the pesticide sodium pentachlorophenate. For the pesticide, the NOEC for zoospore germination was less than 32 ppb. With preliminary wastewater tests, the NOEC was 0.56 percent for primary treatment effluent and 18 percent for secondary treatment.

The short-term mysid bioassay 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.

In addition, as described in Section 5 of this report, preliminary work was done with other organisms including mussels, squid, fish, and another species of mysid shrimp.

Appendix 1 describes the selection of zinc as a reference toxicant. The evolving preliminary bioassay protocols for abalone, kelp, and mysids are presented in three separate appendices. In addition to work performed at the DFG laboratory during Phase 2, a preliminary validation of the evolving procedures was conducted by the Southern California Coastal Water Resource Project (SCCWRP) to identify problems and omissions in the bioassay protocols.

Phases Three and Four (Future Work):

Phase 3 of the Marine Bioassay Project will modify, as necessary, the protocols developed in Phase 2 for testing seawater dilutions of complex effluent from municipal ocean dischargers. Two representative municipal complex effluents will each be tested twice during 1988. Project staff will work closely with laboratory personnel from the two dischargers. At its conclusion, Phase 3 will provide laboratory specifications and requirements for chronic marine bioassay systems that can be used by ocean dischargers.

SUMMARY OF NO OBSERVED EFFECT CONCENTRATIONS
FOR ABALONE, KELP, AND MYSID TOXICITY TESTS

	TEST NUMBER		
	1	2	3
A. Zinc Reference Toxicant Tests (ppb)			
Abalone 48-Hour Repetitive Tests	40	41	37
Abalone 9-Day Calibration Test	19		
Mysid 48-Hour Repetitive Tests	182	175	320
Mysid 96-Hour Test	100		
Mysid 21-Day Calibration Test	45		
Kelp 48-Hour Repetitive Tests			
(germination)	2033	5495	1732
(length)	<1090	<589	<553
Kelp 16-Day Calibration Test	1071		
B. Interlaboratory Zinc Tests (ppb)	MBP[1]		SCCWRP[2]
Abalone 48-Hour Test	37		18
Mysid 96-Hour Test	89		66
Kelp (germination)	957		--[3]
(length)	<538		<559
C. Effluent Tests (percent)	Primary		Secondary
Abalone 48-Hour Test	3.2		10
Mysid 96-Hour Test	1.0		32
Kelp (germination)	0.56		18[4]
(length)	n.d.[5]		32[4]

[1] MBP = Marine Bioassay Project

[2] SCCWRP = Southern California Coastal Water Research Project

[3] No significant difference due to poor germination in controls

[4] NOEC derived by comparison with dilution water control because comparisons with brine controls gave no significant difference.

[5] n.d. = not determined

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

1. Develop a bioassay protocol for a vertebrate species (a fish).

2. Select and test an organic reference toxicant. (An inorganic reference toxicant was used during previous phases.)

3. Develop procedures manual containing the necessary protocols to conduct bioassays on marine organisms.

4. Train Regional Board and discharger personnel to conduct marine bioassays.

RECOMMENDATIONS FOR PHASES THREE AND FOUR (FUTURE WORK)

The Marine Bioassay Project was established to develop and implement better methods to regulate ocean discharges of materials that are toxic to marine life. The major objective of the Project is the development of sensitive biological tests that evaluate effluent toxicity. Section 13170.2 of the California Water Code requires the State Water Resources Control Board to approve and adopt suitable bioassay protocols to evaluate effects of discharges to marine waters by January 1, 1990. To develop reliable and sensitive tests by this date, the following recommendations have been made by the Marine Bioassay Project and members of the Scientific Review Committee:

1. CONTINUE EFFLUENT TESTING. Complex municipal effluents are highly variable in their physical and chemical composition. Bioassays used for toxicity testing must perform reliably with all types of effluents. Continued effluent testing to verify test performance is recommended.

2. ASSESS THE FEASIBILITY OF COMPLIANCE. As more sensitive tests are used to determine Ocean Plan toxicity limits, ocean dischargers will need to know what must be done to comply with changes in discharge permit limitations. Testing a variety of effluents will indicate the level of treatment necessary to achieve compliance. As a first step, testing two types of effluent is recommended: one from a well-run secondary treatment plant that can meet new permit requirements, and another from a plant producing a more toxic effluent. This testing can be incorporated into the effluent testing work described above.

3. CONDUCT QUARTERLY REFERENCE TOXICANT TESTING. Quarterly testing with a stable reference toxicant is recommended to assess seasonal variability in the toxic responses of new test organisms. The variable toxicity of effluents precludes their use in studies of seasonality. Conducting reference toxicant tests concurrently with effluent tests is recommended to distinguish seasonal effects from effluent toxicity variability.

4. CONTINUE TEST DEVELOPMENT. The program should use the best tests available to detect effluent toxicity. The following work to improve the tests presented in this report is recommended.

    a. The existing mysid short-term test measures lethality in juvenile organisms (Holmesimysis). The project should investigate sublethal effects on this species or another indigenous mysid. Growth inhibition in Holmesimysis and reproductive tests with other mysid species are possible variations.

    b. Compare relative sensitivities of larval mysids of different ages. Tests presented in this report used mysids of identical age. If mysids of various ages are similarly sensitive to toxicants, they can be used interchangeably, and it will be easier to supply testing laboratories with suitable test organisms.

c. Investigate the sensitivity and variability of the short term kelp test using a different reference toxicant. Kelp is very tolerant to zinc, making data on sensitivity and variability difficult to interpret. A reference toxicant to which kelp is more sensitive is necessary to determine the sensitivity and reliability of the kelp tests.

5. DEVELOP TESTS THAT USE OTHER SPECIES. Coastal ecosystems contain a diversity of organisms with widely varying tolerances to toxic substances. Possible effects on the entire biological community can only be assessed by testing with a variety of species from different phyla. Tests using an alga, mollusc, and crustacean are described in this report. Bioassay development using an indigenous marine fish species is recommended. Freshwater fish have been used in acute bioassays for several years, and there is widespread public recognition of the need to protect fish populations. Although several fish species have been proposed, no standardized tests using marine fish are ready for use in California.

6. CONDUCT INTERLABORATORY TESTING FOR VERIFICATION OF PROTOCOLS. Tests must be suitable for use by a number of people in a variety of monitoring laboratories. To insure wide applicability, the tests developed by the Marine Bioassay Project should be conducted concurrently by qualified laboratories to determine interlaboratory variability. Through interlaboratory testing, the requirements and constraints of the participating laboratories can be recognized, and necessary modifications of test protocols can be made.

7. COMPARE MARINE BIOASSAY PROJECT PROTOCOLS TO ESTABLISHED TESTS. Existing tests developed by the U.S. EPA and others should be conducted alongside the abalone, mysid, and kelp tests. This comparison will help determine the relative sensitivities of several toxicity tests and will allow the Marine Bioassay Project staff to evaluate the suitability of these tests for use in California.

8. PROVIDE A RELIABLE SUPPLY OF TEST ORGANISMS. A reliable supply of test organisms from clean reference sites will be necessary to support a large scale testing program. A central culture laboratory should be established to supply organisms to the participating bioassay laboratories.

9. PROVIDE ADEQUATE TRAINING OF TECHNICIANS. A comprehensive program should be developed to ensure that laboratory technicians have adequate training to conduct effluent testing.

10. PREPARE FOR IMPLEMENTATION OF TOXICITY TESTING. Several steps are necessary to implement the tests developed by the Marine Bioassay Project and other groups such as the U.S. EPA. First, the dialogue with the discharger community must be expanded to include demonstrations of the evolving test protocols. Second, the staff and others associated with the Marine Bioassay Project need to continue and accelerate efforts to inform dischargers, consultants, and other interested parties of progress in test development. Finally, after it has been verified that the protocols can be used sucessfully by

independent laboratories for testing complex effluents, the bioassays need to be implemented by the appropriate regulatory agencies. Implementation of bioassays includes (1) incorporation into the California Ocean Plan by the State Board and (2) placement in marine discharge permits by the affected regional water quality control boards. The early implementation steps should begin in 1988 and be accelerated in 1989. Marine bioassay protocols will be required in the permits of large marine dischargers by January 1, 1991.

SECTION 1
INTRODUCTION

Two billion gallons of wastewater are discharged into the coastal waters of California every day. Many wastewater constituents are toxic to living marine resources. Because these resources are valued by Californians and play an important role in the state's economy, their protection is a major priority. The Water Quality Control Plan for the Coastal Waters of California (SWRCB Ocean Plan, 1983) states that "marine communities, including vertebrate, invertebrate, and plant species, shall not be degraded."

A number of studies have indicated, however, that marine communities have not been protected from pollution (see reviews by Reish, *et al.*, 1984, 1985). Municipal effluents contain sewage, trace metals, and hundreds of synthetic organic contaminants. The fate of these materials in the ocean is the subject of ongoing research and debate. Many contaminants are toxic substances that accumulate in marine sediments. The flux of lead, zinc, copper, cadmium, silver and chromium into sediments of offshore marine basins is measurably greater today than in historic times prior to the existence of municipal waste outfalls (Bruland *et al.*, 1974). At least some of these elements reach the sediments by way of incorporation in marine plankton (Martin and Broenkow, 1975). Accumulation by plankton and macroalgae makes pollutants available to other members of marine food webs (Stewart and Schulz-Baldes, 1976), and higher organisms may also absorb contaminants directly (Martin *et al.*, 1977). Some toxic substances bioaccumulate, others apparently do not (Young and Mearns, 1978); but a number of studies have shown increased levels of heavy metals (Young and Moore, 1978; Jan *et al.*, 1977; Alexander and Young, 1976) and synthetic organic contaminants (Brown *et al.*, 1984; Ladd *et al.*, 1984) in a variety of marine organisms exposed to effluent discharges. Marine mammals, which generally occupy positions near the top of the trophic pyramid, have shown increased body burdens of synthetic halogenated hydrocarbons (Schafer *et al.*, 1984), and increased levels of mercury in feces (Flegal *et al.*, 1981).

Toxic substances appear throughout exposed marine ecosystems, and there is evidence that they affect organisms and community structure. Long term surveys of benthic communities have shown marked decreases in species diversity and abundance near outfalls (Grigg, 1978). Fish populations have declined from a combination of causes, and there is evidence that effluent discharges play a role in this decline. Tumors in Dover sole are significantly more common in fish collected near outfalls (Cross, 1984), and white croakers in contaminated waters off Los Angeles have exhibited reduced reproduction rates (Cross and Hose, 1986). Reduced reproductive capacity, fecundity, and gametic viability in striped bass have been correlated with the presence of toxic pollutants (Whipple et al., 1983). Where chemical

contaminant concentrations in effluent discharges have been reduced, there has been a corresponding recovery observed in affected biological communities (Swartz *et al.*, 1986).

To protect marine life, efforts have been made to reduce or remove toxic constituents from wastewater effluents. This has been an evolving process. Traditionally, engineering and economic considerations were the basis for a strategy of treating wastewater using the best technology available. The U.S. Environmental Protection Agency has recently emphasized a water quality-based approach that evaluates biological impacts to determine the amount of treatment necessary. But the evaluation of effluent toxicity to exposed biological communities is a difficult task. One approach has been to develop discharge limits based on toxicological information for each toxic constituent, and then to monitor the effluent concentrations of each constituent individually (Branson *et al.*, 1981). Effluents, however, contain hundreds of substances, many of them toxic. For many contaminants there is no toxicity information, and chemical analysis, especially for synthetic organic compounds, can be difficult and expensive. Reliance on chemical monitoring alone has the additional disadvantage that additive, synergistic and antagonistic interactions between the effluent constituents cannot be measured (Livingston *et al.*, 1974).

An approach that circumvents many of these problems uses biological toxicity testing to directly measure the toxic effect of whole complex effluents (Sprague and McLeese, 1968; Woelke, 1972). Risebrough *et al.* (1974) discussed the need for biological tests to determine the ecological effects of synthetic organic contaminants, but Oshida and Goochey (1980) observed that no standardized bioassays were available for testing effluent discharges to California waters. Many types of marine toxicity experiments have been designed (see reviews by Reish *et al.*, 1984, 1985; and Eagle, 1981), but Eagle (1981) described the general lack of standardization in previous bioassay testing. He suggested that future tests include information on temperature, salinity, pH, dissolved oxygen, season, age and general health of the test organisms.

The present work is designed to help fill the need for a series of standardized biological toxicity tests for routine examination of whole effluent toxicity. To estimate effluent impacts on biological communities, sensitive, reliable bioassay tests must be available that use ecologically important species indiginous to the impacted areas. Established tests of this kind using California marine species include the mussel larval development test (Dimick and Breese, 1965), the sea urchin embryo test (Oshida and Goochey, 1980), and the echinoderm sperm cell test (Dinnell *et al.*, 1987). Species vary in their tolerances to effluent constituents, and no single species is most sensitive to the variety of effluents discharged to coastal waters. To accurately assess toxicity, tests using a number of different species must be employed. The tests described here include species from three major groups (molluscs, crustaceans, and algae). The selection of species used by the Marine Bioassay Project is described by Linfield *et al.* (1985), and early test development using the pesticides endosulfan and sodium

pentachlorophenate is described by Martin *et al.* (1986).

This report is comprised of three main sections, each dealing with work on one of the test species: the red abalone *Haliotis rufescens*, the mysid *Holmesimysis* (=*Acanthomysis*) *costata* and the giant kelp, *Macrocystis pyrifera*, plus a short discussion of preliminary work with additional test species. Each section contains an introduction to that species, materials and methods, test results, and discussion. This format is intended to give a complete description of each of the toxicity tests, but causes some overlap in the discussion of topics relevant to all three. A short term test to estimate chronic effluent toxicity is presented for each species; these are the tests designed for regulatory applications. Detailed protocols for conducting the short term tests are found in the appendix. In addition, a long term test is presented for each species. Long and short term toxicity is compared to evaluate the relative sensitivity of each short term test. All procedures described are considered preliminary, and incorporation of these tests into waste discharge permits is subject to approval by the State Water Resources Control Board. Further testing and the coordinated implementation of tests developed by the Marine Bioassay Project are planned for the project's next phase.

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SECTION 2

RED ABALONE TESTS

John W. Hunt
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INTRODUCTION TO RED ABALONE TESTS

The red abalone, *Haliotis rufescens* (Swainson 1822), has been studied extensively because of its economic value. Much of the information on abalone ecology (Cox, 1962; Sakai, 1962), life history (Boolootian *et al.*, 1962; Leighton, 1967, 1974; Morse *et al.*, 1979) and culture (Morse *et al.*, 1977; Morse, 1984; Ebert and Houk, 1984) has been directed toward enhancing its production for the seafood industry. Recently, however, the red abalone hasbeen used as a bioassay organism in studies investigating the effects of toxic pollutants on marine life (Martin *et al.*, 1977; Morse *et al.*, 1979). The work presented here is part of an effort by the California State Water Resources Control Board to develop sensitive biological toxicity tests for routine monitoring of complex effluents discharged into California's coastal waters.

The red abalone is important to the ecology and economy of the California coast. It is the largest of the west coast marine gastropods, and is an important food for the threatened sea otter. In areas of suitable habitat it constitutes a large fraction of theinvertebrate biomass and production (Hines and Pearse, 1982). Commercial landings of red abalones in California peaked in 1957 at about 5 million pounds, then declined to just over 800,000 pounds (worth $2.4 million) in 1985. The annual sport harvest exceeds 1.5 million pounds, and ranks as the most important recreational fishery in northern California (Cal. Dept. of Fish and Game, unpublished data).

There is evidence that abalones are exposed to and assimilate toxic substances from effluent discharges. Concentrations of silver, chromium, nickel, and zinc were 2 to 10 times greater in the edible tissues of abalone collected near large southern California municipal outfalls than in edible tissues of abalone from control sites (Jan *et al.*, 1977) .The distribution of abalones has been affected by effluent discharges, with abundance declining near outfalls (Grigg, 1978). High abalone mortality was associated with high copper concentrations in cooling water discharged from the Diablo Canyon nuclear power plant (Martin *et al.*, 1977).

A number of other molluscs have been used in marine toxicity studies. Among these are various species of clams (Calabrese and Nelson, 1974; Calabrese *et al.*, 1977), oysters (Woelke, 1972; Connor, 1972; Calabrese *et al.*, 1973; Martin *et al.*, 1981), mussels (Dimick and Breese, 1965; Martin *et al.*, 1981), and scallops (Nelson *et al.*, 1976). The red abalone was chosen as a potential molluscan bioassay species in the State Water Resources Control Board's Marine Bioassay Project because it has a combination of characteristics that make it exceptionally well suited for toxicity testing. It is ecologically and economically important, it has been affected by effluent discharges, it is easily manipulated in the laboratory to reliably produce spawnable broodstock and fertilized eggs for testing year-round, it has free swimming larvae that are visible to the naked eye, it is sensitive to a variety of toxicants (Martin *et al.*, 1977; Morse *et al.*, 1979; Martin *et al.*, 1986), and it is distributed throughout California coastal waters.

This report describes a short term sub-lethal bioassay test that estimates the chronic toxicity of complex effluents to the red abalone, and presents the results of toxicity tests using zinc sulfate and samples of primary and secondary treated effluents. Also presented are the results of a nine-day metamorphosis test used to calibrate the sensitivity of the short term test.

MATERIALS AND METHODS

Two tests are described here: a short term test designed for routine use by dischargers and regulatory agencies, and a long term test used to calibrate the sensitivity of the short term test. In the short term test, we exposed abalone embryos to toxicant solutions immediately after fertilization. After 48 hours, we examined the veliger larvae for shell abnormalities. In the long term test, the exposure continued for 9 days, allowing the larvae to pass through their entire larval stage and metamorphose into the adult form (Figure 1). Failure to undergo metamorphosis was the indication of long term toxic effect.

The following are brief descriptions of the two toxicity tests. Included are methods for culturing broodstock, obtaining embryos, conducting toxicity tests, evaluating larval abnormalities, and analyzing the results statistically to determine the toxicity of effluent samples. Step by step instructions for conducting the short term test are presented in the abalone test protocol appended to this report.

Facilities

All of the work was done at the Marine Pollution Studies Laboratory at Granite Canyon, located on the exposed rocky coast of central California, 20 km south of the Monterey penninsula. Seawater for culturing abalones and for dilution water in toxicity tests was drawn directly from the ocean and flowed continuously through the laboratory in a system using stainless steel pump impellers and PVC pipes. The laboratory is remote from sources of pollution. Culture facilities were physically separated from the mobile bioassay laboratory to prevent contamination of organisms prior to testing.

Abalone Culture

We cultured the abalone broodstock in 15 liter opaque plastic containers and fed them *ad libitum* with fronds of giant kelp (*Macrocystis pyrifera*). The nocturnally foraging animals were kept in 24 hour darkness to enhance feeding (Ebert and Houk, 1984). Water flowed continuously through the broodstock containers at a rate of about 2 to 3 liters per minute. Each container held 10 to 20 abalones averaging 8 cm in length.

To obtain eggs and larvae, we routinely induced spawning of ripe broodstock using the ultraviolet irradiation technique developed by Kikuchi and Uki (1974). We assessed the sexual maturity of potential spawners by examining the gonad, which is easily visible under the right posterior edge of the shell. Two days before spawning induction, three ripe males and three ripe females were placed in separate, clean 15 liter

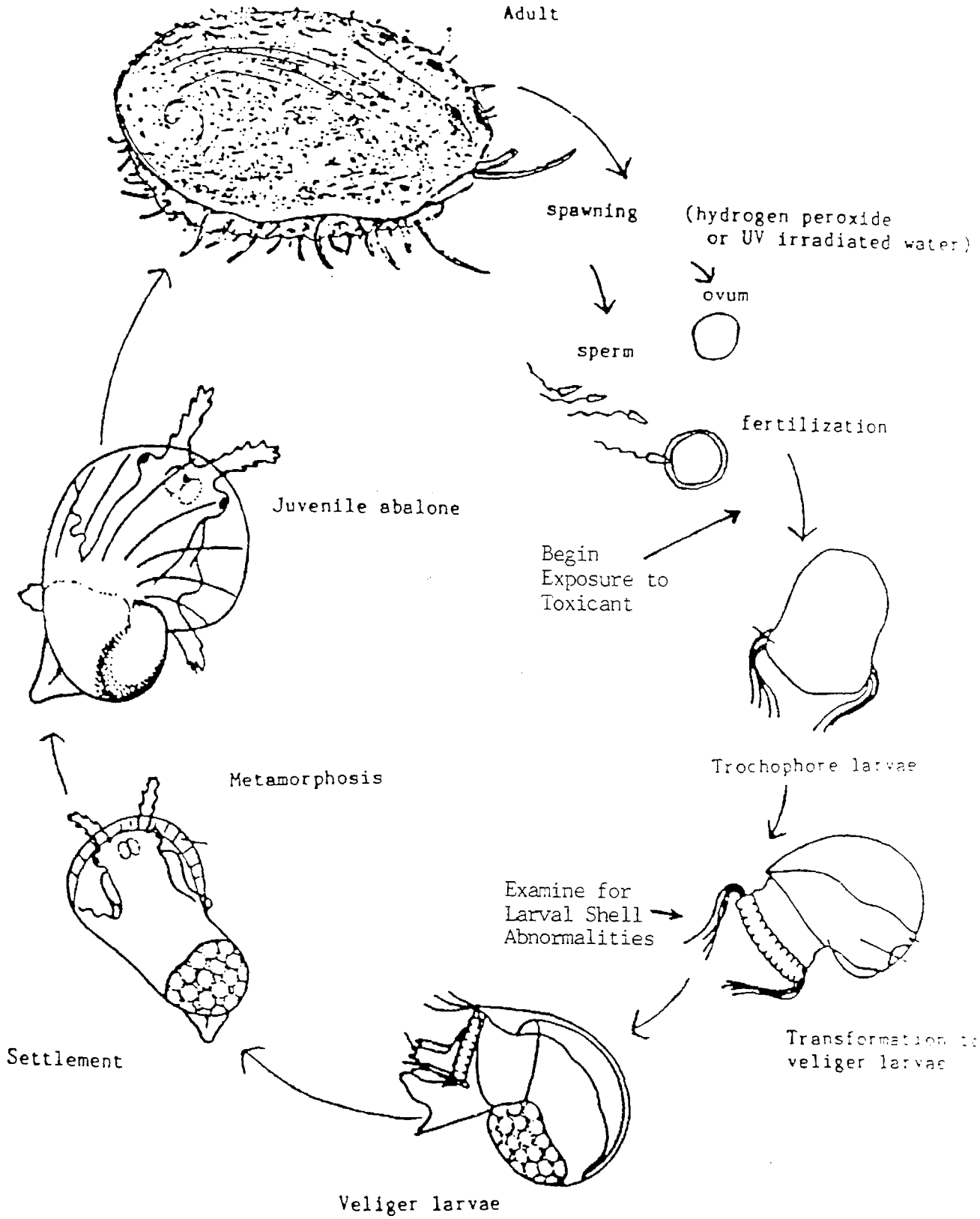


Figure 1. Life history of the red abalone, Haliotis rufescens (Swainson, 1822).

polyethylene buckets with continuously flowing filtered seawater. These animals were kept for two days without food to allow acclimation and the elimination of feces. Two to three hours prior to spawning, the buckets were emptied, wiped clean of accumulated wastes, and refilled slowly (150 ml/min) with one micron filtered seawater from the UV sterilization unit. This flow rate was maintained until the UV irradiated seawater triggered the spawning response (about 2.5 hours). Spawning induction was successful in 15 of 16 trials (94%).

Following spawning by both groups, we siphoned the eggs into a third clean polyethylene bucket and added 300 ml of sperm suspension to allow fertilization. Fertilized eggs were rinsed and concentrated. Five 1 ml samples of the concentrated egg suspension were counted to estimate embryo density (see Appendix 2 for details).

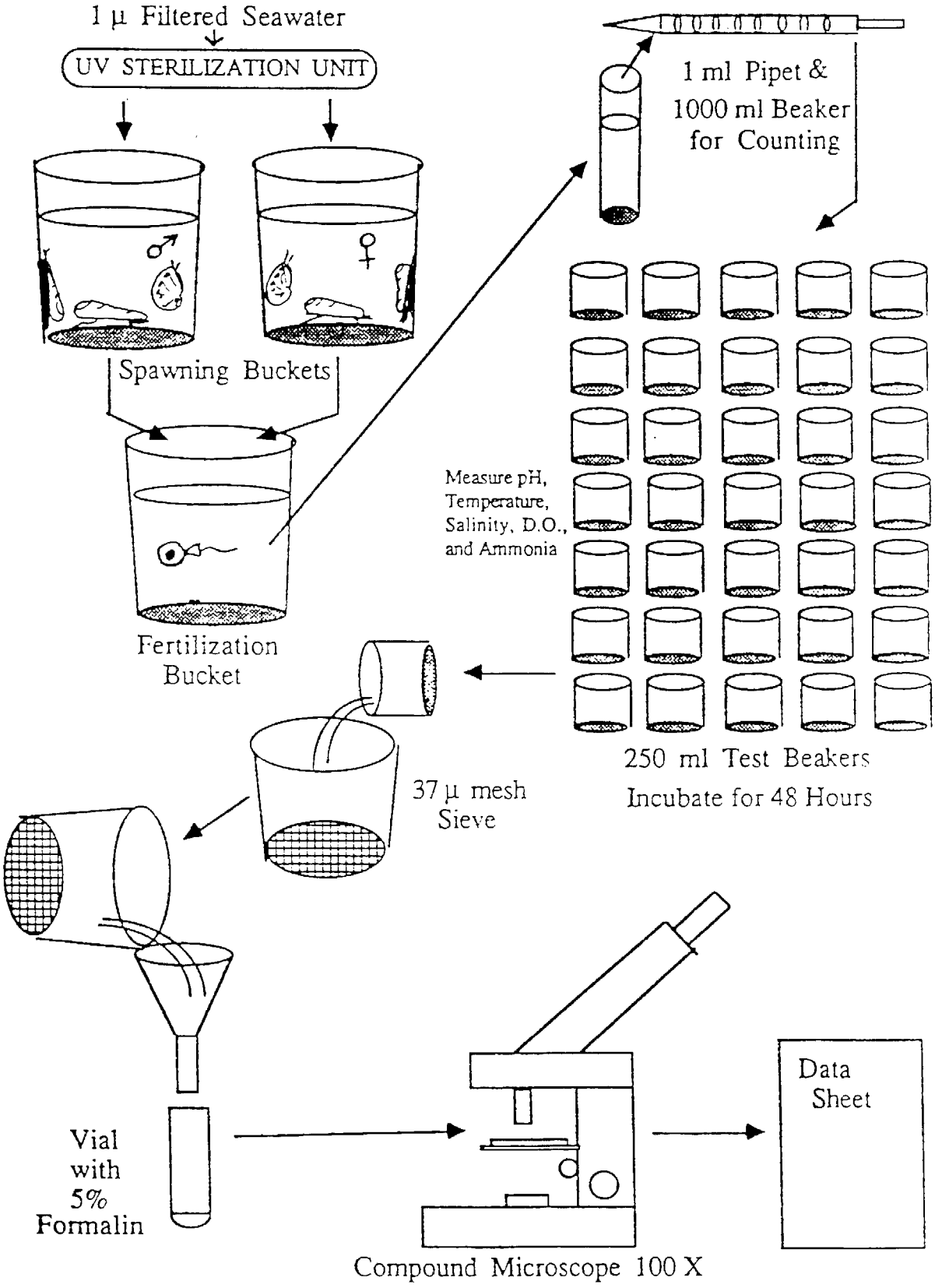
Short Term Toxicity Test

One thousand eggs were pipetted into each toxicity test container. Polypropylene 250 ml beakers were used as test containers in zinc tests, and 600 ml borosilicate glass beakers were used in effluent tests. Each short term test used 5 toxicant concentrations and a control, each replicated 5 times, for a total of 30 test containers (effluent tests included 5 more containers for brine controls). All containers were arranged randomly in a water bath prior to addition of the embryos.

The embryos incubated in the test solutions for 48 hours. During this time they developed into trochophore larvae, hatched from the egg membrane, and transformed into veliger larvae (Figure 1). We measured the dissolved oxygen concentration, pH, salinity, and temperature daily in one randomly selected test container from each concentration.

After 48 hours, we poured the entire contents of each test container through a 37 micron mesh Nytex sieve that retained the larvae. Larvae were then washed with a squirt bottle from the sieve, through a funnel, and into 10 ml glass vials, where they were fixed in 5% buffered formalin. We pipetted all larvae from the vials onto a Sedgewick-Rafter counting slide, and examined 200 larvae at 100x under a compound light microscope (Figure 2). Larvae with smooth snail-shaped shells were scored as "normal". Larval shells with severe deviations from the snail shape or large indentations were scored as "abnormal" (Figure 3). We compared marginally deformed shells with a series of photographs to eliminate as much subjectivity as possible. Comparisons between analysts for all replicates were made using a paired sample T-test. There were no significant differences in the interpretation of this endpoint in three trial tests ($p > .05$; Hunt and Anderson, unpublished data).

FIGURE 2. ABALONE TOXICITY TEST PROTOCOL



NORMAL VELIGER LARVA

Figure 3A. "Normal" 48-hour old red abalone veliger larva (100 x magnification)

ABNORMAL VELIGER LARVA

Figure 3B. "Abnormal" 48-hour old red abalone veliger larva (100x magnification).

Statistical Analysis

For each replicate of each concentration we determined the percentage of normal larvae out of the 200 counted. This percentage was transformed to the arcsine of its square root to normalize the distribution of the percentage data. The transformed data were then analyzed using an Analysis of Variance and a Dunnett's multiple comparison test to compare each concentration to the control (Zar, 1980; Glass, 1972; Sokal and Rohlf, 1969). The highest concentration not significantly different from the control was defined as the No Observed Effect Concentration (NOEC).

Long Term Toxicity Test

The long term test was not designed as a test for routine toxicity evaluation, but was used to calibrate the sensitivity of the short term test. A stable reference toxicant (zinc sulfate) was used to provide verifiable test concentrations for comparisons with short term tests using the same toxicant. We used a proportional diluter system (Brungs and Lemke, 1978) to continuously replenish the toxicant at the desired concentration. Six concentrations and a control were used, each replicated 4 times. The concentrations were 0 (control), 5.6 10, 18, 32, 56 and 100 ug/l zinc. These nominal concentrations were verified by chemical analysis (see discussion of toxicants, below).

Broodstock, spawning, fertilization, counting and delivery of abalones into the test containers were as described for the short term tests. The test containers were of two parts, designed for use in a flow-through system. A polycarbonate cup with a 90 micron mesh Nytex screen bottom was placed half-way into a 250 ml polypropylene beaker. A 1/4 inch diameter plastic tube delivered test solution from the diluter system into the cup, where the abalones were retained above the screen in water 4 cm deep. Test solution gradually flowed through the screen and out the beaker pour spouts.

Larvae were kept in these containers until day six, when they had developed four lobes on the cephalic tenticles and sensory cilia around the margin of the foot, characteristics that indicate their competence for settlement. On day six the abalones were transferred to polycarbonate cups that had been prepared as suitable substrate for settlement. The settling cups were prepared by growing films of naturally occurring benthic diatoms in them, then allowing 1 cm long juvenile red abalones to graze the diatoms. Substrates prepared in this way have been shown to induce settlement of larval abalones, who are chemotactically attracted to the mucus trails left by the grazing juveniles (Seki, 1980). The abalones were left to settle in these cups for the remaining 3 days of the test. On day nine we immersed the cups for 1 minute in 5% buffered formalin to fix the abalones, then washed them from the cups into vials where they were kept in 5% formalin. The abalones were examined microscopically as described above, and were scored as either metamorphosed or non-metamorphosed. Metamorphosis is indicated by the existence of the juvenile shell, with its characteristic radiating lines (Morse, *et al.*, 1979; Figure 4).

METAMORPHOSED JUVENILE ABALONE

Figure 4. Nine day old juvenile red abalone, 2 days post-metamorphosis. Note the juvenile shell with its characteristic radiating lines. Note also that the remnant larval shell is densely striated and free of any indentations.

For each replicate of each concentration we counted 200 abalones, and calculated the percentage that had successfully undergone metamorphosis. These data were then treated as above to calculate the NOEC value.

Toxicants Used

Zinc sulfate was used as a reference toxicant in developing this toxicity test. We used zinc because it is stable in solution (see Table 8 in Appendix 1), easy to analyze chemically, relatively non-hazardous, and found in high concentrations in target effluents (Schafer, 1986). The reference toxicant was used for making comparisons among replicate tests, between long and short term tests, between laboratories, with tests using other organisms, and with the literature.

All zinc concentrations were verified by chemical analysis on a Perkin Elmer 5000 atomic absorbtion spectrometer. One random replicate of each concentration was sampled at the beginning and end of each test. Samples were stored in 30 ml polyethylene vials with 1 % by volume of 14 N double quartz-distilled nitric acid. Unless otherwise noted, all zinc concentrations reported in figures, tables, and text are chemically verified concentrations.

Effluents used in the experiments were samples of primary and secondary treated municipal effluents from publicly owned treatment plants discharging to California coastal waters. Primary effluent was from a single grab sample collected prior to chlorination, secondary effluent was from a 24 hour composite sample collected after chlorination and dechlorination.

Effluent salinities ranged from 0 to 2 ppth, and required additions of hypersaline brine to achieve the test salinity of 34 ppth. Brine was made by heating 1 micron filtered seawater to between 40° and 100°C with constant aeration to produce a brine salinity of 75 to 85 ppth. The higher temperatures were used only because of logistical necessity, and produced brines with elevated pH levels that were measurably toxic to the young abalones. Brines produced at temperatures above 40°C are not recommended for salinity adjustments in toxicity tests.

Effluent ammonia concentrations were not measured during testing. Ammonia levels are measured quarterly by the treatment plants. Total ammonia concentrations for the primary effluent had an annual range of 20 to 49 mg/l. The annual range of total ammonia concentration for the secondary effluent was from 18 to 22 mg/l. We have no data for unionized ammonia concentration.

We also tested samples from inside and outside the zone of initial dilution (ZID) above the secondary plant outfall. One sample was collected at low tide in the boil directly above the outfall, and another was collected nearby but outside of the discharge boil. Both had salinities of 32 ppth and did not need salinity adjustment.

Cleaning of Test Equipment

All sample vials, pipets, test containers, and mixing flasks were cleaned by sequential 24 hour soaks in detergent, 3N hydrochloric acid, and deionized water prior to use. Equipment was rinsed 3 times with deionized water between each soak. Containers used with effluents were triple rinsed with fresh reagent grade acetone before soaking.

Interlaboratory Calibration

The short term test was conducted by two different laboratories to determine if similar results would be produced by different investigators at another facility using the written test protocols developed at the Marine Pollution Studies Laboratory (MPSL).

We transported adult broodstock abalones from MPSL to the Southern California Coastal Water Research Project (SCCWRP) laboratory in Long Beach, California. Three ripe male and three ripe female abalones were packed in moist sponges in bags filled with oxygen, and placed in an ice chest with two blue ice blocks wrapped in newspaper. Within 12 hours they were put in seawater aquaria at SCCWRP. MPSL broodstock were packed in the same way in another ice chest, and kept at MPSL for 12 hours to simulate the adventures of the transported abalone.

All test containers, mixing flasks, culture equipment, and zinc sulfate was brought to SCCWRP from MPSL. Both labs used SCCWRP's seawater, which was originally pumped from the head of the Redondo submarine canyon in Santa Monica Bay. Test temperatures were 15° $\pm$ 1°C at both laboratories. We conducted the test at MPSL one week after it was done at SCCWRP. The participating investigators at SCCWRP were experienced bioanalysts who have developed similar tests with other species, although none had previously conducted the abalone test. Test data were analysed with the same statistical tests at the same significance level (alpha =.05).

Species Comparisons with Secondary Effluent

A study coordinated by the San Francisco Regional Water Quality Control Board gave us the opportunity to compare the effluent sensitivity of the red abalone test with that of the 48 hour mussel test (Dimick and Breese, 1965) and the 1 hour echinoderm sperm cell test (Dinnell et al., 1987). A sample of secondary effluent was split, with subsamples given to MPSL and to investigators from the EPA Environmental Research Laboratory at Newport, Oregon. We conducted the red abalone test at MPSL, and EPA investigators conducted the mussel and echinoderm tests at temporary facilities near the outfall. All samples were transported in ice chests with blue ice, and all tests were initiated on the day of effluent collection. No Observed Effect Concentrations (NOECs) derived with the same statistical methods (ANOVA and Dunnett's) were used to compare results between test species.

RESULTS

The results of 9 toxicity tests using the red abalone are presented here. Three are replicate short term tests with zinc that are compared to examine test variability. One is a long term exposure used to calibrate the sensitivity of the short term test. Two are interlaboratory tests using zinc, and three are effluent tests: one primary, one secondary, and one from the zone of initial dilution above a secondary plant outfall. All No Observed Effect Concentrations (NOECs) derived from these tests are summarized in Table 1.

TABLE 1. NO OBSERVED EFFECT CONCENTRATIONS FOR ABALONE TESTS

Zinc Sulfate Tests (µg/l)	TEST NUMBER		
	1	2	3
Abalone 48 Hour Repetitive Tests	40	41	37
Abalone 9 Day Test	19		
Interlaboratory Zinc Tests (µg/l)	**MPSL**		**SCCWRP**
Abalone 48 Hour Test	37		18
Effluent Tests (percent)	**PRIMARY**		**SECONDARY**
Abalone 48 Hour Test	3.2		10

MPSL = Marine Pollution Studies Laboratory; SCCWRP = Southern California Coastal Water Research Project.

Water Quality Measurements of Test Solutions

Measurements of dissolved oxygen, pH, temperature, and salinity for all test solutions from all abalone tests reported here are presented in Table 2. All values are within normal ranges, except for pH values for brine controls in the secondary effluent test.

TABLE 2. WATER QUALITY RANGES FOR ABALONE TESTS

Test	Dissolved Oxygen mm./Hg.	pH	Salinity ppt.	Temperatrue degrees C.
Short-term #1	-----	-- 8.55 --	-- 36.0 --	-- 13.0 --
Short-term #2	145 - 155	-- 8.55 --	-- 33.0 --	-- 14.5 --
Short-term #3	112 - 141	8.55 - 8.60	33 - 35	14.5 - 16.0
Long-term	138 - 162	8.50 - 8.60	33 - 36	14.0 - 17.5
Inter-Lab	152 - 158	8.50 - 8.60	-- 35 --	14.8 - 16.5
Primary	149 - 161	8.30 - 8.60	35 - 36	14.5 - 15.0
Secondary	148 - 157	8.55 - 8.70*	34 - 36	-- 15.0 --
ZID	151 - 154	8.55 - 8.60	32 - 33	-- 15.0 --

* Brine control pH was 8.90

Replicate Short Term Tests

There was very close agreement between the three replicate short term tests with zinc (Figures 5, 6, and 7). Graphs of the data show negligible variability for all concentrations except the intermediate (56 µg/l nominal) concentration, in which there was a 47% difference in abalone response between tests 1 and 2 (Figures 5-7). The No Observed Effect Concentrations (NOECs) for the three tests were the same nominal concentration, and the verified zinc concentrations for these NOEC values were 40, 41, and 37 µg/l. The mean NOEC was 39 µg/l, and the coefficient of variation was 5.3%. This level of test variability indicates a stable toxic response to zinc by abalone larvae from different parents spawned at different times.

Variability among replicates within tests was also low, with standard deviations ranging from 0 at some of the highest and lowest toxicant concentrations to 11% at the most variable intermediate concentration (Figure 5).

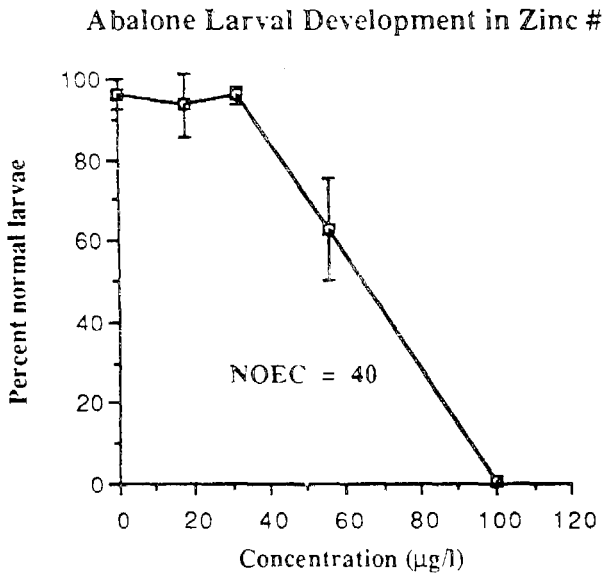


Figure 5. Mean percentage of normally developed abalone veliger larvae (± 1 standard deviation) for each zinc concentration in short-term test #1. N = 5 replicates per concentration.

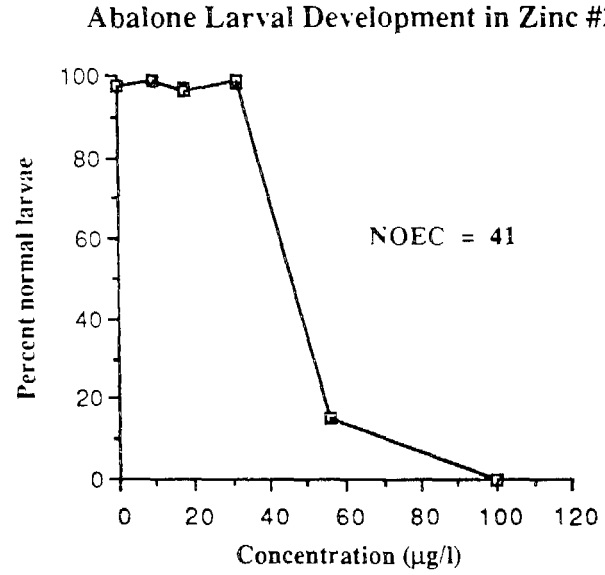


Figure 6. Mean percentage of normally developed abalone veliger larvae (± 1 standard deviation) for each zinc concentration in short-term test # 2. N = 5 replicates per concentration.

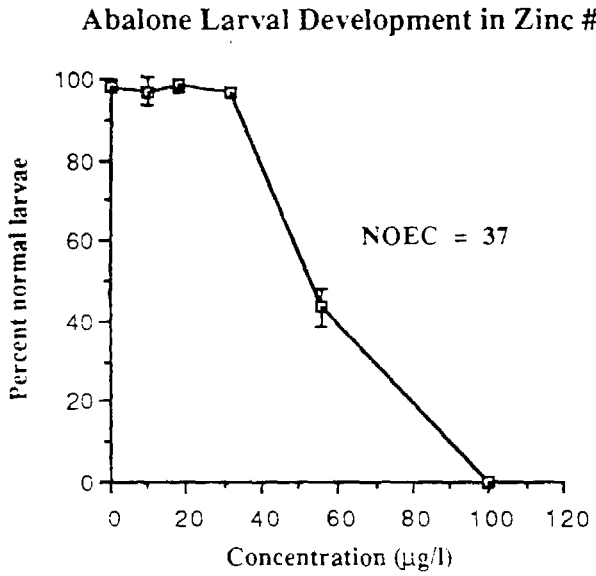


Figure 7. Mean percentage of normally developed abalone veliger larvae (+ 1 standard deviation) for each zinc concentration in short-term test # 3. N = 5 replicates per concentration.

Long Term Test

Larval success at metamorphosis was about twice as sensitive to zinc exposure as was larval shell development. The 9 day metamorphosis test had a NOEC value of 19 µg/l zinc (Figure 8), about half that of the mean short term NOEC of 39 µg/l zinc. Control metamorphosis was about 85%.

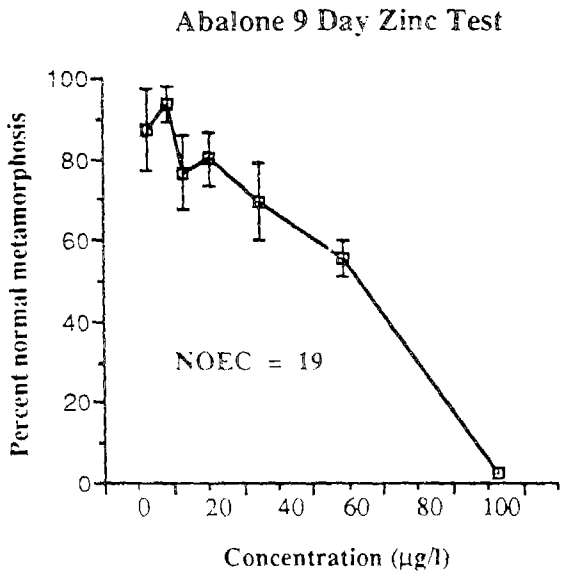


Figure 8. Mean percentage of successfully metamorphosed abalone larvae (± 1 standard deviation) for each zinc concentration in the 9 day flow-through test. N = 4 replicates per concentration.

Interlaboratory Tests

The short term tests conducted at two different laboratories produced similar data (Figures 9 and 10). The mean difference between laboratories at each concentration was 4.2%. The largest difference (12.2%) is at the intermediate (32 µg/l nominal) concentration, and this difference is responsible for the difference in NOECs between the two tests (Figures 9 and 10). SCCWRP reported an NOEC of 18 µg/l (nominal concentration) and MPSL reported an NOEC of 37 µg/l. The presence or interpretation of fragmented preserved larvae in the 32 µg/l nominal concentration probably caused the disparity (Steven M. Bay, SCCWRP, personal communication).

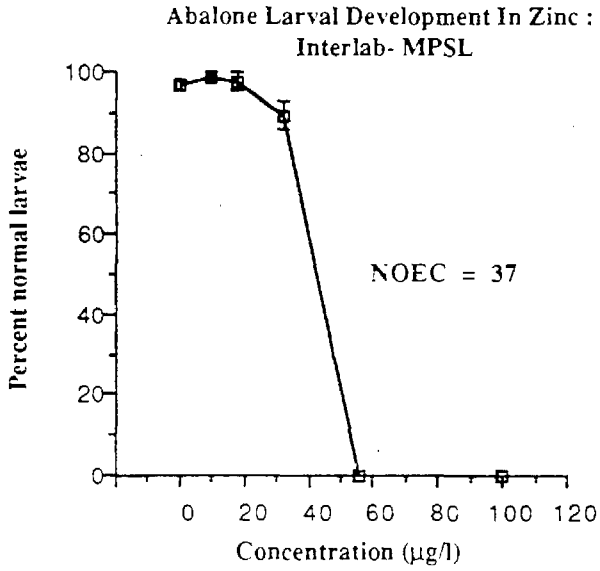


Figure 9. Mean percentage of normally developed abalone veliger larvae (± 1 standard deviation) for each zinc concentration in the interlaboratory test conducted at the Marine Pollution Studies Laboratory. N = 5.

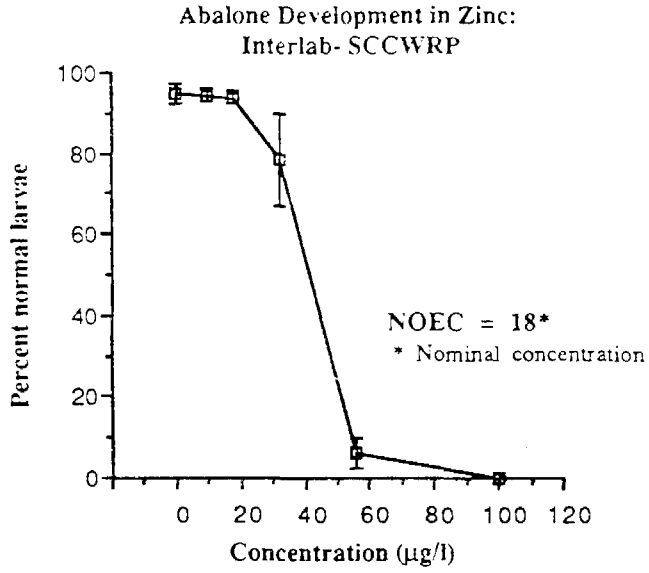


Figure 10. Mean percentage of normally developed abalone veliger larvae (± standard deviation) for each zinc concentration in the interlaboratory test conducted at the Southern California Coastal Water Research Project Laboratory. N = 5.

Primary Effluent

Primary effluent caused the same type of larval shell abnormality as zinc. Particulates in the effluent did not alter the performance or interpretation of the test. There was, however, an additional abnormality found only in the highest effluent concentration. Veliger larvae were found fully encased in the egg membrane 24 hours after they would normally have hatched as trochophores (Figure 1). Because this abnormality was not the same as the shell development abnormality upon which this standardized test is based, these encased larvae were counted separately. An average of 18.4% of the larvae counted in the 10% effluent concentration were encased in egg membranes. Of the remaining larvae in this highest effluent concentration, an average of 95.3% had developed abnormal shells similar to those seen in other tests (Figure 11). The appearance of encased larvae had no effect on the end result of the test. The NOEC value for this primary effluent sample was 3.2%.

Brine used in this test was not heated to more than 40°C, and pH values were within normal ranges. No brine controls were used in this test.

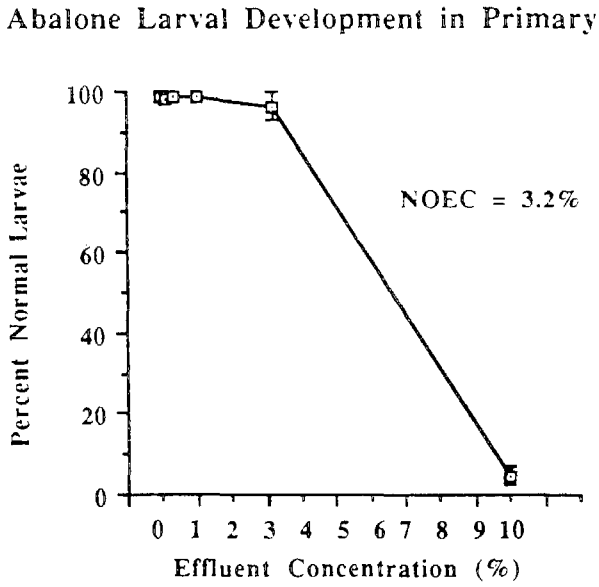


Figure 11. Mean percentage of normally developed abalone veliger larvae (± 1 standard deviation) for each primary effluent concentration. N = 5.

Secondary Effluent

Secondary effluent concentrations above 10% significantly inhibited larval shell development (Figure 12). Effluent concentrations of 18, 32, and 56% had significantly more abnormal larvae than did controls or brine controls.

Brine used in tests with secondary effluent had a noticable effect on the larval abalones (Figure 12). This brine had been boiled, and had a pH of 9.3. Brine controls had the same amount of brine (400 ml per liter) as did the highest effluent concentration (56%).

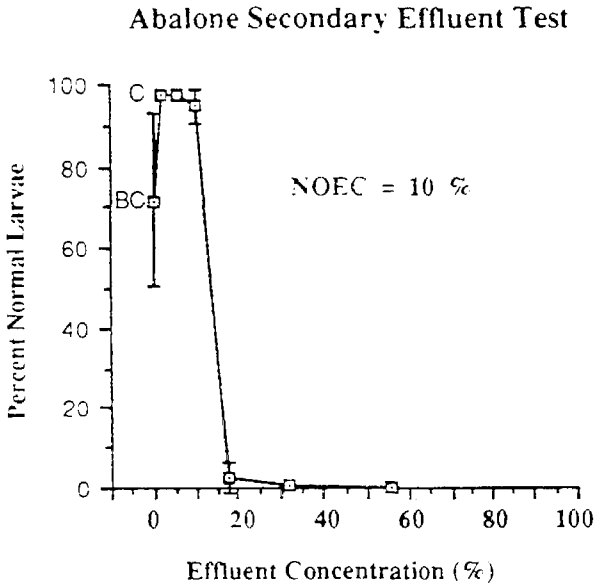


Figure 12. Mean percentage of normally developed abalone veliger larvae (± 1 standard deviation) for each secondary effluent concentration. N = 5. "C" represents dilution water control, "BC" represents brine control.

Abalone embryos exposed to samples collected inside and outside the zone of initial dilution (ZID) above the same secondary plant's outfall developed into normal veliger larvae. There was no significant difference from the controls in this test (Figure 13). ZID sample salinities were 32 ppt, indicating at least an 8 to 1 dilution in the receiving water (salinity $\leq$ 34 ppt), ZID samples did not need brine additions.

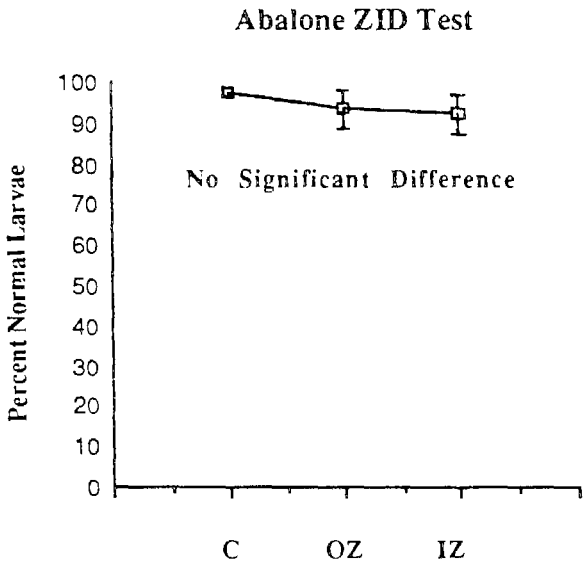


Figure 13. Mean percentage of normally developed abalone veliger larvae ($\pm$ 1 standard deviation) in effluent receiving water. N = 5. "C" represents control water from the Marine Pollution Studies Laboratory, "OZ" represents water collected outside the zone of initial dilution (ZID), and "IZ" represents water collected inside the ZID above a secondary waste treatment plant outfall.

Species Comparisons with Secondary Effluent

The NOEC value for secondary effluent derived using the 48 hour red abalone test was 10%. The same NOEC was obtained for the same effluent sample using the 48 hour mussel larval development test, and the NOEC value obtained for the same effluent sample using the echinoderm sperm cell test was 3.2% (Gary Chapman, U.S. EPA, Newport, personal communication).

DISCUSSION

Direct biological toxicity testing is being used increasingly to evaluate the potential impact of complex effluents on the marine environment. The red abalone toxicity test is being evaluated for this purpose. By describing the variability and sensitivity of this new sublethal toxicity test, the results presented here give an initial indication of the test's suitability for effluent toxicity monitoring.

The reliability of a test is indicated by its inherent variability. In a large scale routine monitoring program, biological tests must detect comparable toxicity levels in different effluents with minimal variability. The results of reference toxicant testing of the 48 hour abalone test indicate low inherent variability both among replicates and between repeated tests. Standard deviations among replicates ranged from 0 to 11% (Figures 5-7), and all tests produced the same nominal No Observed Effect Concentration (NOEC), with chemically verified NOECs varying by 5.3% (coefficient of variation).

Estimations of test variability with complex effluents are more difficult because effluent composition and toxicity change constantly. However, the low between-replicate variability within the effluent tests suggest that larval shell development responds reliably to effluent exposure (Figures 11 and 12).

The ecological implications of abnormal larval shell development are unknown. The planktonic larval stage is important to abalone populations as a means of dispersal (Tegner and Butler, 1985), and the physical and biological stresses on abalone larvae in the planktonic community suggest that this life stage is vulnerable ecologically. Visibly obvious abnormalities at this stage can be expected to have a significant impact on the animals' ability to survive and reproduce. Nevertheless, the ecological importance of toxicant exposure at concentrations causing shell deformities is more clearly established by determining the effect that those concentrations have on metamorphosis of the larval abalones. Failure at metamorphosis causes extended existence in the plankton, delayed initiation of feeding, and possible loss of opportunities to settle on habitable substrates (Morse *et al.*, 1979). Larvae that never undergo metamorphosis will never reproduce.

Zinc concentrations half as great as those causing abnormal shell development were capable of significantly reducing successful metamorphosis (Figure 8). No successfully metamorphosed juveniles were observed with deformities in their larval shells, indicating that shell deformity precludes survival past the planktonic stage.

The fact that the NOEC values for the 48 hour test (39 µg/l) were only about twice that of the NOEC for the 9 day test (19 µg/l) indicates that the short term test gives a reasonable estimation of chronic toxicity.

The results of interlaboratory testing suggest that the short term test can be successfully conducted by different technicians at different facilities with minimal effect on test performance. The mean difference between laboratories at each concentration was 4.2%, with a maximum of 12.2% in the intermediate concentration (Figures 9 and 10). This 12.2% difference, however, was enough to affect statistical significance and cause a difference in NOEC values. The NOEC for the MPSL test was 37 µg/l (verified

concentration), while the NOEC for the SCCWRP test was 18 µg/l (nominal concentration; chemical analyses of SCCWRP zinc concentrations were unsuccessful. All other concentrations were analyzed as discussed in the Methods section).

The sensitivity of the short term test can be assessed by comparisons with published values for zinc toxicity to other organisms. Forty eight hour zinc LC 50 values for other larval molluscs include: 340 µg/l for eastern oysters *Crassostrea virginica* (Calabrese *et al.*, 1973); 119 µg/l for pacific oysters *Crassostrea gigas*, and 175 µg/l for mussels *Mytilus edulis* (Martin *et al.*, 1981). A graphically estimated EC 50 value for the present 48 hour abalone test is about 55 µg/l zinc (Figures 5-7), less than half that of the other reported values. Growth of adult mussels *M. edulis* may be the most sensitive indicator of toxicity to molluscs, with a 48 hour NOEC of 10 µg/l zinc (Stromgren, 1982), lower than the 39 µg/l NOEC level reported here. Reported zinc toxicity values for species from other phyla are generally higher, with 96 hour LC 50s ranging from 499 µg/l for mysids (Lussier *et al.*, 1985) to 10700 µg/l for the polychaete *Capitella capitata* (Reish, 1978). Other sensitive tests exist that do not have reported values for zinc and are not mentioned here, although some comparisons have been made using split samples of complex effluent, as described below.

The 48 hour abalone test worked well with samples of whole complex effluents. No changes in methods or endpoint determination were necessary, despite the difference in physical and chemical composition between effluent and the reference toxicant. Abalone were more sensitive to primary effluent than to secondary effluent; but because these two effluents came from different sources, and because of the variable nature of complex effluents in general, no comparison based on treatment can be made.

Based on comparisons using replicate samples of secondary effluent, the 48 hour abalone test was as sensitive to secondary effluent as the 48 hour mussel test of Dimick and Breese (1965), and less sensitive than the echinoderm sperm cell test of Dinnell *et al.* (1987) (Gary Chapman, personal communication). Both of the latter tests have been recommended for routine use in effluent toxicity evaluations (APHA Standard Methods, 1985; Peltier and Weber, 1985).

The 48 hour abalone test is a simple and sensitive bioassay. Broodstock conditioning and spawning can be accomplished year-round (Morse *et al.*, 1977, Ebert and Houk, 1984), and this is an important advantage in a routine monitoring program. The relatively low variability and low NOEC values in this study demonstrate the potential reliabilty and sensitivity of the test, while comparisons with the long term test suggest that the short term abnormalities are indicative of ecologically significant effects. Information on effluent toxicity obtained using the abalone test is useful both for discharge monitoring and for protection of an economically and ecologically important species in California.

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SECTION 3

GIANT KELP TESTS

Brian S. Anderson
John W. Hunt

Introduction To Kelp Tests

The giant kelp, *Macrocystis pyrifera* is the dominant canopy-forming alga in southern and central California. Giant kelp forms extensive submarine forests along the coast and these are home to a rich diversity of marine life. *Macrocystis* forests are among the largest and most complex ecosystems on earth, and provide the state of California with a valuable economic and ecological resource. Because of the proximity of kelp forest ecosystems to near shore pollution sources, there is increasing concern over the effects of waste discharge on *Macrocystis* and its associated marine communities.

There is reasonable indirect evidence that sewage has contributed to declines in kelp forests in Southern California. Wilson (1980) described a correlation between increased solids discharged by the City of Los Angeles at the Whites Point seawge outfall, and the disappearance of kelp forests on the Palos Verdes Penninsula. This decline was followed by a reappearance of kelp with the concurrent decrease in suspended solids discharged (see also Meistrell and Montagne 1983, Figure 7). Explanations for the disappearance of kelp forests in Southern California are confounded by the influence of other factors such as warm water temperatures, and sea urchin grazing. Declines in the Point Loma kelp forest have been attributed to these factors (North, 1976).

Several factors related to wastewater discharge have been suggested to have adverse effects on *Macrocystis*. These include decreased irradiance due to the presence of suspended solids , increased sedimentation, and poisoning by toxic chemicals. While not demonstrating a direct effect on kelp, Eppley *et al.* (1972) showed that there was increase sedimentation and decreased irradiance around ocean outfalls. Devinney and Volse (1978) demonstrated in laboratory experiments that suspended solids can kill *Macrocystis* reproductive stages. Others (Luning and Neushul 1978; Deysher and Dean 1984, 1986a, and 1986b; Dean and Jacobson 1984) have shown that light is important for kelp reproduction and sporophyte growth, and Grigg (1978) suggests that the potential area for growth of *M. pyrifera* off Palos Verdes has been suppressed by low light levels associated with the Whites Point sewage outfall. No direct correlations have been demonstrated between levels of toxicants associated with seawage discharge and declines in *Macrocystis* field populations. Clendenning (1958, 1959, 1960) used laboratory experiments to show that elevated levels of sewage effluents and various chemical compounds associated with them inhibit photosynthesis in *Macrocystis* blades. Smith (1979) found that copper inhibits the growth of *Macrocystis* gametophytes in the laboratory; copper is found in high concentrations in some sewage effluents (Schafer 1980, 1982, 1984). Because effluents constituents have a demonstrated effect on kelp, it is possible that declines in kelp beds near outfalls may be due to waste discharges.

We selected *Macrocystis* as a macroalgal bioassay test species because of its economic and ecological importance, and its amenability to laboratory culture. *Macrocystis* has been cultured extensively in the laboratory and its life history has been well described (North 1971, Luning and Neushal 1978, Deysher and Dean 1984, see review

by Foster and Schiel 1985). Its use in bioassay tests has been limited to the above mentioned studies (Clendenning 1958-1960, and Smith 1979), and studies on the trace metal requirements of early life stages of kelp by Kuwabara (1980), and Kuwabara and North (1981).

This paper presents methods and results of bioassays being developed at the State Water Resources Control Boards' Marine Bioassay Project. Results are presented on the effects of sewage effluents, and organic and inorganic toxicants on germination, growth, and fertilization of early life stages of *Macrocystis* .

METHODS

Facilities

All of the experiments were done between June, 1986 and July, 1987 at the California Department of Fish and Game's Marine Pollution Studies Laboratory (MPSL) at Granite Canyon. The laboratory is located on the Big Sur coast in Monterey County. The MPSL seawater intake is located at least 4 km from any known pollution source.

Lifecycle

These experiments used the early life stages of *Macrocystis pyrifera.* Like all Laminarian algae, *Macrocystsis* has an alternation of generations life cycle that alternates between a haploid microscopic gametophyte stage and a diploid macroscopic sporophyte stage (Figure 14). It is the sporophyte stage that forms the giant kelp forests. These plants produce reproductive blades, called sporophylls, at their base. The sporophylls develop patches (sori) in which biflagellate spores are produced. The spores are called zoospores because they swim. The zoospores are released into the water column and eventually settle onto the bottom 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 sperm which swim through the water and fertilize eggs produced by the female gametophytes. The fertilized eggs develop into sporophytes, completing the lifecycle. The entire process from zoospore release to sporophyte production can be completed on a microscope slide in the laboratory in approximately 12-16 days.

Toxicity Tests

The two bioassay protocols developed for *Macrocystis* at MPSL focus on three endpoints: germination and growth of the settled zoospores, and sporophyte production through fertilization of female gametophytes. The germination and growth endpoints are used in a short-term 48 hour bioassay. The sporophyte production endpoint is used in a long-term, 16 day, bioassay that focuses on reproduction. The short-term *Macrocystis* test is intended for

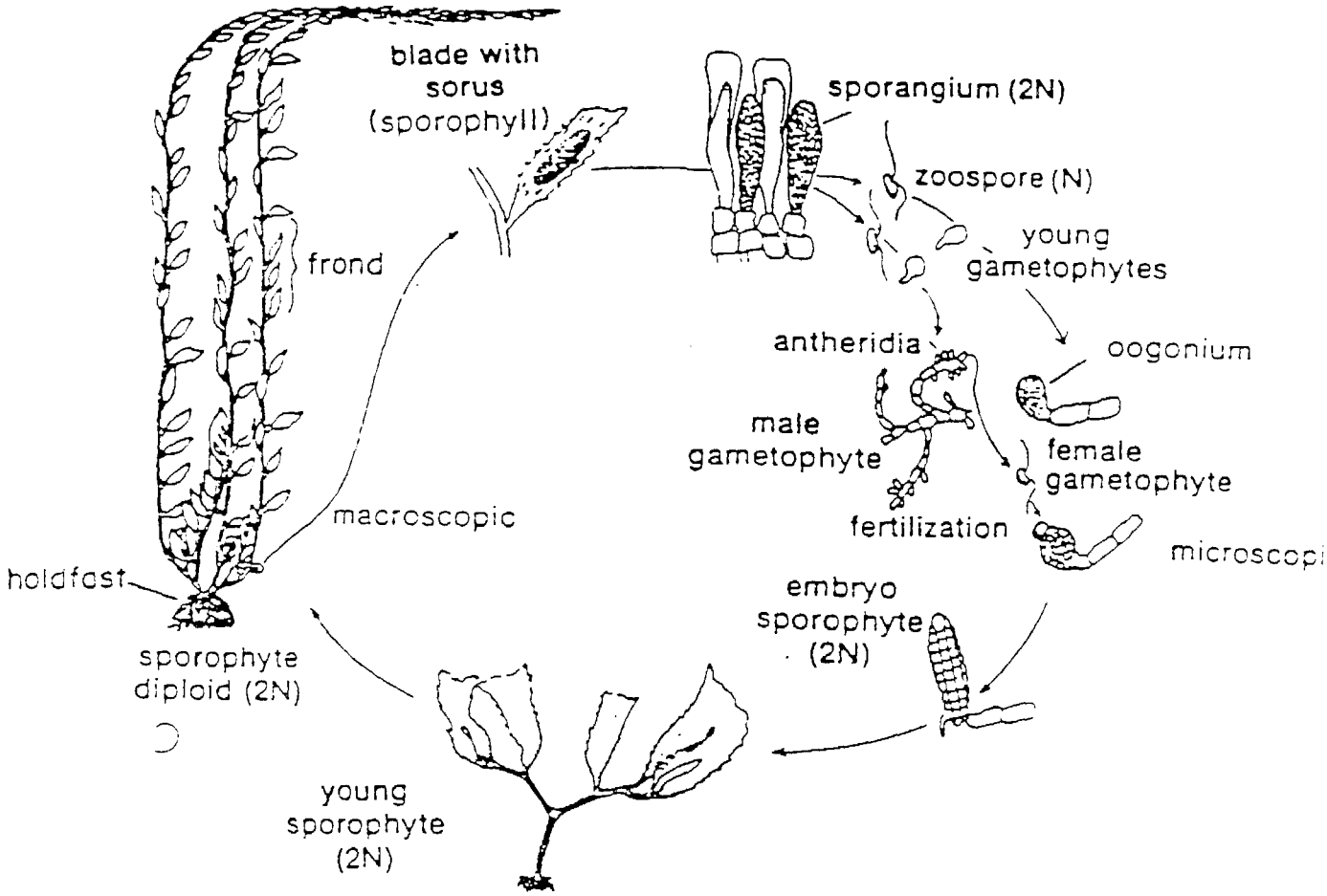


Figure 14 . The morphology and life history of Macrocystis pyrifera.j (from Foster and Schiel, 1984)

use in routine monitoring of effluents. The long-term kelp test was designed to calibrate the sensitivity, and demonstrate the ecological significance of the short-term test endpoints. A schematic diagram showing the two bioassay protocols is given in Figure 15. Detailed instructions for conducting the short-term test are given in Appendix 3. Giant Kelp Bioassay Protocol. A more detailed discussion of the long term protocol is given in Martin *et al.*, 1986.

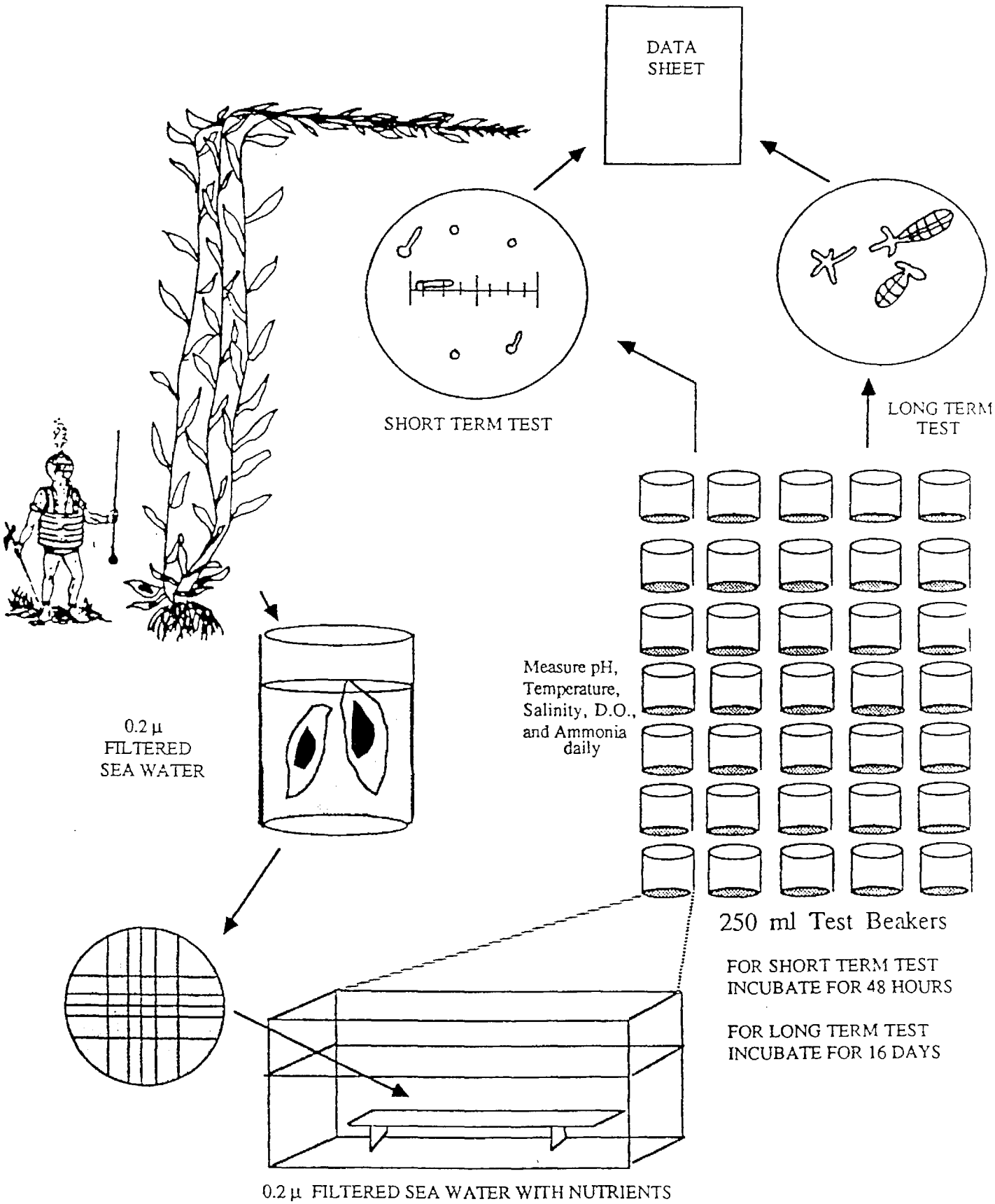
Short-term test methods

Macrocystis reproductive blades (sporophylls) were collected by skindivers from adult plants located off Granite Canyon or off Monastery Beach, Monterey County, California. The blades were brought back to the lab and rinsed with 0.2 µ filtered, U.V. irradiated seawater to remove epiphytic organisms. To induce zoospore release, we blotted the sporophylls with paper towels, let them sit in the air for 1 hour, then placed them in 15 °C filtered seawater. Zoospores were released usually within an hour. Swimming zoospores were used for all tests. The zoospore densities were measured with a hemocytometer. Four hundred and fifty thousand spores were added to each test container. For every test, we calculated the volume of spore solution necessary to give the required density of spores, then randomly distributed this volume to the test containers via pipet. To avoid test water dilution, we tried to obtain high densities of zoospores by using several ripe sporophylls in 1 l of filtered seawater. No more than 10 milliliters of zoopore solution was added to each container. A glass microscope slide was placed on the bottom of each test container, and this served as a substrate on which the spores settled and developed. At the end of the test the slide was removed and observed under 400x on a light microscope.

For all tests we used six test concentrations (5 toxicant dilutions and 1 control) each replicated five times. The test concentrations were assigned in a logarithmic sequence: 0, 10, 18, 32, 56, 100, at an order of magnitude depending on the toxicity of the toxicant. The dilution water for the short-term tests was 1 µ filtered, U.V. irradiated seawater. We used 250 mL of test solution for all tests. Water quality (dissolved oxygen, pH, temperature, and salinity) was monitored daily. Although we did not monitor the ammonia levels in the test containers during the effluent experiments, their yearly ranges are presented in Table 3. The lights used in the short-term tests were cool white flourescent lights adjusted to give 150 µE/meter sq./second at the test solution surface. These have been shown to be optimal light conditions for sporophyte production (Luning and Neushul, 1978; Deysher and Dean, 1984). All tests received continuous light. The test duration was 48 hours; the solutions were not renewed.

The test containers were either 350 ml capacity polyethylene plastic food containers (for metal bioassays) or 600 ml capacity borosilicate glass beakers (for complex effluent and organic toxicant bioassays). Plastic test containers were cleaned by rinsing with hot tap water followed by 24 hour sequential soaks in solutions of mild detergent, 3N HCL, and deionized water. Glass test containers were cleaned by triple rinsing with hot tap water and

FIGURE 15. KELP TOXICITY TEST PROTOCOLS



reagent grade acetone, followed by 24 hour soaks in 3N HCL, and deionized water.

The endpoints measured after 48 hours were germination and germ-tube length (see Figure 16). Germination was considered to be unsuccessful if no germ tube was visible. To differentiate between germinated and non-germinated spores we determined whether they were circular (non-germinated) or had a protuberance of at least 1 spore radius (at least 3 μ = germinated). The first 100 spores encountered while moving across the microscope slide were counted for each replicate of each treatment. The growth endpoint was the measurement of the total length of the spore and germination tube. For this endpoint only germinated spores were measured. The spores to be measured were randomly selected by moving the microscope slide to a new field without looking through the ocular lens, spinning the ocular lens, then measuring the length of the spore that was touching the micrometer. If more than one spore was touching the micrometer, both (or all) were measured. A total of 10 spores for each replicate of each concentration were measured.

Interlaboratory tests

To assess the ability of contract laboratories to use the *Macrocystis* short-term protocol, we conducted an interlaboratory test of the short-term (48 hour) kelp protocol with the Southern California Coastal Water Research Project (SCCWRP) bioassay laboratory, using zinc (0 - 5,600 μg/l) as the toxicant. For this exercise we collected *Macrocystis* sporophylls at Monastery Beach and held them in a refrigerator (5 ° C) for 12 hours, then transported them in an ice chest (~ 8 °C) to the SCCWRP lab. The total holding time of the sporophylls was 36 hours. The SCCWRP personnel then conducted a 48 hour short-term kelp bioassay. Within the same week a second 48 hour kelp test was done at MPSL with a separate set of sporophylls held under the same conditions for the same amount of time. We attempted to duplicate the experimental conditions for both tests by adhering to the written protocols, and by using the same zinc, test containers, lighting, and temperature. To control for any variation caused by using different dilution waters, we ran a duplicate control for the MPSL interlab test with SCCWRP dilution water.

Long-term test methods

The test conditions were the same for the short-term and long-term tests except for the following modifications. The dilution water used was 0.2 μ filtered, autoclaved seawater. The long-term tests were sixteen day static renewal bioassays with media renewals at 4 day intervals. For supplemental nutrients we added full strength PES (Provasoli 1968) enriched growth solution to the test media (minus EDTA chelated iron; 20 mL PES /l test solution). One hundred thousand spores were added to each test container at the start of the test. The lights used in the long-term tests were Duro-lite® Vitalights, a full spectrum light suitable for inducing gametogenesis. These were adjusted to give 150 μE/ meter sq./ second.

The endpoint for the long-term test was sporophyte production (see Figure 17). After 16 days the

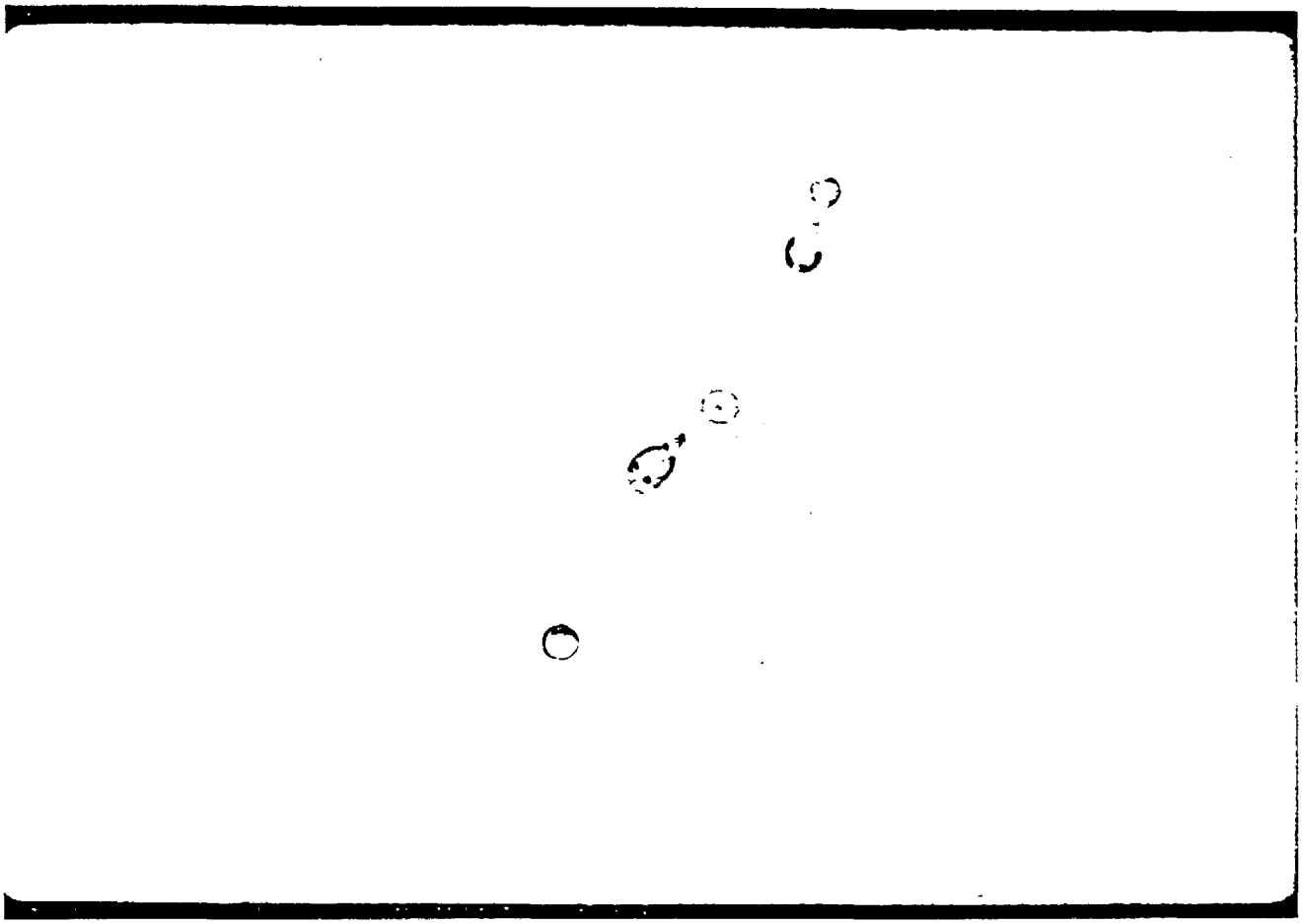


Figure 16. Germinated (upper two) and non-germinated (lower) spores of *Macrocystis* after 48 hours (400x magnification)

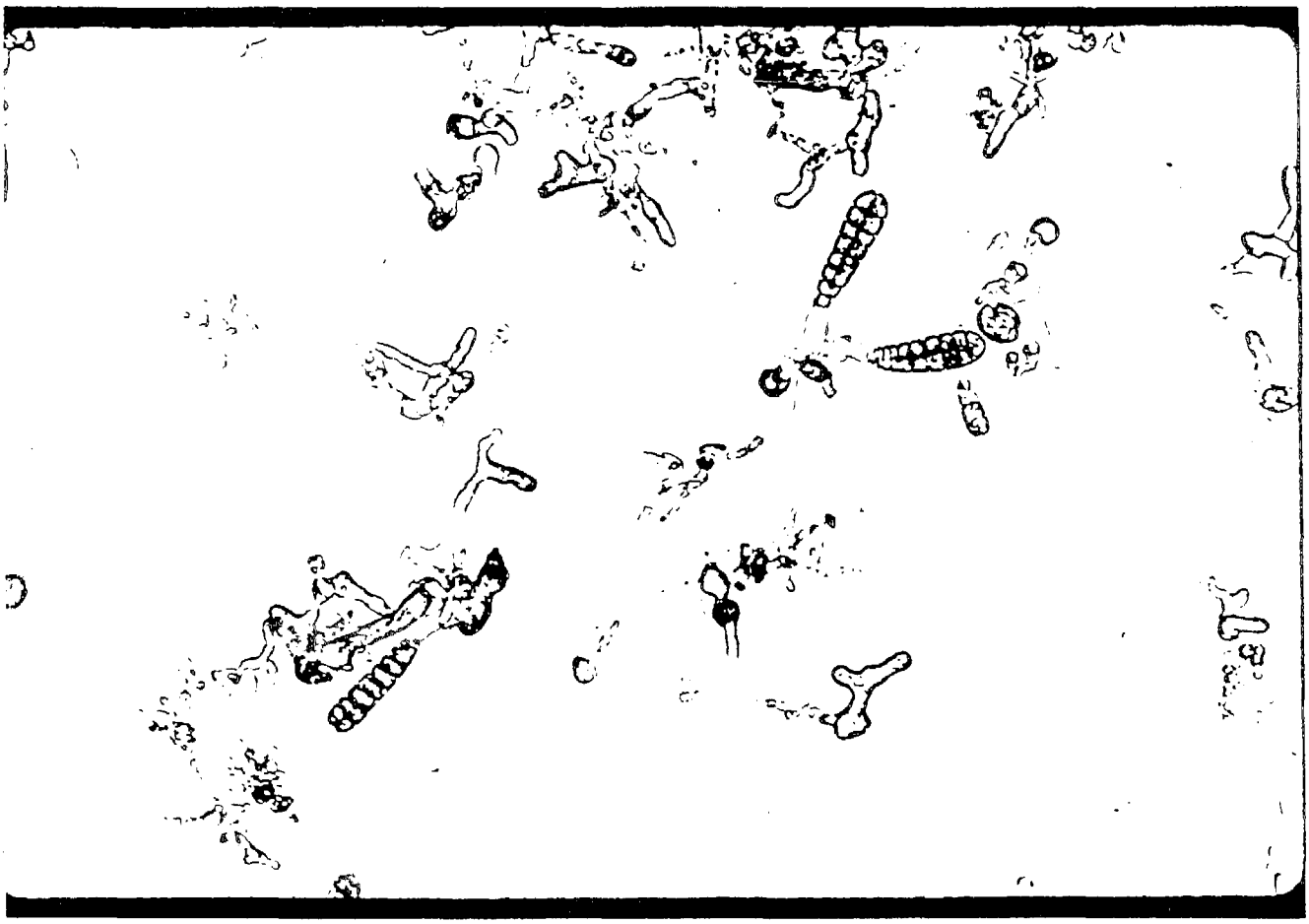


Figure 17. Sporophytes of *Macrocystis* in 16 day old cultures. The sporophytes are the multicellular structures (upper right and lower left). The other structures are non-reproductive male and female gametophytes.

microscope slide was removed from the test container and observed under 100x magnification on a light microscope. We counted the number of sporophytes visible in three 18mm vertical swaths on each replicate, then took a mean of the 3 swaths to give us the replicate mean. Only sporophytes with at least 3 or more cell divisions were counted.

The long-term test protocol was modified for the secondary effluent test. For this test we cultured the gametophytes in clean (control) seawater for 8 days then exposed them to the effluent. These gametophytes were just at the stage when they were beginning to produce gametes. After a 4 day exposure to the effluent, we transferred the slides back into clean seawater and let them continue to develop for 4 more days. By exposing the reproductive gametophytes to the effluent for 4 days instead of the usual 16 day chronic exposure, we hoped to focus on the effect of effluent on fertilization of the eggs. The experimental conditions of this test were otherwise unmodified from the above protocol.

Toxicants

We tested the effects of four different toxicants on the early life stages of *Macrocystis* . We tested one metal salt, one organic toxicant, and two sewage treatment plant effluents: one from a primary treatment facility, and one from a secondary facility. The metal salt tested was zinc sulfate (see Appendix 1 for a discussion of the criteria used to select zinc sulfate as a reference toxicant). We conducted 3 short-term zinc tests (@ 0 - 10,000 µg/l zinc) to assess between-test variability, and 1 long-term zinc test (0 - 3,200 µg/l zinc) to calibrate the short-term tests. Zinc was also used as the toxicant in the short-term interlaboraory tests. Zinc concentrations were verified using a Perkin Elmer model 603 atomic absorption spectrometer at the California State Mussel Watch analytical facility. The zinc solutions were sampled from 1 replicate of each treatment concentration at the start and end of each experiment. Unless otherwise noted, all reported zinc values are analytically verified concentrations.

To assess the toxicity of an organic toxicant to *Macrocystis* we conducted 1 short-term and 1 long-term test with sodium pentachlorophenate (0 - 320 µg/l).

In addition, we did one short-term test each with a primary and a secondary effluent, and 1 long-term test with the secondary effluent (all @ 0 - 56% effluent) . The primary effluent was from a single grab sample. The secondary effluent was from a 24 hour composite sample of chlorinated-dechlorinated effluent.

Statistics

All of the data were analyzed using analysis of variance (ANOVA) followed by Dunnett's multiple comparison test to compare each concentration to the control (Zar, 1974; Sokal and Rohlf, 1969). The percentage data (germination and sporophyte production) were first transformed to the arcsine of their square root before the ANOVA. The length data were not transformed. For each test, the No Observed Effect Concentration (NOEC) was calculated as the highest concentration not significantly different from the control at $p = .05$.

RESULTS

The results of all of the toxicity tests with kelp are summarized in Table 3. The NOECs for the zinc and NaPCP tests are given as µg/l. The NOECs for the complex effluent tests are given as percent effluent.

Table 3. NO OBSERVED EFFECT CONCENTRATIONS FOR PHASE 2 KELP TESTS

Short-Term Zinc Tests (µg/l)	TEST NUMBER		
	1	2	3
48 Hour Repetitive Tests - Germination	2033	5495	1732
- Length	<1090	<589	<553

Interlaboratory Zinc Tests (µg/l)	MPSL	SCCWRP
Short-term (48 hour) - Germination	957	§
- Length	<538	<559

Long-Term Zinc Test (µg/l)	
16 Day - Sporophyte Production	1071

Sodium Pentachlorophenate Tests (µg/l)	
Short-Term (48 hour) - Germination	<32
Long-Term (15 day) - Sporophyte Production	<32

Effluent Tests (percent)	PRIMARY	SECONDARY
Short-Term (germination)	0.56	18**
(length)	n.d.	32**

MPSL = Marine Pollution Studies Laboratory; SCCWRP = Southern California Coastal Water Research Project
§ No significant difference due to poor germination in controls.
** NOEC derived by comparison with dilution water control because comparisons with brine controls gave no significant difference due to brine toxicity.

Water Quality

The water data for all of the experiments are presented in Table 4. In general, the water quality parameters for all of the tests were within normal ranges. The exception was a certain degree of variability in the pH and temperature values in some of the tests, in particular the long-term zinc test and the effluent tests.

TABLE 4. WATER QUALITY RANGES FOR KELP TESTS

Test	Dissolved Oxygen mm.Hg.	pH	Salinity ppt.	Temperature degrees C.
Short-term #1	136 - 159	8.40 - 8.55	34 - 35	16.0 - 17.0
Short-term #2	148 - 161	8.50 - 8.60	34 - 35	11.5 - 15.5
Short-term #3	150 - 161	8.50 - 8.60	34 - 36	13.0 - 14.5
Inter-Lab	156 - 161	8.40 - 8.55	33 - 37	14.8 - 16.4
Long-term	147 - 159	8.55 - 8.90	32 - 39	11.5 - 17.0
Primary	140 - 153	8.50 - 8.85	35 - 36	11.0 - 12.5
Secondary S.T.	148 - 158	8.50 - 8.60*	35 - 37	14.8 - 15.5
Secondary L.T.	150 - 158	8.35 - 8.70†	32 - 36	13.8 - 16.5

* Brine control 8.65 - 9.10

† Brine control 8.15 - 9.15

Kelp Multiple Zinc Tests

The results of the multiple short-term kelp tests show that zinc sulfate has less of an inhibitory effect on germination of *Macrocystis* spores than it does on the elongation of spore germ tubes (Figures 18 - 23). Germination of *Macrocystis* spores was significantly inhibited at concentrations above 2033 µg/l zinc in test #1, 5495 µg/l in test #2, and 1732 µg/l zinc in test #3 (fig.s 18 - 20 respectively). These values represent the No Observed Effect Concentrations (NOECs) for tests 1 - 3 respectively (mean = 3087 ± 2091 µg/l (s.d. for 3 tests). The reason for the relatively high variability in germination NOECs between the 3 tests is unknown, and could be related to several factors (see discussion). Tests #1 and #3 were had the same nominal (not analytically verified) NOEC.

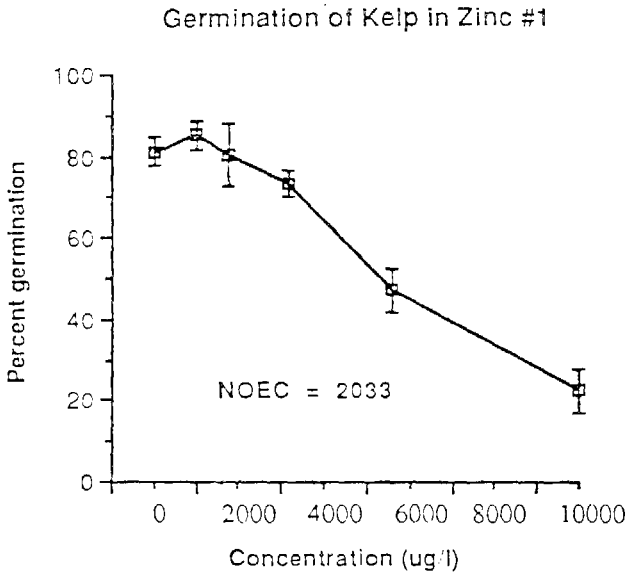


Figure 18. Germination of *Macrocystis* zoospores after 48 hours in zinc, test #1. Mean ± 1 standard deviation. N =5.

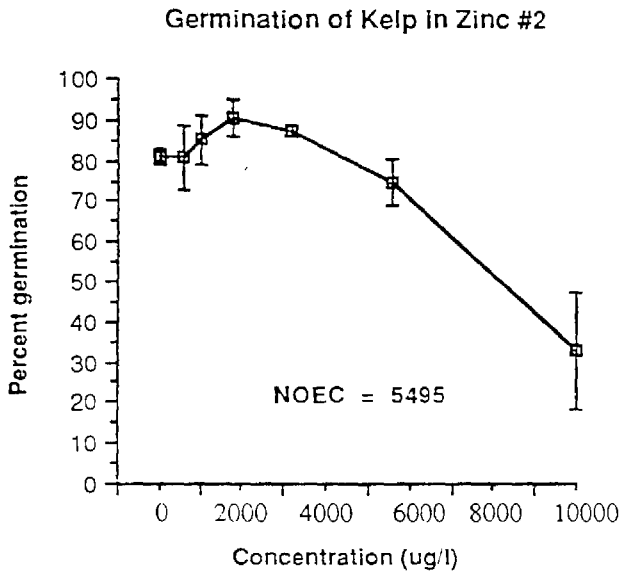


Figure. 19. Germination of *Macrocystis* zoospores after 48 hours in zinc, test #2. Mean ± 1 standard deviation. N= 5.

Although the 560 µg/l treatment (nominal concentration) was significantly different from the control treatment in test #3 (Fig. 20), subsequent concentrations were not. The NOEC was therefore set at 1732 µg/l (= 1800 µg/l nominal concentration) because all concentrations greater than this were significantly different from the control.

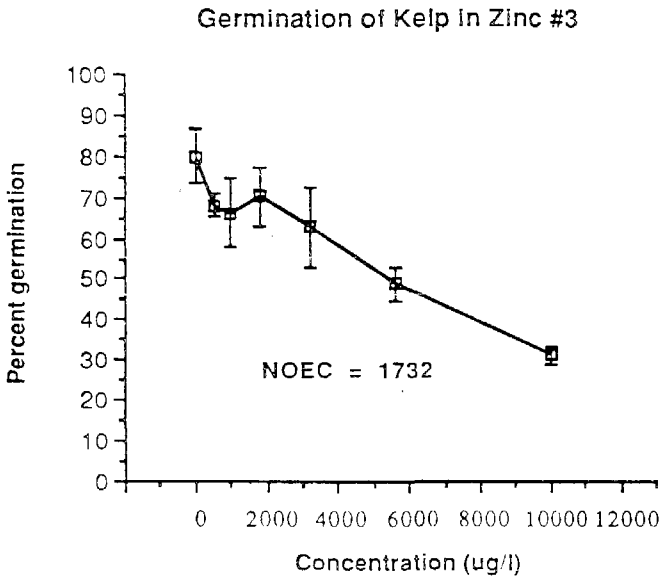


Figure. 20. Germination of *Macrocystis* zoospores after 48 hours in zinc, test #3. Mean ± 1 standard deviation. N = 5.

Elongation of the *Macrocystis* spore germination tubes was significantly inhibited at the lowest zinc concentrations for all three tests (Figures 21 - 23). The germination tube length NOECs were <1090, <589, and <553 µg/l for tests #1 - #3 respectively (mean = < 571 ± 25.5 µg/l; Note that this is the mean of tests 2 and 3. Test #1 did not include a 560 µg/l nominal concentration).

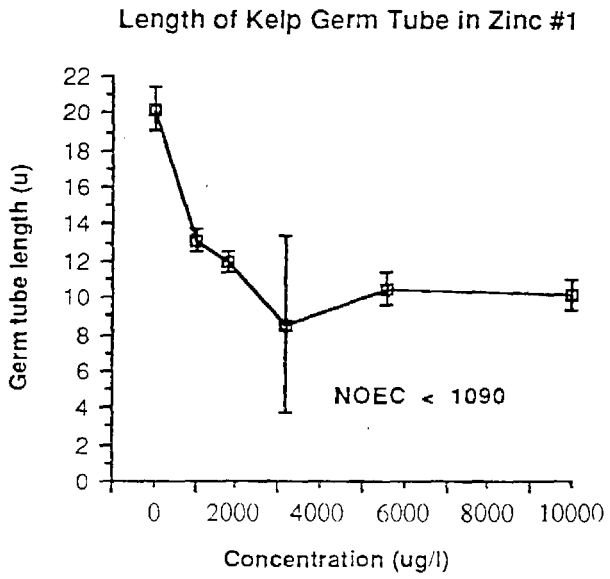


Figure 21. Length of *Macrocystis* germination tubes after 48 hours in zinc, test #1. Mean ± 1 standard deviation. N = 5. *Note: 1090 µg/l was the lowest zinc concentration in this test.

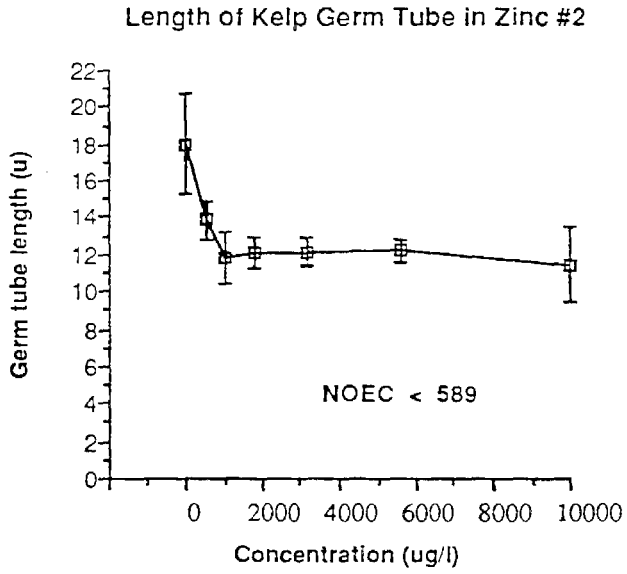


Figure 22. Length of *Macrocystis* germination tubes after 48 hours in zinc, test #2. Mean ± 1. standard deviation. N = 5.

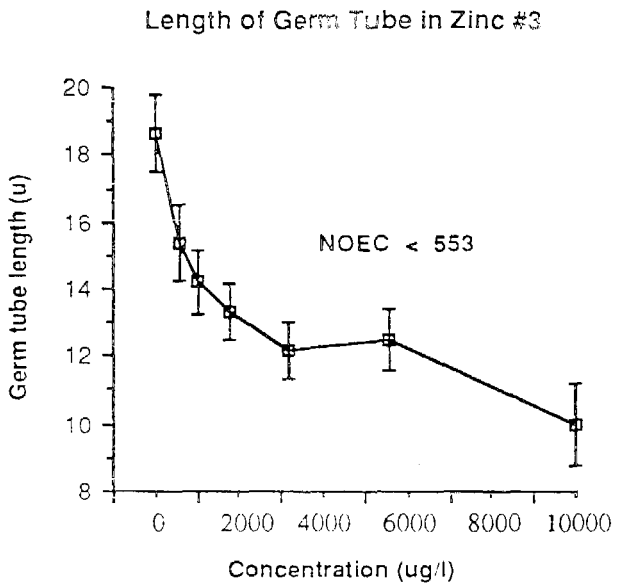


Figure 23. Length of *Macrocystis* germination tubes after 48 hours in zinc, test #3. Mean ± 1. standard deviation. N = 5.

Kelp Interlaboratory Zinc Tests

The results of the interlaboratory zinc tests indicate that the short-term kelp protocol can be conducted by other laboratories. Germination of kelp in the short-term interlaboratory test at MPSL was significantly inhibited above 957 µg/l zinc (= NOEC, Figure 24). There was no significant difference in germination in the SCCWRP short-term kelp test due to unusually low germination in all treatments (< 25%, Figure 25, see discussion for possible reasons for the low germination success in the SCCWRP test).

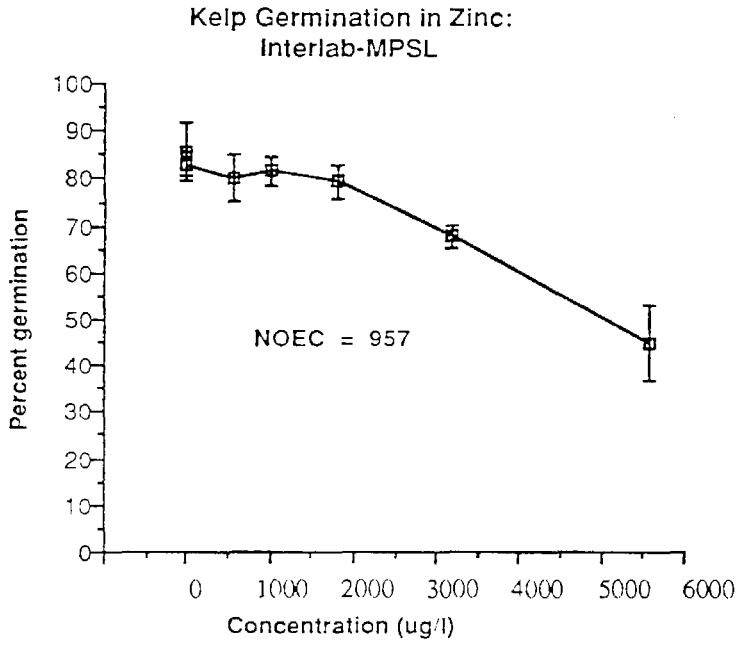


Figure 24. Germination of *Macrocystis* zoospores after 48 hours in the zinc- interlaboratory test at MPSL. Mean ± standard deviation. N = 5.

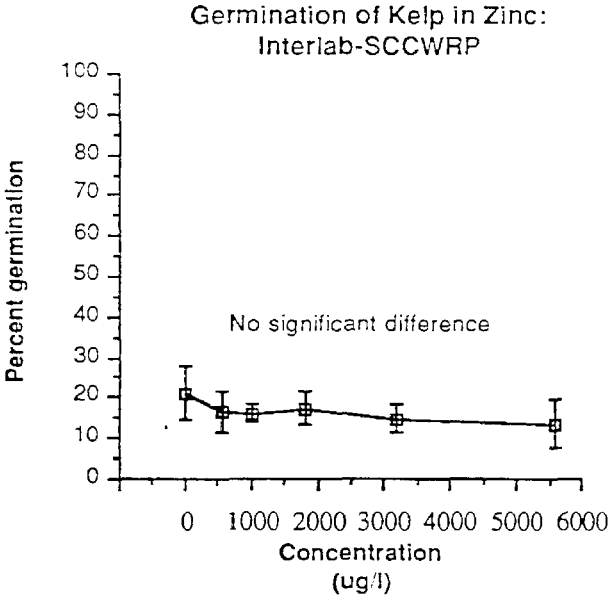


Figure 25. Germination of Macrocystis zoospores after 48 hours in the zinc- interlaboratory test at SCCWRP. Mean ± standard deviation. N = 5.

Elongation of the spore germination tubes were inhibited by relatively low concentrations of zinc in both the MPSL and SCCWRP interlab tests despite the low germination success in the SCCWRP test (Figures 26 and 27). Germ tube lengths were inhibited at less than 538 µg/l zinc in the MPSL test, and at less than 559 µg/l zinc in the SCCWRP test. The lengths of the germination tubes were similar in both tests.

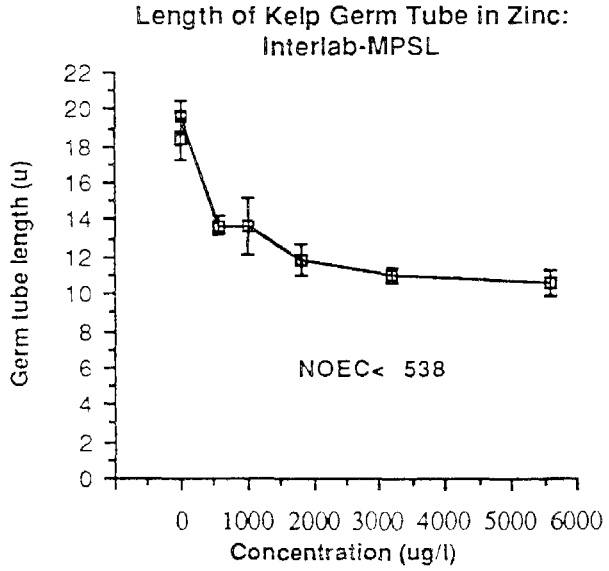


Figure 26. Length of *Macrocystis* germination tubes after 48 hours in the zinc- interlaboratory test at Granite Canyon. Mean length in microns ± standard deviation. N = 5.

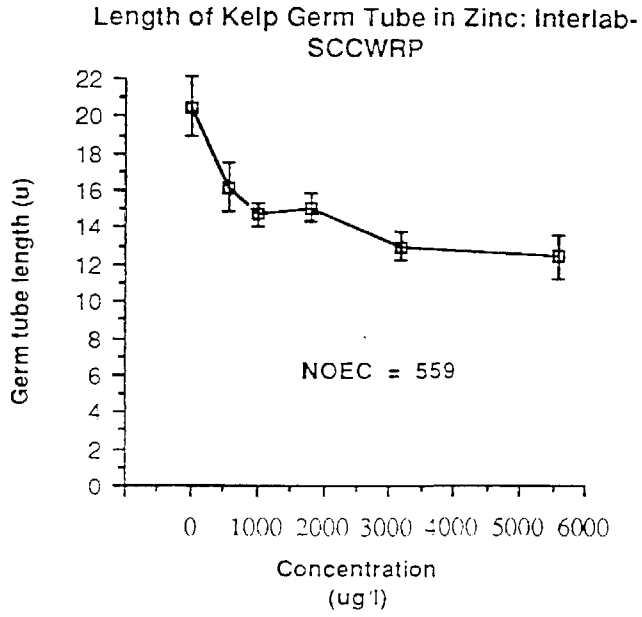


Figure 27. Length of *Macrocystis* germination tubes after 48 hours in the zinc- interlaboratory test at SCCWRP. Mean length in microns ± standard deviation. N = 5.

Long-Term Zinc Test

The production of *Macrocystis* sporophytes in the long-term test was significantly inhibited at concentrations above the NOEC value of 1071 µg/l zinc (Figure 28). This long-term test NOEC was lower than the short-term germination NOEC (1071 µg/l for sporophyte production vs 3087 ± 2091 µg/l for germination) but higher than short-term germ tube length NOEC (1071 vs <571 ± 25.5 µg/l). Although the lowest zinc treatment (320 µg/l = nominal) was significantly different from the control treatment, subsequent treatments were not. The NOEC was therefore set at 1071 µg/l because, as discussed for the third short-term germination test, all of the treatments greater than this were significantly different from the control.

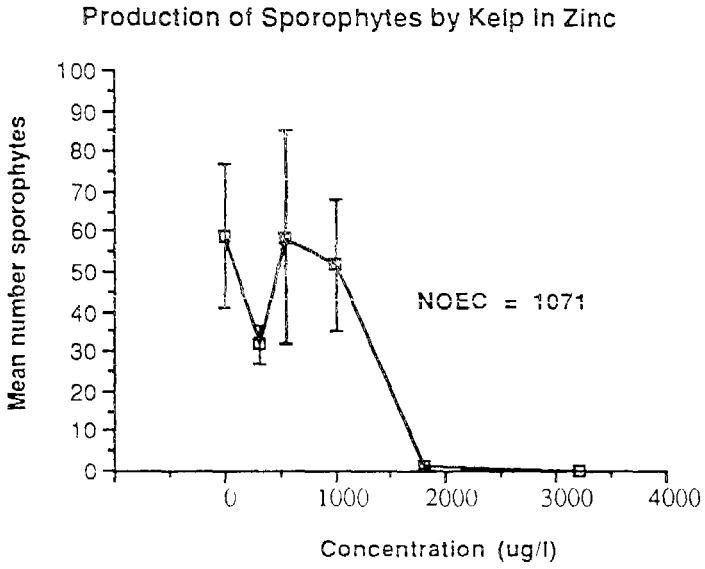


Figure 28. Production of sporophytes by *Macrocystis* gametophytes exposed to zinc for 16 days. Mean ± 1 standard deviation. N = 5.

Kelp NaPCP Tests

The organic toxicant sodium pentachlorophenate (NaPCP) significantly inhibited germination of *Macrocystis* zoospores in the short-term test, and sporophyte production in the long term-test at concentrations much lower than those of zinc. The NOECs for both tests were less than 32 µg/l NaPCP. The NOECs for both NaPCP tests (Figures 29 and 30) are from nominal, not analytically verified, concentrations.

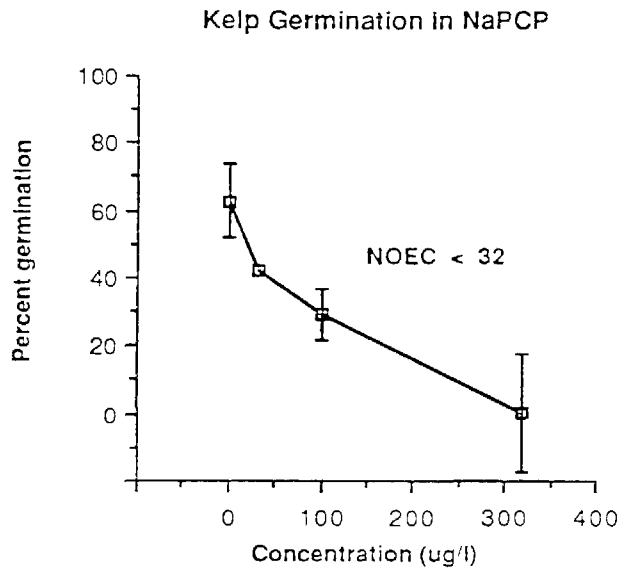


Figure 29. Germination of *Macrocystis* zoospores after 48 hours in sodium pentachlorophenate. Mean ± standard deviation. N = 3.

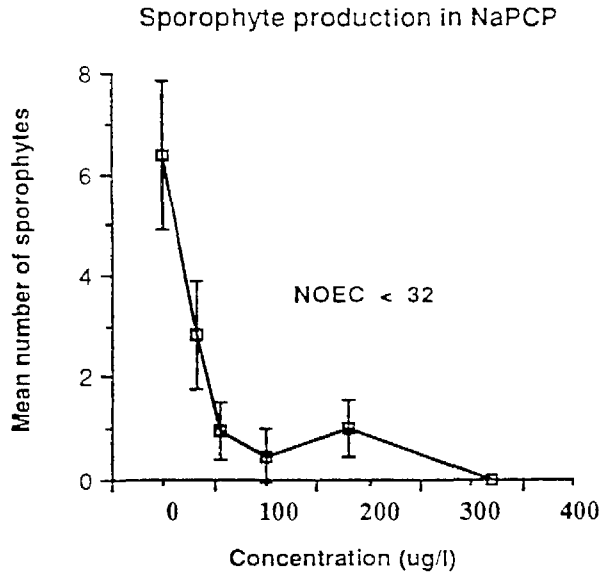


Figure 30. Production of sporophytes by *Macrocystis* after 15 days in sodium pentachlorophenate. Mean ± standard deviation. N = 5.

Kelp Effluent Tests

Primary treated sewage effluent significantly inhibited the germination of *Macrocystis* zoospores at concentrations above 0.56% effluent after 48 hours (Figure 31). No length data were taken during this experiment.

Primary effluent concentrations greater than 1% effluent had salinities adjusted to 34 µg/l using a hypersaline brine. The brine was prepared by aerating and heating MPSL seawater to no more than 40 °C (as recommended by the US EPA). No brine control was used in the primary effluent test.

Although the ammonia levels in the effluent tests were not measured, the yearly total ammonia levels ranged between 20-49 mg/l in the primary effluent (as communicated by the treatment plant staff).

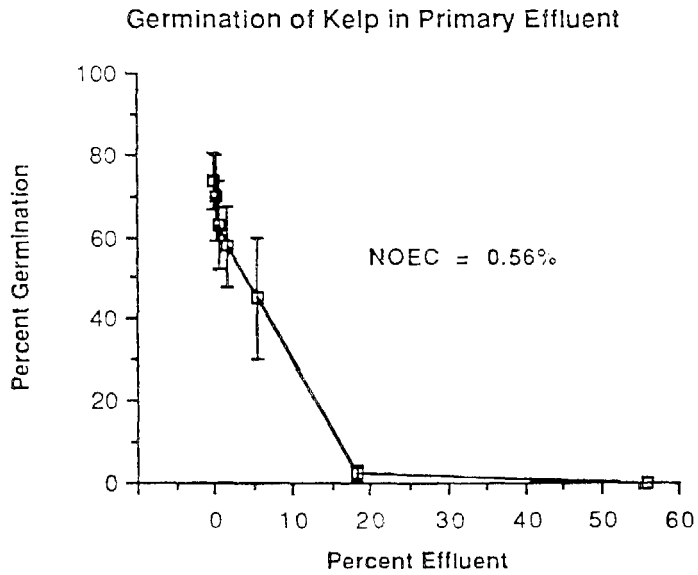


Figure 31. Germination of *Macrocystis* zoospores after 48 hours in primary effluent. Mean ± standard deviation. N = 5.

Because of toxicity problems in the brine controls, we cannot distinguish between effects caused by the effluent and those caused by the brine. Brine was used to adjust the salinity in all of the secondary effluent concentrations, and a brine control was used in these tests. The brine controls were prepared by diluting the maximum

amount of brine used at the highest concentration (56% effluent = 400 mL/l brine) to 34 ppth with distilled water. Because of logistical constraints, the brine for the secondary effluent tests was aerated and heated at 100 °C for several days to rapidly increase its salinity (to 85ppth). Apparently heating the brine at this temperature drove off the carbon dioxide, increasing the pH. The pH values for the brine and brine controls were above normal ranges (Table 4), and this may have caused a toxic effect.

The NOEC values reported here for the secondary effluent tests are based on comparisons with the dilution water controls. Because of brine problems these tests are not valid. Based on comparisons with the dilution water controls, secondary effluent (or the brine) inhibited zoospore germination at concentrations above 32% effluent (Figure 32). The same effluent (or brine) inhibited germ tube elongation at concentrations above 18% effluent (Figure 33). Regardless of the brine problems, the secondary treated sewage effluent was apparently less toxic to *Macrocystis* zoospores than was the primary effluent.

The yearly total ammonia levels ranged between 18 to 22 mg/l in the secondary effluent (as communicated by the treatment plant staff).

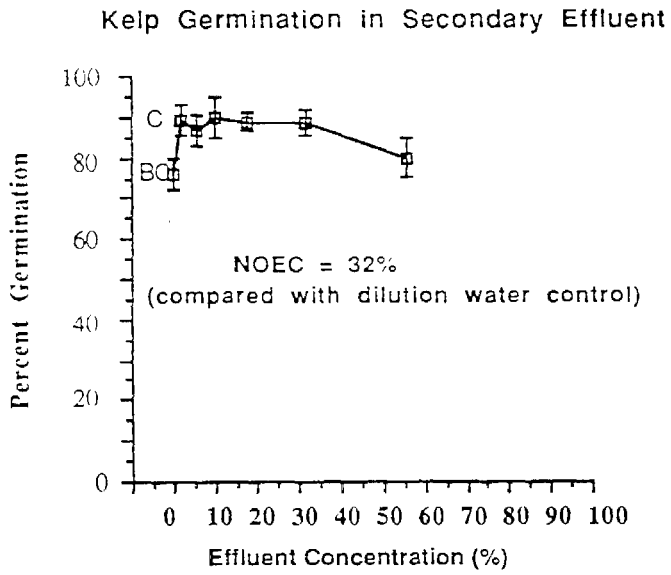


Figure 32. Germination of *Macrocystis* zoospores after 48hrs in secondary effluent. Mean ± s.d.. N = 5.

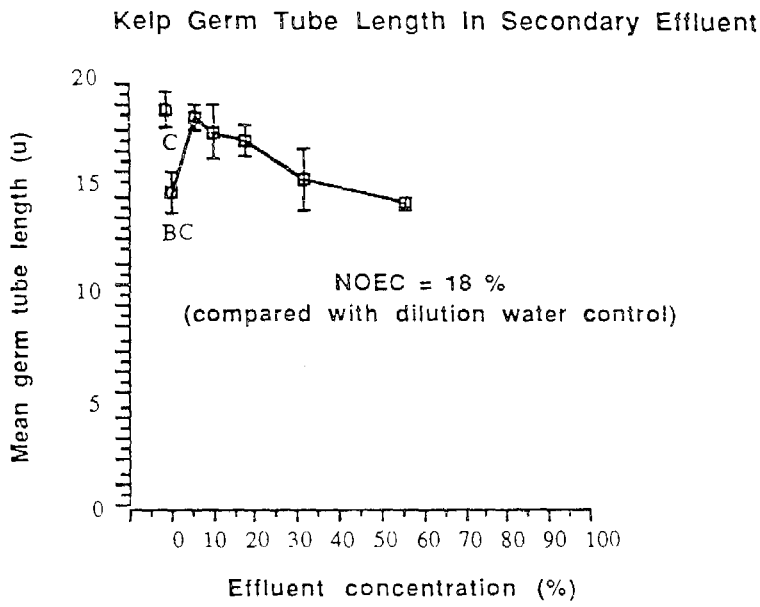


Figure 33. Length of *Macrocystis* germination tubes after 48 hours in secondary effluent. Mean ± standard deviation. N = 5.

Sporophyte production was apparently not siginificantly inhibited by the secondary effluent based on ANOVA statistics (Figure 34). Although the average number of sporophytes was highest in the controls, problems with the brine controls, and high variability in sporophyte production within replicates of each treatment concentration make any interpretation of these results speculative. As in the 16 day sporophyte test with zinc sulfate, the within replicate variability was relatively high in the secondary effluent test, and was probably a reflection of an insufficient number of swaths counted per replicate slide (see discussion). As described in the methods section, this test was a 4 day exposure of 8 day old pre-reproductive kelp gametophytes; after 4 days the gametophytes were transferred to control water and cultured for an additional 4 days before terminating the test (see discussion for implications of this variation in the long-term kelp test methods).

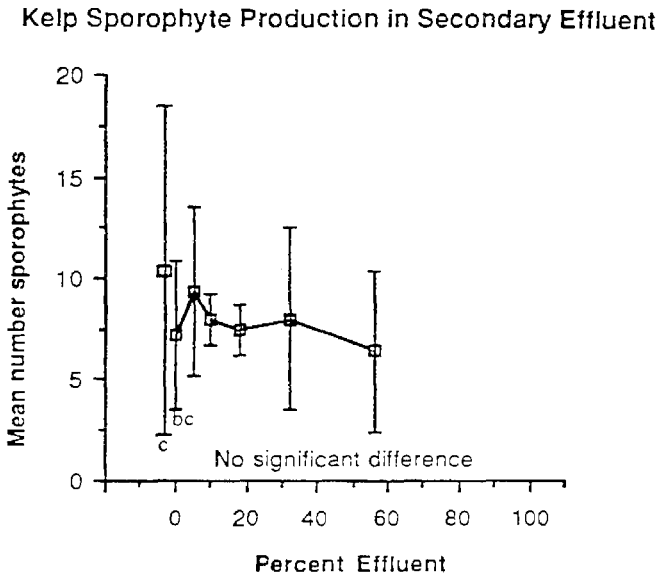


Figure 34. Production of sporophytes by 8 day old *Macrocystis* gametophytes after 4 days in secondary effluent. Mean ± standard deviation. N = 5. C = dilution water control; BC = brine control.

Discussion

The results of the experiments presented here indicate that the early life stages of *Macrocystis pyrifera* are suitable for use in bioassay testing, and vary in their sensitivity to different toxicants (Table 3). Zinc sulfate is apparently less toxic to kelp zoospores and gametophytes than is the organic toxicant sodium pentachlorophenate. Complex sewage effluents vary in toxicity to kelp depending on the level of waste treatment. It was not possible to conclusively interpret the results of the secondary effluent tests because high variability and problems with the hypersaline brine confounded the results of these experiments.

Zinc Toxicity

The results of the multiple short-term (48 hour) zinc tests indicate that the two endpoints, zoospore germination and germ tube elongation, are significantly inhibited at different concentrations of zinc. Although between replicate variablity was low for the percent germination data (Fig.s 18-20), between test variability for the 3 tests was relatively high. The NOECs for the 3 tests ranged between 1732 and 5495 µg/l zinc (2033, 5495 and 1732 µg/l zinc for tests #1-3 respectively). The NOECs for the first and third tests were similar; both had the same nominal NOEC (1800 µg/l). The NOEC for test # 2, however, was more than twice this value (nominal NOEC = 5600 µg/l). The reason(s) for this variability are not known. All 3 tests were done within one month, so seasonal effects are unlikely. It is possible that there is variability in tolerance to zinc among reproducive stages of different adult kelp plants, but this is an unlikely cause of between-test variability because the sporophylls used in each test came from several different adult plants. Another possible explanation is variability in test methods or test conditions. In particular, the timing of spore release and test duration were slightly different for the 3 tests. It is not clear whether these slight changes affected the results of the experiments. Although the water quality data for the 3 tests were all within normal ranges (Table 4), there was some variability between the tests, especially in temperature. In addition, variability in all of the kelp tests with zinc may simply be a reflection of the insensitivity of *Macrocystis* to this trace metal.

Apparently it is the free ion form of trace metals (in this case the divalent cation of zinc) that are biologically more available and therefore toxic to marine algae (Sunda and Guilliard ,1976; Anderson and Morel, 1978; Kuwabara and North,1981). However, free ion concentrations of zinc vary by as much as an order of magnitude from total concentrations (Kuwabara and North 1981). This might explain some of the variability in the germination data. Differences in complexation of zinc ions might have occurred in the three tests because of different organic ligands present in the dilution water or possible zinc percipitates due to high concentartions of zinc.

It is also uncertain what physiological effect high concentrations of zinc might be having on zoospore

germination. Zinc is an important micronutrient for kelp. Kuwabara (1980) suggests that the primary biochemical role of zinc is in auxin metabolism, and that zinc is a cofactor in numerous enzyme systems. Whether high levels of zinc affect these biochemical processes is not known. The results of the first two short-term zinc tests indicate that germination of kelp zoospores increased initially with increasing zinc concentrations, before declining at higher concentrations (Figures 18 and 19). Although this indicates that zinc, an important micronutrient, was limiting in the dilution/control water, this type of initial increase in response to a toxicant followed by a decline is typical of dose-response curves.

Germ tube elongation is apparently a more sensitive and less variable endpoint than germination. The NOECs for length in all of the zinc tests were lower than the lowest concentrations tested (Figures 21-23). Even though germ tube length is a more sensitive and less variable endpoint than zoospore germination, length may not be as relevant as zoospore germination from an ecological perspective. Germination is obviously relevant because if the spore does not germinate it will not develop. The relevance of germ tube elongation is less clear. The elongation of the germination tube precedes the process of cytoplasm extrusion, in which the contents of the spore are transferred to the first gametophytic cell. The length of the germination tubes in the control spores were noticeably longer, but it is not clear if this means they were more "healthy" than spores with shorter germ tubes. Growth is an integration of a number of physiological processes, and for many organisms it has been considered an important indication of toxic effect and nutrient deficiency. Growth has been used as an endpoint in previous experiments involving *Macrocystis*. Kuwabara and North (1981) used gametophyte growth as an indicator of trace metal depletion and toxicity. They found significant decreases in gametophytic growth at 250 nMol (16.35 µg/l) zinc chloride. In addition, Smith (1979) used elongation of developing gametophytes as an indicator of copper toxicity. She found that vegetative growth of kelp gametophytes was siginificantly inhibited at 50 µg/l copper.

Interlaboratory Tests

The results of the interlaboratory zinc tests between the Marine Pollution Studies Laboratory and Southern California Coastal Water Research Project demonstrate that the short-term *Macrocystis* test can be conducted by other laboratories. The written short-term protocol was apparently easy to follow, and the endpoint for the test was relatively straight forward (personal communication, Mr. Steven Bay, SCCWRP). The NOEC for the germination test at Granite Canyon was 957 µg/l. Germination in the SCCWRP test was too low to detect a toxic effect. Germination was low probably because of temperature shock to the sporophylls due to low holding temperatures. *Macrocystis* sporophylls are apparently sensitive to long or adverse holding conditions, so a better transport method needs to be devised for future interlab tests.

The NOECs for the length endpoint was less than 559 µg/l for both tests (MPSL NOEC < 538 µg/l; SCCWRP NOEC < 559 µg/l). The NOEC values for the interlab tests were close to those calculated for the 3

short-term length tests (Fig.s 21-23, and 26-27). The lengths of the germination tubes were similar in both tests. The control germ tube lengths were approximately 20 μ in both tests, and the germ tube lengths in the highest (5600 μg/l = nominal) zinc concentration declined to approximately 11 or 12 μ in both tests (Fig.s 26-27).

Long-Term Zinc Test

Sporophyte production in the long-term test was significantly reduced above the NOEC of 1071 μg/l zinc. Reproduction in the long-term test was apparently more sensitive to zinc than zoospore germination, but less sensitive than germ tube elongation. The mean NOEC for the germination endpoint in the 3 short-term zinc tests was 3087 ± 2091 μg/l. The mean NOEC for the germ tube length endpoint in short-term tests #2 and 3 was <571 ± 26 μg/l. Chung and Brinkhuis (1986) found that sporophyte production by *Laminaria saccharina* was more sensitive to copper than was meiospore settlement and germination.

It is possible that the variability in the long-term test resulted in a higher apparent tolerance to zinc (Fig. 28). This variability resulted from differences in sporophyte density among swaths on individual slides. The number of vertical swaths counted on each replicate slide was probably insufficient to eliminate within replicate variability, and this may have reduced the apparent sensitivity (increasing the NOEC) in the long-term test. If this was the case, it might be that the short-term germ tube elongation NOECs would more closely correspond to the long-term NOEC in the absence of this variation. Conversely, the short-term effect of germ tube elongation might not lead to a long-term effect in reproduction and sporophyte production. A more thorough long-term test with a greater number of swaths read per replicate is planned. By reducing within replicate variability in the long-term test, we hope to answer this question.

As in the short-term test, it is not clear what toxic effect high levels of zinc have on the reproduction process. One hypothesis for the mechanism of toxicity in *Macrocystis* reproduction is that toxicants interfere with fertilization of the female egg by the male sperm. Maier and Mueller (1986) have shown that in another laminarian alga (*Laminaria digitata*) the sperm is attracted to the egg by a pheromone. It is possible toxicants somehow interfere with the ability of the sperm to find the egg. This might be true especially for organic toxicants (personal communication, Dr. R. Steele, U.S. EPA , Narragansett R.I.). However, it is not known if zinc interferes with fertilization by disrupting chemotaxis of the sperm to the egg.

Sodium Pentachlorophenate Toxicity

Macrocystis is more sensitive to sodium pentachlorophenate than it is to zinc sulfate. Germination in the short-term test was significantly inhibited at or below 32 μg/l NaPCP, the lowest test concentration. Germ tube length was not measured in this test because this endpoint had not yet been developed at this point. Clendenning (1959) also found NaPCP to be more toxic to kelp blades than zinc sulfate; he found a significant reduction in

Macrocystis photosynthetic activity after a 48 hour exposure to 1 mg/l NaPCP.

Sporophyte production was also significantly inhibited at or below 32 µg/l in our 15 day long-term kelp test. Replicate short-term tests with an organic toxicant are now being planned to further examine between-test variability, and the sensitivity of germ tube elongation to an organic toxicant. Germination and sporophyte production exhibited similar sensitivities to NaPCP, suggesting the short-term test is a good indicator of the chronic toxicity of this compound.

Effluent Toxicity

The results of the kelp bioassays with complex effluents indicate that the short-term bioassay protocol using *Macrocystis* zoospores can be successfully used for effluent monitoring. Germination of settled zoospores was significantly inhibited by primary effluent at less than 1% effluent (NOEC = 0.56% effluent, Fig. 31). The length of the germination tubes were not measured in this test because this endpoint had not yet been developed at the time of this experiment. The settled zoospores were clearly visible in all but the highest concentration of effluent. At 56% effluent however, particulates in the test solution covered the slides and obstructed the spores. Because the toxicity of this effluent was well below this concentration, particulates were not a problem in this test.

No brine control was used in this test because it was preliminary, and our interest was primarily in evaluating the effects of effluent particulates on the kelp zoospores. Because no brine was needed to adjust the salinity in the lower concentrations (including the lowest effect concentration, 1% effluent), the lack of a brine contro did not effect the test results.

It is not clear what component(s) of the effluent were toxic to the zoospores. Total ammonia levels in this effluent ranged between 20 and 49mg/L (20,000-49,000 µg/l). Based on this range, the ammonia level in the effluent concentration that was inhibiting zoospore germination (1%) could have been between 200 and 500 µg/l. The concentration of unionized ammonia is not known but was certainly far less. Salinity, temperature, pH, and dissolved oxygen were within normal ranges in this test.

The brine controls appeared to be toxic in the secondary effluent experiments. Note that the pH levels in the brine controls were much higher than in the dilution water and higher effluent concentrations (Table 4). The brine in this test was heated to 100 °C to quickly raise its salinity. This apparently drove off the carbon dioxide, raising the pH to 9.35. In addition, the distilled water that was used to adjust the salinity of the brine controls had a relatively high pH value (9.1) which contributed to the pH problem. It is likely that a high pH contributed to the brine's toxicity. Because the brine itself appeared to be toxic, we cannot say that the toxicity of the effluent was due to the effluent itself or to the brine.

However, the secondary effluent was less toxic to kelp than the primary effluent (Figures 32-33). Zoospore germination was significantly reduced in 56% secondary effluent (NOEC = 32%). Zoospore germ tube

elongation was significantly inhibited in 32% secondary effluent (NOEC = 18%). Again, these NOEC values are based on statistical comparisons with the dilution water controls, and are therfore not valid.

As in the 16 day zinc test, the variability in sporophyte production within the replicates of each treatment was relatively high in the long-term secondary effluent test. This was probably due to an insufficient number of swaths counted per replicate slide (see previous discussion of 16 day zinc test). Because of this, the apparent difference in sporophyte numbers between the controls, brine controls, and higher concentrations (Fig. 34) was masked, preventing detection of any statistical significance. In addition, the brine controls appeared to be toxic for the same reasons described for the short-term secondary effluent test. This negates any intepretation of toxicity in this experiment. Thus, it is impossible to say whether the secondary effluent was toxic to the reproductive stages of *Macrocystis* (Fig. 34); there was no significant difference in sporophyte production between the controls and effluent treatments. Another possible explanation for the lack of effect in this experiment was the length of exposure of the gametophytes to the effluent. The gametophytes used in this test had been cultured in control water for 8 days prior to the test, exposed to the effluent for 4 days, and placed back in control water to develop for 4 more days. At the time the gametophytes were exposed to the effluent they had not yet produced gametes. It is possible that fertilization of the eggs and sporophyte production occurred after the cultures were returned to clean seawater.

The ecological and economic importance of *Macrocystis pyrifera* makes it a valuable resource to the state of California. The proximity of *Macrocystis* forests to pollution sources, the ability to routinely culture *Macrocystis* in the laboratory, and its sensitivity to effluent toxicity make it an appropriate algal species for bioassay testing of complex effluents. The results of the experiments presented here show that bioassays being developed with early life stages of giant kelp can be used for evaluating effluent toxicity. Although the long-term, 16 day reproductive bioassay may not be practical for the routine testing of complex effluents, it can be used to calibrate the short-term test. Some questions need to be answered before the short-term test is implemented on a wide-spread basis. The question of between-test variability of the germination endpoint can hopefully be resolved by doing multiple short-term tests with a more toxic, organic compound. The question of ecological relevance of the germ tube elongation endpoint can be resolved by calibrating this endpoint with a conclusive long-term reproduction bioassay that focuses on sporophyte production. We recommend that both endpoints continue to be measured in the short-term bioassay.

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SECTION 4

MYSID TESTS

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INTRODUCTION TO MYSID TESTS

The opposum shrimp *Holmesimysis (=Acanthomysis*) costata* (Holmes 1900) occurs in the surface canopy of the giant kelp *Macrocystis pyrifera*. *H. costata* ranges the length of California where it is one of the most numerous near shore mysid species (Clutter 1967, Clarke 1971, Hobson and Chess 1976). Mysids in general are an important component of estuarine and marine trophic webs (Nimmo *et al.* 1977; Mauchline 1980). *Holmesimysis* is ecologically important in kelp forest ecosystems as a food source to kelp forest fishes, particularly juvenile kelp canopy species (Clarke 1971, Hobson and Chess 1976, Mauchline 1980). Their ecological importance, amenability to laboratory culture, and relative sensitivity to a variety of toxicants, make mysids logical candidates for use in bioassay tests (Nimmo *et al.* 1977; Gentile *et al.* 1982).

Holmesimysis costata was chosen as a potential bioassay test species for use in the State Water Resources Control Board's Marine Bioassay Project. The Project is developing routine bioassay tests for monitoring the toxicity of municipal sewage effluents discharged into California waters (Linfield *et al.* 1985). The biology of this species has not been well described (Holmquist 1979; Clutter 1967, 1969, Green 1970), and there have been no published reports on its use in toxicity testing. However, mysids in general are considered to be excellent test organisms for bioassays, and some species have been used extensively for this purpose (Benfield and Buikema 1980). One example is the eastern estuarine mysid *Mysidopsis bahia*, which is a key species in several toxicity testing programs (Nimmo *et al.* 1977; Gentile *et al.* 1982; Breteler *et al.* 1982; Lussier *et al.* 1985).

Little is known of the effect of marine pollution on the ecology of *H. costata*, or mysid populations in general. The predicted effects of thermal discharge from the San Onofre nuclear power plant in southern California include a reduction of mysid shrimp in and around kelp forests (Murdoch *et al.*, 1980). Declines in *Macrocystis* forests attributed to factors associated with sewage pollution (Wilson 1982) would presumably impact mysid populations by, at the very least, eliminating habitat. Other mysid species have been shown to be susceptible to whole effluent constituents (Nimmo *et al.*, 1977). Because *H. costata* occurs near shore, there is concern that populations are threatened by marine pollution.

This paper presents bioassay tests being developed for the juvenile stages of *Holmesimysis costata*. Results are presented on preliminary testing of the effects of zinc sulfate and sewage effluents on the survival of juvenile *Holmesimysis*.

* Note: There are questions concerning the taxonomy of *Holmesimysis/Acanthomysis* species. We are considering Holmquist's (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).

METHODS

Facilities

All of the experiments were done between June, 1986 and July, 1987 at the California Department of Fish and Game's 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 4 km from any known pollution source.

Lifecycle

These experiments used juvenile *Holmesimysis costata*. Adult female *H. costata* carry between 30 and 40 embryos in an abdominal broodpouch (the marsupium) and these develop *in vivo* and hatch as juveniles (Figure 35). The generation time for this species is 60 - 70 days, depending on feeding rates and water temperatures. Because of its long generation time, lifecycle tests with *Holmesimysis* are not practical for routine effluent testing. The following experiments are based on two short-term and one long-term protocol. The short-term protocols are 48 and 96 hours long. The long-term protocol is 21 days long. All protocols focus on mysid mortality as the indication of toxicity, although growth was considered as a possible endpoint in the long-term zinc test. For detailed instructions on how to conduct the short-term protocol see Appendix 4.

Short-term test methods

Adult *Holmesimysis* were collected from the kelp forest canopy at Pacific Grove, Monterey County, California. The mysids were collected by pulling a small mesh net just under the surface canopy blades of the giant kelp *Macrocystis pyrifera*. The mysids were transported back to the lab in plastic buckets and placed in 20 liter acrylic aquaria that were provided flowing seawater at approximately 1 l per minute. Water temperatures fluctuated between 11 - 15 °C. All broodstock mysids were fed *ad libitum* a diet of newly hatched *Artemia* nauplii (San Francisco Brand ®) supplemented with Tetramin® fish food.

All of the experiments began with 3 day old juvenile mysids (Figure 36). To obtain mysids of this age, brooding female mysids were isolated from the others and placed in their own "hatching" aquarium. Gravid female *Holmesimysis* are identified by their large, extended marsupia filled with eyed larvae. The marsupia of females that are close to hatching are grey in color. Three hundred juvenile mysids were needed per experiment, so approximately 50 gravid females were isolated for each. After the gravid females were transferred, the hatching aquarium was kept free of incidental hatches (< 300 juveniles) by siphoning. When a hatch sufficiently large enough to conduct an experiment occurred (≥ 300 juveniles) these animals were siphoned into their own holding tank. This resulted in the combination of juveniles from several females to give enough animals for one test. These juveniles were fed a diet of flake food (Tetramin ®) until they were old enough to effectively eat *Artemia* (~ 2-3 days), at which time they were fed brine shrimp exclusively.

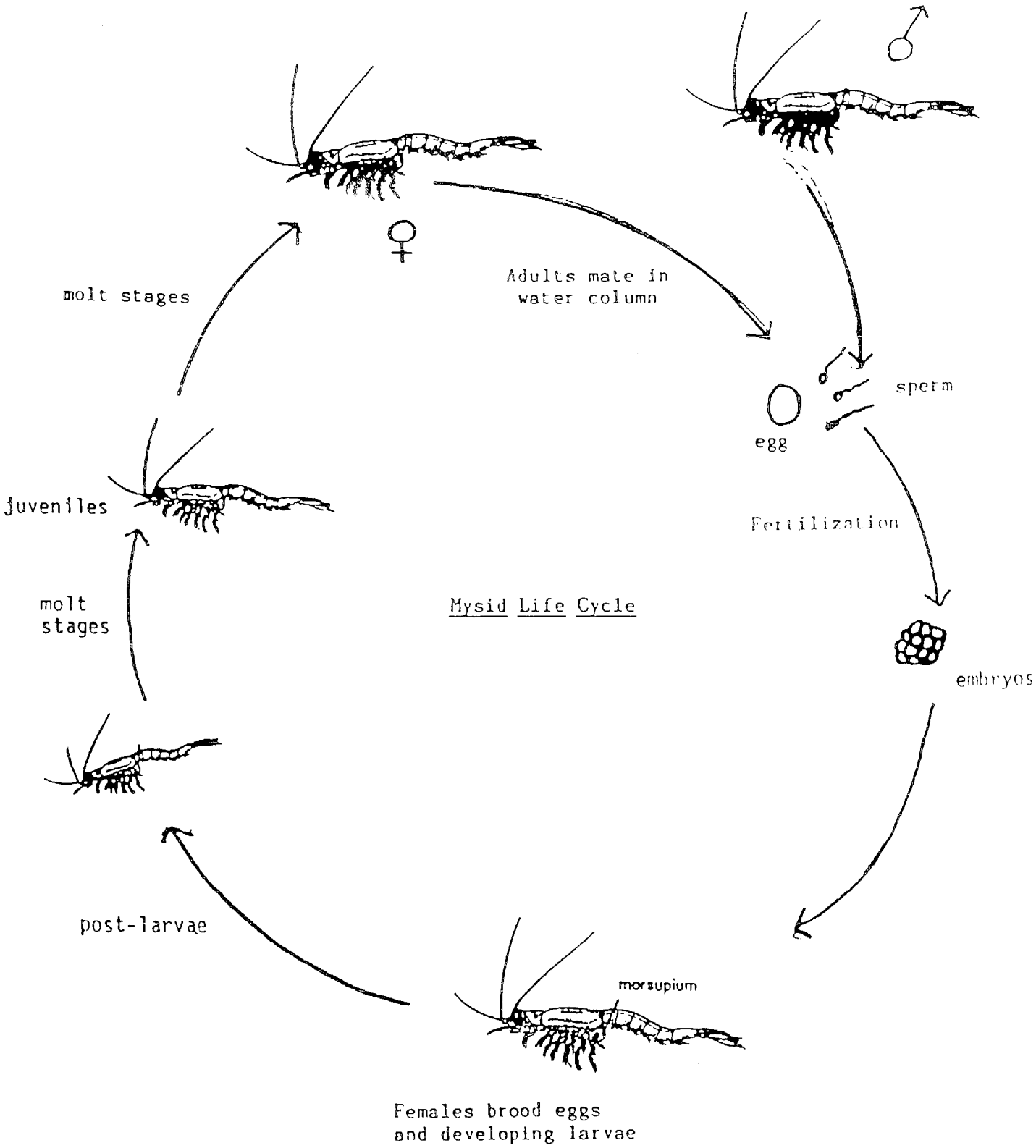
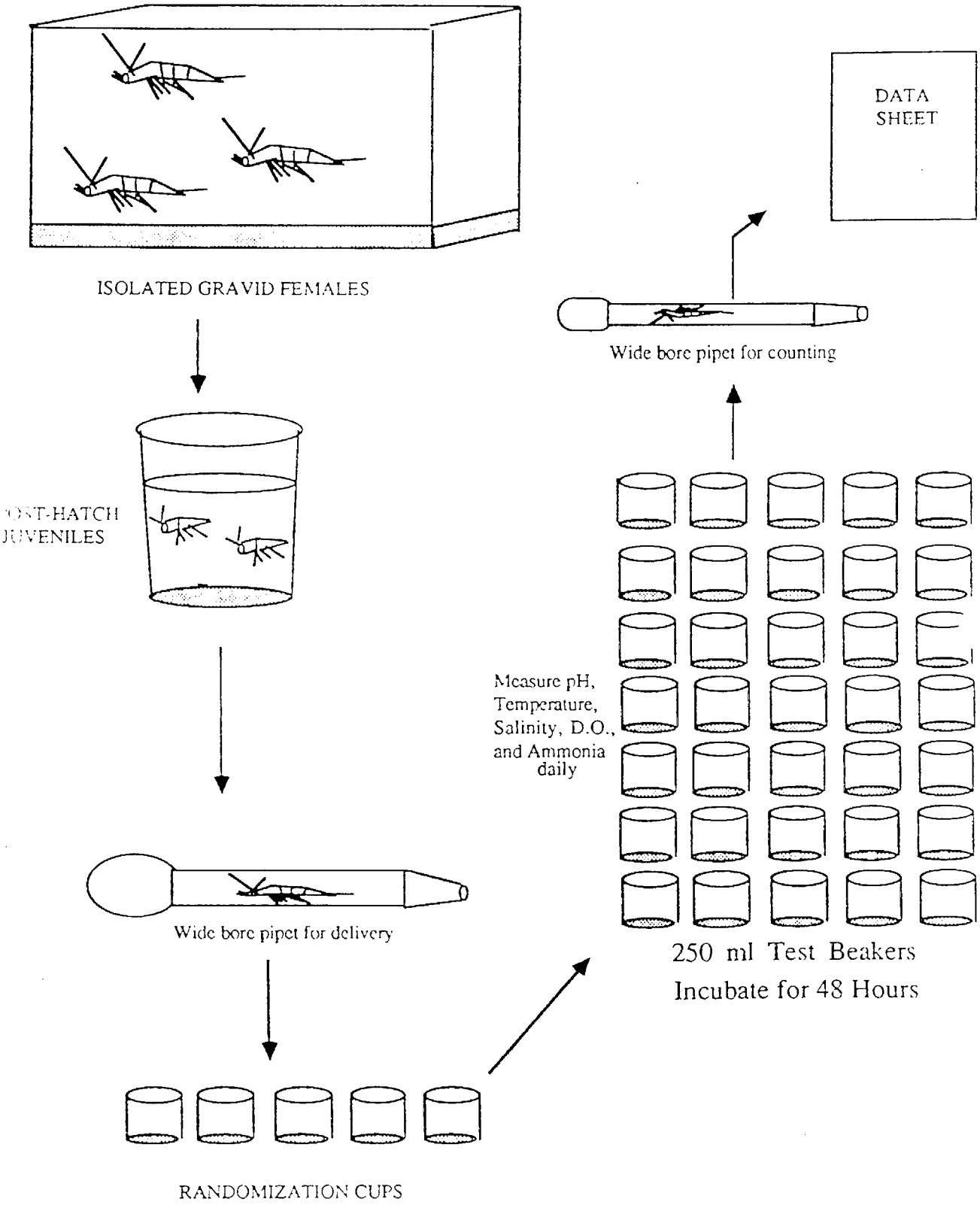


Figure 35 Summary diagram of a mysid life cycle.

FIGURE 36. MYSID TOXICITY TEST PROTOCOL



Three day old juveniles were used in these experiments because this is the youngest age able to eat *Artemia*, a preferable food for use in bioassays, and because we have found control survival to be better than for mysids less than 3 days old.

For all tests we used six test concentrations (5 dilutions and 1 control) each replicated five times. Effluent tests had an additional 5 containers for brine controls. The test concentrations were assigned in a logarithmic sequence: 0, 0.1, 0.18, 0.32, 0.56, 1.0, at an order of magnitude depending on the toxicity of the toxicant. The dilution water was 1μ filtered, U.V. irradiated natural seawater. For all tests dissolved oxygen, pH, temperature, and salinity were monitored daily. The short-term tests were static bioassays with two hundred and fifty milliliters of test solution in each test container.

For the 48 hour short-term tests, five 3 day old juveniles were exposed in each replicate test container. For all other tests 10 juveniles were exposed. The mysids were randomly transferred to the test containers with a wide bore 10 ml pipet. The test animals were randomized by pipeting them 2 at a time into 30 randomization cups. When each of the 30 cups contained 2 animals, 2 more were pipeted. This process was repeated until each cup had 10 animals in it. The mysids were then randomly transferred to the test containers with a minimum volume of water. The test animals were fed 24 hour old *Artemia* nauplii *ad libitum*.

The endpoint for all tests was death. To ascertain whether or not a mysid was dead, we sucked the animal up into a pipet and observed its appendages with a magnifying lens. Death was defined as the total lack of appendage movement. The number of living and dead mysids were recorded daily for each test, and dead animals was removed daily to prevent fouling.

The test containers were either 350 ml polyethylene plastic food containers (for metal bioassays) or 650 ml borosilicate glass stacking dishes (for complex effluent and organic toxicant bioassays). Plastic test containers were cleaned by rinsing with hot tap water followed by 24 hour sequential soaks in solutions of mild detergent, 3N HCL, and deionized water. Glass test containers were cleaned by rinsing with hot tap water then reagent grade acetone, followed by 24 hour soaks in 3N HCL and deionized water.

Long-term test methods

To calibrate the sensitivity of the short term test, a long term test was conducted using mysid growth and survival as indicators of toxic effect. The long-term test methods were exactly the same as those used in the short-term tests except for the following modifications. The long-term test was a flow-through bioassay that used a proportional diluter system (Brungs and Lemke 1978) to continuously supply the toxicant to the test containers. The dilution water was 1μ filtered seawater. Six concentrations and a control were tested, each replicated 4 times. The volume of test solution was 250 ml, and the diluter replaced 50% of this volume every 20 minutes.

We used photography to monitor mysid growth. Pictures were taken weekly with a Ricoh® 35mm SLR camera equipped with a 105mm zoom/macro lens. We constructed a small acrylic chamber into which all of the mysids from a test container could be poured. A ruler was glued to the bottom for reference. Each replicate was

poured into the chamber and photographed. The test containers were cleaned weekly during each photographic session

Toxicants and Tests

We tested the effects of three different toxicants on juvenile *Holmesimysis*: one trace metal salt, one municipal sewage effluent from a primary treatment facility, and one effluent from a secondary facility.

The trace metal salt tested was zinc sulfate (see section 6 for a discussion of the criteria used to select zinc sulfate as a reference toxicant). We conducted 3 short-term, 48 hour, zinc tests (0 - 1,000 µg/l zinc) to assess between test variability. We also did one short-term, 96 hour, zinc test (0 - 1,000 µg/l zinc) to compare survival after 4 days of exposure to survival after 2 days. Zinc concentrations were verified using a Perkin Elmer model 603 atomic absorption spectrometer at the California State Mussel Watch analytical facility. The zinc solutions were sampled from one replicate of each treatment concentration at the beginning and end of each experiment. All zinc No Observed Effect Concentrations (NOECs) are expressed as verified analytical concentrations.

The primary sewage plant effluent was from a grab sample taken prior to chlorination, and the secondary effluent was from a 24 hour composite sample collected after chloination and dechlorination.

Interlaboratory Calibration

To assess the ability of contract laboratories to use the *Holmesimysis* short-term protocol, we conducted an interlaboratory test of the 96 hour short-term protocol with the Southern California Coastal Water Research Project (SCCWRP) bioassay laboratory, using zinc (0 - 1,000 µg/l) as the toxicant. For this exercise we collected adult *Holmesimysis* at Pacific Grove and brought them back to our laboratory. We then isolated juvenile mysids using the above methods and transported these to the SCCWRP laboratory. The mysids were transported in plastic bags containing seawater which had been saturated with oxygen by bubbling. The mysids were transported in coolers (~ 8 °C). The transporting was timed so that the SCCWRP personnel could conduct a 96 hour short-term mysid bioassay on 3 day old mysids. The following week a second 96 hour mysid test was done at MPSL with a separate set of mysids held under the same conditions for the same amount of time. We attempted to duplicate the experimental conditions for both tests by adhering to the written protocols, and by using the same zinc, test containers, and temperature. To control for any variation caused by using different dilution waters, we ran the tests at both laboratories with seawater supplied by the SCCWRP laboratory.

Statistics

The percentage survival data were tallied at the end of each experiment. These data were transformed to the arcsine of their square root, then analyzed using analysis of variance (ANOVA) followed by a Dunnett's multiple comparison test to compare each concentration to the control (Zar, 1974; Sokal and Rohlf, 1969). For each test, the No Observed Effect Concentration (NOEC) was calculated as the highest concentration not significantly different from the control at $p = 0.05$.

RESULTS

The results of all of the toxicity tests with mysids are summarized in Table 5. All of the results are given as No Observed Effect Concentrations. The NOEC's for the zinc sulfate tests are given as µg/l; the NOEC's for the complex effluent tests are given as percent effluent.

Table 5. NO OBSERVED EFFECT CONCENTRATIONS FOR PHASE 2 MYSID TESTS

Zinc Sulfate Tests (µg/l)	TEST NUMBER		
	1	2	3
Mysid 48 Hour Repetitive Tests	182	175	320
Mysid 96 Hour Test	100		
Mysid 21 Day Test	45		

Mysid Interlaboatory Tests (µg/l)	MPSL	SCCWRP
Mysid 96 Hour Test	89	66

Effluent Tests (percent)	PRIMARY	SECONDARY
Mysid 96 Hour Test	1.0	32

MPSL = Marine Pollution Studies Laboratory; SCCWRP = Southern California Coastal Water Research Project.

Water Quality

The water quality data for all of the experiments are represented in Table 6. All of the water quality parameters for all of the zinc tests were within normal ranges, except the brine control.

TABLE 6. WATER QUALITY RANGES FOR MYSID TESTS

Test	Dissolved Oxygen (mm./Hg.)	pH	Salinity (ppt.)	Temperature (degrees C.)
48 Hr. #1	146 - 157	8.50 - 8.55	34 - 36	13.5 - 15.5
48 Hr. #2	146 - 157	8.50 - 8.55	34 - 35	-- 14.5 --
48 Hr. #3	143 - 150	8.50 - 8.55	-- 36 --	13.0 - 13.5
96 Hr.	149 - 160	8.35 - 8.65	-- 35 --	12.0 - 16.0
Long-term	134 - 166	8.30 - 8.55	34 - 36	13.1 - 17.9
Inter-Lab	145 - 161	8.45 - 8.70	34 - 36	14.5 - 15.5
Primary	146 - 160	8.40 - 8.75	34 - 35	12.5 - 15.0
Secondary	146 - 158	7.85 - 8.65*	31 - 37	14.1 - 14.7

* Brine control 8.15 - 8.90.

Mysid Zinc Tests

Juvenile mysid survival was significantly inhibited after 48 hours at concentrations of 320 µg/l zinc and greater in tests 1 and 2, and at 560 µg/l and greater in test #3 (Figures 37-39). The No Observed Effect Concentrations (NOECs) for the 3 tests were 182, 175, and 320 µg/l zinc, respectively.

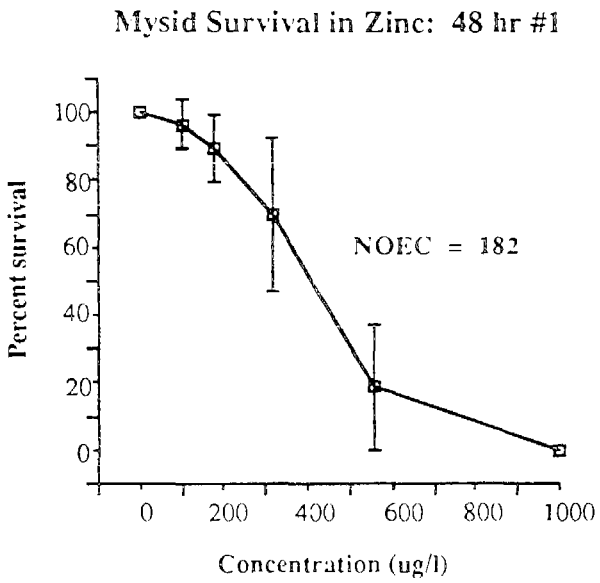


Figure 37. Survival of juvenile mysids after 48 hours in zinc sulfate, test #1. Mean ± standard deviation. N = 5. Five animals in each replicate.

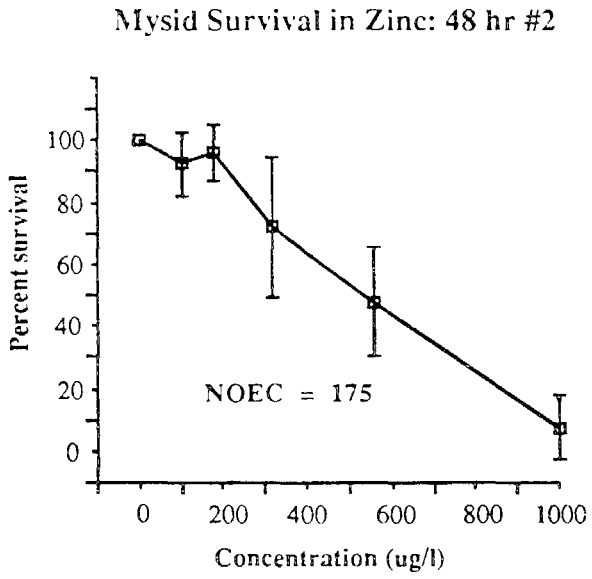


Figure 38. Survival of juvenile mysids after 48 hours in zinc sulfate, test #2. Mean ± standard deviation. N = 5. Five animals in each replicate.

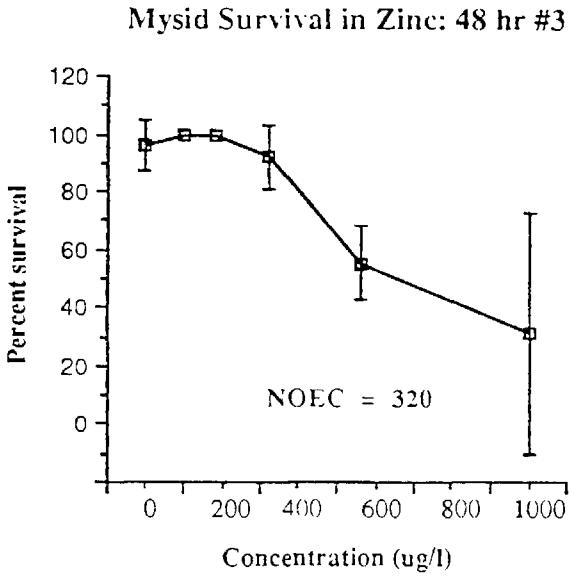


Figure 39. Survival of juvenile mysids after 48 hours in zinc sulfate, test #3. Mean ± standard deviation. N = 5. Five animals in each replicate.

Juvenile mysid survival was inhibited at lower zinc concentrations after a 96 hour exposure (Fig. 40). Mysid survival after 96 hours was significantly reduced at concentrations of 180 µg/l zinc and greater.

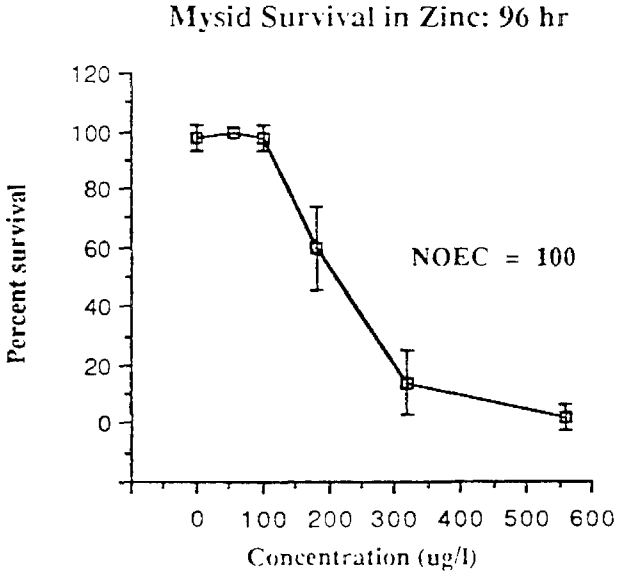


Figure 40. Survival of juvenile mysids after 96 hours in zinc sulfate. Mean ± standard deviation. N = 5. Ten animals in each replicate.

Mysid Long-Term Zinc Test

Because of low control survival the results of the long-term mysid test are tentative. Juvenile mysid survival after a chronic (21 day) exposure to zinc sulfate was significantly reduced at tested concentrations of 100 µg/l and greater (Figure 41). The NOEC for the long-term exposure to zinc is given as 45 µg/l. However, this test will have to be repeated before this NOEC can be considered definitive. In addition, the photographic chamber used for monitoring mysid growth was poorly designed, and most of the mysids hid in the shadows along the edge of the container. The projections of these photographs proved inadequate, so these data are not presented.

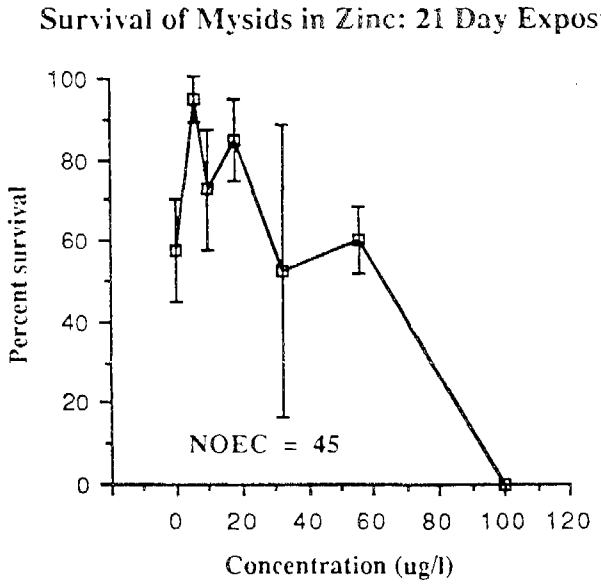


Figure 41. Survival of juvenile mysids after a 21 day chronic exposure to zinc sulfate. Mean ± standard deviation. N = 5. Ten animals in each replicate.

Mysid Interlaboratory Tests with Zinc Sulfate

The results of the interlaboratory mysid tests with the Marine Pollution Studies Laboratory and the Southern California Coastal Water Research Project bioassay laboratory indicate that the 96 hour mysid bioassay protocol is relatively straight foward and amenable for use by laboratories new to the test. Mysid survival was significantly reduced after 96 hours at concentrations of 100 µg/l zinc and greater in the MPSL and SCCWRP tests (MPSL NOEC = 89 µg/l; SCCWRP NOEC = 66 µg/l, Figures 42 and 43). The NOECs for both tests were the same nominal concentration (56 µg/l). Note that the X axis in this and all other zinc figures is given in nominal zinc concentrations.

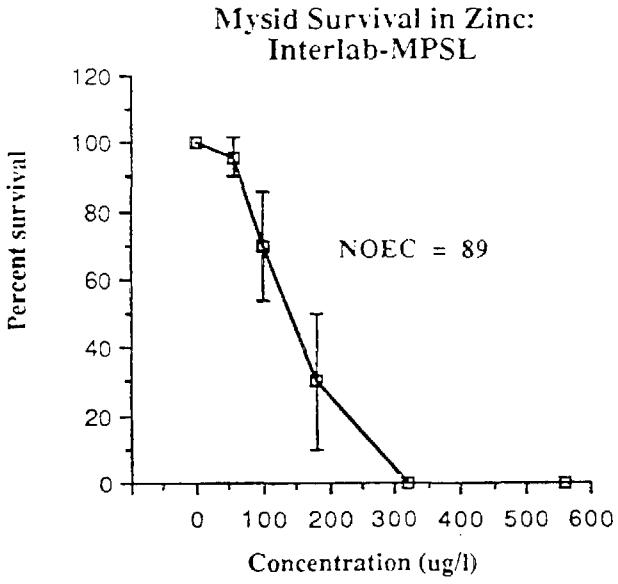


Figure 42. Mysid survival after 96 hours in zinc sulfate- interlaboratory test at MPSL. Mean ± 1 standard deviation. N = 5. Ten animals in each replicate.

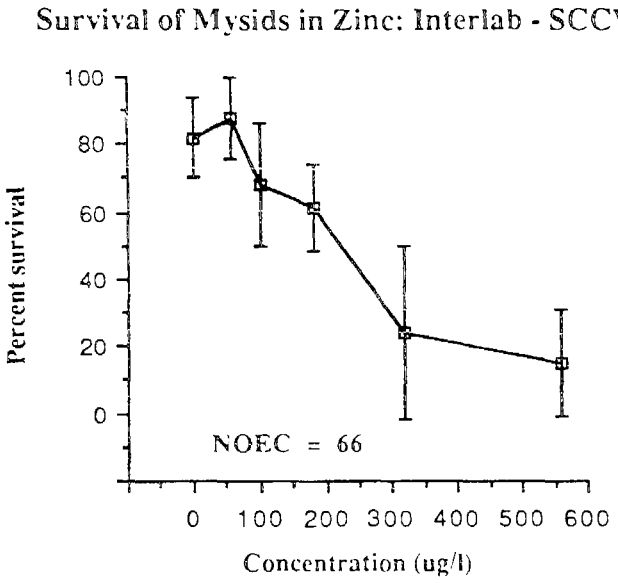


Figure 43. Survival of juvenile mysids after 96 hours in zinc sulfate- interlaboratory test at SCCWRP. Mean ± 1 standard deviation. N = 5 (~ 8 animals in each replicate).

Mysid Effluent Tests

Primary sewage treatment plant effluent greater than 3.2% effluent significantly inhibited mysid survival after 96 hours (NOEC = 1%, Figure 44).

All primary effluent concentrartions greater than 1% effluent had salinities adjusted to 34 ppt using a hypersaline brine. The brine was prepared by aerating and heating MPSL seawater to not more than 40 °C (as recommended by the US EPA). No brine control was used in the primary effluent test (see discussion).

Although the ammonia levels were not measured during testing, the yearly total ammonia levels ranged from 20-49mg/L for the primary effluent (as communicated by the treatment plant staff).

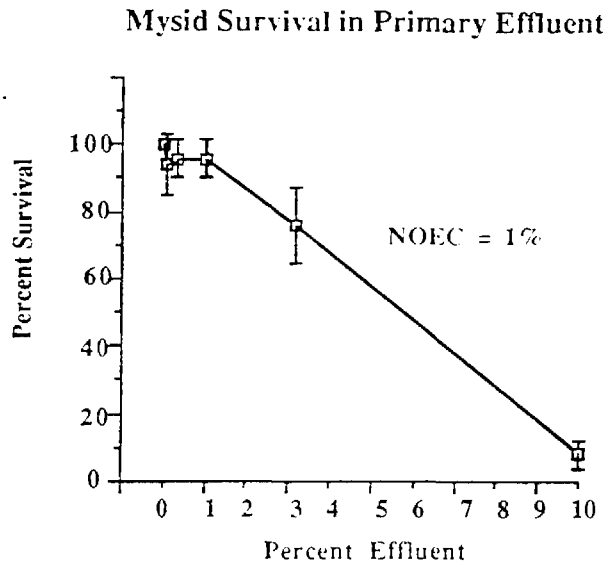


Figure 44. Survival of mysids after 96 hours in primary effluent. Mean ± s.d.; N=5. Ten animals per replicate.

Juvenile mysid survival in secondary sewage effluent was significantly inhibited at 56% effluent (NOEC = 32%). Brine was used to adjust the salinity of all of the effluent concentrations in the secondary effluent test. Brine controls were used in the secondary effluent tests and were prepared by diluting the maximum amount of brine used at the highest concentration (56% effluent; ~400 ml per liter of brine) to 34 ppt with distilled water. Because of logistical constraints, the brine for the secondary effluent test was aerated and boiled at 100 °C for several days to rapidly increase its salinity (to 85ppt). Apparently heating the brine above this temperature drove off the carbon dioxide, increasing the pH above the normal range (Table 6, see discussion). Additionally, poor mixing of effluent and brine in the 56% effluent concentration resulted in salinities as high as 40 ppt. This was not discovered until after the animals were placed into the test containers, and all of the mysids in the initial 56% treatment died rapidly. This treatment was duplicated on the second day of the test with new 3 day old juvenile mysids and effluent from the same sample. The data from this duplicate 56% treatment are presented in the secondary effluent test results (Fig. 45). The relatively high standard deviation in the 5.6% effluent treatment was caused by unusually high mortality in one of the replicates.

The yearly total ammonia levels ranged between 18-22 mg/l in the secondary effluent (as communicated by the treatment plant staff).

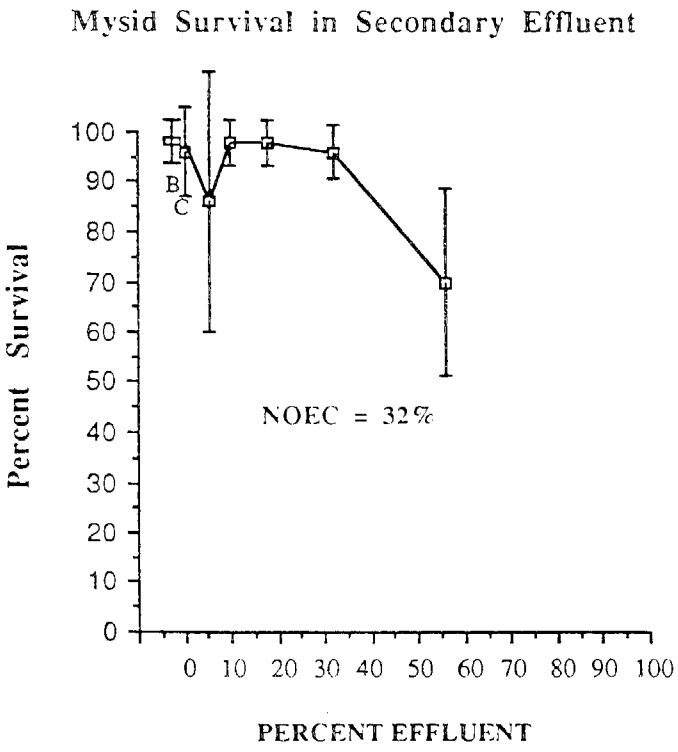


Figure 45. Survival of juvenile mysids after 96 hours in secondary effluent. Mean ± standard deviation. N = 5. Ten animals per replicate. B= brine control; C= dilution water control

DISCUSSION

Although the long generation time of *Holmesimysis costata* makes lifecycle toxicity tests with this species logistically impractical for use in routine monitoring, juvenile *H. costata* are amenable to short-term toxicity testing. This combined with their relative sensitivity to a variety of toxicants make them an appropriate species for use in routine bioassay testing. The short-term bioassay protocol developed for this species with zinc sulfate performs equally well testing the toxicity of complex effluents.

The results of the 48 and 96 hour short-term tests with zinc sulfate show that both exposure time and the number of animals per replicate affect the results of toxicity tests with *H. costata* Mysid survival in the three 48 hour zinc tests was significantly inhibited above 175 µg/l zinc (Figures 37-39). There was some between- test variability in the the 48 hour tests. The NOEC's for tests 1 and 2 were 182 and 175 µg/l, respectively (the same nominal concentration: 180 µg/l); the NOEC for test 3 was 320 µg/l. This was due, in part, to the fact that only five mysids were exposed in each replicate. We had originally developed this protocol with five animals per replicate because the mobility of mysids makes it difficult to accurately count them. However, with 5 animals per replicate, 1 death represents a 20% decline in survival, and one or two deaths in a replicate can significantly affect the results. The low number of animals per replicate also affected the between-replicate variability in the 3 tests. This is illustrated by the relatively high standard deviations, especially in the mid- and higher concentrations (Figures 38-40).

The short, 48 hour exposure period may also have contributed to the between-test variability. The 48 hour mysid tests were originally developed to fit easily into a five day work week. However, because of the kinetics of toxicant uptake by mysids, it takes longer than 48 hours for them to reliably demonstrate the toxic effect (personal communication, Howard Bailey, UC Davis). Many of the animals were scored as alive even though it was obvious to us that they were near death. We therefore decided to extend the exposure period to 96 hours; this also allowed comparison of our data with published mysid toxicity data. In addition, we increased the number of animals per replicate to ten in an attempt to reduce between-replicate variability.

Mysid survival was significantly inhibited at zinc concentrations above 100 µg/l after a 96 hour exposure, a lower NOEC than in the 48 hour tests. The between-replicate variability was relatively low in this test (Figure 40), probably because we used 10 animals in each replicate.

Mysid survival was significantly inhibited at test concentrations of 100 µg/l zinc and greater zinc after 21 days (NOEC = 45 µg/l, Figure 41). However, the results of this test should not be considered definitive because we

experienced several technical difficulties during the course of this experiment. We originally intended to incorporate both growth and survival as endpoints by taking weekly pictures of each replicate. Technical problems with this technique produced unusable data on growth (as described in the results section). Another difficulty in the 21 day test was contamination of some of the replicates. The diluters were equipped with a poor grade of stainless steel (304) originally designed for fresh water bioassays. The steel corroded after prolonged contact with seawater and contaminated some of the containers. This affected mysid survival and explains much of the high replicate variability in this test, particularly in the control, 18 and 32 µg/l replicates (Figure 41). Because of high control mortality, these results should be considered tentative. We intend to refit our diluter system with non-corrodible materials, and repeat this test.

The results of the interlaboratory zinc tests with SCCWRP indicate that the 96 hour mysid test can be conducted by other laboratories having limited familiarity with the protocol. The NOEC was 89 µg/l for the MPSL test (Figure 42), and 66 µg/l for the SCCWRP test (Figure 43); both tests had the same nominal (not analytically verified) NOEC of 56 µg/l. However, the response curves on the two graphs have marked differences (Figures 42 and 43). For example, in the MPSL test, all of the mysids were killed at the two highest concentrations, while there was 20 to 30% survival at these concentrations in the SCCWRP test. The reasons for this are not certain. Note that mysid survival was also low at the higher zinc concentrations in the other 96 hour mysid test at MPSL (Figure 40). One problem noted by the SCCWRP personnel was the relative subjectivity of the criteria for determining mysid death (personal communication, Steven Bay, SCCWRP). A more easily defined procedure for determining toxic response is needed before this test is implemented in any permitting process.

The acute and chronic sensitivities of *Holmesimysis* to zinc are comparable to values reported for other mysid species. Lussier *et al.* (1985, her Table III) reported a 96 hour acute LC50 for *Mysidopsis bahia* of 499 µg/l zinc. A 96 hour LC50 for *Holmesimysis* (estimated graphically from Figure 40) was 205 µg/l. In a 35 day flow-through bioassay that focused on survival, reproduction, and fecudity, the same authors reported a LOEC (Lowest Observed Effect Concentration) of 166 µg/l for *Mysidopsis*. The LOEC in the long-term test presented here was 100ppb zinc (Figure 41). For other crustacea, Martin *et al.* (1981 Table 1) reported a 96 hour EC50 of 456 µg/l zinc for Dungeness crab (*Cancer magister)* zoea, and Ahsanullah et al. (1981) reported a 96 hour LC50 of 10,200 µg/l zinc for adult marine shrimp *Callianassa australiensis*.

The mysid bioassay protocol needed no modification for testing the toxicity of complex effluents. The results of the whole effluent bioassays indicate that mysids have variable sensitivities to sewage effluents depending on the level of sewage treatment. Mysid survival was inhibited by primary treated effluent at concentrations of 3.2% and greater (NOEC = 1%, Figure 44). The primary effluent test was the first complex effluent bioassay preformed with the MPSL protocol for *H. costata*. No brine control was used in this test because it was preliminary, and our interest was primarily in evaluating the effects of effluent particulates on the juvenile mysids. Despite the lack of a proper control for the effects of brine, the water quality parameters for this test were within normal ranges (Table 6).

Mysid survival in secondary treated effluent was inhibited at 56% effluent (NOEC = 32%). For reasons

discussed above, the 56% concentration was duplicated on day 2 of the test with the same effluent used for the other treatments. Because this effluent was a day older, and presumably equally or less toxic, we feel that the NOEC derived for this test is probably correct. The variability in the 5.6% effluent was due to unexplainably high mortality in one replicate.

It is not clear what component(s) of the effluent were toxic to the mysids. Total ammonia levels in this effluent ranged between 20 and 49mg/L (20,000-49,000 µg/l). Based on this range, the ammonia level in the effluent concentration that was inhibiting mysid survival (32%) could have been between 6,400 and 15,680 µg/l. The concentration of unionized ammonia is not known but was certainly far less. Salinity, temperature, pH, and dissolved oxygen were within normal ranges in this test.

Overall, the results indicate that *Holmesimysis costata* is an appropriate mysid species for use in routine bioassay monitoring of complex effluents. However, some modifications would improve the short-term test protocol. The endpoint needs to be better defined so that technicians with minimal prior experience can easily and accurately determine if the mysids are affected. Another long term test needs to be run with a refitted diluter system to accurately assess chronic toxicity. Age dependent toxicant sensitivity needs to be investigated. By determining age-dependent sensitivity, we can improve the test by making it simpler (if no differences exist) by allowing use of mysids from a broader age range.

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SECTION 5

PRELIMINARY WORK WITH ADDITIONAL SPECIES

Preliminary Work With Additional Test Species

Although the emphasis in Phase 2 of the Marine Bioassay Project was on further developing the bioassay test protocols for abalone, mysids, and kelp, some preliminary culture work and test development was explored with alternative species. Some of these species are ones that have previously been used in bioassay tests, others are new. This section contains results and observations on work with vertebrates and invertebrates being considered for the future.

Mussels- *Mytilus californianus* and *Mytilus edulis*

Bioassay protocols are available for the mussels *Mytilus californianus* and *Mytilus edulis*, and these species have been used extensively for toxicity testing. The 48 hour mussel bioassay focusing on larval shell development (Dimick and Breese, 1965; APHA Standard Methods, 1986) is almost identical to the red abalone protocol described in this report. The red abalone protocol was developed as an alternative to the short-term mussel bioassay because, unlike mussels, laboratory cultured abalone are consistently spawnable year round. Our intention was to make sure we could spawn mussels first, and later do a zinc sulfate bioassay with mussels to compare the sensitivity of this test to the short-term abalone test.

We collected *M. californianus* on the rocks adjacent to the Granite Canyon Laboratory in August, 1987. We scrubbed their shells clean, and placed them in moist paper towels in the refrigerator (6° C) overnight. The next morning we put them in individual plastic cups and fed them 100 ml of phytoplankton (*Tetraselmus suicceca* , 3 x 10^6 cells/ml). We then placed the cups in a water bath at 23° C for 2 hours. No spawning occured . We attempted a second spawning later but only one female spawned. Apparently these animals had recently spawned in the field. Sampled mussels had empty gonads. We will attempt this test again next winter after the field populations have had a chance to ripen.

We also attempted a spawning of bay mussels (*M. edulis*) in August. These we collected from Sandholt Bridge in Moss Landing Harbor. Sampled mussels from this group had well developed gonads. To induce spawning we fed phytoplankton to the mussels then placed them in a warm (23° C) water bath for 1 hour. After 1 hour we transferred the mussels to 15° C water. Several females spawned immediately. Because no males spawned, we stripped sperm from one of the ripe males and added this to the eggs. We then placed the fertilized eggs in a 37 µ screen tube and supplied them with ambient 1µ-filtered seawater at a low flow rate. After 2 days the mussels had developed into normal veliger larvae. Although no bioassay was attempted, we feel confident that if fertilized eggs can be obtained, we can conduct a 48 hour mussel bioassay with either *M. edulis* or *M. californianus* in Phase 3 of the Project.

Squid- *Loligo opalescens*

We obtained gravid female market squid from a local trawler for display during our public open house. The squid laid eggs in one of the display tanks and these were close to hatching after about 2 weeks. We took this opportunity to do a short-term zinc bioassay on squid juveniles. We used the same experimental design as for all of our other short-term static bioassays except that each concentration was replicated 3 times instead of the usual 5. We tested 1 control and 6 concentrations (0, 10, 18, 32, 56, 100, and 180 µg/l zinc). This range included zinc concentrations that we have shown to inhibit larval abalone development (56 µg/l) and juvenile mysid survival (180 µg/l). One squid egg case was placed in each test container. The developing embryos were easily visible through the cases and we were able to count their numbers in each egg case. We intended to monitor percent hatching success and survival after a 96 hour exposure period. The squid began hatching in the containers after 1 day, and by day 2 almost all had hatched. We fed each container approximately 500 *Artemia* nauplii at this time. After 96 hours there was good survival in all replicates. On the fifth day there was a drastic decline in survival in all of the containers. Young squid are known to be highly selective feeders, and they did not appear to be feeding on the *Artemia*. Although the water quality parameters (disolved oxygen, pH, temperature, and salinity) were within normal ranges, we did not measure the ammonia levels, and these may have been high due to the large number of juvenile squid and food organisms.

Apparently the newly hatched *L. opalescens* juveniles are not as sensitive to a short-term exposure to zinc sulfate as are larval abalone and juvenile mysids. This suggests that our short-term bioassays are sensitive enough to protect an early life stage of at least one other species. This statement must be qualified however, because the squid embryos were exposed after they had developed for 2 weeks. It may be that exposure at an earlier stage of development or a long-term exposure of newly laid squid embryos would result in a greater sensitivity to zinc.

Fish- *Atractoscion nobilis*, *Paralabrax clathratus*, and *Engraulis mordax*.

In addition to work with invertebrate species, we also began preliminary work with 3 fish species, white sea bass *Atractoscion nobilis*, kelp bass *Paralabrax clathratus*, and northern anchovy *Engraulis mordax*.

We obtained 24 hour old white sea bass larvae from Donald Kent at Hubbs Research in San Diego (with appreciation). Our intention was to assess the feasibility of shipping sea bass larvae by air, and if possible, conduct a short-term bioassay using zinc sulfate. The sea bass larvae survived the shipping, but because of a scheduling problem at the airport, the larvae sat for 48 hours at another laboratory with lower salinity and poor water quality. This resulted in poor survival. The remaining larvae were set up in flowing, heated seawater at Granite Canyon and survived for one week on a diet of rotifers *Brachionus plicata* and *Artemia* nauplii. White sea bass are apparently tolerant to shipping and may possibly become a useful test species in the future. Other researchers are contemplating developing a bioassay protocol with this species, so we intend to focus on exploring other possibilities rather than duplicating effort.

In addition to white sea bass, we conducted preliminary experiments with the kelp bass *Paralabrax clathratus* in collaboration with Jo Ellen Hose and her staff from the Vantuna Research Group in Los Angeles. Dr. Hose provided fertilized kelp bass eggs and these were transported to Granite Canyon in plastic bags held in ice chests (~16° C). The eggs appeared to survive the trip well and were immediately transferred to 15 liter polyethylene buckets set

in a water bath at 19° C. The bioassay protocol developed by Dr. Hose involves exposing 24 hour post-fertilization kelp bass eggs to a toxicant, then monitoring hatching success, survival, and developmental abnormalities after a 96 hour exposure period. We conducted two bioassays with kelp bass: one with zinc sulfate (0 - 1000 µg/l), and one with a secondary treated complex effluent (0 - 56%). Unfortunately, control survival was poor in both tests, preventing any evaluation of toxicity. The poor control survival was probably due to trauma suffered during egg transportation. Dr. Hose has offered to supply us with kelp bass broodstock so that we can have a ready supply of eggs at our laboratory. We hope to devote some research time to this species during the next phase of the Project. We extend our gratitude to Jo Ellen Hose for her cooperation.

The third fish species that we have been working with is the northern anchovy *Engraulis mordax*. We have held anchovy broodstock at our lab for over 18 months now, and survival has been remarkably good. We have attempted one spawning from these fish using the hormone injection techniques of Roger Leong of the National Marine Fisheries Service laboratory in La Jolla. We obtained a few eggs from this trial, but most of the fish appeared to be immature. We have since modified our holding tank conditions and feeding regime, and the fish that we have sampled since then are developing satisfactorily. We will attempt another spawning with this species sometime during the next phase.

Mysids- *Metamysidopsis elongatus*

Metamysidopsis elongatus is an epibenthic mysid that occurs in large numbers over sandy bottom habitats in Southern California. Because this species has a relatively short life cycle (~28 days), development of lifecycle and/or reproductive toxicity tests may be possible. With the help of Steven M. Bay at SCCWRP, we obtained a small number of these mysids at Granite Canyon. These animals are apparently quite sensitive to temperature extremes, and most of the animals did not survive the trip because of cold temperatures in the transport container. Those that did survive were set up in a flowing seawater aquarium at 18° C. These animals survived well on a diet of *Artemia* nauplii, but after a week our heat exchanger failed (for the first time in years), and they succumbed to the drop in temperature. Because other researchers are working with this species, we have elected to discontinue working with them to avoid duplicating effort.

APPENDIX I

SELECTION OF AN INORGANIC REFERENCE TOXICANT

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1.0 INTRODUCTION

In order to further standardize the short-term and long-term bioassay protocols developed for red abalone, mysids, and kelp, an inorganic reference toxicant was chosen before the initiation of toxicity testing in phase II of the Marine Bioassay Project. The reference toxicant was used to compare the results of the short-term and long-term toxicity tests, and to standardize the bioassay test methods intended for implementation in phase III of the project. The reference toxicant was also used for inter-laboratory toxicity tests, and intra-laboratory quality assurance tests designed to look at variability between multiple runs of the bioassay tests, and variability between test analyst results. This report describes the selection of this inorganic (trace metal) reference toxicant.

2.0 INITIAL SCREENING

Several criteria were used to evaluate the potential trace element candidates. The principal criteria used in the initial screening process were: toxicity to marine organisms, ease of analyses in seawater at toxic concentrations, and potential health hazard to laboratory personnel. In the initial screening, 10 toxic trace elements (Ag, As, Cd, Cr, Cu, Hg, Ni, Pb, Se, Zn) were considered. These 10 trace elements are routinely monitored at six major southern California municipal wastewater outfalls [see Section 3.2 (page 2) for list of outfalls]. Six trace elements were initially eliminated (As, Cr, Hg, Ni, Pb, Se) and four were considered in further detail (Ag, Cd, Cu, Zn). The following specific factors were used to eliminate the six elements as reference toxicants: As - difficult to analyze; Cr - low toxicity, difficult to analyze; Hg - difficult to analyze, difficult to remove from laboratory wastewater, hazardous to laboratory personnel, ease of volatilization; Pb - difficult to analyze, difficult to remove from wastewater, hazardous to laboratory personnel; Se - difficult to analyze.

3.0 SELECTION CRITERIA

The criteria used in the screening of the remaining four trace elements were: toxicity to marine organisms, concentration in discharge effluent, bioaccumulation potential, speciation, analyzability/treatability, previous use in bioassays, potential health hazard to laboratory personnel, and recommendation by the Scientific Review Committee. These criteria are discussed in more detail in the following section.

3.1 Toxicity to Marine Organisms.

The toxicity of the reference metal is of primary concern because of its effect on marine biota. Toxicity is also a concern because, if low, it will require higher concentrations to elicit effects or responses, and these may be at concentrations that are rarely found in coastal waters. The acute LC50 and chronic EC50 values for Ag, Cd, Cu, and Zn were compiled and evaluated (Table 1). An attempt was made to compare values from toxicity tests on the same species under similar experimental conditions. The references for the values were compiled from the reviews by Bryan (1984) and Reish et al.(1982).

Toxicity values from Mysidopsis bahia were taken from Lussier et al. (1985). Finally, an overall acute and chronic toxicity value for each metal was taken from the State Water Quality Control Plan (Ocean Plan, State Water Resource Control Board, 1983). The latter reference incorporates toxicity data derived from a modification of the data base compiled in 1976 (Klapow and Lewis, 1979). Toxicity data for marine species were combined to give general acute/chronic toxicity values for each metal. A general summary of relative toxicities of each metal by Bryan (1984) is provided in Table 2.

Bryan (1984) considers the more rapidly absorbed metals (Ag, Cu) to be generally the more toxic, although the toxicities are variable depending on the permeability of the metal to the organism, and the ability of the organism to detoxify the metal. The following are considered important factors moderating toxicity of a particular metal:

a. Form of the metal in the environment; this affects its potential to bioaccumulate;
b. Presence of other metals which can be additive or antagonistic to its toxicity;
c. Environmental factors (temperature, pH, dissolved oxygen, light) can change the metal species or the degree of physiological stress on the organism and influence toxicity;
d. The condition of the organism (life cycle phase, sex, age, size, nutritional state);
e. Ability to avoid contact (e.g. shell closure);
f. Ability to adapt to metals (e.g. detoxify, tolerate through genetic adaptation).

3.2 Presence in Wastewater Effluent.

For this criterion, the four metals were evaluated for their presence in the primary effluent of the six southern California municipal dischargers: Joint Water Pollution Control Plant (Whites Point), Hyperion 5 mi and Hyperion 7 mi (Santa Monica Bay), Oxnard, Point Loma (San Diego), and Sanitation Districts of Orange County. The average concentrations for the years 1978-1983 and 1985 were tabulated (Table 3) and an overall ranking for each metal was derived. The sources of this information was the Southern California Coastal Water Research Project (Biennial Reports 1979-1984, S. Bay, pers. comm. 1985 data).

3.3 Bioaccumulation.

The bioaccumulation potential of the four metals was also considered important because the accumulation of metals in commercial species could have public health implications, as well as affecting other food web organisms in marine and estuarine areas (Bryan, 1984). The metal concentrations in tissues of some representative marine species are listed in Table 4. In general, the bioaccumulation of the four metals under consideration for final selection is directly related to the concentrations of exposure. Evidence from most phylogenetic groups suggests that uptake of metals from water is a passive process, and, therefore, is related to concentrations of exposure (Bryan, 1984).

3.4 Speciation In Seawater.

Because the various species of each metal may react differently in seawater, the toxicity values of each species may differ. The ideal reference toxicant would be the one that has the least number of species. For each of the four metals, the relative percentages of the known species in seawater are listed in Table 5 (Goldberg, 1975). Since few data are available on the toxicity of individual metal species, except in the case of copper, a more detailed discussion of copper and its application in toxicity testing is considered in section 4.3.9.

3.5 Analyzability/Treatability.

The analyzability and treatability of the metal in seawater is another important practical consideration, because all toxicant test solutions require chemical verification for quality assurance purposes, and because it will be necessary to remove the metal from the wastewater generated in the toxicity tests. The sources for information reported in this section were Standard Methods for the Examination of Water and Wastewater (1985), communications with laboratory responsible for trace metal confirmation (CDFG Mussel Watch Laboratory, Moss Landing Marine Laboratories), communication with a seawater-trace metal chemist (A.R. Flegal, University of California, Santa Cruz, pers. comm.), discussions with bioanalysts familiar with the four metals (D. Smith, CDFG Mussel Watch and J. Oakden, Moss Landing Marine Laboratories).

3.6 Previous Use in Bioassays.

Metals were ranked in order of previous use in metal bioassays, because of the obvious advantages of have a better data base for comparison and the expediency of having previously tested experimental procedures and techniques. A thorough literature review will be completed for the metal which is selected as the reference metal for this program. A partial literature list for each metal is included in Table 6.

3.7 Health Hazard to Laboratory Personnel.

Because of obvious problems with handling and shipping hazardous substances, an evaluation of the safety to laboratory personnel was made. The major sources for this information was the Material Safety Data Sheets (J.T. Baker, 1978) and the NIOSH/OSHA Pocket Guide to Chemical Hazards (1978).

3.8 Recommendations of the Scientific Review Committee.

The Scientific Review Committee initially suggested further evaluation and consideration of two metals as an inorganic reference toxicant: Zn and Cd.

4.0 EVALUATION OF INDIVIDUAL TRACE METALS

4.1 Silver

4.1.1. Toxicity

The relative toxicity of silver to a variety of marine organisms is

compared with the other three metals in Table 1. In general, silver is less toxic than copper but more toxic than cadmium and zinc. Silver was particularly toxic to oyster (Crassostrea gigas) embryos and was considered more acutely toxic than the other three metals in the Ocean Plan toxicity review (SWRCB, 1983). A general summary of toxicity study results is given in Table 2. One reason silver may be more toxic is that it is not an essential trace element, unlike copper and zinc.

4.1.2 Presence in Effluent

Silver concentrations in the effluent of southern California wastewater discharges are lower than the other three elements (Table 3). Two point source locations with relatively high silver concentrations in mussel tissues are Whites Point and Point Loma (Martin et al., 1984).

4.1.3 Bioaccumulation

Silver has been found to accumulate in a variety of marine species (Table 4). Silver concentrations are relatively lower than the other three metals possibly because it is not a micronutrient, and occurs in generally lower concentrations in the environment (Table 3). Calabrese et al(1984) found that silver accumulated in Mytilus edulis only at the highest test concentration (10 ppb) after 12 months, but at 18 and 21 months significant levels were accumulated in decreased concentrations (1 and 5 ppb). Young and Moore (1978) showed that rock scallops (Hinnites) at White's Point were accumulating significantly more silver than Channel Island control animals.

4.1.4. Speciation

The different species of silver known to occur in seawater are listed i Table 5. Silver occurs as three different chloride bound species, most common as $AgCl$ (Ahrland, 1975).

4.1.5. Analyzability

Silver is considered relatively easy to analyze and to remove from wastewater.

4.1.6. Previous Use in Bioassays

Silver has been used less in toxicity testing than the other three metals. A partial list of silver toxicity references is given in Table 6.

4.1.7. Health Hazard to Laboratory Personnel

Silver is more toxic than the other three metals. The permissible exposure limits for $AgNO$ is 0.01 mg/m (NIOSH/OSHA, 1978). It can be caustic and irritating to the eyes, skin and mucous membranes, but is safe if handled with routine safety precautions.

4.1.8. Scientific Review Committee

Not recommended by the Scientific Review Committee.

4.2 Cadmium

4.2.1. Toxicity

The relative toxicity of cadmium to a variety of marine organisms is compared with the other three metals in Table 1. In general, cadmium has been found to be more toxic than zinc, but less toxic than silver and copper. Cadmium is particularly toxic to post-hatch juveniles of Mysidopsis bahia, especially in chronic life cycle tests (Lussier, 1985). Cadmium was not particularly acutely toxic to the bivalves Mytilus edulis and Crassostrea gigas (Martin et al., 1981) or Crassostrea virginica (Calabrese et al., 1973). However, the literature did not include many chronic studies. Because cadmium is not rapidly absorbed, it is possible that its toxicity is only observed in chronic studies (Bryan, 1984; Lussier, 1985; and SWRCB Ocean Plan, 1983). A general summary of toxicity study results by Bryan (1984) is given in Table 2.

4.2.2. Presence in Effluent

Cadmium ranks third in concentration in the effluents of the six major southern California waste discharges (Table 3).

4.2.3. Bioaccumulation

Cadmium has been found to accumulate in a variety of marine species (Table 4). High concentrations have been found in rock scallops at Whites Point, California (Young and Moore, 1978). In general, cadmium concentrations in tissues rank third of the four metals surveyed, but uptake rates for cadmium are slower than for the other three metals (Bryan, 1984).

4.2.4. Speciation

The different species of cadmium known to occur in seawater are listed in Table 5. Cadmium occurs as three different chloride species, the form $CdCl_2$ is most commonly found in seawater (Zirino and Yamamoto, 1972; Dryssen and Wedborg, 1974).

4.2.5. Analyzability/Treatability

Cadmium is considered easy to analyze and easy to remove from laboratory wastewater.

4.2.6. Previous Use in Bioassays

Cadmium has been used extensively in toxicity studies. A partial list of references is given in Table 6.

4.2.7. Health Hazard to Laboratory Personnel

Cadmium ranks second in toxicity of the four metals to humans behind silver. The permissable exposure limit (OSHA, 1977) for $Cd\ Cl_2$ is 0.2 mg/m^3. Cadmium is an irritant to eyes and mucous membranes (NIOSH/OSHA, 1978).

4.2.8. Scientific Review Committee

Cadmium was recommended by the Scientific Review Committee.

4.3 Copper

4.3.1. Toxicity

The relative toxicity of copper to a variety of marine organisms is compared with the other three metals in Table 1. Copper toxicity varies from species to species with greatest toxicities recorded for Crassostrea gigas, Mytilus edulis, and Cancer magister (Martin et al., 1981), but less toxic than silver to Crassostrea virginica (Calabrese et al., 1973) and less acutely and chronically toxic to Mysidopsis bahia than cadmium (Lussier, 1985). In other toxicity tests comparing copper, cadmium, and zinc, copper was consistently more toxic to a variety of species (Arnott and Ahsanullah 1979, Ahsanullah and Arnott 1978, Reisch 1978). Copper is considered less acutely toxic than silver but more toxic than cadmium and zinc for Ocean Plan considerations (SWRCB, 1983). Copper is particularly toxic to marine phytoplankton (Sunda and Guillard, 1976). A general toxicity summary by Bryan (1984) is given in Table 2.

4.3.2. Presence in Effluent

Copper ranks second in concentration in the effluents of 6 major southern California wastewater discharges (Table 3).

4.3.3. Bioaccumulation

Copper has been found to accumulate in a variety of marine species (Table 4). High concentrations have been found in mussels (Stephenson et al., 1979), rock scallops (Young and Moore, 1978) and abalone (Martin et al., 1977).

4.3.4. Speciation

The different species of copper and the relative percentages of each in seawater are shown in Table 5. The majority of copper (83%) occurs as Cu(OH) (Zirino and Yamamoto, 1972). Dyrssen and Wedborg (1974) found the majority of copper (65%) to occur as CuOHCl. The effect of copper speciation on toxicity is discussed in Section 4.3.9.

4.3.5. Analyzability/Treatability

Copper is considered easy to analyze and remove from seawater.

4.3.6. Previous Use in Bioassays

Copper toxicity has been studied more than the other three metals. A partial list of references is given in Table 6.

4.3.7. Health Hazard to Laboratory Personnel

Copper ranks third of the four metals in toxicity to humans and is equivalent to zinc. The permissable exposure limit for $CuSO_4$ is 1 mg/m (NIOSH/OSHA, 1978). Copper dust is a strong irritant to skin and mucous

membranes.

4.3.8. Scientific Review Committee

Copper was not recommended by the Scientific Review Committee.

4.3.9. Problems with Copper as a Reference Toxicant

The toxicity of copper, like all heavy metals, depends on the physiochemical form of the metal. Sunda and Guillard (1976) showed that copper toxicity to the marine phytoplankton, Thalassiosira pseudonana, is a function of cupric ion activity and not the total copper concentration. Gnassia-Barelli et al. (1978) found that organic substances (Mol. Wt. 500-100,000) released into the culture media by laboratory reared phytoplankton are able to complex and detoxify copper. Ahsanullah (1984) found that water-soluble ligands decreased copper toxicity by decreasing free ionic copper, while lipid-soluble ligands increased copper toxicity, presumably by disrupting the cell membranes.

Because the species of a particular metal affects its toxicity, the number of species present are important considerations in toxicity testing. This is particularly true when using a metal as a reference toxiciant for interlaboratory calibration. One possible cause for variation is the fact that different laboratories may use different dilution water sources. This will be less of a problem if the same artificial seawater is used for dilution by all the participating laboratories. Also, it is now possible to analyze for total and labile copper; this will allow for tighter control on the variation between laboratories.

As a reference toxicant, copper poses some additional problems. For example, because copper is so ubiquitous it can be a contamination problem. This is also true of zinc, but less true for cadmium and silver (D. Smith, CDFG, pers. comm.; J. Oakden, Moss Landing Marine Laboratories, pers. comm.). Another consideration is that copper adsorbs to glass container walls. Inert plastic containers may reduce the adsorption rates of copper (and other metals).

4.4 Zinc

4.4.1. Toxicity

The relative toxicity of zinc to a variety of marine organisms is given in Table 1. In general, zinc is the least toxic of the four metals, and Bryan (1984) considers zinc the least toxic of the four metals (Table 2). Zinc is more toxic to Crasssostrea gigas embryos and seaweed growth rates than cadmium (Table 2).

4.4.2. Presence in Effluent

Zinc occurs in higher concentrations in the effluents of the six major southern California wastewater discharges than the other three metals (Table 3).

4.4.3. Bioaccumulation

Zinc has been found to accumulate in a variety of marine species (Table 4). High concentrations of zinc have been found in Haliotis rufescens (Anderlini, 1974), Mytilus edulis (Stephenson et al., 1979), decapod crustaceans (Bryan, 1976b), and the alga Fucus vesiculosus (Fuge and James, 1975). Young and Moore (1978) found more zinc than the other three metals in ridgeback prawns and sea urchins near the Whites Point outfall. One reason for zinc bioaccumulating in such high concentrations is the fact that it occurs in seawater in greater concentrations. Zinc is also an essential trace element in marine organisms.

4.4.4. Speciation

The species of zinc known to occur in seawater are listed in Table 5. The majority of zinc (50%) occurs as $Zn(OH)$ (Zirino and Yamamoto, 1972). Dryssen and Wedborg (1974) found the majority of zinc bound as $ZnCl$.

4.4.5. Analyzability/Treatability

Zinc is considered easy to analyze and remove from seawater.

4.4.6. Previous Use in Bioassays

Zinc has been used extensively in toxicity studies. A partial list of refernces is given in Table 6.

4.4.7. Health Hazard to Laboratory Personnel

Zinc ranks third (with copper) in toxicity of the four metals to humans. The permissable exposure limit is 1 mg/m . Zinc dust is an eye irritant and can irritate mucous membranes if inhaled.

4.4.8. Scientific Review Committee

Zinc was recommended by the Scientific Review Committee.

5.0 Summary.

The choice of reference toxicants is critical to the success and efficiency of the Marine Bioassay Project. The characteristics of a desirable reference toxicant are stability in solution, ease of analysis, low potential for human health hazard and ease of removal in laboratory waste treatment systems. In addition, the relative toxicity, presence in effluents, and bioaccumulation potential are important considerations because these indicate the potential hazard to the marine environment posed by each toxicant and it is the purpose of the bioassay testing program to detect in effluents the presence of such hazardous substances.

Silver, cadmium, copper, and zinc could all conceivably be used as reference toxicants, though all have unique disadvantages.

Silver is suitably toxic and is present in tissues of marine organisms sampled near outfalls. It is found in effluents in the lowest concentrations of the four trace elements considered here. It is suitable as a reference toxicant because it is easy to analyze and remove from wastewater, but it presents a relatively high health hazard to laboratory personnel and has been used in relatively few previous bioassays. Its stability in solution is dificult to determine, but its presence in seawater in only three common chemical species is a possible advantage.

Cadmium is not as toxic as copper or silver and would have to be used in higher concentrations to elicit an effect in bioassay tests. Cadmium is far less abundant in effluents than copper or zinc, but has been found in high concentrations in marine organisms near outfalls. Little is known about its stability in test solutions, but, like silver, it has only three common chemical species in seawater. Cadmium is relatively easy to remove from wastewater, but it presents a potential health hazard to laboratory personnel similar to the threat posed by silver. Cadmium has been used extensively in previous bioassay work.

Copper is suitably toxic to marine life and is found in high concentrations in waste effluents and in the tissues of marine organisms. In the laboratory it is easy to analyze, easy to remove from laboratory wastewater, relatively safe to laboratory personnel,and has been widely used in previous bioassays. Its stability in solution and speciation characteristics are potential problems, but of the four trace elements considered here copper has been the most extensively studied in this regard. Because copper is so widely used in plumbing and other common applications, the potential for contamination of test solutions is relatively high.

The last trace element to be discussed is zinc. Zinc is found in the highest concentrations in waste effluents of the four elements, but is also the least toxic to most organisms. This lower toxicity would require that higher concentrations be used in laboratory testing. Zinc does accumulate in marine organisms. Little is known about speciation characteristics or zinc's stability in test solutions, but it is considered easy to analyze and easy to remove from laboratory wastewater. Zinc has been used extensively in previous bioassay studies.

The material presented in this report has been drawn from a thorough search of published literature to determine the suitability of trace elements as reference toxicants. Final selection of the inorganic trace element to be used in the Marine Bioassay Program will be made by the staff and Scientific Review Committee based on the information contained here and on the experience and expertise of the Committee members.

Results of Scientific Review Committee Meeting June 11, 1986: Selection of an Inorganic reference Toxicant.

The SRC members listed the four reference toxicant candidates in order of preference: Zn > Cd > Cu > Ag. Zinc was chosen as the most preferred reference toxicant for the following reasons:

1) Of the 4 metals, zinc is the easiest to analyze for with atomic adsorption spectrometry.

2) Initially, the project staff considered higher toxicity to be a desirable attribute of the metal reference toxicant. However, the relatively lower toxicity of zinc means that it will require higher test concentrations. This will result in a higher signal to noise ratio in the test containers thereby decreasing the relative effect of backround (ambient) concentrations of zinc.

3) Zinc does not sorb onto container walls as easily as copper, any any zinc that does adsorb and remain after washing would be less toxic, thereby decreasing the possibility of contamination.

4) The toxicity of zinc to a variety of species is relatively consistent.

The SRC considered the other 3 metals less desirable for reasons listed in this report as well as the following:

Ag- expensive, photoreactive.

Cu- Contamination problems.

Cd- Inconsistent toxicity, variable uptake ratyes between species.

Table 7 gives a summary of the four metals in a selection matrix.

After the selection of zinc sulfate as the inorganic reference toxicant to be used in phase II of the project, we conducted an experiment to assess the stability of zinc in solution over time. A brief description and the results of this experiment are given in Table 8.

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Table 1. Effects of Trace Metals on Marine Organisms. Compiled from: Bryan 1984, Lussier 1985, Reish et al. 1982, and SWRCB 1983.

Species*	Test	Metals (ppb)				Reference
		Ag	Cd	Cu	Zn	
Crassostrea virginica	48h LC50	9	3800	128	340	Calabrese et al. 1973
Crassostreas gigas	48h EC50	22	611	5.3	119	Martin et al. 1981
Mytilus edulis	48h EC50	14	1200	5.8	175	Martin et al. 1981
Mysidopsis bahia	96h LC50	249	110	181	499	Lussier et al. 1985
Mysidopsis bahia	chronic	59	7.1	104	166	Lussier et al. 1985
Cancer magister	96h LC50	55	247	49	456	Martin et al. 1981
Agropecten irradians	96h LC50	33	1480	310	-	Nelson et al. 1976
Acartia simplex	24h LC50	-	1370	200	1860	Arnott et al. 1
Paragrapsus sp.	96h LC50	-	490	170	1230	Ahsanullah et al. 1978
Capitella capitata	96h LC50	-	5800	200	10700	Reish 1978
Ocean Plan	Acute	8	200	28	210	SWRCB 1983
Ocean Plan	Chronic	-	7.6	6.4	56	SWRCB 1983

* all tests were on larval or embryonic life stages

Table 2. Experimental Toxicity Studies Summary
From Bryan, 1984

Species	Toxicity of Metals	Source
Natural Phytoplankton (growth)	Hg))Cu)Pb)As(V))Zn=Cd)Ni, Cr(VI), Sb, Se, As(III)	(HOLLIBAUGH and co-authors, 1980)
Phytoplankton: *Ditylum brightwellii* (growth)	Hg)Ag)Cu)Pb)Cd)Zn)Tl	(CANTERFORD and CANTERFORD, 1980)
Seaweeds (growth)	Hg(org.))Hg(inorg.))Cu)Ag)Zn)Cd)Pb	(BRYAN, 1984)
Hydroid *Eirene viridula* (morphlolgical abnormalities)	Hg)Cu)Cd)Pb)Zn	(KARBE, 1972)
Polychaete: *Ctenodrilus serratus* (reprod.)	Hg=Cr(VI))Cu)Al)Zn)Ni)Pb)Cd	(REISH, 1978; PETRICH and REISH, 1979)
Mollusc embryos: *Crassostrea virginica* (48hLC50)	Hg)Ag)Cu)Zn)Ni)Pb)Cd)As(III))Cr(III))Mn	(CALABRESE and co-authors, 1973)
Echinoderm larvae: *Pergonella japonica* (develop.)	Hg=Cu)Zn)Cr(VI))Cd	(KOBAYASHI, 1979)
Copepod: *Nitrocra spinipes* (96hLC50)	Hg)Zn)Cd=Cu)Co)Ni)Al)Fe)Mn	(BENGTSSON, 1978)

Table 3. Mean Concentrations (ppb) of Effluent Characteristics from 1978-1983 and 1985 (compiled from SCCWRP)*

Metal	Overall Rank	JWPCP	Hyperion		O.C.	P.L.	Oxnard
			5	7			
Ag	4	11.8	26.1	697.8	13.3	13.4	14.8
		(3.7)	(9.7)	(44.1)	(1.7)	(7.3)	(10.8)
Cd	3	17.8	15.6	797.8	28.1	9.3	12.2
		(8.7)	(4.2)	(267.7)	(17.1)	(2.8)	(5.7)
Cu	2	163.7	177.1	9,961.7	230.8	114.8	61.8
		(64.8)	(22.9)	(2,336.9)	(80.3)	(21.8)	(37.1)
Zn	1	564.4	268.1	15,766.7	237.6	233.1	121.6
		(226.3)	(82.9)	(5011.5)	(85.7)	(101.8)	(84.3)

* - Standard Deviation in Parentheses

Table 4. Metal Levels in Some Marine Species
compiled from Bryan 1984 and Fuge and James 1975.

Species	Location	Metals (ppm dry wt.) Ag	Cd	Cu	Zn	Source
Hinnites rufescens (digestive gland)	California	13.90	184.00	11.20	556.00	Anderlini 1974
(muscle)		2.80	0.17	2.60	42.00	
Mytilus edulis	California	0.18	11.20	18.80	161.00	Stephenson et al. 19
Various Decapod Crustaceans+	-	0.40	1.00	70.00	80.00	Bryan 1976
Fucus vesiculosus	Bristol channel, U.K	-	13.81	9.10	195.30	Fuge and James 1975
Hinnites giganteus*	Whites Pt. CA.	2.30*	520.00*	190.00	130.00	Young and Moore 1978
	Channel Is. CA.	0.26	540.00	64.00	100.00	Young and Moore 1978
Sicyonia ingentis*	California	(.01	0.03	2.00	9.80	Young and Moore 1978
S. [illegible]ciscanus*	California	(.01	0.13	0.27	4.20	Young and Moore 1978

+ - geometric mean of lit. values
* - mg/Kg wet wt.

Table 5. Metal Speciation
(from The Nature of Seawater; Goldberg, 1975 ed.)

Metal	Species	Source
Cu^{2+}	$Cu(OH)_2$ 83%, $CuCO_3$ 11% or	Zirino and Yamamoto (197
	CuOHCl 65%, $CuCO_3$ 22%, CuCl 6%, CuOH 4%	Dyrssen and Wedbory (197
Zn^{2+}	ZnCl+ 44%, Zn^{2+} 16%, $ZnCl_2$ 15% or	Dyrssen and Wedborg (197
	$Zn(OH)_2$ 50%, Zn^{2+} 22%, ZnCl 8%, $ZnCO_3$ 6%	Zirino and Yamamoto (197
Ag	$AgCl_4^{3-}$ 54%, $AgCl_3^{2-}$ 24%, $AgCl_2^-$ 17%	Ahrland (1975)
Cd^{2+}	$CdCl_2$ 50%, CdCl 40%, Cdcl 6% or	Zirino and Yamamoto (197
	$CdCl_2$ 38%, CdCl 29%, CdCl 28%	Dyrssen and Wedburg (197

Table 6. List of References for Toxicity Tests

Metal	Group	Species	Reference
Ag	Oysters	Crassostrea gigas	Martin et al. 1981
	Mussels	Mytilus edulis	Martin et al. 1981
	Crabs	Cancer magister	Martin et al. 1981
	Mysids	Mysidopsis bahia	Lussier et al. 1985
	Crabs	Carcinus maenas	Amiard 1976
	Oysters	Crassostrea virginica	Calabrese et al. 1973
	Clams	Mercenaria mercenaria	Calabrese and Nelson 1974
	Scallops	Argopecten irradians	Nelson et al. 1976
	Snails	Nassarius obsoletus	Mactinnes and Thurberg 1973
Cd	Oysters	Crassostrea gigas	Martin et al. 1981
	Mussels	Mytilus edulis	Martin et al. 1981
	Crabs	Cancer magister	Martin et al. 1981
	Mysids	Mysidopsis bahia	Lussier et al. 1985
	Crabs	Cancer irroratus	Tucker and Matte 1980
	Shrimp	Palaemonetes pugio	Pesch and Steward 1980
	Hermit Crab	Pagurus longicarpus	Pesch and Steward 1980
	Larval Crab	Rhithropanopeus harrisii	Rosenberg and Costlow 1976
	Copepod	Tigriopus japonicus	D'Agostino and Finney 1974
	Copepod	Pseudodiaptomus coronatus	Paffenhofer and Knowles 1978
	Lobster	Homarus americanus	Thurberg et al. 1977
	Oyster	Crassostrea virginica	Calabrese et al. 1973
	Scallop	Argopecten irradians	Nelson et al. 1976
	Amphipods	Rhepoxynius abronus	Oakden et al. 1984
	Amphipods	Eohaustorius sencillus	Oakden et al. 1984
	Copepod	Eurytemora affinis	Sullivan et al. 1983
	Various	various australian invertebrates	Ahsanullah 1976
	Shrimp	Callianasa australiensis	Ahsanullah 1976
Cu	Oysters	Crassostrea gigas	Martin et al. 1981
	Oysters	Crassostrea virginica	Calabrese et al. 1973
	Mussels	Mytilus edulis	Martin et al. 1981
	Crabs	Cancer magister	Martin et al. 1981
	Amphipods	Gammarus duetzeni	Moulder 1980
	Clams	Mercenaria mercenaria	Calabrese et al. 1977
	Scallops	Argopecten irradians	Nelson et al. 1976
	Shrimp	Pandalus danae	Young et al. 1979
	Mysids	Mysidopsis gahia	Lussier et al. 1985
	Copepods	Acartia tonsa	Reeve et al. 1977
	Copepods	Tigriopus japonicus	D'Agostino and Finney 1974
	Copepods	3 species	Arnott and Ahsanullah 1979
	Amphipod	Allorchestes compressa	Ahsanullah and Florence 1984
	Phytoplank.	Cricosphaera elongata	Gnassia-Barelli et al. 1978
	Phytoplank.	Thallassiosiia pseudonana	Sunda and Guillard 1976
	Diatom	Asterionella japonica	Fisher et al. 1981
	Abalone	H.rufescens	Martin and Stephenson 1977

	Abalone	H.cracheroclii	Martin and Stephenson 1977

Table 6, Continued

Cu	Anchovy	Engraulis mordax	Rice et al. 1980
	Herring	Clupea harengus	Rice and Harrison 1978
	Oyster	Ostrea edulis	Connor 1972
	Shrimp	Crangon crangon	Connor 1972
	Crab	Carcinus maenas	Connor 1972
	Lobster	Homarus gammarus	Connor 1972
	Kelp	Macrocystis pyrifera	Smith and Harrison 1978
Zn	Oysters	Crassostrea gigas	Martin et al. 1981
	Oysters	Crassostrea virginica	Calabrese et al. 1973
	Mussels	Mytilus edulis	Martin et al. 1981
	Crabs	Cancer magister	Martin et al. 1981
	Clams	Mercenaria mercenaria	Calabrese and Nelson 1974
	Mysids	Mysidopsis bahia	Lussier et al. 1985
	various	various australian invert.	Ahsanullah 1976
	various	various estuarine invert.	Eisler and Hennekey 1977
	Herring	Clupea harengus	Somasundaram et al. 1984
	Shrimp	Callianas australiensis	Ahsanullah et al. 1981
	Sea urchins	Temnopleurustoreumahius	Lee and Xu 1984
	Fish	Arbacia lixula	Castagna et al. 1981
	Copepods	3 species	Arnottand Ahsanullah 1979
	Diatom	Asterionella japonica	Fisher and Jones 1981
	Crab	Paragrapsus quadridentatus	Ahsanullah and Arnott 1978
	Oyster	Ostrea edulis	Connor 1972
	Shrimp	Crangon crangon	Connor 1972
	Crab	Carcinus maenas	Connor 1972
	Lobster	Homarus gammarus	Connor 1972

Table 7. INORGANIC REFERENCE TOXICANT SELECTION MATRIX

	Metal			
	Ag	Cu	Cd	Zn
Criteria				
Toxicity	2	1	3	4*
Presence in effluent	4	2	3	1
Bioaccumulation	1	1	1	1
Speciation	1	1	1	1
Analyzability/Treatability	3	2	3	1
Previous use in Bioassays	3	1	2	1
Health Hazard	2	1	2	1
SRC Recommendation	4	2	3	1
$\bar{x}$ ranking	2.5	1.4	2.3	1.4

Ranking Legend: 1 = Most preferred
4 = Least preferred

* A lower toxicity ranking may be considered desirable in some cases, see SRC discussion, page 10.

Table 8. The Stability of Zinc in Solution.

Time (Hours)	Concentration 1 (µg/l) (Mean ± 1 SD; N = 3)	Concentration 2 (µg/l) (Mean ± 1 SD; N = 3)
0	366 (61)	2161 (11)
1	334 (28)	2158 (4)
2	352 (29)	2146 (18)
4	352 (22)	2179 (11)
8	341 (44)	2182 (28)
12	337 (34)	2191 (7)
16	341 (33)	2170 (7)
24	330 (31)	2198 (25)
36	326 (39)	2182 (15)
48	341 (40)	2186 (4)
72	348 (52)	2191 (0)
96	337 (34)	2175 (21)
Mean of All Time Intervals	342	2176
Standard Deviation Among Time Intervals	11	16
Coefficient of Variation Among Time Intervals	3.2%	0.7%

As part of the evaluation of zinc sulfate for use as a reference toxicant, we measured the zinc concentration over time in 3 replicate test containers at two zinc concentrations. Samples of the zinc solutions were taken from the test containers at the indicated time intervals and stored in polyethylene vials. Stored solutions were acidified by adding 1% by volume of 14 N double quartz distilled nitric acid. Samples were analyzed on a Perkin Elmer 603 atomic absorbtion spectrophotometer. Concentration of zinc varied by 3.2% at 342 µg/l, and by 0.7% at 2176 ppb. Hennig and Greenwood (1981) found that zinc concentrations between 0.2 and 3 µg/l decreased by 4% after 50 hours in glass and polystyrene containers.

APPENDIX II

RED ABALONE
SHORT TERM BIOASSAY PROTOCOL

ABALONE LARVAL DEVELOPMENT
SHORT TERM TOXICITY TEST PROTOCOL

Marine Bioassay Project
Coast Route 1, Granite Canyon
Monterey, California 93940

This 48 hour toxicity test uses the early development of abalone larvae as an indication of wastewater toxicity. Abalones 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 (15° to 18° C)
  or Constant Temperature Water Bath
Ultraviolet Water Sterilization Unit (4 to 5 foot UV bulb)
Compound Microscope (100x)
Meter and probes for Dissolved Oxygen, pH, and Ammonia
Salinity Refractometer
Thermometer
Analytical Balance

1 L Volumetric Flasks (2)
Volumetric Pipets 1 mL, 5 mL, 10 mL, 25 mL, 50 mL, 100 mL (1 each)
Graduated Pipets 1 mL, 10 mL (1 each)
Wide-bore Pipets 1 mL, 10 mL (1 each)

25 mL Screw-capped test tubes (35 per test)
250 mL Beakers (borosilicate glass for effluents and organics,
  polypropylene for metals, 35 per test)
37 micron mesh sieve
15 L Polyethylene Pails (3)
10 L Polyethylene Water Bottle
1000 mL Beaker (tall form)

Perforated Plunger (for stirring eggs in the 1000 mL beaker, see Section 2.6)
Polyethylene funnel (with spout to fit into test tubes)
Sedgewick-Rafter counting cell
Hand Counters (2)
Stainless Steel Butter Knife, smooth edged (for handling adult abalones)

2 or more darkened Aquaria for broodstock
Supply of Macrocystis or other brown algae (if broodstock are to be held at
  the lab longer than 5 days)
Flowing 20 Micron-filtered Seawater (2 L per minute, for maintaining
  broodstock prior to testing; if flowing water is not available, static
  aquaria equipped with aeration, temperature control, and sand and activated
  charcoal filters can be used)

10 L of 5 Micron-filtered Seawater (for dilution)
60 L of 1 Micron-filtered UV-sterilized Seawater (for spawning)
Reagent Grade Acetone (1 L per test)
3N Hydrochloric Acid (15 L per test; can be reused 3 times)
37% Buffered Formalin (200 mL per test)
Ethyl Alcohol (750 mL per test)

Data Sheets

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 indigenous to California waters, sensitive to toxicants, important economically, and spawnable year-round.

Broodstock should be positively identified to species. Epipodial characteristics provide the surest means of identification. All California Haliotids have a lacey epipodial fringe, except for the red and black abalones, 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 abalones captured in the wild can be induced to spawn, those grown or conditioned in the laboratory are more dependable. A centralized culture facility can provide a dependable supply of spawnable broodstock, and should be considered as an integral part of any large scale monitoring program. In any case, obtain broodstock from sources free of contamination by toxic substances to avoid genetic or physiological preadaptation to pollutants.

2.3 BROODSTOCK CONDITIONING

Transport broodstock from the field or supply facility in sealed, oxygen-filled plastic bags containing moist (seawater) polyfoam sponges.

Cut sections of polyfoam and soak them in seawater. After 24 hours, remove the polyfoam and wring it well so that it is just moist. Place the polyfoam in double plastic trash bags and then place the abalone on the moist foam. It is important that there is no standing water in the bag. Put the bag with the abalones in an ice chest with two blocks of blue ice wrapped in newspaper. Fill the bags with oxygen, squeeze the bags to purge all the air, then refill with oxygen. Inflate the bags until they fill the

ice chest. Monitor the temperature within the ice chest, making adjustments to keep it within 3 or 4 degrees C of the source water. Ice chest temperatures usually range from 11° to 13° C under these conditions. Abalones can be maintained this way for 24 to 30 hours.

At the testing facility, place the abalones in aquaria with flowing seawater (1-2 L/min). Water temperature should be within 3 degrees of the temperature of the source water. Ideal maintenance temperature is 15° C. If broodstock are to be held for longer than 5 days at the testing facility, feed broodstock ad libitum with blades of the giant kelp Macrocystis. 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 abalones immediately, and drain and scrub their aquarium.

Broodstock can be held in static 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 sand and charcoal filters, and monitor the ammonia content of recirculating seawater to check that filters are removing metabolites.

Assess the reproductive condition of the broodstock by examining the gonads, located under the right posterior edge of the shell. 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. Large (20 cm) abalones ripen at least once a year and provide up to 10 million viable eggs per spawning. Smaller abalones (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).

2.4 SPAWNING INDUCTION

Ripe abalones can be induced to spawn using a number of techniques, the most reliable of which involve stimulating the synthesis of prostoglandin-endoperoxide in the reproductive tissues. This can be done in two ways: addition of hydrogen peroxide (4 mM) to seawater buffered to a pH of 9.1 with 6 mM Tris buffer (see Morse et al., 1977), or irradiation of seawater with ultraviolet light (Kikuchi and Uki, 1974). The latter method has been used in the development of this protocol, and is described briefly below.

Select three ripe male abalones and three ripe females. Clean their shells of any debris. Place the males in one clean polyethylene pail and the females in another. Cover the pails with a tight fitting perforated lid, supply the containers with flowing or recirculating (1 L/min) 20 micron-filtered seawater (15°C), and leave the animals without food for 24 to 48 hours to acclimate and eliminate wastes. Three hours prior to spawning time, drain the pails, wipe and rinse out mucus and debris, and refill with just enough water to cover the abalones (which should all be placed in the bottom of the pail). Begin slowly filling the pails with 1 micron filtered seawater that has passed through an ultraviolet sterilization unit. Flow rates to each of the pails should be 150 mL/min. This low flow rate is necessary to permit

sufficient irradiation of the seawater in the sterilization unit. (The sterilization unit should be cleaned and the UV bulb replaced at least once annually.) Place the pails 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 h), abalones 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 eminating from the respiratory pores.

2.5 FERTILIZATION

Slowly siphon eggs into a third clean polyethylene pail containing one or two liters of 1 micron-filtered seawater. 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. Keep this container in the water bath (15° C). Make sure that temperatures differ by no more than 1° C when transferring eggs or sperm from one container to another.

Using a small beaker, collect about 200 mL of sperm laden water from the pail containing the spawning male abalones. The sperm concentration does not have to be exact, but the beaker should appear cloudy, and sperm concentration in the beaker should be on the order of 50 million cells per mL.

Pour the sperm solution into the third pail containing the clean isolated eggs. Add 1 um filtered seawater at a low flow (1 L/min) to this fertilization pail using a hose fitted with a clean glass tube about 50 cm long. Make sure incoming seawater is within 1° C of the water in the pail. Use the flow from the glass tube to gently roil the eggs up in the pail to allow mixing with sperm and complete fertilization. When the pail is about two- thirds full and eggs are evenly mixed, allow them to settle to the bottom of the pail (about 15 min). At this time fertilization is complete. Pour off the water above the settled eggs to remove sperm laden water. Slowly refill the pail with 1 um filtered seawater of the same temperature. Allow the eggs to settle, and decant again. Repeat again if necessary to remove any excess sperm and obtain clean eggs. After supernatent water has been decanted away, gently pour the eggs into a tall 1000 mL beaker for counting.

2.6 ESTIMATION OF EMBRYO DENSITY

Evenly mix eggs in the 1000 mL beaker by gentle vertical stirring with a perforated plunger. The plunger is a plastic disk, slightly smaller in diameter than the beaker, that is drilled full of small (approx. 5 mm diameter) holes. A plastic rod is fixed vertically to the center of the disk as a handle. 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. 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 100 and 200 eggs per mL.

3.0 TOXICITY TEST PROCEDURE

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), 0.10% 0.18%, 0.32%, 0.56%, and 1.00% effluent; or, as an example of a wider range, 0% (control), 0.10%, 0.32%, 1.00%, 3.2%, 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 concentrations from 0 to 100% effluent may be necessary when nothing is known about the toxicity of the target effluent.

3.2 TEST CONTAINERS

For tests using complex effluents or organic toxicants, use 250 mL borosilicate glass beakers as the test containers. For tests using trace metal toxicants, use 250 mL polypropylene beakers.

3.3 TEST SOLUTIONS

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.

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 um in diameter, unless the effects of dilution water particulates are being specifically addressed in the study. Dilution water salinity should be 34 ppt $\pm$ 2 ppt.

3.3.2 Salinity Adjustment

Adjust the salinity of effluent dilutions to 34 ppt by adding hypersaline brine. Make the brine in advance by heating dilution water to 40° C and aerating until about half the water has been evaporated away. Do not use temperatures in excess of 40° C, or concentrate the brine to salinities greater than 100 ppt. To calculate the amount of brine to add to each test solutions, 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:

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

This calculation assumes that dilution water salinity is 34 ppt. If dilution water is other than 34 ppt, adjust it using distilled (not deionized) water or hypersaline brine.

Use brine controls in all tests where brine is used. Make brine control solutions with the same amount of brine as is used in the highest effluent concentration. Add distilled water to adjust the salinity to 34 ppt, then add dilution water to fill the mixing flask. To determine the amount of distilled water to add, use the above equation, set SE equal to zero, and solve for VE.

3.3.3 Example Test Solution

Two hundred milliliters of test solution are added to 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 100 ppt, add 5.2 mL of brine using a 5 mL volumetric pipet and a 1 mL graduated 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 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 WATER QUALITY ANALYSIS

Measure the temperature, salinity, pH, dissolved oxygen, and ammonia concentration daily in one randomly chosen replicate of each test concentration. Prior to testing, compile a list of containers in which to measure water quality, so that each day one container from each concentration is measured.

Pour or pipet about 10 mL of test solution from the sampled test container into a clean 50 mL beaker. Use this sample for measurement so that probes do not contaminate test solutions. After eggs hatch and larvae

begin swimming, the loss of some larvae during sampling is inevitable but should be minimized. If loss of larvae is likely to occur, swirl the contents of the container before sampling so that swimming and non-swimming larvae are equally subject to removal.

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 or mmHg using an oxygen probe accurate to 0.5 mg/L or 5 mmHg. 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

Based on the estimation of egg density in the 1000 mL beaker, 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. Make sure the temperature of the egg suspension is within 1° C of the temperature of the test solution.

3.6.2 Incubation

Incubate the test organisms for 48 hours in the test containers at 15° C. Fertilized eggs become trochophore larvae, hatch, and develop into veliger larvae in the test solutions during the exposure period. Monitor water quality daily as described above.

3.6.3 Sampling

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 micron mesh screen. The test solution is discarded and the larvae are retained on the screen. Using streams of filtered seawater from a squeeze bottle, wash 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 less accurate and more difficult. Add enough buffered formalin to preserve larvae in a 5% solution. If larvae are to be preserved for longer than 48 hours, decant away the formalin solution after 24 hours and refill vials with 90% ethyl alcohol to avoid chemical deterioration of larval shells.

3.6.4 Counting

Decant away the supernatant solution from the 25 mL vial, leaving only about 5 mL of solution with the preserved larvae. Shake the vial gently to suspend the larvae, then pipet a sample from the vial to a Sedgewick-Rafter counting cell. Examine 200 larvae from each vial under a 100x compound microscope, counting the number of normal and abnormal larvae using hand

counters. After counting, return the larvae to the vial for future reference.

3.6.5 Endpoint

Examine the shape of the larval shell to distinguish normal from abnormal larvae. Normal veliger larvae have a smoothly curved, snail-shaped larval shell that is striated and somewhat opaque. Larvae having clear, thin shells with obvious dents are scored as abnormal. Refer to the accompanying photographs for classification of marginally deformed larvae.

One celled eggs are not counted because arrested development may have occurred before exposure. Any eggs that have stopped developing at the two or more cell stage are counted as abnormal. Some larvae are found remaining in the egg membrane as veligers after 48 hours. These are not counted. Larvae with broken shells or shells separated from rest of the animal are not counted, as these are most likely the result of physical damage during handling.

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 hot tap 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 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 hot tap 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 deionized water, 7) rinse 3 times with deionized water, 8) dry in a clean oven at 50° C.

5.0 DATA ANALYSIS

Add the number of normal and abnormal larvae to get the total number counted for each replicate. Calculate the number of normal abalone 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 (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 Level (NOEL) as the highest concentration that

is not significantly different from the control. Use an alpha level of p = 0.05 to determine statistical significance.

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TOXICITY TEST PROTOCOL USING RED ABALONE LARVAE

1. Induce 3 male and 3 female abalones to spawn using seawater that has passed through a UV sterilization unit at a flow rate of 150 mL per min to each spawning container. Keep containers in 15°C water bath.

2. Mix the eggs and 200 mL of sperm laden water in a third container to produce fertilized eggs. Wash the fertilized eggs at least twice by 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 one mL pipette to estimate egg density.

4. Fill 35 test containers (5 effluent concentrations, a dilution control, and a 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. Pipette 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 micron mesh screen. Wash larvae from the screen into 25 mL vials. Add buffered formalin to preserve the larvae in a 5% solution. Replace preservative solution with 90% ethyl alcohol if larvae are to be stored longer than 48 hours.

7. Pipet a sample from each vial onto a Sedgewick-Rafter counting slide and examine 200 larvae. Return the larvae to the vials for future reference.

8. Record the number of normal and abnormal larvae in each count using larval shell development as the endpoint.

9. Calculate the percentage of normal abalone 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 NOEL value as the highest concentration that is not significantly different from the control.

BIOASSAY DATA SHEET

DATE:

ORGANISM:

TOXICANT:
(for effluent samples give source and date of collection)

EXPOSURE PERIOD:

BEAKER NUMBER	NOMINAL CONCENTRATION ppb = ug/l	NORMAL (alive)	ABNORMAL (dead)	OTHER	PERCENT ABNORMAL	NOTES

APPENDIX III

GIANT KELP
SHORT TERM BIOASSAY PROTOCOL

GIANT KELP BIOASSAY PROTOCOL

Marine Pollution Studies Laboratory
Coast Route 1, Granite Canyon
Monterey CA, 93940

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 glass beaker
(2) 1 L volumetric flasks

pH meter
DO meter
Thermometer
Refractometer
Microscope (w/ocular micrometer)
Analytical balance
Light meter
Cleaning liquids

Pipets (vol. 1 ea. 1- 100 mL)
(grad. 1 ea. 10 mL)
4 cool white flourescent lights
1" PVC frame to hold lights 2' above test containers
Waterbath (15°C)
(or)
Temperature control room
0.2 u filtered seawater

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. Macrocystis forests support a rich diversity of marine life and provide habitat and food for hundreds of invertebrate and vertebrate species. Macrocystis is recommended as a bioassay test species because of its availability, economic and ecological importance, history of successful laboratory culture (North 1976, Dean and Deysher 1983), and previous use in toxicity testing (Clendenning 1958; Smith and Harrison 1978). Like all kelps, Macrocystis exhibits 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 (sporophyll) at their base. The sporophyll develop patches in which biflagellate spores are produced. The spores are called zoospores because they swim. The zoospores are released into the water column where they swim and eventually settle onto the bottom 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 swim through the water and fertilize eggs produced by the female gametophytes. These fertilized eggs develop into sporophytes, completing the lifecycle. This entire process from zoospore release to sporophyte production can be completed on a microscope slide in the laboratory in approximately 12-16 days.

The bioassay protocol described here focuses on germination of the zoospores. 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 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. The two endpoints measured after 48 hours are germination success and elongation of the germination tube.

2.2 COLLECTION

Macrocystis zoospores are obtained from the reproductive blades of the adult plant. The reproductive blades, sporophyll, are located near the base of the plant just above the 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. 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. Sporophyll 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*.

2.3 PREPARATION

After collection, the sporophyll should be kept damp. Avoid immersing the blades in seawater, however, to prevent premature spore release. The sporophylls should be rinsed in 0.2 u 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 and refrigerated (5-6°C) until needed (note: the sporophylls must be used within 24 hours to insure zoospore viability).

2.4 TEST CONDITIONS

2.4.1 Lighting

This test must be done under controlled temperature and lighting. 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 flourescent light tubes.

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

The light fixtures should be adjusted to give 100 uE/m²/s at the top of each test container. It is important that each test container receive the same (± 10 uE/m²/sec.) quanta of light. Areas of increased light can be eliminated by taping the outside of the light diffuser or wrapping the fluorescent bulbs h aluminum foil.

2.4.2 Temperature

Adjust the level of the water bath for maximum cooling, but low enough to prevent floating the test containers. The water bath temperature should be between 14-16°C. If preferred, the test containers can be placed in a temperature room (14-16°C).

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), 0.10% 0.18%, 0.32%, 0.56%, and 1.00% effluent; or, as an example of a wider range, 0% (control), 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. 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

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 food storage containers. Place one standard microscope slide in each test container to serve as substrate for the settled zoospores.

3.3 TEST SOLUTIONS

Prepare test solutions by diluting the effluent with an approved dilution water using volumetric flasks and pipet. Mix test solutions by combining effluent, hypersaline brine (if necessary, see below), and dilution water in a 1 L volumetric flask.

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 0.2um in diameter, unless the effects of dilution water particulates are being specifically addressed in the study. Dilution water

salinity should be 34 ppt $\pm$ 2 ppt.

3.3.2 Salinity Adjustment

Adjust the salinity of effluent dilutions to 34 ppt by adding hypersaline brine. Make the brine in advance by heating dilution water to 40° C and aerating until about half the water has been evaporated away. Do not use temperatures in excess of 40° C, or concentrate the brine to salinities greater than 100 ppt; heating seawater above 40°C causes pH changes. To calculate the amount of brine to add to each test solutions, 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:

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

This calculation assumes that dilution water salinity is 34 ppt. If dilution water is other than 34 ppt, adjust it using distilled (not deionized) water or hypersaline brine.

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. Add distilled water to adjust the salinity to 34ppt, then fill the remainder of the mixing flask with dilution water. To determine the amount of distilled water to add, use the aboce equation, setting SE equal to zero, and solving for VE. Mix the brine control solutions thoroughly.

3.3.3 Example Test Solution

Two hundred milliliters of test solution are added to 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 100 ppt, add 5.2 mL of brine using a 5 mL volumetric pipet and a 1 mL graduated 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. After the test solutions are poured into the test containers, the microscope slides can be added.

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 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 WATER QUALITY ANALYSIS

Measure the temperature, salinity, pH, dissolved oxygen, and ammonia concentration daily in one randomly chosen replicate of each test concentration. Prior to testing, compile a list of containers in which to measure water quality 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 ppt. Measure oxygen in mg/L or mmHg using an oxygen probe accurate to 0.5 mg/L or 5 mmHg. 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 on their maturity, usually 10-15 blades are sufficient. After 1 hour the blades can be placed in a 2 L glass beaker filled with 0.2 u filtered seawater at ambient (15-16°C) temperature. It is important that the release water does not exceed 18°C. The release beaker is placed in the dark to prevent premature germination of the zoospores. To minimize the amount of mucus exuded into the zoospore solution, the blades are placed in the jar so that the torn ends are drooped over the outside of the jar. 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 be sure that zoospores are present, periodically sample the solution and observe the sample microscopically for the presence of swimming zoospores). To insure that the zoospores 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 (at least 1,500,000 zoospores in no more than 10 ml of water), repeat the release process with new sporophyll.

3.7 ZOOSPORE COUNTS

After the zoospores are released from the sporophyll determine their density using a hemacytometer. A sample of swimming zoospores is taken from the zoospore release beaker and placed on the hemacytometer. Take the sample from the top of the release beaker to avoid sampling the dead zoospores and kelp exudate that have settled to the bottom. Give the zoospores enough time to settle onto the hemacytometer (approx. 10 minutes) then count them. 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 of zoospores is determined, calculate the volume of zoospores necessary to give 1,500,000 zoospores per test container. To prevent over-dilution of the test solution, this volume must not exceed 10 mL.

3.8 EXPOSURE OF TEST ORGANISMS

After the zoospore density has been determined and the volume of solution needed to give 1,500,000 zoospores is calculated, add this volume of zoospore solution 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 of zoospores under magnification to insure that they are swimming before adding the zoospores to the test containers.

3.9 ENDPOINT DETERMINATION

After 48 hours the test is terminated. The endpoints measured for the 48 hour Macrocystis bioassay are germination success and germ tube length. Germination is considered successful if a germ tube is present on the settled zoospore when observed under 400x magnification. 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 radius (about 2.0 u) from the edge of the spore (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. For this endpoint only the germinated spores 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, spinning the micrometer ocular lens, and measuring the length of the germination tube that is touching the micrometer in each field. If more than one spore is touching the micrometer, both (or all) are counted. A total of 10 spores for each replicate of each treatment are measured.

4.0 DATA ANALYSIS

Add the number of germinated and non-germinated spores at 48 hours 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 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.

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

5.0 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 hot tap 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 at 50 °C.

Test Containers (plastic): All plastic test chambers used in metals bioassays should be cleaned as follows: 1) rinse 3 times with hot tap 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 deionized water, 7) rinse 3 times with deionized water, 8) dry in a clean oven at 50°C.

6.0 REFERENCES

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

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Smith, B.M. and F.L. Harrison. 1978. Sensitivity of Macrocystis gametophytes to copper. U.S. Nuclear Regulatory Commision, NUREG/CR-0694; UCRL-52461.

MACROCYSTIS BIOASSAY PROTOCOL

1) Collect sporophyll and rinse in 0.2 u filtered seawater. Refrigerate if necessary (5-6°C), no more than 24 hours.

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

3) Place 10-15 sporophyll blades in 2 L of 0.2 u filtered seawater for no more than 2 hours (keep in the dark). 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 1,500,000 spores. This volume should not exceed 10 ml.

5) Check to make sure that the zoospores are swimming, then pipet the volume of water necessary to give 1,500,000 spores 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 NOEL value as the highest concentration that is not significantly different from the contol (at $p < 0.05$). Do an ANOVA on the (untransformed) length data and determine the NOEL using the Dunnett's test as above.

BIOASSAY DATA SHEET

DATE:
ANISM:
TOXICANT:
EXPOSURE PERIOD:

ONTAINER NUMBER	CONC. (ug/l)	NUMBER GERM.	NUMBER NOT GERM.	LENGTH MEASUREMENTS L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	% Germ.

APPENDIX IV

MYSID
SHORT TERM BIOASSAY PROTOCOL

MYSID BIOASSAY PROTOCOL

Marine Bioassay Project
Marine Pollution Studies Laboratory
Coast Route 1, Granite Canyon
Monterey, Ca 93940

1.0 EQUIPMENT

140u Nitex screen tube
Magnifying lens
350 mL glass stacking dishes (35)
(or)
250 mL polyethylene food storage containers
1u filtered seawater
Volumetric flasks 1000 mL
Volumetric pipets: 1, 5, 10, 25, & 100 mL
Wide bore pipet: 10 mL

pH meter
DO meter
Refractometer
Thermometer
15°C water bath
(or)
Temp. control room
Graduated pipets: 1 & 10 mL
Spectrophotometer

2.0 TEST ORGANISM

2.1 SPECIES

The test organisms for this protocol are the juvenile stages of the mysid shrimp Holmesimysis (= Acanthomysis*) costata (Holmes 1900). Although there have been no published reports on the use of this species in toxicity testing, mysids in general are considered to be excellent bioassay 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). Holmesimysis costata occurs in the surface canopy of the giant kelp Macrocystis pyrifera. Little is known of the ecology of this mysid species (Holmquist, 1979; Clutter, 1967 & 1969; Green 1970).

Adult female H. costata carry between thirty and forty embryos in an abdominal broodpouch (the marsupium) and these develop in vivo and hatch as juveniles. The generation time for Holmesimysis costata is approximately 60 - 70 days, depending on feeding rates and water temperature (Anderson and Hunt, unpublished data). Lifecycle tests are impractical with Holmsimysis because of its' relatively long generation time. The following protocol is a simple 96 hour mortality bioassay designed for testing the toxicity of single chemicals and complex effluents.

(* 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 synonomous with H. costata (see also Mauchline 1980).

2.2 COLLECTION

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 come from waters free of point and non-point source pollution to minimize the possibility of genetic or physiological adaptation to toxicants. Brooding adult mysids can usually be obtained by pulling a small mesh net through the water just under the surface canopy blades of Macrocystis*. Mysids can be transported short distances (< 3 hours) in lidded 12 L plastic buckets. They should be well aerated in transport and handled gently to reduce mortality. For longer transport times the mysids can be shipped in sea water in sealed plastic bags. The following transport procedure has been successful: 1) fill the plastic bag with 1 L of sea water, 2) saturate the seawater with pure oxygen, 3) place 25-30 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 by a taxonomist prior to testing; for a review of the taxonomy of this genus consult Holmquist (1979, 1981).

2.3 BROODSTOCK ISOLATION

After collection, broodstock should be transported quickly to the laboratory and placed in 20 L aquaria equipped with flowing seawater with a flow rate of approximately 1 L per minute. The water temperature should be below 17°C. A few kelp fronds can be added to the aquaria to serve as substrate and supplemental food for the animals. The aquaria used at the Marine Pollution Studies Laboratory are made of acrylic plastic 50cm W x 80cm L x 50cm H. The aquaria are fitted with a 1/2" PVC inflow on the top end of one side, and a 1/2" outflow at the bottom end of the opposite side. The outflows have two drainpipes which are lengths of 1/2" PVC with 3/16" holes along their length. These drain pipes are attached to a 1/2" PVC tee on the inside of the aquaria. This arrangement gives two lengths of drain pipe along the bottom of the aquaria which serve to diffuse the suction over the entire bottom of the aquarium. A layer of plastic mesh is placed on plastic light diffuser panel over the drain pipes. Two layers of substrate are layered on top of the mesh. The first layer (directly on top of the mesh) is a layer of pea gravel, the second layer, on top of the pea gravel, is a layer of coarse river sand. The sand and gravel layers filter the water and prevent the mysids from escaping. Mysids can also be maintained under static seawater conditions if necessary. If static conditions are required, cool (15 °C) water temperature must be maintained, and 50% of the culture water should be replaced weekly. It is not necessary to include sand and gravel layers in static seawater aquaria.

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

Newly captured mysids should be acclimated gradually to the holding aquaria to prevent temperature shock. Once in the laboratory, brooding females should be isolated from the other animals and placed in a separate aquarium. This female hatching aquarium does not need a sand/gravel filter system because it is a temporary holding tank. Brooding female mysids can be easily identified by their large, extended marsupia filled with eyed juveniles. The marsupia of females that are close to hatching are grey in color. For this protocol 350 juveniles are needed. Approximately 60 brooding adults should be isolated to provide enough juveniles for one test. The purpose of a separate hatching aquarium is to provide a hatching habitat to isolate juvenile mysids from cannibalistic adults. Cannabalism can also be greatly reduced by keeping the gravid female broodstock well fed (approx. 100 Artemia nauplii per adult mysid per day; Artemia can be supplemented with additions of Tetramin flake food, see Section 2.5). Because several kelp canopy mysids co-occur and are similar in appearance, care should be taken to isolate brooding females of the correct species. After the gravid females release their brood they can be transferred back to the general broodstock aquarium.

2.4 ISOLATION OF TEST ANIMALS.

The newly hatched young can be separated from the broodstock adults by siphoning. Hatchings generally occur at night, so the broodstock tank should be checked each morning for the presence of newly hatched juvenile mysids. The hatching aquarium should be kept free of incidental hatches (350 juveniles), until a sufficiently large enough hatch (>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. The next morning it is checked for juveniles. It is monitored until enough (>350) juveniles have hatched for a test. When large numbers of juveniles are present, they can be siphoned into there own holding container. The juvenile mysid holding container used at the Marine Pollution Studies Laboratory is a 12" diameter PVC screen tube with 140u Nitex screen. This screen tube sits inside of a plastic pail so that seawater flows into the screen tube (0.25 l/minute), through the screen, then out over the top of the pail. This arrangement keeps the juvenile mysids 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, so the newly hatched mysids are kept in their own separate screen tube for three days.

2.5 FEEDING

All broodstock mysids should be fed ad libitum a diet of newly hatched Artemia nauplii (approximately 100, 24 hour post-hatch nauplii/mysid/day), supplemented with flake food (A pet store flake fish food with > 5% lipid content). The amount of supplemental flake food depends on the density of animals in the brood aquarium. In general the mysid broodstock at the Marine Pollution Studies Laboratory are fed approximately 0.1 grams of finely ground flake food/20 L aquarium at the end of the day. . Newly hatched mysids are fed only flake food until they are three days old. After three days they can be fed Artemia.

Feeding rates in the test beakers should be closely controlled. To reduce the accumulation of debris in the test containers, test animals should be fed Artemia only. A feeding rate of twenty nauplii per test animal every 24 hours

is sufficient. Artemia can be delivered to the test beakers 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).

2.6 AQUARIA MAINTENANCE

Broodstock holding tanks should be cleaned at least once per week to prevent accumulation of organic matter and bacteria. The sand and gravel layers can be siphoned with a low-flow aquarium siphon. It is not necessary to clean the bioassay containers during the test.

3.0 TOXICITY TEST PROCEDURE

3.1 EXPERIMENTAL DESIGN

This protocol is based on the use of 5 effluent concentrations and 2 controls, each replicated 5 times. Thus, 35 test containers are needed for each test. Effluent concentrations are usually assigned in a logarithmic sequence as 0% (control), 0.10% 0.18%, 0.32%, 0.56%, and 1.00% effluent; or, as an example of a wider range, 0% (control), 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. 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 [illegible] control is needed in addition to a dilution water control.

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

Prepare test solutions by diluting the effluent with an approved dilution water using volumetric flasks and pipettes. Mix test solutions by combining effluent, hypersaline brine (if necessary, see below), and dilution water in a 1 L volumetric flask.

3.3.1 Dilution Water

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 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 um in diameter, unless the effects of dilution water

particulates are being specifically addressed in the study. Dilution water salinity should be 34 ppt $\pm$ 2 ppt.

3.3.2 Salinity Adjustment

Adjust the salinity of effluent dilutions to 34 ppt by adding hypersaline brine. Prepare the brine in advance by heating dilution water to 40° C and aerating until about half the water has been evaporated away. Do not use temperatures in excess of 40° C, or concentrate the brine to salinities greater than 100 ppt; heating seawater above 40°C causes pH changes. To calculate the amount of brine to add to each test solutions, 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:

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

This calculation assumes that dilution water salinity is 34 ppt. If dilution water is other than 34 ppt, adjust it using distilled (not deionized) water or hypersaline brine.

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. 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 Solution

Two hundred milliliters of test solution are added to 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 100 ppt, add 5.2 mL of brine using a 5 mL volumetric pipet and a 1 mL graduated 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 30 (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 WATER QUALITY ANALYSIS

Measure the temperature, salinity, pH, dissolved oxygen, and amm a concentration daily in one randomly chosen replicate of each c concentration. Prior to testing, compile a list of containers in which to measure water quality 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 ppt. Measure oxygen in mg/L or mmHg using an oxygen probe accurate to 0.5 mg/L or 5 mmHg. 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 Age of Test Animals

Because post-hatch mysid juveniles are not large enough to eat 24 hour post-hatch Artemia nauplii, it is recommended that 3 day old juvenile mysids be used for toxicity testing. Because the mysid bioassay is a 96 hour test, it is practical to start the test on Monday in order to conform to a 5 day work week. Therefore, newly hatched juveniles should be isolated on Friday of the previous week in order to have 3 day old juveniles for the start day of the test.

3.6.2 Exposure of test organisms

After juvenile mysids are 3 days old they are consolidated into a 1000 ml (tall) beaker. They can then be more easily transferred to the randomization cups. Juveniles are counted and tranferred by pipeting in a wide-bore 10 ml pipet. The test animals should be randomized by pipeting them 2 at a time into 35 randomization cups. The randomization cups should sit in the same w baths as the test containers. They can be elevated on to a pedestal to prevent floating. After the first 2 animals are placed in each of the randomization cups, 2 more animals are transferred to each cup. This process is repeated until each randomization cup has 10 juvenile mysids in it. They can then be transferred to the (randomized) test containers. The minimum amount of water should be used for this process to prevent dilution of the test solution. When transferring animals, care should be taken to prevent contaminating the delivery pipet. It is important that each randomization cup has exactly ten juvenile mysids in it to prevent counting errors during the course of the experiment. Verify the number in each test container.

3.6.3 Endpoint determination

The endpoint for the 96 hour mysid bioassay is death*. Because mysids are often found immobile on the bottom of the test container, it is difficult to determine whether or not they are dead. Death is defined as lack of appendage movement. Individual mysids can be sucked up into a widebore pipet or glass tube and observed with a magnifying lens. If its' appendages are not moving when viewed under magnification, an individual is considered to be dead.

* A sublethal endpoint based on a righting reflex or an inability to respond to stimuli is now being considered for this protocol. Until this endpoint is perfected, mortality will be used as the indication of a toxic response.

The dead mysids are removed after each counting to prevent fouling of the testcontainers. 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.

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 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 hot tap water, 4) soak 24 hours in 3% 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 at 50 °C.

Test Containers (plastic): All plastic test chambers used in metals bioassays should be cleaned as follows: 1) rinse 3 times with hot tap water, 2) soak for 24 hours in a mild detergent, 3) rinse 3 times with deionized water, 4) soak for 24 hours in 3% 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.

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TOXICITY TEST PROTOCOL USING MYSID SHRIMP

1. Isolate approximately 50 gravid female mysids into a static sea water hatching aquarium (15°C).

2. Siphon clear the hatching aquarium to remove incidental hatches. When a significant (>35%) hatch occurs, isolate the newly hatched individuals into their own container.

3. Maintain juveniles for 3 days on finely ground flake fish food (> 5% lipid).

4. Fill 35 test containers (5 effluent concentrations and 2 controls, one for dilution water and one for the brine, 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. 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 (eg. 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 <u>exactly</u> 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 <u>Artemia</u> nauplii (100 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 NOEL value as the highest concentration that is not significantly different from the contol (at $p < 0.05$).

BIOASSAY DATA SHEET

DATE:
ORGANISM:
TOXICANT:
CONCENTRATION RANGE (ppb or %):

Container number	Conc. (ppb or %)	Number Dead				Number Alive	Percent Alive	Notes
		Day 1	Day 2	Day 3	Day 4			

WATER QUALITY DATA SHEET

ORGANISM: TOXICANT:

TE	BEAKER NUMBER	NOMINAL CONCENTRATION (ppb = ug/l)	DO (mmHg)	pH	SALINITY (ppt)	TEMPERATURE (degrees C)

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