CULTURE AND TOXICITY TESTING

OF WEST COAST MARINE ORGANISMS

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ACRONYMS AND SYMBOLS

ASTM	American Society for Testing and Materials
ANOVA	Analysis of Variance
BKME	Bleached Kraft Mill Effluent
BML	Bodega Marine Laboratory
cm	Centimeter
CV	Coefficient of Variance
CI	Confidence Interval
DNA	Deoxyribonucleic Acid
DO	Dissolved Oxygen
EC	Effective Concentration
EDTA	Ethylene diaminetetracetate
EPA	U.S. Environmental Protection Agency
ERL	Environmental Research Laboratory
IC	Inhibition Concentration
LBL	Lawrence Berkeley Laboratory
LC	Lethal Concentration
l	Liter
LOEC	Lowest Observable Effect Concentration
MPSL	Marine Pollution Studies Laboratory
μE	Microeinstein
μ	Micron
μg/l	Microgram Per Liter
m	Meter
mg	Milligram
mg/l	Milligram Per Liter
ml	Milliliter
mm	Millimeter
NCASI	National Council of the Paper Industry for Air and Stream Improvement
NOEC	No Observed Effect Concentration (used variably for both lethal and sublethal endpoints)
OEPER	Office of Environmental Processes and Effects Research
ORD	Office of Research and Development
OSU	Oregon State University
ppt	Parts per Thousand
PCB(s)	Polychlorinated Biphenyl(s)
PAH	Polynuclear Aromatic Hydrocarbons
KCl	Potassium chloride
QA	Quality Assurance
SCCWRP	Southern California Coastal Water Research Project
SD	Standard Deviation
TL	Total Length
USACOE	United States Army Corps of Engineers

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DAY 1 -- ALGAE AND INVERTEBRATES

1. MACROALGAE SUMMARY

Two test procedures have been developed for important species of kelp on the West Coast of the United States. The life cycle of each kelp contains both an asexual and a sexual reproductive phase. The test developed for *Laminaria saccharina* assays the effect of a pollutant on sexual reproduction of the kelp; the test developed for *Macrocystis pyrifera* assays the effect on zoospore germination and germ-tube growth (but can be extended to include sexual reproduction as well). Both test procedures, however, should be applicable to either of these species, and probably to some of the many other kelp that populate the West Coast.

The *Laminaria* sexual reproduction test, developed at the U.S. EPA laboratory in Narragansett, Rhode Island, has not been utilized to any extent outside of that laboratory. The *Macrocystis* test was developed at the California Department of Fish and Game Marine Pollution Studies Laboratory and has seen considerable testing outside of that laboratory. *Macrocystis* has been successfully cultured by several laboratories in California for aquacultural applications.

Comparative sensitivity of the two test procedures (and the two species of kelp) has not been sufficiently studied. Indications are that the two tests are generally comparable, but studies designed to ascertain sensitivity to a broader range of toxic materials should be conducted.

The two test procedures differ in the source of the test organisms. The *Laminaria* test organisms are laboratory cultures of isolated male and female gametophytic cells; the *Macrocystis* test organisms are spores released from recently field-collected kelp plants. The *Macrocystis* spore test is limited primarily by distributional or meteorological factors constraining access to spore-bearing populations of the kelp. The *Laminaria* procedure is limited by the maintenance of reasonably pure cell cultures of male and female gametophytic cells. The *Macrocystis* test (48-h)

is shorter in duration than the *Laminaria* test which includes a several day growth period following the 48-h exposure. The two tests could be used in a complementary manner, with sexual tests used when spores could not be collected.

The *Macrocystis* spore test is easier to export to other laboratories because it does not require the equipment and expertise needed for the *Laminaria* cell culture. Further development of these test procedures requires the following activities: (1) interlaboratory studies of the *Macrocystis* spore test using several classes of reference toxic materials; (2) export of the *Laminaria* gametophyte cultures and test procedure to other laboratories to assess the level of culture success attained; and (3) comparative sensitivity tests with the two procedures in at least one laboratory utilizing several classes of reference toxic materials.

Summary of Toxicity Test Development with the Kelp
Laminaria saccharina

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We first isolated *Laminaria saccharina* from Narragansett Bay in late 1985, but the work I will be reporting on is that accomplished in the last couple of years since we've been able to grow it in artificial seawater and have had good repeatability in obtaining eggs and sperm.

Laminaria gametophytes are very easy to culture either in natural seawater (in red light) or in artificial seawater (in white light) without added iron in the medium. Occasionally, a culture will become contaminated with microalgae and will require cleaning treatment. The cultures are most at risk at this time, and may be lost due to excess toxicity of the treatment on plants in weakened condition. It is for this reason that we <u>never</u> treat all of one clone but retain backup cultures in two to three other growth chambers. As long as the protocol is followed and no iron or Tris (TRIZMA 7.8) is added in white light, the cultures remain vegetative.

Once we worked out the culture requirements for the kelps, we really haven't had any unsuccessful culture experiences, except with contamination. When not being actively cultured, clones may be stored in quiescent culture conditions (12 - 14°C, and red light at 10 - 15 $\mu E \cdot m^{-2} \cdot s^{-1}$).

We have conducted several tests, both on effluents and on known toxicants. The metals copper and silver have been tested. These metals are toxic to other algae (copper at < 10 $\mu g/l$ and silver at < 1 $\mu g/l$) such as *Champia*. *Laminaria*

appears to be somewhat less sensitive to metals (copper at < 50 μg/l and silver at < 5 μg/l). However, *Laminaria* does appear to be somewhat more sensitive to organic toxicants.

Since *Laminaria* gametophytes can be cultured year-round in artificial seawater, it is not subject to the vagaries of nature. It need only be maintained in active growth conditions and kept free of contaminants. Tests theoretically can be run every day, about one to two per day. This includes set-up and tear-down (counting). However, since weekends do occur, and the initiation time (6 days), exposure (2) and recovery (4 - 6 days) require that these days be in sequence or at least be timed so that personnel will be available to do the work, about 3 - 4 tests (per week) are practical. During the course of a week, other tasks must also be performed, such as glassware treatment and cleaning, medium preparation, and other ancillary tasks so that the tests themselves are possible. Approximately 12 - 16 man-hours per week are required to run one test per week.

The major drawback to these tests is the fact that in all kelps tested so far, parthenogenesis occurs. This means that in any test, parthenogenesis controls must be established and counted, and the average number of sporophytes obtained must be subtracted from the total to derive those formed by sexual fusion. Some knowledge/experience is needed in culturing algae -- the first tests and/or cultures may not be as good as those performed after some experience is gained in handling the cultures.

We have looked at other genera and species. Of the three *Macrocystis* species, *M. pyrifera* forms eggs most easily with the *Laminaria* technique, with a slight modification (higher iron-chelator). Even so, the very high percentage of cells forming eggs (80 - 90 percent in *Laminaria*) does not occur in *Macrocystis* (20 - 40 percent). The other two species, *M. augustifolium* and *M. integrifolium*, form hardly any eggs at all. *Egregia mensiezii* is difficult to culture in white light, limiting the growth rate (it forms some eggs even without added iron). *Nereocystis lutkeana* appears to have a high percentage of parthenogenesis. Perhaps with further research on egg production in *Macrocystis* we will have a better test, as parthenogenesis appears to be very infrequent, at least in the isolates tested.

DISCUSSION

MR. ANDERSON: How many times have you run the Laminaria test for copper?

MR. STEELE: About 10 times, I think.

MR. ANDERSON: Do you usually obtain consistent results between tests?

MR. STEELE: Yes.

MR. ANDERSON: Do you generally find the NOEC for copper to be 45 μg/l?

MR. STEELE: Yes. If we're getting a good test, the results are pretty consistent. We're developing this particular test, and, at the same time, we are testing, so we have not always gotten good runs with respect to the amounts of eggs and sperm produced, especially in our earlier tests. But lately, we're getting really good results. Of course, that's due to experience in growing the gametophytes.

MR. CHERR: Do you run the tests in polystyrene plastic?

MR. STEELE: Yes.

MR. CHERR: Do they seem to have a preference for polystyrene, glass, or polypropylene?

MR. STEELE: No. When we look at gametogenesis, that is the eggs and sperm, and what's on the glass and what's on the polystyrene itself, there seems to be no difference that we can detect. We have noticed some difference between the companies that manufacture the dishes and what type of dish it is. We've tried some Falcon[R] dishes. In some of them, the surface tension was very high and you could see that a bead of water had a really high meniscus where other dishes would pool out. And the difference seemed to be the manufacturer. Lab Tek Brand seems to work pretty well, I think.

MR. CHAPMAN: I have a couple of questions. Do you run a parthenogenesis control with no added males?

MR. STEELE: Yes, we do. Once we found that it was a problem, then we ran a parthenogenesis control for each test.

MR. CHAPMAN: You add no sperm?

MR. STEELE: Yes, only eggs are present.

MR. CHAPMAN: What sort of variability do you see between the density of potential eggs for fertilization between different dishes?

MR. STEELE: Well the most difference was between different experiments rather than between different dishes. We disperse the gametophytes (male and female) in a liter of water. And then we dispense them with an Ace dispenser or squeeze bottle, so we get a fairly good distribution of male and female gametophytes in the dishes. In runs where I've had too many females, we don't get the nice results that we do if we have a less dense dispersion. If you get too few, you don't get enough pheromone released to cause the males to react. About 100 - 150 gametophytes on the small glass slide is optimum. So it was determined that in the tests we're talking about, the number of gametophytes are about right.

MR. DEAN: When you do a whole effluent test, what kind of problems do you see with contamination at that point and can you differentiate toxic effects from effects of contaminant growth?

MR. STEELE: Well, since the initiation portion of the experiment is not in natural sea water, there's only a 2-day exposure in the effluent. Gametophytes are then taken out and put into another media. We may see a few diatoms or other organisms developing, but they're not dense enough at the end of 4 days to cause any problems. If they were in there the whole 11 or 12 days, you wouldn't be able to see the gametophytes. In the short time of the test, the contaminants don't have time to take over. However, you do see some growth and things come up pretty fast.

MR. HUNT: I'd just like to follow up on Gary Chapman's question. Have you tried looking at the results in terms of a ratio between eggs and female gametophytes present as opposed to just absolute sporophyte numbers?

MR. STEELE: We consider it not a good run unless we get at least an egg for every gametophyte particle. What I meant to say is sporophytes per gametophytes, as opposed to absolute sporophyte numbers. Is that what you (Gary Chapman) were asking?

MR. CHAPMAN: Not quite. I was concerned about having, let's say, 5,000 eggs on 1 plate and 4,000 on another so that you'd end up with differential sporophyte counts as a function of different egg densities; that was my concern.

MR. HUNT: Right. I know that we've tried looking at ratios. We usually count absolute sporophyte numbers but, sometimes, we've done tests where we look at a ratio of sporophytes to gametophytes.

MR. STEELE: In a lot of our experiments, we've counted everything; for instance, we counted the number of gametophytes present, the number of eggs, and the number of sporophytes; we just haven't done much with that data yet. We have a lot of numbers, but we haven't worked them up yet. That's one thing we've got to look at.

MR. ANDERSON: That would eliminate the problem of having different densities of female gametophytes between replicates.

MR. STEELE: Yes. With our technique, we get fairly nice repetition between dishes because it's a highly dispersed small fragment, not much bigger than a piece of phytoplankton, and if you're keeping it stirred it disperses quite evenly. So you don't really have dishes where you have a lot in one dish and very few in the next. If you noticed, we filter the females through a 45 μ filter, and the males through a 75 μ filter. We want to get an excess of males because we want a lot of sperm. And the reason we use a smaller filter for the female is, we want the fragments smaller. We get less production from our culture of female fragments, but they're smaller 2- or 3-cell particles rather than 8- or 10-cell particles that we would get with a larger filter. That's the reason we went to the smaller filter. They also stay in suspension a lot better.

MR. ANDERSON: Has anybody else had success conducting this test? Have you conducted interlaboratory tests?

MR. STEELE: We aren't really to that spot in development yet. We've had other people in the lab try it but we haven't farmed it out. We're still working on some of the problems.

MR. ANDERSON: It might be a good idea to try that.

MR. STEELE: Yes, I agree, that comes next, we'll have round-robins -- of course, in developing these tests, these steps have to come one after the other.

MR. ANDERSON: I agree.

MR. CHAPMAN: In that respect, Dick Steele will be moving to our lab in Newport late this year. This will allow us an opportunity to do some interactive work.

MR. STEELE: Yes, and I will be able to go out and get gametophytes that I isolate. A lot of these I have are from people like Neuschel down at Santa Barbara; and some of these are at least 10 years old. I may not be working with the best isolates because some of these have been 8 and 10 years in culture. I'd like to isolate some of my own so that maybe I can get better results with fresher, genetically newer clones, if that's appropriate terminology.

MR. CHERR: I'm just curious about using Tris, it being a weak base. Did you try other weak bases?

MR. STEELE: Yes, I tried several of the Sigma products. I tried Tris Bis-propane and a couple of others; Tris was the only one that really worked well. However, one of my techniques for cleaning up cultures is the use of 2, 3, 5 triphenyl tetrazolium chloride. It's a compound that's used for determining seed germination percentage in higher plants. And I'm using it as a method to clean up cultures because it tends to kill green algae and not the kelp gametophytes. However, at very low levels, say from 100 - 500 μg/l, it has the same effect as Tris. I don't know what the mechanism is; it's just a serendipitous observation, but it works. Maybe I can replace Tris.

MR. CHERR: It should be noted that Tris increases the intracellular pH.

MR. ANDERSON: What was that chemical?

MR. STEELE: It's written in my protocol. It's 2, 3, 5 triphenyl tetrazolium chloride. It's a seed germination indicator. The full name is given in the protocol that I handed out; it's one of the cleaning techniques. That tends to be the biggest problem in culturing -- contaminants -- not so much in the culturing itself, but during the tests. If the cultures are contaminated, the contaminants can interfere with the test, especially if the contaminant is algae.

MR. DEAN: What do you see as the effort involved in maintaining daily cultures and performing the clean-up work on cultures?

MR. STEELE: Well, if you have dirty ones, it can be quite a task at the beginning. I think every one I've gotten through the mail has been contaminated when I got it, with at least one thing. Once they're cleaned, then it's just a matter of transferring them about once every 2 weeks. We keep enough cultures around, and we know how frequently we're going to be using them. For instance, if we're going to use 1 every day, we keep 7 to 10 cultures so that we don't have to reuse that culture for at least a week; 2 weeks growth is preferable. And usually about 1 day out of 2 weeks, I'll spend the morning transferring cultures.

Summary of Toxicity Testing With Giant Kelp

Macrocystis pyrifera

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We began working with the laminarian alga *Macrocystis pyrifera* in 1986 as part of research funded under the California State Water Resources Control Board's Marine Bioassay Project. The Project is developing sensitive-life-stage toxicity tests that are intended to be short-term indicators of chronic toxicity. Our initial toxicity tests with this species were relatively long-term (12 - 15-day) experiments that focused on kelp reproduction (sporophyte production). This test proved to be impractical for routine effluent testing for a number of reasons, so our focus shifted to a short-term (48-hour) toxicity test. The short-term test has two endpoints: zoospore germination and gametophyte growth. Although we have continued to use the long-term sporophyte production test for reference toxicant testing, we consider the short-term test to be more practical for routine effluent testing, and the majority of our efforts have been devoted to developing this test.

In the 48-hour kelp test, motile kelp zoospores settle onto glass microscope slides in test solutions. The slides are examined microscopically after 48 hours to determine both the proportion of spores that fail to germinate, and the length of the embryonic gametophytes. Zoospores are induced to release from reproductive blades (sporophylls) collected from adult plants in the field. Fertile sporophyll blades are available year-round, but must be collected by skin divers prior to each toxicity test because zoospore production decreases approximately three days after collection. We generally collect sporophylls the day before starting a test and induce zoospore release the next morning. Out of 44 experiments conducted since 1986, 43 have had

successful spore releases and greater than 80 percent germination in the controls. Using these criteria, 98 percent of the tests were successful. An unsuccessful test occurred during an interlaboratory experiment in July 1987 when there was low control germination rates at one of the laboratories, presumably due to receiving water toxicity or shipping stress (see Table 1).

TABLE 1. KELP 48-HOUR TOXICITY TESTS

Month	Successes/Attempts	Comments
January	2/2	
February	6/6	
March	3/3	
April	1/1	
May	3/3	
June	1/1	
July	1/2	Low control germination
August	3/3	
September	11/11	
October	2/2	
November	3/3	
December	6/6	

*Success is defined as successful spore release and greater than 80 percent control germination.

Because fresh kelp sporophylls are needed for each experiment, there are some logistical constraints involved in the scheduling of collection and delivery of sporophylls and effluent. *Macrocystis* forests occur from Baja to Santa Cruz, California, so laboratories north of Santa Cruz must depend on southern suppliers for their test material. This has not been a problem because mature sporophylls are easy to collect and ship. There are several research institutions that are willing to supply sporophylls, and consulting firms will presumably fill the supply void when the market develops. Sporophylls transport well, and past interlaboratory experiments using shipped sporophylls for complex effluent and reference toxicant experiments have shown comparable results between laboratories. It may be possible that

northern laminarian species can be substituted in locales where *Macrocystis* does not occur, but more research with other genera needs to be completed before this becomes common practice.

We have conducted experiments with a number of reference toxicants, but most of our bioassays have been with zinc sulfate and copper chloride. NOECs in three tests with zinc ranged from 1800 - 5600 μg/l for germination and < 560 μg/l for germ-tube growth. With copper, germination NOECs ranged from 32 - 100 μg/l in multiple tests, and from <10 - 32 μg/l for germ-tube growth. In general, germination has been less sensitive and more variable than germ-tube growth in repeated reference toxicant tests. Vegetative endpoints (such as germination and germ-tube growth) appear to be less sensitive than reproductive endpoints (such as sporophyte production).

We estimate that it would require approximately 40 man-hours to conduct one effluent and one concurrent reference toxicant test (30 containers each = 60 containers total). This estimate covers everything from sporophyll collection to test container clean-up. Assuming a 39-test container design for the purposes of this workshop, it would require between 25 - 30 man-hours per test. Two people could conduct two tests per week; one test would require one person.

The 48-hour kelp test can be conducted year-round, and our research indicates a greater than 95 percent success rate based on spore releases and germination rates. The protocol is limited by the need for fresh, field-collected test material for each test, and the lack of *Macrocystis* populations north of Santa Cruz. In addition, test organisms are analyzed live and the two endpoints require a considerable amount of microscope time on the last day of each test (approximately 10 - 12 man-hours).

DISCUSSION

MR. BAY: One of the difficult things about the test, I think, is that you need to count the samples immediately at the end of the 48-hour exposure period. Do you think there's any potential way to fix the slides so you have a little more leisure in determining the endpoint?

MR. ANDERSON: Yes, I think there is. I know Gary Cherr at Bodega Marine Laboratory has fixed kelp tests with glutaraldehyde. The data from one of the interlaboratory tests that I showed earlier are from a copper test that Gary conducted at Bodega. He fixed the test at 48 hours then read it the next day, and his results were consistent with ours.

MR. CHERR: We started fixing in 1.0 percent glutaraldehyde and sea water and we've gone down to 0.1 percent now. I think there is still a small amount of shrinkage in the germinated tube. So, if you measure it non-fixed and then after fixation, there is a slight difference in morphology but that's all the way across the board. However, that difference is only seen after about 24 hours of fixation. During the first 4 or 5 hours, it seems, they look identical.

MR. ANDERSON: I agree. I don't think shrinkage will be a problem as long as the test is read soon after fixing (within 24 hours). We have fixed kelp gametophytes in formalin and let them sit for a week before reading; after this much time they appeared bleached-out. We would not recommend fixing the test with formalin. We need to design an experiment that looks specifically at what effect fixing the gametophytes has on the results, particularly effects on germ-tube growth.

MR. STEELE: One of the things in my protocol with glutaraldehyde -- do you buffer it?

MR. ANDERSON: We buffer it with sea water.

MR. STEELE: We buffer with phosphate. Tris works pretty well with buffering, too, but keep it in the cold and in the dark. Our gametophytes are a little more robust than the spores. Another question. Do you collect your water through your lab system? We've found that we get more comparability in our lab using water samples collected only on an incoming tide. Of course, our situation is just a little different than yours; we're in sort of a bay. I was just thinking that maybe you ought to try collecting some offshore for awhile.

MR. ANDERSON: Why do you think that is? Because of variations in the chelation capacity of the seawater?

MR. STEELE: It's coming down from Providence on an outgoing tide; we try to get the cleaner water. Some samples will dry and everything is dead, even including controls if we use outgoing water. There's a lot of crap that comes down from Providence.

MR. ANDERSON: I think it might be a good idea to try using artificial seawater for the metal reference toxicant tests. That would eliminate variability due to variation in the chelation capacity of the seawater. I assume that chelation differences are partially responsible for differences between inter- and intra-laboratory tests. An alternative approach would be to use an organic reference toxicant. However, we have not found an organic toxicant that suits all of our needs. It should be noted that Steve Bay's original point is right, the kelp test is only 48 hours long, but on the final day of the test it takes three people most of the day to read a standard 75-container test. By preserving the test, we could spread the microscope time over a couple of days.

MR. DEAN: Do you end the test at 48 hours and then start counting or do you end it at 46 and go plus or minus two hours?

MR. ANDERSON: We bracket it so that we start reading at 43 - 45 hours and usually finish reading at 48 - 51 hours. I don't think there is a significant amount of germ-tube growth during that period.

MR. BERGER: I was wondering, have you run this with the effluent both using hypersaline and artificial sea salts to adjust salinity?

MR. ANDERSON: Yes.

MR. BERGER: And do you see any differences?

MR. ANDERSON: No, of all of the species we work with, *Macrocystis* is the least sensitive to any kind of salinity changes. We've had salinities up to 44 g/kg and not seen any effect. We sometimes encounter problems with particles obscuring the nongerminated spores when using the *Macrocystis* test to assess effluent toxicity. The kelp gametophytes grow on microscope slides on the bottom of the test containers. At higher sewage effluent concentrations, for example, there can be a considerable amount of particles settling on the slides; these can hide the spores. In some cases particles may look like nongerminated kelp spores. It takes a certain amount of training to know how to differentiate kelp spores from other objects.

MR. CHERR: Could you comment on your experience with illumination and, specifically, the lack of it? I remember, at some point, you had mentioned that you had extra-long germ-tubes without light.

MR. ANDERSON: Yes.

MR. CHERR: What are your thoughts about, for example, running the whole test in the dark? In particular, working with toxicants that may undergo breakdown under light.

MR. ANDERSON: Our initial experiments used constant light in order to accelerate the reproductive process; this was the lighting regime reported in the literature for research on factors important for sporophyte production in kelps. We carried this lighting regime over when we developed the short-term, 48-hour kelp test. We later changed the lighting regime to a more environmentally realistic regime: 16 hours light/8 hours dark. We conducted one test under complete darkness in order to investigate possible mechanisms of germ-tube growth inhibition. We do not intend to conduct more tests in dark, however, because it is not environmentally relevant.

MR. DEAN: Brian, just as a comment. I think that when we did those interlaboratory tests, the differences that we saw in germ-tube length may have been due to the differences in light levels. Because, at that time, there was confusion about what light levels to use; and we were using incredibly high light levels.

MR. ANDERSON: Yes, that's a good possibility. We have seen similar shifts in some of our other interlaboratory tests. However, I don't think it is a significant problem because the response curves are generally similar between laboratories.

MR. CHAPMAN: What is the ecological significance of germ-tube length?

MR. ANDERSON: That is a good question. Our research, and the work of developmental biologists working with similar plant systems, indicates that toxicants inhibit germination-tube elongation by disrupting the germ-tube membrane. This, in turn, may influence the ion gradient across the membrane. It is thought that an ion gradient is necessary to establish an electro-chemical gradient across the membrane, which, in turn, is necessary for germ-tube elongation. It is

difficult to say what inhibition of germ-tube elongation means to kelp populations in nature. We consider germ-tube growth to be an indicator of toxic effect. We have found that sporophyte production is generally a more sensitive endpoint than germ-tube growth. Therefore, if a sewage effluent significantly inhibits germ-tube growth, it will likely affect sporophyte production at similar or lower concentrations.

MR. CHAPMAN: Another question. How do you randomly select spores for measuring? Is there a table of random numbers (e.g., 4th, 12th, and 13th)?

MR. ANDERSON: No, instead of a random numbers table, we change field of views under the microscope without looking through the eyepiece. We then look through the eyepiece and measure the germinated spore nearest the ocular micrometer. In addition, all containers are read blind to eliminate any bias that might result from the reader knowing the concentration being read. There are thus two layers of randomization.

MR. CHAPMAN: One last comment -- and maybe Dick Steele can confirm this --when he was visiting Newport about 3 years ago, and we did a few *Laminaria* tests, it seems to me that our copper effect levels were down around 10 μg/l. Do you have any recollection of it?

MR. STEELE: Not really.

MR. CHAPMAN: My feeling was they were low like yours (Anderson).

MR. ANDERSON: From what I have read, copper NOECs reported for sporophyte production in *Laminaria saccharina* and *Laminaria hyperborea* are around 10 μg/l. Chung and Brinkhuis recently reported a NOEC of 10 μg/l for inhibition of sporophyte production by copper chloride in *Laminaria saccharina*.

MR. LANGDON: Do you find that there's a positive correlation between germ-tube length and the viability of gametophytes? Have you looked at that at all in terms of producing fertilization tubes?

MR. ANDERSON: Are you asking whether spores that have reduced germ-tube length develop into gametophytes which successfully produce gametes?

MR. LANGDON: Yes.

MR. ANDERSON: We have not looked at that specifically. We do, however, find that NOECs for sporophyte production are generally lower than NOECs for germ-tube length. We have conducted experiments with copper where we placed two microscope slides into each test container, and removed one slide after 48 hours to look at germ-tube growth. We then removed the second slide after 21 days and looked at sporophyte production. The NOEC for germ-tube growth in this case was 10 μg/l. The NOEC for sporophyte production was less than 10 μg/l. Thus, although spores in the 10 μg/l treatment did not have significantly shorter germ tubes, copper inhibited the reproductive process at this copper concentration. It is not clear whether or not individual spores having slightly shorter germ-tubes eventually are able to develop into gametophytes and produce gametes. It may be that the sporophyte production is inhibited at some other point in the reproductive process, during fertilization for instance.

Short-term Chronic Toxicity Tests with *Macrocystis pyrifera*

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We have been culturing the various microscopic life-stages of giant kelp, *Macrocystis pyrifera*, in our laboratory for the past 12 years, and have been conducting kelp spore germination tests, as developed by B. Anderson and J. Hunt of the Marine Bioassay Project Laboratory at Granite Canyon, for almost 2 years. In that time, we have conducted 22 tests. In all cases, spores were obtained from sporophylls of giant kelp collected from the field the day prior to the test. No attempt has been made to hold sporophylls in the laboratory for extended periods.

In our toxicity tests and in prior culture work with *Macrocystis*, we have experienced no difficulty in obtaining spores on a year-round basis. We have collected sporophylls for release on over 200 occasions. The collections have been made in all months under a variety of oceanographic conditions. We have obtained successful release of spores on all occasions except for several collections made during the height of El Nino in the late summer and fall of 1983. During El Nino, we made several unsuccessful attempts to locate "ripe" sporophylls in the field and had no success in obtaining sufficient release of spores from the several marginal sporophylls collected at that time.

We have conducted tests with various wastewaters and with a copper reference toxicant. While there are, as yet, no established standards for acceptance of kelp spore tests, we have adopted "in house" standards that require that reference toxicant tests, using filtered sea water from an unpolluted site for dilution, be run in conjunction with each wastewater test. Test acceptance is based on the following criteria: (1) germination in the reference control shall exceed 70 percent and average germ tube lengths will exceed 10 μm (2) NOEC levels for germination and germ-tube lengths will not exceed 80 and 40 μg/l copper, respectively; and (3) standard errors for Dunnet's tests shall not exceed 30.0 (for arcsin transformed percent germination)

or 1.0 (for germ-tube length). Of the nine tests that we have run using these criteria, only one has failed to meet the acceptance standards. We expect about a 90 percent success rate using our acceptance standards.

For the nine copper reference tests performed since March 1989, the NOEC values for germ-tube length averaged 23 μg/l and ranged from 40 to <10 μg/l copper (see Table 1). NOEC values for germination were generally higher and more variable, ranging from 10 to 160 μg/l and averaging 52 μg/l copper. EC10 values averaged 79 μg/l for germinations and 24 μg/l for germ-tube length. These are comparable to values for chronic toxicity obtained for sensitive life stages of other marine plants and animals (see Table 2).

TABLE 1. SUMMARY OF REFERENCE TOXICANT TEST RESULTS FOR GIANT KELP SPORES TESTS (NOEC AND EC10 VALUES ARE μg/l COPPER)

Percent Germination

Test Date	Control Mean	Dunnett's std. err.	NOEC	EC10	Upper 95% CI
2/14/89	96.6	3.3	56	81	125
2/14/89	95.2	2.9	56	107	139
2/28/89	98.4	2.1	10	65	84
2/28/89	96.8	2.7	18	77	107
3/15/89	93.4	2.2	32	66	108
3/21/89	92.8	2.0	56	88	113
6/20/89	92.4	3.0	56	124	150
7/11/89	19.4	3.5	160	-	-
12/27/89	74.4	2.9	20	20	26
Mean	84.4	2.7	51.6	78.5	106.5
CV	30%	20%	87%	40%	36%

Germ-Tube Length

Test Date	Control Mean	Dunnett's std. err.	NOEC	EC10	Upper 95% CI
2/14/89	11.8	0.5	18	32	39
2/14/89	11.7	0.7	18	25	31
2/28/89	13.4	0.6	18	18	23
3/15/89	12.2	0.5	18	23	30
3/21/89	11.9	0.7	32	19	24
6/20/89	13.6	0.5	18	27	34
7/11/89	11.3	0.5	40	36	43
12/27/89	12.1	0.5	<10	4	8
Mean	12.0	0.6	22.7	23.9	31.4
CV	9%	24%	42%	40%	40%

TABLE 2.   AVERAGE NOEC VALUES FOR COPPER TOXICITY (g/l) AND COEFFICIENTS OF VARIATION OF TESTS PERFORMED IN A SINGLE LABORATORY FOR SEVERAL MARINE SPECIES

Test Species	End Point	Mean NOEC	CV
Macrocystis pyrifera	Germination	52	45%
	Length	23	40%
Champia parvula	Reproduction	0.8	32%
Arbacia punctulata	Fertilization	12.2	61%
Cyprinidon variegatus	Growth	105	40%
Menidia beryllina	Growth	94	47%

With a staff of three persons, we can comfortably conduct six kelp spore tests per week (four wastewater and two reference toxicants).   One test per week would require two persons working half-time.   The limiting factor for conducting more tests is the ability to count and measure germinated spores at the culmination of the test. At present, there is no way of preserving spores, and all must be counted within several hours of the 48-hour test period.   It takes approximately 2.5 hours for a trained person to count and measure spores from 30 slides (5 replicates for each of 6 treatments).

There are few major problems in conducting this test on a routine basis.   The only potential problem would be the inability to collect spores during extremely bad weather, or the inability to find ripe sporophylls during El Nino periods.   While we prefer to collect sporophylls from attached plants using SCUBA, we have successfully obtained spores from beached plants when storms precluded diving.

DISCUSSION

MR. HALL: How would the *Laminaria* 48-hour test and the *Macrocystis* 48-hour test compare? Is there any comparative data base? And, if you

the way these things occur in terms of sporophytes, would there be one that would be a likely first choice?

MR. DEAN: By the *Laminaria* 48-hour test, you mean the one that Dick Steele talked about?

MR. HALL: And the one you just described for *Macrocystis*.

MR. DEAN: I don't know of anyone that's done those tests so I guess I can't answer your question.

MR. HALL: From what you know of the biology of that species, would it probably --

MR. DEAN: It should be comparable.

MR. ANDERSON: I believe that David James at Cal Tech has used a 96-hour test with endpoints similar to our growth endpoint to look at the relative sensitivity of several kelps such as *Nereocystis*, *Pterygophora*, *Egregia*, and others. He found a difference between species; I believe that *Nereocystis* was more sensitive to PCBs.

MR. DEAN: Yes, but I don't think there's anybody that's done germination and germ tube length tests with any of the other laminarians that I know of.

MR. ANDERSON: Yes.

MR. DEAN: One of the problems with a lot of the other laminarians is that they are more seasonal, so you're not able to obtain viable sporophylls at all times of the year. Also, other laminarians have other factors that affect spore release. *Nereocystis*, for example, seems to release spores relative to lunar cycles. I've been able to get spore release and do comparable kinds of experiments with some of the *Alaria* species, although it's not quite as simple as it is with *Macrocystis* because of the release problems.

MR. STEELE: Do you have comments on the particular biology of *Macrocystis* that lends it to this kind of test? In New England, I would be able to do *Laminaria* only, say, 4 months of the year.

MR. ANDERSON: Right.

MR. STEELE: But we essentially have an El Nino every summer.

MR. HUNT: Tom, is that the same situation on the West Coast, that *Laminaria* has a limited reproductive season?

MR. DEAN: Yes. Most of the other *Laminaria* do have seasonal release of spores.

MR. CHERR: What season is that? Winter?

MR. DEAN: It depends on where you are; but in southern California, peak time of release for laminarians other than *Macrocystis* is in late winter or early spring. As you go north, when you get into Alaska, for example, it's May-June, or maybe even somewhat later. I think Vadas' data for *Agarum* shows peaks in spore production in July and August in Puget Sound.

MR. STEELE: Egg production occurs mostly in the winter; about 12° C is optimum for egg and sperm production.

MR. HALL: I have one more question. Would you expect *Macrocystis* to have year-around availability farther north -- like off the Washington-Oregon Coast?

MR. DEAN: The northern limit of distribution for *Macrocystis pyrifera* is probably the Humboldt area.

MR. HALL: So you're talking about a particular species of *Macrocystis* then?

MR. DEAN: I haven't looked at *Macrocystis integrifolia*, which occurs farther north. Dick (Steele), do you know anything about *integrifolia*?

MR. STEELE: No, *pyrifera* would be more advantageous for egg and sperm production; under my technique, it produces more eggs than sperm. But that's the only experience I have with that.

MR. DEAN: I think it would work fairly well with *integrifolia*. Again, my guess is it would be fairly limited to the seasons you get spores. Have you (Mr. Anderson) looked at *integrifolia*?

MR. ANDERSON: We've done spore releases on *M. integrifolia* and had no problem. We've never conducted a toxicity test using this species. The other solution to the supply problem is to ship *Macrocystis* sporophylls; they transport well and finding a supplier would be no problem.

MR. CHERR: Is anything known about temperature effects with respect to culturing -- specifically, temperature effects on germ-tube length in strains of *Macrocystis* coming from areas of different temperature?

MR. DEAN: We collected sporophylls from Mexico, near its southern limit of distribution; Monterey, which is near the northern limit; and then from our area in southern California. We did see slight differences in sporophyte production among the different strains. The results were pretty much as you would expect, with plants from the south doing slightly better at higher temperatures. But it was a really minor difference; there was a 1°C shift in the maximum temperature for sporophyte production, from 18°C in San Diego plants to 19°C in plants from Mexico. Also, I don't think there will be any effect of plant source on sensitivity to toxicants. Brian Anderson compared sensitivity of spores from plants collected at various locations along the California coast and found no differences in sensitivity.

MR. ANDERSON: It should be noted that the experiment Tom Dean is referring to was conducted only once. It may be that if there are differences in sensitivity to toxicants due to physiological differences between kelp populations, they may become evident if comparisons were made over the course of a year.

MR. CHERR: We have only one comparison of Santa Barbara versus Monastery Beach samples at 15°C, but, we saw marked differences in germ-tube length and the stage they were at. The two zoospores from Monastery were much farther along than the ones from Santa Barbara. What I was wondering is, if at 15°C, which is a little bit cooler than the Santa Barbara region, at least in the summer, has germination been slowed down?

MR. DEAN: I don't know. It seems possible, but first I would look at the shipping conditions and other factors as a possible explanation.

MR. STEELE: You said you worked with culturing *Macrocystis* for about 12 years?

MR. DEAN: Yes.

MR. STEELE: Have you any idea or feeling for how many spores that produced germ tubes go on to produce a viable egg and/or sperm and sporophyte?

MR. DEAN: We don't have quantitative data for that; but I suspect it's very high, it's probably on the order of 90 percent or higher. When we carry spores through to the gametophyte stage, we almost never see dead eggs or gametophytes that haven't produced eggs; and we almost always get greater than 90 percent sporophyte production.

MR. BAY: Have you done any work with kelp from Catalina Island? I've heard there's a different population there that's potentially adapted to different nutrient conditions and in some transplant experiments they do much worse in waters along our coast.

MR. DEAN: No. I know that it is a little more difficult to find ripe sporophylls in plants from Catalina in mid-summer because they have somewhat warmer conditions out there and there's a potential that there could be some differences with these plants in terms of sensitivity to toxicants. Dick Zimmerman has looked at physiological effects in adult sporophytes and found differences among island and mainland plants, but we haven't looked very carefully at gametophytes or spores.

MR. BAY: Are there some recommendations on holding times for the sporophyll? I know the typical protocol talks about using them within 24 hours. What has been your experience in holding for periods of days?

MR. DEAN: We haven't. We don't have any experience. We've always felt it's best to get them to release within 24 hours of collecting. We considered trying to play with holding methods, keeping sporophylls in flowing sea water systems for example, although we don't really have the facilities to do that. If you could do it under the proper temperature control conditions, I think you could probably keep them in the laboratory for several days, at least, before you released them. There's also a possibility of looking at cryopreservation as a possible means of preserving spores, but that's down the road.

MR. LANGDON: Just to follow up on that, has anyone cryopreserved these?

MR. DEAN: No, but it should be simple to do. Unicellular and green algae have been cryopreserved fairly routinely with good success, and I think it would be relatively simple to do with spores.

MR. ANDERSON: We've held sporophylls for as long as 3 days and had reasonable spore releases; beyond that, we see decline.

MR. DEAN: And you've maintained those in a flowing sea water system?

MR. ANDERSON: No, we maintained them in damp paper towels.

2. MYSIDS SUMMARY

Toxicity testing of West Coast mysids has been limited, with most being acute tests with *Holmesimysis costata* (formerly *Acanthomysis sculpta*). Attempts to develop a reproductive test with this species have been thwarted by its long life cycle (about 70 days), but 7-d growth tests show some promise. Recent investigations by Oregon State University at the Hatfield Marine Science Center in Newport, Oregon, have identified several other species of West Coast mysids with sufficiently short life cycles that they show promise as candidates for tests similar to those developed for *Mysidopsis bahia* on the East and Gulf Coasts.

Limited data are available on the relative sensitivity of the species that have been tested; these data indicate that the acute tests with *Holmesimysis costata* may be more sensitive (to zinc) than chronic tests with the other species (*Metamysidopsis elongata* and *Mysidopsis intii*). These comparisons must be considered guardedly at this time because they do not include tests conducted at the same laboratory and with the same dilution water.

Development of a reproductive stage test with *Mysidopsis intii* will continue at the Oregon State University Laboratory under an EPA cooperative agreement. Relative toxicity data for marine and freshwater organisms indicate the importance of inclusion of a sensitive crustacean in any array of test species. Until such a chronic test has been developed, interim inclusion of the acute test with *Holmesimysis*, use of the East Coast mysid *Mysidopsis bahia*, or even the use of the freshwater *Ceriodaphnia*, should be considered in marine effluent monitoring.

As soon as the *Mysidopsis intii* test has developed to the degree that interlaboratory testing is feasible, such testing should be expedited. It is feasible that such testing will be possible by 1991. Additionally, several reference toxic materials need to be tested to develop a better picture of the relative sensitivity of this and other species of mysid.

Although not included in this workshop, developmental work is underway by California Fish and Game to develop culturing and testing procedures for the low salinity mysid *Neomysis mercedis*. This organism is found in the Sacramento-San Joaquin delta and appears to represent a good low-salinity test species for estuaries. The life cycle of this species may be too long (being similar to that for *Holmesimysis*) for development of a short-duration test to estimate chronic toxicity.

Summary of Toxicity Testing with the Mysid, *Holmesimysis costata*

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As part of the State Water Resources Control Board's Marine Bioassay Project, we began to experiment with the mysid crustacean *Holmesimysis costata* to determine its suitability for effluent toxicity testing in 1985. From 1985 to 1988 we used reference toxicants and effluents to evaluate a simple 96-hour mortality test using 3-day old juveniles. This test has been sensitive to a variety of toxicants, but it lacks a sublethal endpoint and cannot be used as a "chronic" test in many regulatory applications. We are currently investigating growth as a sublethal endpoint in longer (approximately 7- to 10-day) *Holmesimysis* tests.

Holmesimysis costata occurs in the canopy blades of the giant kelp, *Macrocystis pyrifera*, and can be collected year-round in areas where the canopy persists throughout the year. Approximately 100 gravid females are needed to produce 600 juveniles for testing (30 effluent containers, 30 reference toxicant containers, and 10 mysids per container). We have maintained mysid cultures in the laboratory, and these cultures have often produced enough gravid females to provide a source of test juveniles. It is important to note, however, that most of the time we rely on field-caught gravid females. There have been times, notably during stormy winters, when difficulties with collecting adults has forced rescheduling of mysid tests. Culture methods developed for other mysid species might be applied to *Holmesimysis* to provide better reproductive output from laboratory cultures. Until then, field collection should be able to supply sufficient numbers of gravid females for testing throughout most of the year.

Juvenile mysids are released from the females in the laboratory, and are cultured for 3 days prior to testing. Juvenile release has always been successful, although we sometimes have to wait a day for a large enough cohort for testing. We have done approximately 25 mysid tests, and none have been cancelled for lack of juvenile test organisms.

Approximately 35 person-hours would be needed to conduct a test using 39 containers. This includes everything from isolation of gravid females through clean-up and data analysis. Tests using disposable tissue culture flasks as test containers save about 3 hours of clean-up time. One technician could do one test per week. Multiple tests are dependent on availability of space, personnel, and gravid females. We have run up to three tests concurrently in our laboratory. We have conducted 20 short-term mortality tests: 3 48-hour tests and 17 96-hour tests. Of these, only one had < 80 percent control survival, the limit we set for test acceptability. Control survival was 90 percent, or better, in 60 percent of the tests.

Results of 96-hour mortality tests with *Holmesimysis* indicate that this mysid is sensitive to a variety of toxicants (see Table 1 below; values are means ± SD; n = number of tests).

TABLE 1. MORTALITY TESTS WITH *Holmesimysis*

Toxicant	NOEC (μg/l)	LC50 (μg/l)
Zinc	58 ± 29 (n = 7)	78 ± 12(n = 6)
Copper	< 11 (n = 1)	27 (n = 1)
Pentachlorophenate	32 (n = 1)	103 (n = 1)
Endosulfan	< 0.1 (n = 1)	0.19 (n = 1)

A number of effluent tests have also been conducted, and NOEC and LC50 values for the effluents tested have increased with increasing levels of effluent treatment.

Although our research indicates that *Holmesimysis* mortality is sensitive compared to other crustacean endpoints, development of a sublethal endpoint is necessary if this

species is to be considered for "chronic" toxicity testing. Because *Holmesimysis* has a 70-day life cycle, reproductive endpoints are difficult to standardize. Juvenile growth appears to be a more feasible endpoint. We have taken daily length measurements throughout the life cycle to estimate molting frequency, and have conducted toxicity tests with juveniles of different ages to incorporate one or two early molts. Control survival over the 7- to 10-day exposure period has so far been unsatisfactory (< 80 percent). New test containers, and new feeding and renewal schedules are being tested now in an attempt to improve test conditions. The growth test with the best control survival to date (78 percent) has indicated that growth is as sensitive as mortality over a seven-day exposure to zinc (NOEC = 18 μg/l). The relative sensitivity of these different endpoints most likely depends on the toxicant used. The advantage of using growth as an additional endpoint is that test sensitivity may be increased for some effluents and toxicants. The disadvantage is the significant amount of additional labor involved with a longer test and an endpoint that takes more time to analyze. Growth in weight has been considered as an endpoint, but the juvenile mysids are too light to weigh accurately (mean juvenile dry weight is 0.05 mg).

We have shipped mysid adults and juveniles within California and across the country with minimal mortality, and adults and juveniles can be easily maintained under static conditions at testing laboratories. Three interlaboratory tests have shown good correlation of results between laboratories using the 96-hour test. Further work needs to be done with broodstock culture to reduce reliance on wild stocks, and sublethal endpoints need to be developed to allow use of this species in "chronic" testing.

DISCUSSION

MR. LANGDON: You mentioned you feed your mysids on *Artemia* and *Tetramin.* Do you use *Tetramin* during the bioassay as well?

MR. HUNT: No, just *Artemia* during the bioassay because we're trying to avoid getting things in there that might chelate or otherwise affect the toxicant availability.

MR. LANGDON: So it's just during the period of juvenile release?

MR. HUNT: Not only during release, actually, but during the culture of juveniles from the time they're released up until they're 3 days old.

MR. DEAN: How long after you collect them till you do the test. How long do you hold them there?

MR. HUNT: Immediately after collection we separate gravid females from other mysids and place them in aquaria where they will release their juveniles. Juveniles are released over the next few nights, and any of the resulting daily cohorts can be used for testing. The minimum holding time is less than 24 hours. On the other hand, we have kept mysids in culture for many months, and can occasionally collect enough gravid females from the cultures to provide juveniles for testing. Mysid culture has always been a low priority because field collection and transport is successful and convenient. If more effort were put into nutrition and cropping of adults, I think cultures would be capable of regular production of gravid females.

MR. LANGDON: In your culture tanks, do you filter the water that goes through the culture tank?

MR. HUNT: We've used both filtered and unfiltered seawater. You've commented in the past about possible nutrition from particles coming into the cultures in the unfiltered seawater. The seems very likely. For that reason we have recently switched to unfiltered seawater for the mysid cultures. Prior to that we used filtered water to help keep the cultures clean.

MR. LANGDON: But you didn't see any differences?

MR. HUNT: We have never done a side-by-side comparison of mysid cultures on filtered and unfiltered seawater. We have seen some differences in the condition and availability of gravid females in the field, and this might be related to particulate availability, the quality of the kelp canopy, or some other environmental factors, but we have no experimental data on the effect of these factors on lab cultures.

MR. LANGDON: Has anyone tried maintaining cultures in the lab for long periods of time?

MR. HUNT: Perhaps Don Reish at California State Long Beach has, but I believe he also regularly collects from the field. I can't be certain anyone has kept cultures for more than a year without supplementing them from the field. We're working on that now at Granite Canyon. Our associate, Ms. Sheila Turpen, has been studying related life history characteristics, and we will soon expand our culture work.

MR. DEAN: Have you been able to collect gravid females from the field at all times of the year?

MR. HUNT: We have always been able to collect enough gravid females from the field, but this involves more effort when there are less gravid females in the population. The number of juveniles released in the lab from a given number of gravid females is also variable throughout the year. Often, enough juveniles for testing will be released the first night, but at other times we have to wait a few days before a large enough cohort is released at one time. If testing has to be scheduled to coincide with effluent delivery, we make sure to collect larger numbers of gravid females to supply sufficient numbers of juveniles for test day.

MR. STEELE: Can you feed them on any *Artemia* from any source?

MR. HUNT: We've been using Argent Gold Label brand *Artemia*. We haven't done controlled experiments with different brands of *Artemia*, but we've had good mysid control survival and the *Artemia* hatching rate appears to be high. We continue using it to be consistent.

MR. STEELE: There's been quite a bit of work done, I think; there's quite a difference in the quality depending on where the cysts are from.

MR. HUNT: Hatching success and nutritional value can vary quite a bit.

MR. STEELE: Salt Lake and San Francisco are two of the worst sources.

MR. BAILEY: This has been my experience with *Neomysis*, which is sort of similar in extended life history to *Holmesimysis*. I've maintained them under continuous culture for 5 years. And, similarly, if the nutrition is not

properly taken care of, the average brood size goes way down, you know, from say about 10 to 15, to 3, or 1.   So it becomes very difficult, if you're not paying attention to the cultures, to obtain enough young.

MR. HUNT: Have you been able to maintain production of juveniles?

MR. BAILEY: Yes, we raise algae for it.   It depends on whether you just want to maintain them and go on forever, accepting that the production from individual females is relatively low.   Or you can really feed them.

MR. HUNT: Chris Langdon has given advice on supplements to boost the nutritional value of the *Artemia*, and has sent us some cultures of T-*Isochrysis* that we have had success with.

MR. BAILEY: For feeding the *Artemia*?

MR. HUNT: That's right, for feeding to the *Artemia* to raise their nutritional value.

MR. BAILEY: You use large diatoms for the mysids so you will take those preferentially?

MR. HUNT: Yes.

MR. BAILEY: But they are not as easy to raise?

MR. HUNT: It seems that they are omnivorous in nature and have quite a varied diet.

MR. BAILEY: Do you have any problems when you pool them?   Like, if you have 10 per individual container, do you have problems with missing pieces?   Any evidence of cannibalism?

MR. HUNT: We do sometimes have missing mysids.   This may be due to cannibalism or counting error.   With 10 tiny mysids darting around each container, it can be difficult to get good counts every time.   We recently decreased the number to eight mysids per container to limit the counting errors.   We count them daily in 96-hour tests, or every other day in longer static renewal tests.   There are usually about five percent of the containers where the daily counts don't add up to the total count at the end, so we suspect that some cannibalism is occurring.

MR. LANGDON: In terms of slowing them down for counting, we've found that MS-222 works very well.   I don't know how it affects the sensitivity of the animal.

MR. HUNT: You wouldn't want to do that before the test, though.

MR. LANGDON: For taking photographs and things like that, it's good.

MR. HUNT: Right.

MR. BAY: And that could be added right at the end of the test, right before counting.

MR. LANGDON: Yes.

MR. HUNT: Right.

MR. ANDERSON: The difficult time to get correct counts is at the start of the test when all 10 mysids are swimming around.

MR. HUNT: It's important to make sure of the counts at the beginning of the test.

MR. DEAN: Do you ever see more at the end than you had at the beginning?

MR. HUNT: Yes, we do.

MR. BAY: Looking at your data on the changes in length with time, there seems to be a lot of variability where the length jumps up and then it drops down to less than several days before. I'm curious what causes that variability; is it real or is it measurement error? And have you tried using dry weight as opposed to length as the endpoint?

MR. HUNT: Using dry weight is difficult because the animals weigh only about five hundredths of a milligram, so even if you pool them they can't be weighed accurately on a typical lab balance. Static electricity compounds the problem, causing negative weight readings. I don't know why the lengths decreased in some cases following molting. We measured 10 mysids each day to get the data for the growth curve you were asking about, and there was some variability, especially in the older mysids. Also, those measurements were carapace length, not total length, and we often had some difficulty seeing exactly where the posterior dorsal edge of the carapace was. This might have been especially difficult after molting.

Short Term Chronic Bioassay Development
for *Holmesimysis costata* (Holmquist 1979)

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We have worked with this species for about 6 months in 1986 at the Hatfield Marine Science Center. Original broodstock animals were obtained from Granite Canyon and Watsonville, California. Three generations were raised in static cultures on a diet of *Artemia* (RAC II) at 30 g/kg and 14°C. There appeared to be no problems in culturing this species except that high mortalities of 50 to 60 percent were observed as mysids grew from the time of release to mature adults. Culture of this species was not pursued because it was considered unsuitable for chronic bioassays, due to its long life cycle of 8 to 10 weeks.

Holmesimysis costata has been used in acute bioassays in California. However, there needs to be a convenient source of animals from the field because its long life cycle and high mortality during development makes it unlikely that laboratory-reared animals could be conveniently raised in sufficient numbers for routine bioassays.

Short-term Chronic Bioassay Development
for *Metamysidopsis elongata* (Holmes 1900)

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We have worked with this species for about 2 years (1987 to 1988) at the Hatfield Marine Science Center. Original broodstock animals were obtained for us by Steve Bay (SCCWRP) from offshore Los Angeles. Initially, we had problems raising this species on an *Artemia* diet under laboratory conditions, even though the *Artemia* were enriched by feeding them on lipid and algal diets. We found that although newly collected females from the field released viable young, the young from females that had been reared throughout their entire life in the laboratory released weak, non-viable young. After many experiments, we found that it was necessary to add small quantities of the harpacticoid copepod *Tigriopus californicus* to mysid cultures to ensure that females released viable young. Subsequent fatty analysis of the enriched *Artemia* and *Tigriopus* suggests that *Tigriopus* was dietarily important to the mysids due to its high content of the omega-3 fatty acid 22:6 w3. At a culture temperature of 20 - 21°C and salinities of 34 g/kg we found that this species had a life cycle of 27 to 30 days and a brood period of 7.5 days, indicating that it was a good candidate species for chronic bioassays. Occasionally we experienced problems with fungal infections of cultured mysids that could be overcome by chlorination and frequent cleaning of culture containers.

We have carried out two acute and one chronic bioassay with *Metamysidopsis elongata* exposed to a range of concentrations of zinc. In 96-hour acute bioassays, adult *M. elongata* showed significantly higher mortality with additions of 0.50 mg/l zinc (background zinc concentration was 0.06 mg/l) than controls with no additions of zinc (Tukey's HSD test, $p < 0.05$); mortality of controls was 13 percent. In a 44-day life cycle bioassay, added concentrations of 0.25 mg/l zinc (background zinc concentration was 0.05 mg/l) resulted in significantly higher mortality of mysids (Tukey's HSD test, $p < 0.05$) than in controls; control mortality was 27 percent. A more sensitive

endpoint in the chronic bioassay was mysid body length after 23 days, where a significant reduction in mysid growth was evident at added concentrations of 0.05 mg/l zinc.

The species could be conveniently used for routine acute and chronic bioassays. Laboratory cultures could be set up to supply animals, when needed, throughout the year, although hygienic culture practices need to be adopted to prevent the spread of fungus within lab populations. One person could run a bioassay of 36 treatments per week -- providing others were responsible for maintenance of food species and broodstock populations.

Short-term Bioassay Development
for *Mysidopsis intii* (Holmquist 1957)

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We have cultured *Mysidopsis intii* for 1 year (1989) at the Hatfield Marine Science Center. The original broodstock animals came to the Center mixed with *Metamysidopsis elongata* collected from Los Angeles. As in the culture of *Metamysidopsis elongata*, we found that it was necessary to supplement a diet of enriched *Artemia* with *Tigriopus californicus* in order to ensure that cultured female mysids released viable young. At a temperature of 2°C and a salinity of 34 to 35 g/kg, we found that *Mysidopsis intii* completed its life cycle in 19 - 24 days and had an average brood period of 4.9 days. Mortality of *Mysidopsis intii* from release to maturity was about 15 percent and, therefore, was less than mortalities of 20 to 25 percent observed for *Metamysidopsis elongata* over this developmental period. Mass flow-through cultures of several thousand *Mysidopsis intii* are presently being successfully maintained at the Center, even though salinity of the flow-through seawater is only about 30 g/kg and, therefore, lower than the optimal salinity of 35 g/kg for mysid culture.

We carried out one acute and one chronic bioassay with *Mysidopsis intii* exposed to a range of concentrations of zinc. Sensitivity to zinc in a 96-hour acute exposure was similar to that for *Metamysidopsis elongata* in that significant effects on mortality (Tukey's HSD, $p < 0.05$) were observed at added concentrations of 0.50 mg/l zinc (background zinc concentration was 0.02 mg/l). In the chronic bioassay, significant reductions in body length (Tukey's HSD, $p < 0.05$) were observed when mysids were exposed for 7 days to added concentrations of 0.05 mg/l zinc (background concentration was 0.02 mg/l zinc). Mysid mortality, after 25 days of exposure, was less sensitive as an endpoint than mysid length at 7 days and significantly higher

mortality (Tukey's HSD, $p < 0.05$) was only observed at concentrations of 0.25 mg/l zinc. The sensitivity of *Mysidopsis intii* to zinc appears to be similar to that for *Metamysidopsis elongata*.

Mysidopsis intii would appear to be the ideal test species. We can maintain large populations of animals for bioassays in the laboratory in flow-through cultures. Production of the broodstock population can be controlled by food supply and temperature. We have had no problems with fungal infections, even though the flow-through tanks are only cleaned once every other week. Survival of controls in acute and long-term chronic bioassays is satisfactory. We have had no failures in the limited number of bioassays that we have carried out. The only major problem we have in raising this species is that the salinity of the seawater supply at the Hatfield Marine Science Center can drop below 30 g/kg during the rainy, winter months and we will probably have to keep broodstock populations on a batch seawater-change mode with additions of seawater and brine or concentrated artificial salts. At present, the 7-day growth bioassay is sensitive (significant effects at 0.05 mg/l zinc) and a bioassay with 36 treatments could be undertaken by one person, providing others were maintaining food species and broodstock populations.

DISCUSSION

MR. OSHIDA: I missed where you collected them. What I heard was that they came as, basically, a contaminant.

MR. LANGDON: Yes, they came in *elongata* and we got them from Steve Bay. Steve, where exactly did you get them?

MR. BAY: We were making our collections in outer Los Angeles Harbor, right near the mouth of the harbor.

MR. LANGDON: You had a dredge-trawl?

MR. BAY: Yes, we had an epibenthic sled that did a pretty good job of picking up mysids. We've also collected *elongata* from the Newport Harbor area although, at that time, we weren't looking for *intii* so I'm not sure of its occurrence there.

MR. LANGDON: That's Newport, California?

MR. BAY: Yes, Newport, California.

MR. LANGDON: So they seem to share the same habitat. And there's a difference in behavior. The *elongata* are more pelagic so they will be swimming around day and night. The *intii* seem to just sit on the bottom during the day and they don't actively swim and feed until darkness. So you should be able to get them fairly easily; they came, I think, in most of the cultures you collected.

MR. BAY: They were not uncommon; we got them on several occasions and I think it was probably more a matter of the collection technique. How well the sled contacted the bottom was probably a big determinant in the numbers we did get because I think they were associated with the sediments more than *Metamysidopsis* was.

MR. OSHIDA: For your experiments, you had to use a fairly large range of concentrations. I was wondering what was the next highest concentration above 22?

MR. LANGDON: Fifty.

MR. OSHIDA: I guess what I'm getting at is that the acute level for *Holmesimysis* may be more similar than 22 and 40 -- excuse me, it's unclear. That you use 22 and 50 as an interval and, because John Hunt and Brian Anderson use, probably, a smaller interval because they don't have to look at such a large range, you're level of NOEC may be closer to --

MR. LANGDON: We may be closer to 50.

MR. OSHIDA: May be closer to 50, may be closer to 40, we don't know.

MR. LANGDON: We don't know for sure.

MR. OSHIDA: It may be a function of what concentrations were used?

MR. LANGDON: Yes, it's obviously between 22 and 50. What were your intervals, John?

MR. HUNT: The concentrations were 10, 18, 32, 56, and 100 μg/l for zinc.

MR. LANGDON: We haven't done a lot of work on bioassays yet; and that's something that we are obviously going to try to do next.

MR. STEELE: The *Tigriopus* that you feed them, do you have to collect them each time?

MR. LANGDON: No. The *Tigriopus*, actually, are very easy to culture; they are extremely hardy beasts. And essentially, we have a couple of trays and water about 40 g/kg at 20°C and fish food is dumped in every now and again and a couple of buckets of algae twice a week; and that's it. So it's really not a lot of work involved in maintaining *Tigriopus*. They are very easy to culture. I think the main work at the moment, is involved in culturing algae to feed the *Artemia*. And I think, if we can get away from the need to culture algae, either by using lipid microcapsules or, perhaps, by being able to feed newly-hatched *Artemia* with *Tigriopus*, that will be a big labor-saving part of the presumptive protocol.

MR. HALL: How are you enriching *Artemia* right now, fatty-acid-wise?

MR. LANGDON: The RAC II *Artemia* that we have, we're enriching with Tahitian *Isochrysis* that has 22:6 w3 in it. So there's some enrichment with the algae. But we also use lipid microspheres that are made out of 80 percent menhaden oil and 20 percent of a monoglyceride/diglyceride mix. And that's a very potent source of omega-3 fatty acids.

MR. HALL: Is that something you buy?

MR. LANGDON: No, we make it in the lab. But it's similar, I think, to your -- was it --

MR. HALL: Well, I know of three products. One from Belgium is called Selco; Aquafauna BioMarine in California sells one they call Omega Booster; and Argent sells Artemote.

MR. LANGDON: Suspensions of fish oil which you dump in?

MR. HALL: Right.

MR. LANGDON: I suspect that some of the improvement that you see in the fecundity of the mysids is due to enhancement of fatty acid by this enrichment process. But my view is that the main beneficial factor of the *Tigriopus* is due to something quite different. My feeling is that it could well be a sterol that they're lacking; it would be a very nice project for a graduate student to try and identify whatever it is *Tigriopus* has that enriched *Artemia* or newly hatched *Artemia*

lack.   But I don't think it's a fatty acid because we have done fatty acid analysis on the enriched *Artemia* and there is a lot of 20:5 and 22:6 w3 fatty acids in the enriched *Artemia*.   So I think it's something else that *Tigriopus* is supplying.

MR. HALL: I also want to mention I've been working with *Mysidopsis bahia* and I've gone through all kinds of problems and somersaults trying to get them to work.   And, in talking to the folks at Gulf Breeze and Narragansett, they have been doing work with the megaboosters.   Their concern is that they won't be able to always get adequate reference *Artemia* cysts in the future.   And the latest advice I got from Gulf Breeze was to suggest using the cheapest quality *Artemia* cysts that I can get, which they think is Salt Lake, and then a megabooster to bring it up to a standard of quality.   So that's one of the things we're working on right now.

MR. LANGDON: Yes, you have to watch out since some of these *Artemia* carry with them residues of organic pesticides.

MR. HALL: Well, they're concerned about that, too; but they are claiming, I think, that bringing them up to nutritional quality is going to far override whatever subtle problem you may have from pollutants.

MR. LANGDON: Right.   Yes, the answer is to develop an artificial diet for these species; and we don't know whether EPA will ever support that.   But there's an obvious need for it.   And there are some artificial diets that are available for rearing penaeid larvae that have been developed in Japan by Kanezawa.   And it would be worth trying to get some of those diets to test out the mysids.   If they work, it would end a tremendous amount of grief and solve a lot of the unknowns in trying to maintain cultures of these organisms for bioassays.

MS. ANDERSON: I just have one comment.   Maybe you could try working with algae that people normally have in their bioassay culture laboratories like *Skeletonema* or *Thalassiosira*; therefore, it would involve almost no work for them because they are routinely culturing them anyway.

MR. LANGDON: Yes.   Well, labs vary in their little pet algae, but they maintain a --

MS. ANDERSON: It's true of research laboratories, but it's not really true of those who are doing routine testing, which is your eventual goal. People are restricted in that regard so you might want to try to target some of those algae that people have tc have around.

MR. LANGDON: You mean those aigal species that are used in bioassays. Is that right?

MS. ANDERSON: Yes. Most of the labs that are doing a lot of work always have algae cultures up. And so, for them, it's no big deal to take splits.

MR. LANGDON: Yes, it's a good point. I think the *Thalassiosira* would potentially be a good food for *Artemia*.

MS. ANDERSON: What do you think about *Skeletonema*?

MR. LANGDON: Well, it could conceivably be okay; it's a little larger than *Thalassiosira* (6-8 μm in size), and has slightly larger spines. But it would be worth trying, anyway.

MS. ANDERSON: Because that's the most frequently used.

MR. STEELE: There is a problem that *Thalassiosira* is a much easier cultured organism than *Skeletonema*.

MR. LANGDON: Yes, we chose *Isochrysis* because it's so easy to culture; we have no problems growing it. We have had problems growing *Skeletonema* in the lab; *Thalassiosira* is a little bit easier.

MR. OSHIDA: One of the questions that's been raised by John Hunt and Brian Anderson in the past has been that they have a test that appears to be as sensitive to the acute type of testing as it does to the chronic type of testing; and they have sort of been at a little bit of a disadvantage in terms of some of the regulatory hoops that people have to jump through or have to meet in terms of criteria. However, we have a test here whose chronic sensitivity may be in the same range as the *Holmesimysis* acute toxicity and I just wanted to pose a question: does it make sense to spend a couple more years to develop the dietary types of things that might have to be developed, to really sensitize this test on a species, particularly *intii*, that only has a limited distribution, and that the chronic test might be substantially harder to do, when it has a similar sensitivity to a test that already exists? I guess my question is: would it be better to start looking at the sensitivity of the chronic test on what you have so far before you start looking at the dietary types of things and trying to refine those so that you find out if, in fact, you already have something that would work?

MR. LANGDON: Yes, dietary refinement is a secondary problem or question. The 7-day chronic test that I showed you data for is only our first attempt at a chronic test. And what I hope to do is either use a 7-day or a 10-day chronic test that includes not only growth but also a phase of their reproduction. I think it's very important to try and understand the effects of these pollutants on reproduction because that's really what dictates what happens to the populations in the field. And, as I mentioned with *Mysidopsis bahia*, Suzanne Lussier says that there are some pollutants that seem to affect reproduction rather than growth. And so I think my primary goal is to try and include reproduction as well as growth in a 7 or 10-day chronic. And then, as you say, secondarily try and simplify the dietary questions so that people that actually have to use the test, or may have to use the test, won't have such a labor-intensive job as we've had.

MR. CHAPMAN: I'd like to comment on that. If it turns out that we have an easy acute test that is as sensitive as several of these chronic tests, and the acute test species are easier to culture in the laboratory, it seems to me that we can get by with the acute test. But, at this point, we have insufficient data to make that judgment. We've got some zinc toxicity comparisons, but until we run a number of different chemicals, and until we start doing interlaboratory comparisons that take into account differences in dilution water, I think it's premature to make that judgment.

MR. BAILEY: I was a little bit concerned about the usefulness of the fecundity as an endpoint because, particularly on the chronic test that you ran, you are only producing an average of two to two and a half?

MR. LANGDON: Somewhere around there.

MR. BAILEY: Yes, which doesn't give you much leeway.

MR. LANGDON: No. I think, using the release of young as an endpoint is not, as you say, a good endpoint. Not only because of that point, which you brought up, but also the cannibalism and carnivorous behavior of the mysids could affect your fecundity endpoint; the adults could consume their young which would obviously affect the accuracy of your endpoint. I think the type of endpoint that Suzanne Lussier and others in Narragansett have is probably better. They use the endpoint of presence or absence of eggs in the brood sac or some alternative. Perhaps, one could use the presence or absence of eye development,

some developmental stage of the juveniles while they're still protected by the adult mysid and haven't been released yet. I think that would be a better and more sensitive type of endpoint.

MR. BAILEY: One observation that I had -- depending on at what stage you count the young in the brood pouch -- the numbers don't necessarily bear any relationship to the number that will actually be dropped.

MR. LANGDON: Oh, yes.

MR. BAILEY: So some of those may be absorbed or the female may strip them out. I don't know whether it's a better endpoint to count the number that are actually dropped or whether you count them when they're in the pouch.

MR. LANGDON: Yes. Well, we certainly saw that with *Metamysidopsis elongata* before we had stumbled upon *Tigriopus* as a dietary component. The females would brood eggs in their brood sac and it wasn't until the later stages of larval development in the brood sac or immediately after release of the larvae, that the very high mortality was observed. So, yes, that's a very good point; we've got to, obviously, be able to relate the number of young in the brood sac with the viability of the larvae once they're released.

MR. BERGER: This is a question for both Brian Anderson and Chris Langdon. Could the different dietary intakes of both organisms be causing an artifact of more sensitivity in Granite Canyon and that you really didn't see that until you started looking at a chronic endpoint in terms of the effect of other dietary inputs?

MR. LANGDON: Yes, I think relating the effects on diet, and stress in general, to the sensitivity of test organisms to pollutants is important.

MR. BERGER: Do you have any plans to compare the effects of different diets on the response to various pollutants?

MR. LANGDON: It's fairly simple to do. One could rear mysids on several different diets and expose them to a range of pollutants and then examine the different effects. That might be something that we need to think about.

MR. HUNT: I was wondering, do you (Chris Langdon) use these diets in the actual toxicity test also?

MR. LANGDON: Yes, we don't use algae; we don't add algae. So we use enriched *Artemia* and some lipid microcapsules but no *Tigriopus*.

MR. HUNT: And that is sufficient?

MR. LANGDON: Yes, because we won't follow the whole life-cycle through in the short-term chronic tests.

MS. ANDERSON: It may be that, if we look at zinc ion concentration as opposed to total zinc concentration, we may see more similarity in sensitivity between the two species. The presence of ligands and organic particles in the test solutions affects ion concentrations. We use filtered seawater and *Artemia* as the food organism, while you used enriched *Artemia* and lipid microcapsules; the presence of the different foods may affect the results by binding free ions. There is a grey literature report published for the Army Corps of Engineers in 1983 by Tatum, et al. They compared the relative sensitivities of *Mysidopsis bahia* and *Holmesimysis costata*, and found a difference in response to metals using 96-hour mortality tests. I think the relative sensitivity depended on the metal being tested; one species was more sensitive to zinc, while the other was more sensitive to lead. It's probable that there is a difference in sensitivity between *Holmesimysis, Metamysidopsis,* and *Mysidopsis.*

MR. CHAPMAN: One other concern, especially with the lipid microspheres, would probably be with any organic chemicals that were partitioned into the lipid; you'd be taking them out of the water and putting them into dietary particles, which could really change things.

MR. HUNT: To return to Mr. Berger's comment, differences in diet may affect the sensitivity of mysid tests, especially chronic tests using growth or reproduction as endpoints. But with short-term tests, it might be more efficient to simply standardize a basic *Artemia* diet that provides good control survival and reasonable between-test precision rather than expending a great deal of research effort developing an "optimal" artificial diet. I agree that more research is needed to develop better diets for long-term mysid tests. This work might also produce a better diet for short-term tests, but the short-term tests are useful and reasonably precise right now using standardized diets. I think the effect of an improved diet on short-term sensitivity would be too small to justify a diversion of effort for this purpose.

MR. LANGDON: I think, for the short-term, you're right; but, obviously, if you're interested in chronic tests that include some reproductive phase, you've got to consider an optimum diet as your control.

MR. HUNT: I agree.

MR. CHAPMAN: From listening to Bob Berger's question, it wouldn't be quite that intensive. I think he was just interested in whether or not the Granite Canyon low numbers, compared to your higher numbers for zinc, might be solely an expression of dietary differences. And, if that were the case, that would be reasonably easy to check out.

MR. LANGDON: I don't know with *Holmesimysis*, for example, whether *Tigriopus* is necessary for reproduction.

3. SEA URCHINS/SAND DOLLARS SUMMARY

The toxicity tests with embryos and larvae of various species of sea urchins is a relatively simple and underutilized test. The test has been conducted utilizing the purple urchin, *Strongylocentrotus purpuratus*, the green urchin, S. *droebachiensis*, and the sand dollar *Dendraster excentricus*. The test is comparable in conduct and duration to embryo/larval tests with oysters and mussels. One or the other of the test species is usually available in spawning condition at any time of the year.

Indications of general sensitivity rank alongside those for mollusc embryo/larval tests. More work is needed to compare the sensitivity of the sea urchin embryo/larval test with that of the shorter, more simple, sea urchin sperm cell test. The primary limitation to the sea urchin embryo test is that few people have had experience in judging the larval aberrations resulting from delay or maldevelopment. This should not represent a significant hindrance to the wide-spread adoption of this test as the developmental stages are easily distinguishable.

This test might be used in lieu of, or in addition to, tests with mollusc embryo/larval tests and sea urchin sperm cell tests. Overall, both interlaboratory testing (of relative sensitivity of this test to mollusc embryo/larval tests and sea urchin sperm cell tests) and intralaboratory testing of the robustness of the test procedure should be conducted.

Summary of Toxicity Testing with Sea Urchin and Sand Dollar Embryos

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Species: *Strongylocentrotus purpuratus* (purple sea urchin), *S. droebachiensis* (green sea urchin), *S. franciscanus* (red sea urchin) and *Dendraster excentricus* (sand dollar).

Most of the sea urchin embryo work in our Puget Sound, Washington, laboratory was carried out from 1979 to 1984 in conjunction with the development, refinement, and validation of a sea urchin sperm/fertilization bioassay.

During this time, we tested the relative sensitivities of sea urchin embryos to metals, pesticides, and sewage effluents. We utilized three species of winter-spawning sea urchins and the summer-spawning sand dollar, *Dendraster excentricus*. All of our work relied on naturally spawning stocks collected during their normal spawning seasons (approximately January to mid-April for sea urchins and April through October for sand dollars). Sea urchins were collected by divers from shallow subtidal areas of the Strait of Juan de Fuca and sand dollars came from intertidal sandy beaches of Puget Sound. Typically, we held the animals in flowing seawater for up to about 30 - 45 days. Sea urchins were fed kelp (various species) and sand dollars were kept on a bed of sand from which they scavenged some nourishment (some food may have also been filtered from the flow-through seawater). We had few problems holding sea urchins and sand dollars, although red urchins were least hardy to collecting and handling stresses and to reduced salinities. Most test animals were spawned once or twice (at approximately 30-day intervals with 0.5 molar KCl) and then returned to the point of collection.

Our testing has assessed the toxicity of metals (silver, cadmium, copper, lead, and zinc), pesticides (DDT, Dieldrin, Endosulfan, and Endrin) and sewage effluents (various stages of treatment). The results of these tests are summarized in Table 1 as EC50s (calculated toxicant concentrations adversely affecting development to a normal pluteus).

TABLE 1. TOXICANT EC50S FOR PURPLE AND GREEN SEA URCHIN AND SAND DOLLAR EMBRYOS

Toxicant	Purple Sea Urchin	Green Sea Urchin	Sand Dollar
Metals			
Cadmium (mg/l)	0.5	1.8	7.4
Copper (μg/l)	6.3	21.0	33.0
Lead (mg/l)	<9.7	<9.7	0.7-1.5
Silver (μg/l)	15.0	24.0	33.0
Zinc (μg/l)	23.0	27.0-51.0	580-820
Pesticides			
DDT (μg/l)	>8.2	>82	>17.2
Dieldrin (μg/l)	143	>111	>68
Endosulfan (μg/l)	227	>549	822
Endrin (μg/l)	221	>359	>362
Sewage (%)			
Influent	NT*	NT*	NT*
Primary	NT*	15.8	NT*
Secondary	NT*	18.1	NT*
Chlorinated Secondary	NT*	8.7	NT*
Dechlorinated secondary	NT*	17.4	NT*

*NT = not tested.

Toxicity testing with winter-spawning sea urchins in the Puget Sound region is limited to January through about mid-April with local stocks.   Spawning can sometimes be extended a number of weeks by holding urchins in a temperature/light-controlled closed system.   Use of mail-order animals from

California can also extend the use of urchins by a month or so on either end. Back-to-back use of sea urchins and summer-spawning sand dollars effectively extends the use of an "echinoid" embryo bioassay to almost year-round. The time of the year when gametes are not available (or marginally available) is from about mid-October through December.

Routine embryo bioassays are easily conducted by a team of two individuals working together, but can also be run by a single individual if necessary. Two people working together can easily run 1 or 2 full bioassays per week (48- to 96-hour exposures) of up to about 100 samples (including most of the sample "reading"). One test per week is easily conducted by a single individual. Analyses of parallel chemical samples, if elected, would require a additional person.

There are few drawbacks to using echinoid embryo assays. Back-to-back use of sea urchins and sand dollars allows testing most of the year from natural spawning stocks (thus, no culture or "conditioning" is necessary); feeding is simple; the assay is quite sensitive to a variety of toxicants; sea urchins are found on both coasts of the United States and in almost all marine waters world-wide; sea urchins have been standard bioassay tools in Europe and Japan for decades; and, in the Puget Sound region, echinoid embryo bioassays are an approved EPA/USACOE bioassay for assessing marine sediment quality.

The success rate for echinoid embryo tests in our laboratory has been 21 of 29 (72 percent success rate) based on controls with ≥ 70 percent survival and ≥ 90 percent normal development to pluteus. However, some of these tests were on the "beginning end of a learning curve." Given a trained, experienced laboratory, I would expect a success rate closer to 85 or 90 percent for this assay.

DISCUSSION

MR. CHERR: I was wondering about the endpoint of the pluteus larvae. Do you look for things like spicula formation, length of the arms, and so on? Do you see much aberration, or any aberration?

MR. DINNEL: There are different endpoints, of course: pigment synthesis; DNA synthesis; length of the arm; the skeleton, as done by Heslinga in Guam a ways back. In our laboratory, we were using it as a secondary assay, to compare with the sperm fertilization assay which was under development. So we didn't get fancy, shall we say, or detailed, in the endpoint. Basically, did it make it to a reasonable pluteus stage? If it was retarded back to a prism stage or earlier, or if it was so grossly abnormal that it was not going to survive, then we considered it abnormal. By using other refined endpoints, the sensitivity would increase.

MR. LANGDON: Do you find embryos of sea urchins and bivalves different in their sensitivities to pollutants? Have you seen that?

MR. DINNEL: It kind of jumps around; but my overall impression would be that an oyster embryo would probably be slightly more sensitive. Now, what the factors are, I don't know. Typically, an oyster embryo is run at 2°C, a pretty warm temperature; whereas, urchin embryos tend to be run at more like 12°C. The temperature difference affects toxicity; but exactly how much depends on the toxicant and the conditions of the testing. It's just my gut feeling that oyster embryos are going to be slightly more sensitive. But, in a practical assay situation, routine testing of effluents or sediments, I think both are quite usable.

MR. HUNT: When you bring sand dollars into the laboratory, do you attempt to feed them or culture them in any way?

MR. DINNEL: What we do is provide them with a bed of sand, which is pretty much their normal habitat, with a flow-through system. And they seem to be detrital filter feeders so they'll get some of the nourishment out of the organic material in the sand as well as the material, either plankton or detrital material, coming through the sea water system. And they seem to be healthy for months at a time.

MR. HUNT: You can spawn them after months in the laboratory?

MR. DINNEL: Yes.

MR. DEAN: Just as a comment, we've been able to maintain urchins in the laboratory pretty much all summer in southern California and get them to spawn just by keeping them in a cold room at 15°C and keeping them fed with a

lot of kelp.   They seem to do quite well.   They don't spawn quite as readily as they do in the winter, but we've always been able to get substantial numbers of eggs and sperm.

MR. DINNEL: Yes, when they are normally unavailable in the Puget Sound region, it's not uncommon to send down to California to some of the supply houses or have someone go out and collect them in different areas and ship them up for use.   So it extends the season in the Puget Sound region by using animals from California.

MR. HALL: We've had real good luck with sand dollars in our lab, have held them upwards to a year, simply raising them on the substrate sand. We've also noticed that they feed voraciously on Tetramin, if you think you need to add a little extra nourishment.   And, by bringing the dollars in early in the spawning season, like April or May, we've been able to run those dollars through the summer period and they'll still spawn; I think the latest we've gone is the first week of December.   By holding them over winter at a slightly warmer than ambient temperature in the lab, we've had them spawn as early as mid-February.   So we've practically closed the window on the season.

MR. BERGER: This is open to the room in general: how many people respawn their organisms and what kind of refractory times are people encountering?

MR. DINNEL: I can answer that for our lab and what we usually do: if we needed to respawn an animal because it's not convenient to go out and get some new ones, we hold them about 30 days with food (in the case of urchins - kelp; in the case of sand dollars - just a bed of sand with flowing sea water), and usually, in about 30 to 40 days, you can respawn.   And you can probably do that a couple of times with sand dollars and, in Puget Sound, I'd say maybe once with urchins.   If you get close to the end of their spawn-out time, once they spawn, they won't spawn again.

MR. DEAN: We've tried to bring urchins back to spawning condition after an initial spawn on several occasions and have been fairly successful. However, we see some mortality when we reintroduce spawned urchins into static

culture conditions. I've been reluctant to reuse urchins based on higher than normal mortality, so we've gone to using them only once and then taking them back to the field.

MR. BAY: That's been pretty much our experience down at SCCWRP, also. We use them just one time because we haven't had an availability problem. Recently, we investigated keeping them for repeated spawnings but we had a little bit of a mortality problem, also, although we haven't really investigated it thoroughly to minimize that.

MR. DINNEL: Was that a static system?

MR. BAY: It's a recirculating static system.

MR. DINNEL: I've always used a flow-through system and never had much problem except with the reds; the reds are finicky.

MR. BAY: I think it might just be related to the handling, perhaps injection.

MR. DINNEL: Yes.

MR. BAY: We use the same hypodermic needle for years at a time; we don't use them just once.

MR. DEAN: I think that the urchins get a bacterial infection.

MR. BAY: Yes, the same thing with our KCl, we make 100 ml of it and it lasts for a few years.

MR. CHERR: In our experience, we've had the same animals in the lab for about 3.5 years. Again, we have a flow-through system. And they have been spawned and recycled through every few months. I think, depending on how you inject them or, if you shake them too much, you can damage internal organs.

MR. DINNEL: One thing that probably hasn't been looked at closely is the quality of gametes coming out of animals that are continually reused in relation to the holding conditions and the food; and I don't really have any information on that because I've never held them for more than a couple injections.

MR. BAY: This is for everyone also: has anyone had much success with the electrical shock method of spawning the purple urchins or the sand dollars on this coast?

MR. (UNKNOWN): Not with purple, that's fairly well established. That's been our experience.

MR. DINNEL: That's routine on the East Coast with *Arbacia.*

MS. HOFFMAN: I've also heard people having some success with injecting sea water.

MR. DINNEL: I found when I was adapting this test to Hawaiian species, if you just looked at them they would spawn -- KCl didn't work very well with those urchins; but salinity shock worked quickly. They were very sensitive to a few parts per thousand. In Hawaii, you've got a different situation because they're not seasonal; they can be ripe any time of the year. There are intermittent spawn-out periods when rains lower the salinity. There's also epidemic spawning and urchins won't spawn for a month or two until they come back into condition again. And that can happen just about any time of the year, I suspect.

MR. BAY: Another question regarding the Puget Sound criteria. Do you agree with their 70 percent and 90 percent limits for an acceptable test?

MR. DINNEL: Well, that's been adapted from the ASTM mollusc embryo standard; and that's what it's always been. But, ironically, in the last few years there has been a very high level of failures with oyster embryo and mussel embryos assays. In the write-up I put together, I identified in our laboratory something like a 50 percent failure rate based on those standards because it was difficult to get 70 percent survival. You weren't knocked out of the water on the abnormality, but the survival. I know it's easier to get a test based on those standards with an echinoderm.

MR. BAY: Yes, I would think that if you have 30 percent mortality, then I would think that this would also be reflected in the degree of abnormalities. I'd expect an even higher incidence of abnormalities.

MR. DINNEL: There isn't necessarily a relationship that I've found. I've looked at that and basically what apparently happens is, for some reason, half the eggs aren't of satisfactory quality to develop and they die. The other half are fine. So, really, there's a fairly good chance you could drop down to 50 percent survival without any problem. I think it's pretty routine with oysters; Jim Lannon at OSU did a lot of work on gamete and embryo quality, looking at different factors that affect viability and survival. Quality can be all over the board. A lot of different factors, such as nutrition and spawning condition were considered.

During this last year, in Puget Sound, for instance, they were having something like an 80 - 90 percent failure rate in some of the labs with oyster and mussel embryos. That's unusual, but still, it can happen causing a lot of frustration.

48 Hour Sea Urchin Embryo Toxicity Test
Strongylocentrotus purpuratus

Steven Bay

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Embryo-larval toxicity tests have been conducted with the purple sea urchin at SCCWRP since 1978. Laboratory cultures have been used successfully to provide organisms for spawning both within and outside of this species' normal spawning season in southern California (approximately November to April). Successful culture methods consist of collecting mature (spawnable) urchins from an intertidal location in Santa Monica Bay and holding them in recirculating seawater aquaria at 13 - 15°C and 34g/kg. Urchins are provided an abundant supply of drift kelp (*Egregia* sp.) as food. Two collections of urchins are usually sufficient to meet our testing needs throughout the year. Animals are usually collected in late November and again in April.

Success of our culture method is illustrated by Table 1, which shows the results of urchin spawning attempts since 1986. Difficulty in obtaining good yields of eggs is occasionally found in late summer (September to October), requiring the injection of more than 10 urchins in order to obtain a satisfactory quantity of eggs. No complete failures in spawning were recorded during this time period, partly because our research schedule enabled us to schedule experiments at times when we were reasonably sure that spawnable urchins were available. Individual urchins are usually only spawned once during holding in the lab.

We have had few unsuccessful culture experiences with this species. Significant mortalities of this species have been rare and were usually caused by failure of aeration or filtration equipment. Spawning success in late summer could probably be improved by more careful attention to feeding and environmental conditions (e.g., light and temperature).

TABLE 1. RECORD OF RECENT SPAWNING ATTEMPTS WITH *Strongylocentrotus purpuratus* FROM JANUARY 1986 TO DECEMBER 1989 (CRITERION FOR SUCCESSFUL SPAWNING IS THE PRODUCTION OF VIABLE SPERM AND EGGS)

Month	Tries	% Success
January	4	100
February	7	100
March	4	100
April	3	100
May	5	100
June	0	
July	3	100
August	5	100*
September	3	100*
October	2	100*
November	2	100
December	2	100

*Occasionally difficult to spawn.

Our culture currently consists of approximately 80 urchins in spawning condition. No significant differences in mortality patterns or culture conditions presently exist, compared to previous years. The availability of organisms from the field is limited by the tides and sea conditions. Spawnable urchins can be reliably collected between early November and late April in southern California.

The test method consists of exposing fertilized eggs to water samples in 250 - 1,000 ml beakers for 48 hours at 15°C. Normal embryos reach a well-defined prism stage at the end of exposure. Subsamples of embryos are removed from each test chamber, preserved in formalin, and examined under a microscope for

malformations and delayed development. Additional endpoints have also been used at SCCWRP to describe toxic effects. We have frequently used measurement of production of the pigment echinochrome by developing embryos to indicate toxic effects. This pigment is easily extracted from a sample of embryos and measured with a spectrophotometer. Reduced pigment production is often correlated with increased abnormal development. Sea urchin embryos have also been examined for cytogenetic abnormalities such as anaphase aberrations and micronucleus formation. This analysis provides greater sensitivity to toxicants producing chromosome damage.

Reference toxicant tests conducted at SCCWRP have utilized zinc, copper, and deionized water (reduced salinity). Two tests conducted with zinc indicate an EC50 of ≤ 50 μg/l for percentage abnormal development. Sensitivity to zinc compares favorably to that reported for 48-hour mollusc development tests (50 - 200 μg/l). The NOEC for zinc was 12 μg/l. Two tests with copper produced NOEC values of ≤ 3 μg/l for percentage abnormal development.

Reduced salinity has been used in our lab more frequently to assess embryo condition. Table 2 summarizes the results of repeated embryo tests with reduced salinity using the echinochrome endpoint. Sensitivity to salinity fluctuations was relatively consistent throughout a 13-month period, producing a mean EC50 of 27 g/kg with a coefficient of variation of 3 percent. The results show little seasonal trend, despite the fact that laboratory conditioned sea urchins were used for tests conducted from May to October.

Two effluent tests per week could be conducted by a team of two persons. Tests with this species can be conducted during any part of the year, provided an adequate supply of urchins is maintained in culture from April to November. One test per week could be conducted by one person working full-time.

**TABLE 2. SUMMARY OF REDUCED SALINITY TEST RESULTS WITH
S. *Purpuratus* EMBRYOS DURING 1981 - 1982 (EC50 VALUES
ARE SHOWN FOR REDUCTION IN ECHINOCHROME
PIGMENT PRODUCTION BY 48-HOUR EMBRYOS)**

	Month	Salinity EC50 (g/kg)
1981		
	July	26
	August	28-29
	September	27
	October	27
	November	28
	December	26
1982		
	January	27
	February	27
	March	27-28
	April	27
	May	28
	June	28
	July	27

I would expect a maximum 20 percent test failure rate due to poor control development (see Table 3). Test rejection due to poor control performance is typically the result of having slightly more than the minimum acceptable 15 percent of otherwise normal control larvae not achieving prism stage in 48 hours. This situation could be caused by variations in test temperature and may not seriously compromise the usefulness of that specific test. Less rigorous criteria for the classification of normal embryos would improve the "success" rate of this test.

TABLE 3. RECORD OF 48-HOUR EMBRYO TEST SUCCESS WITH
Strongylocentrotus purpuratus **EMBRYOS SINCE 1986**
(CRITERION OF A SUCCESSFUL TEST IS LESS THAN 15
PERCENT ABNORMAL OR RETARDED EMBRYOS IN
CONTROLS)

Month	Tries	% Successes
January	4	100
February	4	75*
March	1	100
April	1	0*
May	4	75*
June	0	
July	0	
August	0	
September	0	
October	0	
November	0	
December	1	100

*All failed tests had less than 20 percent abnormal embryos.

The availability of test organisms throughout the year, high percentage of test success, and the good sensitivity of urchin embryos to toxicants are important advantages of this test method. The embryos are also amenable to examination for other endpoints in addition to normal development. This flexibility permits the test to be adapted to the needs of specific monitoring programs.

The most significant drawback to the test relates to the potential for variable interpretations of normal versus abnormal embryos between analysts. Toxicant effects can be expressed as both malformations and reductions in the rate of development, with each type of effect requiring a somewhat arbitrary determination of the acceptable normal range of variation. The potential therefore exists for

inconsistent interpretations of the test endpoint. This possibility can be reduced by the establishment of standardized rating criteria and the use of more objective measures of embryo condition, such as echinochrome production or DNA content.

DISCUSSION

MS. ANDERSON: When you raise the embryos for 48 hours, do they have to be paddled, you know, agitated?

MR. BAY: No, they don't. Originally at SCCWRP, back in the early 80s, our method consisted of a beaker with both a stirring paddle and a source of gentle aeration. Over the years, we have streamlined the method. First we eliminated the aeration and felt good about those results; and recently we've eliminated the stirring. For the past 3 years, we have generally not used any paddling.

MS. ANDERSON: Do you know why that was the conventional wisdom for so long? It's a pain in the neck to have that kind of equipment around and the potential for contamination in between.

MR. BAY: I think it's a matter of taking a research tool and turning it into a monitoring tool. I think in research, for culturing the embryos over a long-term time period -- through their larval stage and metamorphosis -- a paddle is needed. And these methods were taken from that kind of work, where they were doing long-term developmental studies, and needed to have gentle agitation of the embryos. The research papers used to develop our test method specified using a paddle-type system, and I think it just worked it's way in from that aspect.

MS. ANDERSON: Do you (Steve Bay) or Phil Oshida have any idea when the cutoff is temporally, and when you have to start paddling?

MR. BAY: I would say when they start feeding, after about 96 hours. In that case you've got food accumulation on the bottom that could interfere with the embryos. You also need to ensure a better distribution of the food for those embryos to feed on.

MR. DINNEL: A comment on the standardization of reading samples, the degree of abnormality: you spell it out pretty clearly for your lab, but each lab looks at it a bit differently.

MR. BAY: Yes.

MR. DINNEL: We found this in the Puget Sound region for testing of sediments with the Corps of Engineers/EPA program. At one point, I noticed that one of the labs had a nice posterboard of different figures with a line down the middle: normal versus abnormal. The Corps of Engineers, then, had those reproduced and sent out to all the different laboratories and were having occasional workshops to discuss exactly how samples are to be read. So that's one thing that can be done, possibly, in terms of interlab standardization.

MR. BAY: Yes, I think that's great. I'd like to participate in those workshops in the future. Generally, when you look at the research papers on this test they're referring back to something like Kobayashi's papers that show examples of grossly abnormal embryos that aren't useful in drawing the fine line. And so, in the Puget Sound case or in my case, we really need to make some arbitrary decisions and then agree upon them. One important aspect of our method is the shortened exposure time. I think this might be a significant benefit for some samples that have variable toxicity with time. Additional intercalibration on the relative sensitivities between the 96-hour and the 48-hour exposures might be in order.

MR. OSHIDA: I have a couple questions and then a comment. Does the echinochrome test work well with your new, lower volume and lower density?

MR. BAY: The density is the same as that we were using earlier with the one-liter volumes. The data that I showed you is based on the smaller volumes. And, from a practical standpoint, that's about the smallest volume that you can use for the echinochrome extraction. Basically, the more embryos you have, the larger the signal. Our current method gives an absorbance value of about .07 absorbance units, so you still have a reasonable range to see reductions in. And that's the main limitation, with a small absorbance, you can't detect differences with much sensitivity. But it works fine. I think we've sacrificed some sensitivity to make it compatible with that volume; but we've just been reluctant to change the density of embryos so that we could have better comparability with previous data.

MR. OSHIDA: It sure sounds a lot easier not having to stir. To answer Susan's question, I think you answered it right on the nose, that the work of the late '70s really involved stirring. Another point was that, in the early days, we

used, and still use, relatively high densities of embryos; and, if they all settled on the bottom, it would create a real fungal problem, even at fairly cold temperatures. At that time, we really weren't looking at a lot of metal sensitivity; we focused on the differences, the gross differences in toxicity between different types of effluent. And, even with slight contamination from the stirrers, if there was any, it probably didn't overshadow the results of the effluent toxicity. One of the things that Steve Bay hasn't mentioned, which is a major advantage that SCCWRP has, is that they have a very, very low turnover of people there. And the fact is that, probably over the last decade or so, the number of people that have been the major reviewers of the embryos, has been a relatively low number; and that makes a lot of difference when you're looking at variability over time, if you have the same person looking at those embryos. You know, if anybody can build that into the system, I bet everybody would appreciate it.

MS. ANDERSON: Can those embryos be fixed, I mean, for those more subtle morphological observations? Can you use an indefinite fixation or are there limitations?

MR. BAY: I neglected to mention that; but all of our ratings of percentage-normal development are based on formalin-fixed animals. And the way we operate at SCCWRP, it's sort of like an indefinite fixation because a lot of the time, we'll do a test and won't look at it for months. So it works out quite well, I would say. We've had many occasions where we've archived our samples and then gone back and looked at them to determine if our endpoint assessment was similar over subsequent experiments. And so it's quite practical. The other thing that I didn't mention is the advantage or the potential to look at a more sensitive endpoint, which is the occurrence of cytologic and cytogenetic abnormalities in these samples. At the 48-hour stage, the cells are still large enough that you can prepare a thin smear preparation from formalin-fixed embryos. So you can archive those embryos and if you're interested in looking at potential cytogenetic effects at a later date, you can pull out the embryos, do a preparation, and look for mitotic aberrations. So, fixation gives you a lot of flexibility in which endpoints to look at.

MR. DINNEL: In the Puget Sound region, we've looked a little bit at the cytogenetic endpoint such as anaphase aberration, and you made a statement that it appears to be more sensitive. In the testing up there, it appeared

to be somewhat less sensitive as far as percentage for a given set of samples. But the point that is often missed is that it is really not a case of more sensitive or less sensitive, but sensitive to different things. The effect you see in cytogenetic endpoints is additive with abnormal development to a certain extent. They're two different endpoints.

MR. BAY: Yes, I would agree. I think any time you have multiple endpoints with a test, you're not going to get perfect correspondence between them. Our particular experience with the samples from San Francisco Bay, when we did elutriates, was that the percentage normal or the echinochrome endpoints were not very responsive at all; but the cytogenetic endpoint did have a clear dose-response relationship that followed the overall contamination, and more specifically, the PAH gradient in the sediments.

4. MOLLUSCS SUMMARY

Included in this section are the tests with embryos and larvae of the Pacific oyster (*Crassostrea gigas*), the mussels (*Mytilus edulis* and *M. californianus*), and the abalone (*Haliotis rufescens*). The oyster and mussel embryo/larval tests are perhaps the oldest chronic toxicity test procedures for marine organisms. However, a canvass of people actively engaged in marine toxicity testing indicates that they are not in common usage and that there are seasonal problems with obtaining adults that will produce viable embryos.

At the other end of the spectrum, the abalone test has been recently developed, with indications that seasonal availability of mature adults is not as severe a problem. The abalone test has been successfully tried at other laboratories, but more work is needed testing the ability to ship, receive, and hold the adults, and testing the interlaboratory variability with reference toxic materials. In addition, experience needs to be gained in reading the developmental endpoint of the abalone test.

Although ASTM has published a procedure for a 48-hour embryo/larval test with four species of molluscs, including the oyster and the mussel, several workers on the West Coast report that 48 hours is often not sufficient for the development of larvae into the prodissoconch or D-hinge larval stage.

Embryo/larval tests with all four species of mollusc appear to show sufficient promise to warrant further investigation and improvement. The species appear generally comparable in sensitivity and might be used interchangeably depending upon the availability of each. Better information is needed on the embryo/larval developmental rate and on the spawning periods of *Mytilus* populations along the West Coast.

All of these test species represent attractive candidates for toxicity testing and effluent monitoring. They are sensitive and relatively easy to obtain, they represent no significant cultural problems, and they have the general public interest.

Resolution of the identified weaknesses in the development of broader data bases and improved test procedures should be pursued quickly. Most of these weaknesses could be overcome within a short period of time.

Summary of Toxicity Testing with the Red Abalone *Haliotis rufescens*

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As part of the State Water Resources Control Board's Marine Bioassay Project, we began in 1985, to develop a short-term test to estimate chronic toxicity of effluents to the red abalone, *Haliotis rufescens*, an economically important species indigenous to California marine waters. The test uses a sublethal endpoint (larval shell development) and sensitive life stage (embryo to veliger larva), and is similar to the 48-hour mussel embryo test. An abalone test offers some advantages over other mollusc tests because of the longer spawning season, ease of assessing sex and gonadal maturity, and larger size of the test embryos. The primary disadvantages of using abalone are that they are not so widely distributed as mussels and their culture is dependent on a reliable supply of macroalgal food. The following is a brief summary of our work to date with the red abalone.

We initially obtained abalone broodstock from the California Department of Fish and Game Marine Culture Laboratory at Granite Canyon, and have used hatchery-produced animals as spawners throughout our work. These abalone have spawned dependably, and limited interlaboratory data indicates that abalone from other hatcheries respond similarly to toxicants. (Further experiments are planned to examine interpopulation variability). Of 36 spawnings attempted in our laboratory over the past 4 years, 34 have been successful (97 percent). Unsuccessful spawnings occurred in March 1987, when the males did not spawn, and in December 1988, when the females did not spawn (see Table 1 below).

TABLE 1. SPAWNING ATTEMPTS/SUCCESSES (1986 - 1990)

Month	Males Successes/Attempts	Females Successes/Attempts
January	3/3	3/3
February	1/1	1/1
March*	4/4	3/4
April	1/1	1/1
May	1/1	1/1
June	3/3	3/3
July	3/3	3/3
August	9/9	9/9
September	4/4	4/4
October	1/1	1/1
November	2/2	2/2
December*	3/4	4/4

*Months in which unsuccessful spawnings occurred.

Irradiation of seawater with ultraviolet light was used to induce spawning in 17 trials; the addition of hydrogen peroxide was used in 19 trials. One failed spawning occurred with each method. Abalone used in the trials were generally rated as "2" using a gonadal maturity index that designates extremely ripe animals as "3" and animals in which the sex cannot be determined as "0." Maturity is assessed by placing the animal shell down and pulling back the foot to view the gonads, located under the right posterior edge of the shell.

Red abalone are relatively easy to culture. Research for the commercial mariculture industry has produced techniques for spawning induction, larval rearing, and culture throughout the life cycle. Broodstock are easily maintained on a diet of macroalgae, primarily the giant kelp *Macrocystis*, which is plentiful and easily collected in central and southern California. It may be difficult to collect sufficient amounts of macroalgal food outside of this area. The Marine Culture Laboratory at Granite Canyon has maintained broodstock continuously for over 15 years. We have maintained spawners at MPSL for 5 years. Prior to 1989, we maintained 100 abalone as broodstock. Last year, we obtained 1,000 more abalone, of which approximately 300 are over 2 years old (generally the youngest spawnable age).

We have only recently begun to keep records of gonadal production, but in the past, enough ripe animals were generally available when needed for spawning. Recently, however, ripe animals have been harder to find among our 300 broodstock abalone during the winter months of December and January. Other abalone hatcheries have reported similar trends, but have been able to meet their spawning needs during these months, and have had enough surplus spawners to supply a limited number of other laboratories. We are presently looking into more intensive conditioning procedures to assure a year-round supply of gametes.

We have conducted 38 toxicity tests, since 1985, using a variety of toxicants. Copper, tributyltin, sodium pentachlorophenate, and endosulfan were tested once each; complex effluents from various sources were tested 11 times; and zinc was tested 17 times. The remainder were range-finding tests or tests investigating brine toxicity. Two additional tests were attempted but abandoned after the unsuccessful spawns mentioned above, and one early test was unsuccessful because of poor fertilization. Control response has been acceptable in all tests (>80 percent normal development), and in most cases >90 percent of the control larvae developed normally.

The following table is a summary of NOEC and EC50 values for 48-hour abalone toxicity tests. NOEC and (especially) EC50 values have not yet been calculated for all tests. Values given are means ± SD (n = the number of tests).

TABLE 2. RESULTS OF 48-HOUR ABALONE TOXICITY TESTS

Toxicant	NOEC (μg/l)		EC50 (μg/l)	
Zinc	28 ± 8	(n = 12)	63 ± 12	(n = 4)
Copper	< 6	(n = 1)	9	(n = 1)
Endosulfan	180	(n = 1)	252	(n = 1)
Pentachlorophenate	32	(n = 1)	59	(n = 1)

We estimate that it would take between 30 and 40 person-hours to conduct one effluent and one concurrent reference toxicant test, depending on which type of test

container is used. Tests can be done more quickly in tissue culture flasks than in open beakers. This time estimate assumes that broodstock are obtained from a supplier rather than by collecting wild stock, and covers everything from spawning to clean-up. The test protocol specifies 30 effluent containers and 20 reference toxicant containers (50 total). If 39 containers were used, for the purpose of this workshop, it would require between 25 and 35 person-hours to conduct the test, again depending on the type of test container used. Two technicians could therefore conduct two or three tests per week. One test per week would require only one technician. Given a reliable broodstock supply, this test can be run during any week of the year. As noted above, however, we have recently had a shortage of spawners from our broodstock during two winter months of this past year. It may also be difficult at present for laboratories to obtain ample broodstock to begin a large scale testing program. This is a logistical problem rather than a biological one. There are between 10^5 and 10^6 spawning-age abalone produced every year by commercial farms in California. What is needed is an intermediate facility that could take large numbers of abalone from growers, condition and inspect them for spawning, and relay them to testing laboratories. Two commercial operations have expressed an interest in this type of operation, but they are waiting to see the market develop before they dedicate resources to such a program. At MPSL, we have about 800 young abalone that will come to spawning age soon, and we hope to act as an interim supplier until the market becomes established.

Abalone can be shipped using 24-hour delivery services, and can be held for several weeks in static aquaria, as long as water is changed twice per week, temperature is controlled, and culture tanks are well aerated. This makes it possible for most labs to obtain and hold broodstock for short periods prior to testing. Interlaboratory tests have been done with university laboratories, public agencies, dischargers, and private laboratories; and other contract laboratories have run the test on their own.

DISCUSSION

MR. LANGDON: I was just wondering, have you tried any artificial sea waters?

MR. HUNT: We've done only preliminary work with some commercial sea salts. We often use trace metals as reference toxicants and it would be helpful to use artificial seawater to avoid chelation problems. Of three commercial sea salts we have tried, all three produced 100 percent mortality of larval abalone.

MR. LANGDON: You might want to look at an artificial sea water mix called Zaroogian's which is one you make up in the lab. It's based on Lymen and Fleming's artificial seawater, except for EDTA. It's an artificial sea water; it works very well with the bivalve larvae. It may work very nicely for abalone larvae as a controlled sea water.

MR. HUNT: Did you say it has EDTA in it?

MR. LANGDON: Yes, it has a chelating agent. For a control sea water it would be useful just to standardize and measure viability.

MR. CHERR: There's a number of artificial seawater formulae available in the literature that use analytical grade salts.

MR. HUNT: Right.

MR. CHERR: There's a book called <u>Artificial Sea Water Formulae</u>, which has numerous formulae.

MR. LANGDON: Natural sea waters are better because the concentration of trace metals in your analytical grade salts is often very high. This was the breakthrough with Zaroogian's; he recognized this and added EDTA as a chelating agent to remove a lot of those trace metals.

MR. ANDERSON: How could it be a true control, if your control water is different from other treatments?

MR. LANGDON: Well, this would just allow you to test the viability of your sperm and eggs so that they would at least give you that information as to whether the mortality that you're seeing in your control in natural sea water is due to the problems with your control natural sea water, or whether it's due to the embryos, or whether it's due to the gametes.

MR. HUNT: Another factor that seems to affect control response in the abalone test is the handling of broodstock prior to spawning. We have seen differences in interlaboratory tests between the control response at labs using their own broodstock and labs using broodstock recently shipped from elsewhere. This does not significantly affect test sensitivity, and controls usually meet acceptability criteria (i.e., greater than 80 percent normal larvae), but broodstock acclimation time is something we want to investigate as a way to improve control response in labs that do not have culture facilities.

MR. BAILEY: One of the things I noticed was that there wasn't any difference between the controls where you both used your own stocks. Your NOECs were only one concentration different.

MR. HUNT: Yes, they were.

MR. BAILEY: Basically the same as what happened when your controls were a little bit offset.

MR. HUNT: Yes. Actually, the control offsets didn't make a whole lot of difference.

MR. BAILEY: Right. But the variation between the labs still seems to be there.

MR. HUNT: In the interlaboratory tests with SCCWRP, the dose-response curves were nearly identical except for a slight offset at an intermediate concentration. This caused the NOEC to vary by one concentration.

MR. ANDERSON: What about the EC50s?

MR. HUNT: I don't remember the numbers offhand, but I think they were fairly close. In five recent interlaboratory tests, the EC50s varied between labs by about 16 percent.

MR. CHAPMAN: Just in general, NOECs are convenient but they're not necessarily the easiest things to work with sometimes. If we were willing to bite the bullet, either scientifically or from a regulatory standpoint, we'd probably go to regressions and say, "This is the EC10 concentration or the EC5 concentration."

MR. HUNT: EC50s are more representative of the whole data set than are NOECs.

Toxicity Testing with Oyster and Mussel Embryos

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Species: *Crassostrea gigas* (Pacific oyster) and *Mytilus edulis* (bay mussel).

Some of the mollusc embryo tests in our laboratory were carried out with metals, pesticides, and sewage, but the majority of the tests evaluated the quality of ambient water samples collected from the central basin of Puget Sound.

Typically, we limited our testing to periods close to the natural spawning time (summer) for oysters and mussels, thus avoiding the need for extensive conditioning to induce gametogenic development. Spawning stocks were collected from field-cultured stocks or obtained from spawning stocks maintained by commercial oyster hatcheries (this was the best source for out-of-season spawning stock). We have found that there are no guarantees that a given animal or group of animals will produce good quality gametes or embryos, even if "properly conditioned." This factor reduced our overall test success rate with this assay to only 43 percent (22/51 successful tests) over a several year period. Success of mollusc embryo testing by commercial labs in the Puget Sound region during 1989 was reported to be even less than this; the factor(s) causing the poor gamete/embryo quality was unknown.

Once brought into the laboratory, oysters (mussels to a lesser degree) usually need to be conditioned to spawn by holding in 20° C seawater with daily feeding of cultured algae. Once conditioned, oysters or mussels should be used within a week or two to avoid over-ripeness. Spawning is usually induced by thermal stimulation (temperature spikes of 25 - 30° C) and/or addition of sperm suspension from a sacrificed male.

One or two individuals can easily run two 48-hour embryo assays per week, not including chemical analyses, if required. The primary drawbacks to a mollusc embryo assay are: (1) the need to condition spawning stock (requires heated seawater

and cultured algae), which can be especially difficult during the winter; and (2) tests often fail the control standards of ≥ 90 percent survival and ≥ 70 percent normal development to a D-shaped veliger. Other than these factors, this test has proven to be quite sensitive to toxicants (possibly slightly more sensitive than an urchin embryo assay) and this assay (using Pacific oysters) has been the basis of a marine water quality monitoring program in Washington State (implemented by C. Woelke) for several decades. Generically speaking, this test is also available on both coasts of the United States and in many other countries.

The sensitivity of this test (48-hour developmental EC50s) to selected toxicants as determined in our laboratory is summarized in the following table:

TABLE 1. RESULTS OF 48-HOUR DEVELOPMENTAL EC50 TEST

Toxicant	Pacific Oyster	Bay Mussel
Metals		
Cadmium (mg/1)	0.12 - 1.20	2.20 - 6.50
Copper (µg/1)	6.1	21 - 35
Lead (mg/1)	0.68	>9.52
Silver (µg/1)	19.0	<4.4
Zinc (µg/1)	206.5	96 - 314
Pesticides		
DDT (µg/1)	>4.6	>17.2
Dieldrin (µg/1)	22.9	48.3
Endosulfan (µg/1)	55.0	212.3
Endrin (µg/1)	152.4	>362
Sewage (percent)		
Influent	4.2	NT*
Primary	5.9	NT*
Secondary	>20	NT*
Chlorinated Secondary	2.3	NT*
Dechlorinated Secondary	>20	NT*

*NT = not tested.

Toxicity testing of *Mytilus edulis* and *Crassostrea gigas*

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We obtain adult molluscs from two West Coast suppliers, Sea Farms West, located in southern California (in Carlsbad) and Cove Mussel Company/Intertidal Aquafarms in Central California (at Tomales Bay), to account for differences in spawning condition between different populations. From our experience and that of laboratories in the San Francisco Bay Effluent Toxicity Characterization Program, both *Mytilus* and *Crassostrea* follow somewhat consistent patterns of spawning seasonality. The best spawning period for the mussel occurs from November to mid-April with more marginal spawning in mid-October and late April. The oyster has a shorter optimal spawning season running from July to September with marginal spawning in May/June and October/November. Fortunately, the months when mussels do not spawn correspond in large part to the spawning season for the oyster. Only in May, June, and October is there a high risk of having neither species in spawning condition. When testing during the marginal spawning periods, it is advisable to order organisms from at least two sources and set up at least 100 animals from each source.

We've run four toxicity tests using sodium azide as a reference toxicant. NOECs are currently being calculated and will be presented at a later date. Results of quality assurance testing from the Effluent Program using copper as a reference toxicant are attached (see Table 1). In the past year, we have conducted four mollusc tests on ambient waters, three of which used bay mussels and one used the Pacific oyster. While we have not yet seen a significant toxic response using the mollusc test, in three of the four tests, other species run simultaneously did exhibit a toxic response.

One standard test (six ambient, five reference toxicant, and two control treatments) takes one person 3 working days to complete. One day is spent preparing the samples, taking water chemistry measurements, and setting-up the test.

The other 2 days are spent taking down the test and counting the embryos. The number of tests that can be performed in a week is dependent on the season and holding tank space. During peak spawning season we need to spawn between 30 and 50 mussels per test in order to use gametes from at least four males and four females. When tests are conducted during the optimal spawning period, the probability of a test failure is low (<10 percent). However, when we attempted to run an oyster test in late September, spawning was either marginal or absent. Below are deviations in our testing methods from the ASTM protocol.

- Instead of holding the adults for two weeks prior to testing, we receive them either the day before or the day of testing and hold them dry in a cooler with ice.

- We have found that both mussels and oysters spawn more readily in water heated to 25°C instead of the ASTM maximum of 20°C. Once an individual commences spawning, it is then removed from the heated water and placed in an individual beaker containing water at the test temperature. This procedure ensures minimal exposure of gametes to water over 20°C.

- We have been using glass scintillation vials instead of beakers for test containers. These vials easily fit into a small water bath and make it possible for the test to be terminated by injecting fix into the test vial itself. Our only concern with these containers is that the increased surface area to volume ratio involved in using a small test volume (10 ml) may result in increased adsorption and a decreased dosage, thereby decreasing the sensitivity of the test.

- For mollusc tests run at 16°C, it is possible that the D-hinge stage will not be attained in the controls after 48 hours; therefore, test termination time is determined using an observation vial, run with the other test vials, in which we monitor control development. This method adds no more than 10 hours to the normal 48-hour exposure time.

- We question the value of the survivorship endpoint. Data from the Effluent Program indicate that survivorship is never a more sensitive indicator of toxic effect than development. If only development were used, scoring time could be decreased by 50 percent.

TABLE 1. OYSTER LARVE DEVELOPMENT TEST INTERLABORATORY PRECISION USING COPPER

Lab	Survival IC50 (μg/l)	Development IC50 (μg/l)
1	>20.5	6.52
2	16.6	11.8
3	>20.5	17.3
4	15.0	14.5
Mean		12.6
CV		37

Note: To supplement our limited experience with these animals, we include information from laboratories participating in the San Francisco Bay Effluent Toxicity Characterization Program.

DISCUSSION

MR. BAY: You did some chemistry on looking at the free ion copper and the availability; did that correspond as you would expect to different water types?

MS. ANDERSON: No, we used a couple of measures of ion activity too. In the cases where we had lysing of the embryos, we detected free cupric ions but NOEC values or LOEC values were below levels at which we actually detected the free ions. So it was really interesting. Our hope was that we could use free cupric ion activity (looking at a variety of measures to do that) and make correlations to suspended solids. Although we realized we probably wouldn't get that lucky; we thought we would try. Copper work is very tricky. It is difficult to get a measurement you can use in a regulatory setting, which is my concern. Different methods give you varying measures of free cupric ion activity. So, while you make a curve for one method, when you make a curve for another method, it varies with different ligands and whether there are organic ligands, or just solids, and the method itself.

MR. DINNEL: Regarding survival-abnormality counting, you were going with just looking at normality, forgetting survival; and I tend to agree with that except for one thing. Conducting this test routinely, Woelke and Cardwell and

others found that, if they got into some of the waters, especially in south Puget Sound that had high phytoplankton populations, they would get, routinely, very poor survival in the test. And they basically showed that, if you had high concentrations of phytoplankton, especially dinoflagellates, in the water, they would survive very poorly. But, those that did develop, developed normally. And that means, if you drop the survival counting, you can get batches of control water that are poor and give you poor results in a test with toxicants and you don't know that you've got that factor affecting the results. So that's one exception to the survival rule.

MS. ANDERSON: What was the factor that caused the mortality? Did they ever explain that?

MR. DINNEL: Phytoplankton metabolites, essentially.

MS. ANDERSON: Have you ever seen anything like that in the sea urchin sperm assay?

MR. DINNEL: There's a funny story about that. The short answer is no. What happened is that the University of Alaska sent down some PSP toxins for the shellfish toxins test and I wanted to look at its toxicity using a sperm assay. I tested it and got a nice dose-response curve with the test and wrote it up for publication, sent it in, had it reviewed, comments back, and some question came up about something and I ended up checking for the concentration of copper -- oh, I know what it was -- I asked the University of Alaska how they extracted the toxin and they said, "Well, we killed all the cells with copper sulfate first." So we took the toxin off the shelf and measured it and compared my results with copper; and it was a copper response curve. There was no toxicity due to the toxin. So, for the sperm assay, it doesn't appear to affect them, but for some unknown reason, the metabolites evidently greatly affect the survival rate of embryos.

MS. ANDERSON: So, even filtration wouldn't help?

MR. DINNEL: No, they tried all sorts of filtration experiments. They tried a lot of different experiments and they tied it down to high concentrations of dinoflagellates.

MS. ANDERSON: I'd love to see that because, having thought a lot about ambient issues, we have some fairly subtle observations in ambient waters with the embryo tests. I'd like to be able to rule out such alternative explanations for the toxicity. Was this ever published in Cardwell and Woelke's reports?

MR. DINNEL: It's in the Washington Department of Fisheries reports.

MR. BAILEY: I notice your brine performance is essentially the same as your sea water performance. Do you make up fresh batches of brine every time you run the test? Have you done any work with holding and aging it?

MS. ANDERSON: No, we've had problems in other assays with holding the brine and found that caused trouble and so we make it up fresh every time. I don't think we've found that out specifically in tests, but, instead of checking further, we just decided to make it up fresh every time.

MR. CHAPMAN: I notice you said "diluted with mineral water?"

MS. ANDERSON: Yes.

MR. CHAPMAN: What are you using?

MS. ANDERSON: It's Arrowhead Mineral Water.

MR. HUNT: Do you have any experience using brine made by freezing seawater?

MS. ANDERSON: No, I don't.

MR. HUNT: Does anyone have any experience with that?

MR. SLATTERY: Yes, it actually works real well.

MR. HUNT: Making brine by freezing seawater was recommended to us by someone at EPA in Narragansett. It has worked well for us and I wondered if others had used it.

MS. ANDERSON: One thing we did find out is that filtering the sea water at 4 or 5 μm before you make the brine really helps. That's the only little trick that we found was necessary and other people have told us, "Oh, yeah, we always do that, too."

MR. HUNT: I would recommend filtering also. Making brine by freezing is faster and easier than evaporation.

MR. CHAPMAN: Time and temperature, please?

MR. HUNT: Our freezer temperature is minus 12°C. We filter seawater to one micron. To make at least one liter of brine we put four one-liter volumes in the freezer for 6 hours, remove the ice, combine the remaining

hypersaline liquid, and refreeze overnight. The next day you may have to thaw it a bit to break up the ice before pouring off the liquid brine, which should have a salinity of about 60 to 80 g/kg.

MS. ANDERSON: With the ambients, the problem you will run into is the dilution that you take when you use any form of natural brine. We did a little bit of work with commercial sea salts and it wasn't too bad but we really didn't have the time to do that kind of development so we're just taking the easy road. When running ambient samples from San Francisco Bay, we're normally looking for somewhat subtle effects. So anything to minimize dilution is really helpful.

Toxicity tests for *Mytilus californianus* (California mussel)

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Our laboratory has been investigating *M. californianus* embryo toxicity tests since 1986 (Cherr, Shoffner-McGee, and Shenker; Env. Tox. and Chem., in press). The majority of the experiments during this time period involved methods development and were not focused on toxicity assessment. We have found that the Sonoma Coast population of *M. californianus* generally spawns from November to May, with sporadic spawning during August and September (see Table 1). However, this time frame can shift dramatically depending on oceanographic conditions (e.g., El Nino). In addition, spawning success was greatly decreased following periods of rain. During the spawning season, approximately 30 - 50 percent of the animals spawned following feeding of *Isochrysis galbana* - Tahitian strain (1-2 x 10^6/ml). We found that the holding of ripe mussels in running seawater for more than 24 hours generally decreased spawning success. As such, we typically collect animals the morning of the test. Some attempts at long-term (30 - 60 days) holding and feeding of California mussels were also unsuccessful in maintaining sexual maturity.

TABLE 1. SPAWNING PATTERNS OF *M. Californianus*

Year	Month	Success	Number Of Experiments
1986	February	Spawn	2
	March	Spawn	2
	April	Spawn	2
	May	None attempted	
	June	Spawn	1
	July	None attempted	
	August	Spawn	2
	September	Poor-good spawn	3
	October	Spawn	1
	November	Spawn	2
1987	February	Spawn	2
	March	Spawn	2

TABLE 1. SPAWNING PATTERNS OF *M. Californianus* (Continued)

Year	Month	Success	Number Of Experiments
	April	Poor or no spawn	1
	May-October	None attempted	
	November	Spawn	2
1988	January	Spawn	2
	February	Spawn	1
	March	Spawn	2
	April	Spawn	1
	April-July	No spawn	9
	August	Spawn	2
	September	Spawn	2
	October	Spawn	2
	December	Spawn	1

Development of the zygote to the veliger stage at ambient temperatures (12° C) takes a minimum of 72 hours. We culture our embryos to 96 hours to ensure complete development and calcification of the shells of veligers in control seawater. We found that predetermination of optimal sperm/egg ratios (ratios resulting in >90 percent monospermic fertilization) greatly facilitated success of the bioassays. The optimal ratio varied from 25 to 200 sperm/egg among 17 females. Even when the same batch of sperm was used on a given day, variability in the optimal sperm/egg ratio occurred. Differences in the optimal sperm/egg ratio between different dates suggests that both eggs and sperm may contribute to this variability. We developed a microscopic method of accurately determining the percentage of eggs exhibiting monospermic as well as polyspermic fertilization prior to fertilization of the entire batch of eggs for the toxicity test.

We found that embryos could be cultured quite efficiently in small volume culture chambers (3 ml) and that the response of embryos to a toxicant (sodium azide: EC50s range from 12.4 - 38.0 mg/l) did not differ between culture beakers (400 ml, 35 embryos/ml) and the 3 ml chambers (35 embryos/ml). This enabled us to fix the embryos and score them in the culture chambers without inducing

subsampling variability. At 96 hours, larvae were assessed using polarization microscopy to take advantage of shell birefringence. This enabled accurate assessments of shell formation and calcification.

We prefer to run this test no more than twice a month since 96 hours of culture is required and assessment may require two people and 2 - 3 days of microscope work, depending on the experimental design. When the optimal sperm/egg ratio was predetermined, we had virtually 100 percent test success. Success was defined as $\geq$ 80 percent development to the veliger stage in control seawater. Two experienced people are needed to conduct this test.

M. californianus could be a useful organism for assessment of open-coast discharges. The only drawbacks are the inability (at least in our hands) of maintaining stocks in culture so the same animals could be shipped to other locations for interlab comparisons. However, mussels can be maintained relatively dry and ship quite easily such that freshly collected animals could be sent to other locations. We believe that in a "typical" year *M. californianus* can be spawned 7 - 11 months of the year in our region. However, this time period may be highly variable from year to year depending on water temperature, rainfall, etc. We do not have comparative data to standard toxicants other than sodium azide. We have also collected data using complex effluents and fractions of the effluents.

DISCUSSION

MR. DINNEL: Have you looked at how critical polyspermy is with oysters?

MR. CHERR: No, we haven't done much work with oysters. My understanding from the literature is that Gould and Stefano published a paper, a year or 2 ago I think, showing that it depends on how you collect the oyster eggs. If you take them from the ovary, you currently have to culture them in sea water for a couple hours and they slowly develop a block to polyspermy. If you fertilize them right away, they become polyspermic. However, I'm not sure about spawned eggs.

You know, in terms of *Mytilus*, we have not taken eggs from the ovary at all; we strictly use *Isocryosis* to induce spawning. In fact, we have tried to use peroxide and temperature shock and we found we get much poorer quality eggs. It seems like they seem to release some of the less mature eggs as well. But I know that with oysters, if you don't hold them in sea water for some time, there's a real problem.

MS. ANDERSON: How about *edulis*?

MR. CHERR: We are not sure. We really haven't done anything with *edulis* along these lines. Do people routinely use the same sperm concentration and always obtain good development?

MS. ANDERSON: No. The ASTM protocol just calls for a range of two orders of magnitude and, as long as people hit within that, we haven't altered that requirement. By virtue of the fact that we get really good control data submitted to us, I would say that we could guess that it's less sensitive to polyspermy; but I'm sure someone has that answer. I routinely run it at lower sperm concentrations than the protocol says, even in the lower, lower range, because I've found the protocol concentration is way too high. So, when I would look under the microscope by focusing up and down and looking at how many sperm I saw on the outside of the egg, I would always shoot for around two. My sperm concentrations were way lower than the ASTM protocol requirements. So I don't know.

MR. CHERR: What we were doing initially was just simply adding sperm. For example, people over in the oyster facility at BML, add sperm, wait 5 minutes, and then count the number of sperm on the egg and say, as long as you have "X" number of sperm, you're okay. With *Mytilus*, we were doing that and we were having tremendous problems. And I think, as we started using the Hoechst method, it became quite clear that the difference between 100 and 150 sperm per egg can result in mono- or polyspermy. At that point, that we labeled PS, that is, just starting to see polyspermy, maybe only 5 to 10 percent of the eggs are polyspermic. But, nevertheless, it's indicating right away that it's a pretty tight range.

MS. ANDERSON: How much influence do you think the eggs sticking to the containers really has? Are you talking about 5 percent level or are you talking about 30 percent level or you don't have a feel for it?

MR. CHERR: I don't know. We haven't really quantified it, we've just observed it on the walls of the tubes, which alarmed us. We immediately tried to look at ways of preventing this adherence.

MS. ANDERSON: It would be easy if someone followed up on it to estimate the number of embryos per milliliter and then the number of milliliters in the tube and then have somebody count. Because it's an important thing; there are a lot of tests being reported that way. If it's really biasing the data, it would be nice to know.

MR. CHERR: Yes. I agree. I agree with the approach of John and Brian in using a single vessel without subsampling with the abalone; it makes a lot of sense since all your embryos are in there and you can assess them all at that point.

MR. CHERR: One thing that's difficult, though, if you want to look at the number of embryos upon seeding in the small volume chambers, you must put them on the microscope and go through and do a count. Even if you have a microscope set up in a cold room, I am leery about putting embryos on the microscope stage with transmitted light. We initially attempted some video-taping and then went back to review the video-tapes in order to count. That can be done but it's very tedious and difficult. All of your embryos are in the chamber. When we do see a reduction in numbers, it is in extremely high concentrations of azide, and is due to lysis and fragmentation. It is difficult to quantitate this. I'm not sure that this is meaningful, since it occurs at concentrations way above the NOEC.

MR. BERGER: Could you get around that by using maybe cold light or did you do that? Was your concern the incandescence or the heat?

MR. CHERR: No, not the heat as much as the wave length.

MR. BERGER: Oh, okay.

MR. CHERR: We could use a red light; but it really becomes tedious to do that.

MR. CHAPMAN: In talking to people that have had anything to do with the development of the ASTM protocols, it's my feeling that there's a lot of data that went into that on oysters and there is perhaps even some regret that some of the other species have been included. So I agree that there's more work that

needs to be done on *Mytilus* from that standpoint. Also from the standpoint of conditioning the organisms, would it help if you bring the *Mytilus* in without taking them off the substrate?

MR. CHERR: Yes, that's an excellent point. We have a graduate student that's going to be, in part, looking at that; she is going to be caging mussels out at the sites. It could be that ripping the animals off the rocks immediately puts them through tremendous stress -- they may resorb, what have you. One needs to look at the ovary to confirm that. It could be that after holding them under the right condition, letting them reattach in the lab, could alleviate this problem. However, the natural environmental stimuli for spawning would be removed.

MR. ANDERSON: I have a question. Do you have any idea about test precision using reference toxicants? How do they compare to Susan Anderson's data for instance.

MR. CHERR: Comparing *M. Californianus* with Mike Martin's data with zinc and *M. edulis*, the response is similar.

MR. ANDERSON: I mean, if you ran repetitive tests in your lab with a reference toxicant.

MR. CHERR: We have not done a lot of reference toxicant testing, other than the azide; and you can see, in those experiments, there was quite a bit of variability between experiments. They were conducted at different times of the year and so on. So, right now, I would say the precision is pretty low between those three tests.

Summary of Culture and Toxicity Test
Experience with the Blue Mussel, *Mytilus edulis*

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CULTURE

We have worked with blue mussels since May 1987, and fairly intensively since January 1988 (essentially one season). Stock has been procured from commercial growers and from the wild in the northern Puget Sound area. In our laboratory, mussels have been held for up to 6 months (January to June) in flow-through seawater tables without significant mortality. We consider tank cleanliness to be important, since a buildup of fecal matter or any accumulation of dead mussels could lead to mortality, a result of bacterial fouling of the water.

Our current culture methods call for obtaining mussels from the wild at monthly intervals starting in late December. Conditioning the mussels to spawn predictably has been a challenge. The mussels are held in flowing seawater at 14° C with a daily feeding of a dense *Isochrysis galbana* suspension until conditioned. Our feeling is that the microalgae is probably important in accelerating the conditioning process and in lengthening the spawning period once conditioning has been completed.

The period of time required for conditioning has varied considerably from as few as 4 days to as long as 2 - 3 weeks or more. Using an earlier procedure of continuing mussel exposure to the warmer conditioning temperatures allowed for spawning for a period of up to 2 weeks after conditioning was completed. Subsequent adoption of the more recent ASTM procedure (E 724-89), calling for a return to ambient seawater temperatures following warm water conditioning, has allowed for continued spawning for 5 - 6 weeks following conditioning. We anticipate that with further refinement of our conditioning procedures we will be able to reliably spawn mussels during the January to June period.

SPAWNING PROCEDURES

Our procedures for spawning for the most part followed those of ASTM, 1980. Seawater was heated from ambient to 20°C and placed in two glass baking dishes (28 x 33 cm). Thirty mussels were placed in each baking dish. Temperature was maintained during the spawning period by placing the aquaria in an environmental chamber. After the addition of mussels to the warmed water a dense suspension of *I. galbana* was added as well as a suspension of either fresh or frozen sperm. A source of great frustration, mussels oftentimes did not spawn according to the ASTM procedure. A follow-up procedure was initiated if spawning did not occur within 2 hours. This involved renewing the water and sperm/algal suspensions. In some cases, this renewal was sufficient in itself to cause spawning. Other procedures which occasionally resulted in spawning included removal from water for 15 - 30 minutes, chilling by placing in ambient seawater for 15 - 30 minutes with a return then to warm water, and injections of 0.5 M KCl. Once spawning had commenced, spawning organisms were transferred to individual beakers for subsequent gamete collection and egg fertilization.

TOXICITY TESTS

Table 1 contains a summary of the control data for mussel toxicity tests we have done. ASTM (1989) protocol for bivalve tests calls for 70 percent normal development of the initial number of control embryos. Only one of the mussel tests we have conducted to date met these criteria. Our control development ranged from 2 to 70 percent. Our procedure of prolonged holding at the elevated conditioning temperature may have resulted in substandard gamete quality. Also, it has been suggested by others, that northern stocks of mussels may not reach full "D" hinge stage development within the 48 hours allowed in the protocol. In some cases we extended the bioassays to 72 hours, and normal development increased during that time. Control performance criteria used by ASTM were most likely established from oyster test data and may not be appropriate for mussels. We believe that useful information can be obtained from mussel toxicity tests that do not meet the ASTM criteria.

TABLE 1. SUMMARY OF CONTROL PERFORMANCE FOR MUSSEL BIOASSAYS

Date	Test Type	% Normal	% Survival
2/23/89	Test spawning - single pair	36.5	50.3
3/29/89	Test spawning - single pair		
	• 48 hour incubation	60.1	78.0
	• 70 hour incubation	71.2	80.0
4/4/89	Single pair spawn		
	• 48 hour incubation	39.3	48.0
	• 72 hour incubation	38.1	41.0
	Pooled spawn		
	• 48 hour incubation	69.5	79.0
	• 72 hour incubation	75.8	79.0
4/12/89	Pooled spawn test A		
	• 48 hour incubation	34.9	83.0
	• 72 hour incubation	42.0	56.0
	Pooled spawn test B		
	• 48 hour incubation	32.6	74.0
	• 72 hour incubation	50.4	70.0
5/3/89	Pooled spawn		
	• 16° C incubation	1.9	21.6
	• 18° C incubation	1.9	19.2
	• 20° C incubation	1.2	11.9
5/9/89	Pooled spawn		
	• 16° C dark incubation	11.3	67.9
	• 18° C light incubation	4.6	47.2
	• 18° C dark incubation	2.3	42.4
	• 20° C dark incubation	0.7	36.9
5/24/89	Single pair spawn		
	• Pair A	55.8	78.9
	• Pair B	28.0	68.7
	Pooled spawn		
	• A + B	38.5	78.5
6/6/89	Single pair spawn	5.4	56.0

The erratic nature of control performance and the inability to satisfy ASTM control criteria has inhibited our ability to precisely determine the relative sensitivity/suitability of this bioassay. Initial work with pulp and paper mill effluents does however suggest it to have the potential for being a relatively sensitive test.

Mussel spawning and test initiation requires about one day's work for one person. Final sampling and reading results requires an additional 1 - 2 days of manpower. Under present conditions, one person could accomplish one mussel bioassay per week. Under ideal conditions, two people could run at least three tests per week if enough conditioned mussels were available. The limiting factors for this species as a test organism are: (1) understanding conditioning and spawning induction so that tests can be started predictably; (2) understanding typical mussel embryo development times and rates so that reasonable criteria for control performance can be established; and (3) the limited spawning season. From our present experience, we would expect nearly all of the tests to fail the ASTM control performance criteria. In addition, we are not at present capable of accomplishing bioassays on an on-demand basis, due to the unpredictable nature of spawning. We are hopeful that this situation will improve with experience and as more is learned about proper conditioning and spawning procedures.

Summary of Culture and Toxicity Test
Experience with the Pacific Oyster, *Crassostrea gigas*

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Rick Haley

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CULTURE

Most of our work with the Pacific oyster has taken place during the 1989 spawning season. All of our broodstock has been obtained from commercial growers. We have attempted to locate sources throughout the Puget Sound region in order to take advantage of the progression of spawning seasons from southern stocks in late spring/early summer to fall-spawning northern stocks. Our current culture methods call for obtaining early-spawning stocks of oysters from southern Puget Sound in the late spring, and obtaining middle and northern stocks later in the summer. Oysters should be available for spawning between early June and late October.

We have successfully maintained oysters in the laboratory for many months in flow-through seawater tables. Attention should be paid to tank cleanliness, especially with regard to the removal of dead oysters. Dead organisms are not always easily detectable until considerable water fouling has already taken place. For oyster conditioning, we have adopted the 1989 ASTM protocol. Oysters to be conditioned for immediate use are held at 20° C, while those to be conditioned later are held at ambient seawater temperature (10 - 12° C). Conditioned oysters not needed immediately are held at 14 - 15° C. Initially, we had followed an earlier ASTM procedure of continuing exposure to elevated water temperature after conditioning but believe now that this prolonged warm temperature exposure reduced the useful spawning period.

In some cases, oysters received from commercial sources spawned with minimal conditioning time (2 days) in the laboratory, but more often required 2 weeks or more. In one instance, we had the problem of mass spawning of one group of oysters after only 2 days of holding in the laboratory. All of our conditioning and long-term

oyster holding tanks are provided as much *Isochrysis galbana* (a microalgae) as we can culture. Under these conditions, conditioning and long-term holding mortality of broodstock has been low (< 5 percent per month).

SPAWNING PROCEDURES

Our procedures for spawning were based on those of ASTM, 1980. Seawater in two 40-liter aquaria was heated from ambient to 25° C, at which point 25 - 30 oysters were added to each aquarium. Once the oysters were open and actively pumping, fresh or frozen sperm suspension was added, either in the water column or by pipeting directly into the incurrent side of each oyster. Oysters spawning were removed to individual beakers to keep the gametes separate until controlled fertilization occurred. If spawning did not occur within 2 hours, the oysters were returned to the holding tanks for 15 - 30 minutes and the whole process repeated with fresh seawater and sperm solution. If spawning still did not occur, other measures, such as removing the oysters from the water for 15 - 30 minutes and the addition of dense suspensions of *Isochrysis galbana*, occasionally instigated a spawning response.

TOXICITY TESTS

Table 1 contains a summary of the control performance data for oyster toxicity tests we have completed. We used the ASTM 1989 protocol for bivalve testing performance criteria of 70 percent normal development of the stocked embryos. Our control performance has ranged from 33 to 96 percent normal development, with only one test meeting the ASTM criteria. Possible explanations for low control performance include inadequate conditioning resulting in immature or overmature gametes, damage to gametes from the thermal stimulation needed to induce spawning, or the inability of northern oyster stocks to reach full prodissoconch ("D" hinge stage) development within the 48-hour test period. Control performance for the Southern Puget Sound oyster stocks generally exceeded that of the more northerly stocks.

Failure to meet ASTM criteria limits our ability to precisely determine the relative sensitivity of the oyster test. Initial work with pulp and paper mill effluents suggests it has potential for being a relatively sensitive test.

Spawning and initiating a test requires about 1 day's work for one person. Final sampling and reading of results requires another 2 days. Under ideal conditions, two people could conduct at least three tests per week. Due to the unpredictable nature of spawning, it would be difficult at this time to accomplish more than one test per week. Tests could be conducted from about June 15 to October 30, a period of about 18 weeks. Current conditions would lead us to expect a high rate of test failure based on the ASTM control performance criteria. In addition, some tests cannot be initiated on schedule due to difficulty in inducement of spawning. The limiting factors to this bioassay are: (1) limited knowledge about specific requirements for optimally conditioning broodstock; (2) poorly defined techniques for inducing spawning with predictable results; (3) extreme variability and general great difficulty in achieving ASTM established control performance criteria; and (4) the limited spawning season.

TABLE 1. SUMMARY OF CONTROL PERFORMANCE FOR OYSTER BIOASSAYS

Date	Test Type	% Normal*	% Survival
10/5/88	Pooled spawn	59.5	64.8
7/13/89	Single pair spawn	95.9	97.7
8/2/89	Single pair spawn	67.4	76.0
8/23/89	Single pair spawn	64.7	72.0
9/14/89	Single pair spawn	51.7	85.0
9/27/89	Single pair spawn	32.6	75.9
10/11/89	Single pair spawn	56.5	94.1
10/30/89	Single pair spawn	38.0	62.2

*Percent of stocked embryos reaching D-shaped larval stage.

DISCUSSION

MR. LANGDON: I just wondered about the problems of mortality that you're finding in your controls. Do you know whether it's the sea water or is it the animals themselves -- that the eggs and the sperm themselves are not of good quality?

MR. HALL: We've never tried any water other than what we have, which is just natural sea water, because it works well for other tests.

MR. LANGDON: Does that natural sea water come into the building through piping or do you actually go out and collect it?

MR. HALL: It comes in through Thompson PVC piping. The lab's been operating for 14 years; so, whatever is there should be well aged, at least.

MR. BERGER: I guess this is more of a general comment and it has to do with most of the presentations this afternoon. I think one of the things I've learned is that there is a real need for the standardization of the endpoint interpretation when we're looking at these developmental tests. And, simply having textbook cases at both ends of the continuum, what's good and what's bad, is not going to suffice when these are going to be used as biomonitoring tools.

MR. HALL: I had a comment regarding folks who have the ability to go out and collect bivalves and bring them back in and spawn them immediately. Our inability to do that, I think, is probably due to our northern location, and the fact that, in many years, like for oysters, there may not be any natural spawning at all and, in other years, it may be very marginal. So I would assume that, farther north where we are, that conditioning is probably something we have to live with and have to do a good job at because we can't take them from the environment and expect them to be in condition to spawn; we have to take them from the environment and build them up to spawning condition.

MS. ANDERSON: A lot of people in our program, including our lab at LBL, just get mussels via Federal Express shipments from animal suppliers all around. So I really think the conditioning issue is not a variant to implementation. And one of the requirements in the program in the Bay Area was actually -- it sounds absurd, but we had to come to a point of defining reasonable effort in

people's level of effort in spawning. And what we've determined is that, when the spawning season is reasonably underway, even then we ask people to order their animals from two sources and to spawn at least 100 animals from any source for *Mytilus* and then, maybe, 50 or 60 oysters from each source, at least. I think, if people cover their bases, the animal costs and the shipping are not the biggest costs in this type of work. We've had a high success rate except for this fall, during what should have been the peak of the spawning season, and mussels from all sources were not spawning: from Southern California; from northern California; straight out of Tomales Bay; and all of that. But normally, and in most years, people can work with shipped samples; they don't have to deal with conditioning and they can get good tests.

MR. HUNT: What other organisms have you (Mr. Hall) tested?

MR. HALL: We've worked extensively with a sperm-egg test; the sand dollar is our favorite, and *Mysidopsis bahia*, the East Coast mysid, and sheepshead minnows. We've got a relatively small lab. What we're trying to do is cycle through all the existing EPA East Coast methods and also try to keep up with what's going on the Pacific Coast. Since we're trying to do both coasts at the same time, we can only handle about two of each at a time, at the most.

MR. CHERR: Do you often see, with mussels or with oysters, skewed sexual shifts?

MR. HALL: I can't say that we have.

MR. CHERR: We've seen that periodically, usually 20 - 24 mussels are collected randomly off the rocks and, for example, recently we had about eight females spawn and no males. Occasionally, we've seen this and I don't know what it correlates to.

MS. ANDERSON: Well, I've never seen it, but I think there is some patchiness with *Mytilus*, although they should be one to one. But you have to order small oysters and big oysters or you don't get both sexes.

MR. CHERR: I guess one thing we did find with *Mytilus*, was that if we took out testes and collected sperm that looked very mature but were immotile, then washed the sperm three or four times in sea water and used them in experiments, we obtained good development rates, in the 90-percent range.

MR. HALL: I think it's the luck of the draw because sometimes you can get 20 males in a row and no females.

MR. CHAPMAN: Gary (Cherr), you are talking about spawning individuals when the percentage spawning is maybe no more than 50 percent?

MR. CHERR: Right.

MR. CHAPMAN: You're not talking about opening them up and determining what the maturation is?

MR. CHERR: No, just in terms of spawnability.

CULTURE AND TOXICITY TESTING
OF WEST COAST MARINE ORGANISMS

Day 2 -- FISH

5. FISH SUMMARY

Development of a satisfactory short-duration test for chronic toxicity estimation for a West Coast fish has been pursued by a number of individuals. Several species have been found to be reasonably sensitive, but a portion of these evidence significant culturing problems leading to high mortality of embryonic and larval fish. All of the species have too narrow a window of availability of spawning adults to provide adequate test material for more than a few months of the year. Taken as a group, the species might provide reasonable year-round availability, but obtaining some of the species might be difficult; shipping of fish or gametes is often untried or unsatisfactory, and interlaboratory testing is very limited.

For the present, we encourage the pursuit of test method development for any of the species discussed at the workshop, but for current or impending programs of effluent monitoring, the only current test species that has sufficient availability is the silverside minnow, *Menidia beryllina*. Although native to the East Coast, this species has been introduced into the San Francisco Bay area and is currently being used as a test species in California, using test procedures developed on the East Coast.

A related West Coast species (also in the Atherinid family) is the topsmelt (*Atherinops affinis*). This species has been used in at least three laboratories on the West Coast and, although considerably larger than *M. beryllina*, appears to hold up well in laboratory culture. Preliminary indications are that the Atherinids are somewhat less sensitive than other species of West Coast fish. Indeed, acute survival of embryonic or larval stages of some other species may be more sensitive than the standard *M. beryllina* short-duration chronic estimation test.

Toxicity Testing of *Atherinops affinis*, (Ayeres 1860)

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The topsmelt is a member of the family Atherinidae, with a range from the Gulf of California, to Vancouver Island (Miller and Lea, 1972). Schultz (1933) reported five subspecies of *Atherinops affinis*: *A.a. oregonia* (from Oregon to Humboldt Bay, California), *A.a affinis* (San Francisco to Monterey, California), *A. a. littoralis* (Monterey to San Diego Bay, California), *A. a. guadalupe* (Cedros Islands, central Baja California), and *A. a. magdalenae* (Magdelena Bay, southern Baja California). Fronk (1969) also reported five subspecies of *A. a. affinis*, all similar to those reported by Schultz, with the exception of *A. a. guadalupe*. Fronk recognized *A. a. insularum* (from the Channel Islands off southern California) as the fifth subspecies. Hart (1973) reported *A. a. affinis* from southern British Columbia to Monterey, California, and *A. a. litorales* south of Monterey.

Schultz (1933) reported that adult topsmelt school at the mouth of Coos Bay, Oregon, during March - April and spawn in the sloughs over mudflats from late May to early July. Eggs are attached by chorionic filaments to eelgrass. Miller and Lea (1972) reported topsmelt as common inhabitants of bays, sloughs, and kelp beds. Individuals can attain a length of up to 14.4 inches (36.6 cm) (Hart, 1973; Miller and Lea, 1972).

Topsmelt spawn single-demersal, clear spherical eggs with six chorionic filaments (White et al., 1984). The eggs are approximately 1.62 mm diameter (White et al., 1984) with a number of variably sized oil globules. Twenty-four-hour old larvae have a mean total length of 6.20 mm with a range of 5.63 - 6.63 mm (present study). The yolk sac is elongate and the larvae are pigmented as follows: (1) two melanophores above the eyes (not present in all individuals); (2) three melanophores behind the two

* Presented by Gary Chapman.

above the eyes; (3) melanophores concentrated on the dorsal surface of the yolk sac; and (4) dispersed melanophores on the ventral surface of the yolk sac (Middaugh et al., unpublished manuscript).

Rearing experiments and a toxicity test were begun in July 1988. Eggs were collected from spawn released in a 250 gallon indoor laboratory holding tank. The adults had been collected prior to spawning from Coos Bay, Oregon. The eggs were removed by hand from catchment material (twisted stands of gill net) suspended into the water of the holding tank. Developing embryos were incubated in a flow-through system consisting of two white hard-plastic containers (diameter = 17.2 cm, depth = 9.6 cm) partially immersed in the holding tank, and connected with tygon tubing to an incoming seawater source. The bottom of each container was perforated to allow for the passage of water. Incubation water temperature was 12 - 14° C, and eggs hatched approximately 24 days post-fertilization. Middaugh and Shenker (1988) reported that topsmelt eggs hatched 14 - 15 days post-fertilization at 21° C, and also have described in detail their method of incubation.

Two rearing experiments involving 16 chambers (including 1 starvation trial) were performed. The following table details the mean percent survival of larvae for all rearing trials:

TABLE 1. MEAN PERCENT SURVIVAL OF LARVAE (16 CHAMBERS)

Days Post-Hatch	Number(n) of Trials	Mean % Survival	SD	Range (%)	n>70%
3-4	14	97.5	4.3	87-100	14
6	14	91.4	8.6	72-100	14
10	14	70.9	20.8	33-100	8
13	14	57.9	21.6	7-87	5
16	14	51.2	24.6	0-87	4
20	10	62.6	14.4	47-87	4
24	10	61.9	15.4	40-87	4

Mean larval survival remained high (i.e., >70.0 percent) through day 10 post-hatch with low variability in percent survival between trials through day 6 post-hatch. The best rate of survival through day 24 post-hatch was 87 percent.

Ten of the sixteen chambers were utilized in examining the effect of prey density and nutritional enhancement of prey on larval survival. Rotifers were provided at a density of either 10 or 20/ml and not enhanced or enhanced with a fatty acid supplement. The mean percent survival for larvae in these trials was:

TABLE 2. MEAN PERCENT SURVIVAL OF LARVAE (10 CHAMBERS)

Treatment	Number (n) Of Trials	Mean % Survival	SD	Range (%)
Not enhanced, 20/ml	2	73.5	19.1	60, 87
Enhanced, 20/ml	2	66.5	19.1	53, 80
Not enhanced, 10/ml	3	53.7	17.2	40, 48, 73
Enhanced, 10/ml	3	59.3	11.8	52, 53, 73

Larvae provided with nonenhanced rotifers at a rate of 20/ml had the best overall mean percent rate of survival. It also appears that rotifer density is a more important variable than enhancement/nonenhancement of prey.

Four trials were performed to examine the effect on larvae of providing *Artemia* as the only prey:

TABLE 3. MEAN PERCENT SURVIVAL OF LARVAE (WITH ARTEMIA ONLY)

Days Post-Hatch	Number(n) Of Trials	Mean % Survival	SD	Range (%)
6	4	96.5	4.0	93 - 100
10	4	45.0	11.5	33 - 60
16	4	20.0	14.4	0 - 33

Larvae fed only *Artemia* did not survive well beyond 6 days post-hatch. It should be noted that Middaugh, et al. (unpublished manuscript) and Middaugh and Shenker (1988) have successfully reared topsmelt larvae using *Artemia* as the only available prey.

One 96-hour copper toxicity test was performed using 16 days post-hatch larvae. The LC50 was 138 μg/l, with 95 percent confidence limits of 116 μg/l and 164 μg/l. Use of 1 day old larvae could possibly reduce the LC50.

Induced ovulation, spermiation, and spawning of topsmelt were also examined. A manipulation experiment was begun on November 4, 1988, at which time water temperature and photoperiod were changed to reflect typical mean late summer/early fall values (12.9° C, 11 hours light/13 hours dark). By day 60 of the manipulation, mean winter values were being provided (9.9° C, 9 hours light/15 hours dark). Temperature and day length were steadily increased until mean summer values were achieved (15.6° C, 15.5 hours light/8.5 hours dark). At day 150 (April 3, 1989), <500 eggs were found attached to catchment material hanging into the water column of the in-laboratory holding tank. Spawning continued through day 188, by which time approximately 7,500 eggs had been released. All embryos examined appeared viable and larvae were hatched that survived through metamorphosis.

The one toxicity test performed demonstrates the relative copper sensitivity of the larvae of this species. Rearing trials show that this species exhibits adequate survival under laboratory conditions. Field work has demonstrated the availability and capturability of topsmelt adults, and has demonstrated that adults survive transport of 100+ miles from estuary to laboratory holding facilities with minimum damage or mortality. Adults have been successfully held under laboratory conditions utilizing minimum effort and space, and have spawned in the laboratory. Further investigation will be required to determine the optimum conditions for continued induced ovulation, spermiation, and spawning. Also, further work will be required to determine the optimum nutritional requirements for developing larvae, and additional toxicity tests need to be performed to determine the toxicant sensitivity of larvae younger that 16 days post-hatch.

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DISCUSSION

MR. ANDERSON: Were the *Artemia* newly hatched?

MR. CHAPMAN: Yes, these were all newly hatched -- less than 24-hours old, anyway. The enhanced ones could have been a little bit older -- towards 24 to 48 hours. They may have gone through one developmental step; but we generally fed *Artemia* that were less than 24-hour old. We didn't measure things like larval mouth opening size and that sort of thing, so I'm not certain the larvae fed on the *Artemia*. But the slower mortality of *Artemia*-fed larvae compared to standard unfed controls leads me to think that maybe the larvae were feeding on *Artemia*. But I know you are able to rear them on *Artemia* alone and not have any problem with it, so I don't think it's a question of what's right and what's wrong. It's certainly possible to use *Artemia*; it's just that it didn't work with this particular fish and temperature. Whether it may have something to do with the mouth size of the northern subpopulation, or nutrition, or the size of our *Artemia*, is just a matter of finding out.

MR. ANDERSON: We've found that the *Artemia* density has to be very high for them to feed.

MR. CHAPMAN: We routinely used 5 *Artemia* per milliliter, often 20 rotifers and 5 *Artemia* per milliliter per day.

MR. KUBO: (Mostly inaudible.)

MR. CHAPMAN: Okay; let me see if I can summarize your comments. Correct me if I'm wrong. What you are saying is that the presence of the rotifers or copepods or things like that, is necessary to sort of stimulate the appetite of the young fish early, when they first begin feeding.

MR. KUBO: The first couple of days, it's okay with brine shrimp. But, after that, they lose interest.

Summary of Toxicity Testing With Topsmelt *Atherinops affinis*

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Work on the atherinid *Atherinops affinis* began in 1988 as part of research funded under the California State Water Resources Control Board's Marine Bioassay Project. The Project is developing sensitive-life-stage toxicity tests that are intended to be short-term indicators of chronic toxicity. Our initial experiments were modeled after the *Menidia* survival and growth protocol, and used topsmelt larvae hatched from laboratory-spawned eggs in 10-day static-renewal assays. Topsmelt larvae adapted well to static bioassay conditions; control survival was excellent and sensitivity to copper appeared to be comparable to *Menidia*. Under the guidance of Doug Middaugh from the EPA's Gulf Breeze Laboratory, our research in 1989 compared the sensitivity of topsmelt larvae to two other early life-stages: embryos and gametes (sperm).

All experiments used laboratory-spawned test animals. Adult topsmelt were seined from a local estuary in March and adapted well to laboratory culture conditions. They were induced to spawn by manipulating water temperature, lighting, and water flow. Temperature increases in the form of 2° C "spikes" over a 12-hour period appeared to be the most important factor for spawning induction. Egg production typically peaked 4 days after temperature spikes, and declined beyond about day eight. Of six temperature spike cycles conducted during the summer of 1989, all were successful in producing enough embryos for toxicity tests.

Preliminary experiments with copper indicate that of the three stages, sperm are potentially more sensitive to toxicants than embryos or larvae in static toxicity tests. Sperm tests used gametes stripped from adult topsmelt. Sperm was stripped from male fish and exposed to toxicant solutions for 15 minutes. Eggs stripped from ripe

females were then introduced to the test solutions, and the sperm and eggs were left to incubate for 48 hours. The mean EC50 using copper was 94 ± 72 μg/l in four preliminary fertilization experiments. Embryo sensitivity was assessed by exposing early-blastula embryos to copper in 12-d static experiments. Two endpoints were compared: hatching success and terata in hatched live larvae. The mean EC50 for hatching success in copper was 147 ± 25 μg/l (n = 3 tests); the mean EC50 for terata in hatched larvae was 155 ± 76 μg/l (n = 3 tests). The relative sensitivity of sperm and early-blastula embryos was compared to that of 10-day old larvae. Larvae were hatched from lab-spawned eggs, fed *Artemia* nauplii for 10 days, then exposed to copper for 96 hours. The mean LC50 for larval tests was 264 ± 58 μg/l (n = 3 tests).

Control fertilization was greater than 70 percent in the sperm tests; control hatching rates in the embryo tests were greater than 85 percent, and control survival in the larval tests was greater than 95 percent. Using these criteria, all of the toxicity tests have been successful to date (see Table 1).

TABLE 1. TOPSMELT TOXICITY TESTS

Month	Stage	Successes/Attempts*
April	Embryo	2/2
May	Sperm/embryo/larva	5/5
June	Embryo/larva	4/4
July	Sperm/larva	5/5

*Successful tests indicated by acceptable control response: survival, hatching success, or fertilization.

Experiments with this species have been limited to spring and early summer months. Mature topsmelt are present locally from late March through August and tests could presumably be conducted during these months. Southern populations may have different reproductive seasons. Assuming availability of test organisms and a 39 test container design for the purpose of this workshop, two people could conduct two 96-hour larval tests per week, or two concurrent, 12-day embryo tests. Two people

could conduct four 48-hour sperm tests per week. One person could conduct one larval or embryo test, or two sperm tests per week. Based on our past experience, we would expect greater than a 95 percent success rate.

Topsmelt are not available from September - February and this is the greatest drawback to working with this species. We have had good success inducing spawning at our laboratory, and techniques used for year-round spawning of *Menidia* and other species can probably be applied to topsmelt. The sperm and embryo tests show the most promise in terms of sensitivity. The fertilization test is potentially more sensitive and involves considerably less time and effort, but needs more background work before it can be used routinely. Future research will emphasize developing toxicity test protocols with these two stages.

DISCUSSION

MR. BAY: You mentioned that the sperm were viable for about 15 or 20 minutes; is that correct?

MR. ANDERSON: About 17.

MR. BAY: Do you foresee that to be a problem in developing a reproducible test in terms of having an adequate exposure time?

MR. ANDERSON: We feel that 15 minutes is an adequate exposure time. The issue here is whether or not the sperm will remain viable long enough to measure their density. We need to be able to measure and adjust sperm densities in order to obtain consistent sperm to egg ratios between tests. Presumably, the variability we've seen in our results is related to inconsistent sperm to egg ratios. Based on your suggestion, we are planning to hold the sperm "dry" until we determine the density. Then we'll dilute the sperm to the appropriate density and introduce them to the test containers. The assumption here is that the 15 - 17 minute viability period starts once the sperm have become activated in the test solutions.

MR. DINNEL: A little bit of confusion. You called the sperm assay a 48-hour test and it's based on 15-minute exposures?

MR. ANDERSON: We expose the sperm to the toxicant for 15 minutes before adding the eggs. We then incubate the fertilized eggs for 48 hours so that we're sure they are fertilized. By 48 hours, the embryos develop optical vesicles and it's obvious that they're developing. I neglected to mention that we've noticed a certain amount of what appear to be eggs developing parthenogenically a short time after fertilization occurs; the eggs appear to be early blastula embryos. If you let the embryos develop, however, embryos that are not truly fertilized do not develop past this early blastula look-alike stage.

MR. DINNEL: Also, do you know what the salinity limitations for those species are?

MR. ANDERSON: They appear to be quite tolerant of a wide range of salinities. Doug Middaugh reports studies that indicate the fish can tolerate a range from 2 - 65 g/kg.

MR. DINNEL: What about the sperm?

MR. ANDERSON: We have not investigated that. That's a good point.

MR. DINNEL: We've done some of that testing with salmon and found definite salinity limitations. The fresh water viability is only a matter of seconds, probably 30 seconds to a minute. And, a saline solution extends the time. So, as you drop the salinity, your sperm viability may go down even though you had embryo development at low salinity.

MR. ANDERSON: That's something we need to investigate, especially if the test is to be applied to estuarine waste dischargers. For marine dischargers, brines can be used to adjust the salinity.

MR. CHERR: When you did the sperm cell test, did you wash the embryos out of the toxicant after fertilization?

MR. ANDERSON: No, we didn't.

MR. CHERR: That's one thing to consider: Is this really -- in addition to the sperm cell test -- also an early embryo test? If you really want to just look at sperm exposure, you might consider, after say 15 minutes of co-incubation, moving the eggs into fresh sea water and then 48 hours later, score them.

MR. ANDERSON: That's a good idea.

MR. CHERR: I'm interested in the sensitivity of the embryos to copper because, when Doug Middaugh (EPA-Gulf Breeze, FL) was at our lab, he used to tell anecdotal stories about the shore birds eating the *Menidia* embryos and the embryos passing through the digestive tract and then hatching afterwards at the other end. We found that when we tried to fix topsmelt embryos, it could take sometimes up to an hour for formalin and glutaraldehyde to penetrate the chorion. It would be interesting to see, when you use other toxicants, if you obtain other responses.

MR. ANDERSON: That's possible. However, Jo Ellen Hose has mentioned that it's possible they may be more sensitive to organic toxicants.

MR. CHERR: That will be interesting.

MR. ANDERSON: Researchers at the EPA's Gulf Breeze laboratory conducted side-by-side larval tests with *Atherinops* and *Menidia* using several different pesticides. They found that *Atherinops* larvae were equally or more sensitive to most pesticides. They conducted one embryo test, and found comparable sensitivity between the two species. We are definitely sacrificing a certain amount of sensitivity using topsmelt, because their eggs are tough. However, this compromise allows us to use a species that is relatively easy to work with, and one that requires a minimal amount of technical expertise. This is important to the people who will be using the test on a routine basis. We're hoping that we can increase the sensitivity of the fertilization test by manipulating some of the procedures I mentioned earlier.

MR. HALL: On your recirculating sea water system, how long has that been maintained and how often do you recharge it with fresh sea water?

MR. ANDERSON: It's not a completely closed system because a small amount of fresh seawater is constantly trickling into the tanks. (To Mr. Hunt) I think it's about a half liter per minute?

MR. HUNT: I think half a liter per minute is about right.

MR. ANDERSON: Doug Middaugh at Gulf Breeze developed the system and has a lot more experience with it. You should consult him if you're curious. I think it would be no problem to hold topsmelt in a closed recirculating system; these fish adapt well to laboratory culture and require very little maintenance.

MR. HALL: And one other question. In terms of measuring DO in 12 ml of solution, how do you go about doing that?

MR. ANDERSON: We sample stock solutions at the beginning of the experiments. At the end of the experiments we combine replicates to give us enough solution to sample; the alternative would be to use some sort of microelectrode which could be dipped into the test tubes.

MR. CHAPMAN: I assume, when you observed this parthenogenesis, that basically then you also carried some eggs along with no sperm to confiim that?

MR. ANDERSON: Right.

MR. CHAPMAN: My second question is sort of directed to Gary Cherr, I suppose. You showed us some pictures yesterday of fluorescence DNA; I was wondering if you could use that as a measure of fertilization in this test and shorten it from 48 hours to something even briefer?

MR. CHERR: I would imagine it could be. In the parthenogenetic eggs, how far do they go? Do they just cleave once or a couple of times?

MR. ANDERSON: They look like early-blastula embryos.

MR. CHERR: In order to assess fertilization, there are a number of available methods if you didn't want to use fluorescence. You could fix the fertilized eggs and there's a number of staining methods, for example, that you could use to look for the fertilizing sperm. So it's a possibility, something to think about.

Summary of Toxicity Testing of Topsmelt (*Atherinops affinis*) and Jacksmelt (*Atherinopsis californiensis*)

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We worked with these atherinid species during the spring/summers of 1987 and 1988 at the Bodega Marine Laboratory. We have had mixed success, but feel that they hold significant promise for utilization as larval fish bioassay organisms. Procedures and data from our work with larval topsmelt in testing complex organic effluents are described in a manuscript currently in press (Shenker and Cherr, Bull. Environ. Contam. Toxicol.).

Our work with topsmelt utilized larvae from egg masses produced both in laboratory culture and from ripe adults collected in the field. While some laboratory spawning was obtained, we did not get consistent production of large numbers of eggs. In a culture maintained throughout the year on an accelerated photoperiod/temperature cycle, we observed a little spawning activity in February (3 - 4 months earlier than wild populations). However, shortly after the spawning activity was noted, disease wiped out the topsmelt culture. Jacksmelt eggs masses were stripped from ripe wild adults, or from egg masses found attached to macrophytes in estuaries.

Incubating the egg masses of both species required some care. The eggs typically required 14 - 18 days to hatch at 18° C, and the eggs were susceptible to fungal infection. This fungus proved completely insensitive to methylene blue and malachite green (indeed, these chemicals stained the fungal tissue nicely, making it easy to observe hyphae penetrating chorions and infiltrating developing embryos). Wild-collected egg masses had the highest degree of infection. The fungal infection was more virulent in the summer of 1988 than in 1987. It was more common than not to find egg masses in the field that were completely riddled with the fungus, without any viable eggs.

*Presented by Gary Cherr.

In addition to the fungal problems, Doug Middaugh found indications that early (< 5 days or so) jacksmelt embryos were sensitive to light, and had to be reared in the dark. Topsmelt embryos did not display a similar sensitivity.

Once the larvae hatched, topsmelt proved to be easy to maintain, and immediately began feeding on newly-hatched *Artemia* nauplii. They could rapidly be acclimated to salinities ranging from nearly fresh water to hypersaline conditions (Middaugh and Shenker, 1987. Calif. Fish Game). In toxicity bioassays, mean control survival at 14 days post-hatch was 88.1 percent. Jacksmelt larvae, on the other hand, underwent an initial period of mortality following hatching. An eyeball estimate of this mortality was 20 - 30 percent.

Test sensitivity data for this species are limited to the use of pulp mill effluent as a toxicant. Experiments were designed to provide estimates of both 96-hour mortality and 7- or 14-day growth rates during continuous exposure to the effluent. The 96-hour LC50 levels ranged from 6 - 10 percent effluent, while growth NOECs during 7- or 14-day tests ranged from 1 - 3 percent effluent. During these longer tests, significant mortality was frequently observed at effluent concentrations lower than those which caused a reduction in growth. As a comparison of larval sensitivity to toxicants, larval English sole had 96-hour LC50s of 1 - 9 percent BKME (these tests were done on different batches of effluent in different seasons). Because of problems with larval jacksmelt survival, we do not have good toxicity data for this species.

We are not doing any current work with these species, but feel that other labs are making significant advances in methodology for culturing and spawning adults. If this can be routinely achieved, the topsmelt is a very valuable potential bioassay species.

If larvae were available, we could set up two full tests/week without difficulty. A lot depends on the experimental design -- if these tests were to run for 14 days each, lots of room in a carefully controlled environment would be necessary to hold the test chambers from different experiments. If we relied on wild-caught and spawned topsmelt, we would be able to run tests from the end of May to the middle of August

(about 12 weeks). Our test failures were due to problems in getting viable eggs and embryos. If we succeeded in setting up a test with topsmelt larvae, the tests were always carried to successful conclusion. It took one person about 3 hours to set up one test, and about 30 minutes per day monitoring mortalities. Of course, additional time was necessary for collecting specimens, culturing embryos/larvae (30 minutes per day), and weighing larvae at the end of an experiment. The primary limiting factors to the use of these species are the need to get adult topsmelt in culture to spawn during an extended season, and the potential fungal/disease problems. Both of these are soluble problems for topsmelt; jacksmelt will require a far greater degree of effort.

DISCUSSION

MR. HUNT: Did you try any embryo exposures? Identifying live and dead embryos would be simple if the live ones float and the dead ones sink to the bottom of the container.

MR. CHERR: We did some additional tests, not with zinc but with a complex effluent we were working with. The embryos were less sensitive than larvae. And, once again, I refer back to the question I had for you on topsmelt since fish chorions are extremely tough. But our results were only based on complex effluent. I think the sole embryos and larvae could be potentially used for microlayer assessment; there are not many organisms available for microlayer bioassays, and these would be appropriate organisms.

MR. DINNEL: You mentioned that one advantage sole has, too, is that the larvae do not feed during the first 96 hours; they depend on the yolk-sac reserves. That may also be somewhat of a disadvantage because I've done some tests with yolk-sac cabazone and squid that hatched and still had a yolk sac and I found that their sensitivity to toxicants was very low. And I think, possibly, that this was simply because they are not reacting to any degree with their environment yet, they are just sitting there using the yolk sac. So the effective exposure to the toxicant during that period of time may be minimal.

MR. CHERR: That could be. One thing -- I didn't show the data -- we had some interesting information in which we pulsed the larvae with effluent for 24 hours and then moved them into clean sea water. It took another 24 to 48 hours before we started seeing mortality, a delayed response. One possibility might be that whatever materials adsorb on the yolk, are becoming bioavailable as the yolk is utilized. But you have a good point. Because they seemed so fragile, we expected them initially to be far more sensitive to zinc. The yolk utilization could be one reason. I should point out that out of 25 experiments, we had control survival between 87 and 100 percent; the average was 95 percent control survival. That included the transferring experiments as well. So I think, once you get used to handling them, it's really quite easy.

MR. BAILEY: What happened in your renewal experiments, was that control survival also for renewal experiments?

MR. CHERR: Yes. That was the same. I think the average was 95 percent. In the renewal experiments was where we had the high 80s to low 90s.

MR. BAILEY: Could you tell, fairly rapidly after the renewal, which larvae had been damaged during the renewal process?

MR. CHERR: Yes, that's why we came up with 2 hours; it takes about 2 hours to see that mortality. And so, in the renewals, we would assess survival 2 hours later.

MR. BAILEY: The same thing with striped bass. I like your idea of transferring larvae. We had more fish, though, and we would end up draining the solutions down with micropore tubing; but some of them would get caught in that air-water interface.

MR. CHERR: If you have a complex experiment set up and there are not that many people that are experienced handling larvae in the lab there can be a problem. You will have somebody locked in a cold room for the whole day doing the transfers. We looked at static versus renewal and we didn't see much difference, at least in the effluent we were working with. There was virtually no difference in LC50; but in other toxicants it may be important.

MR. HUNT: Did you see any or quantify any of the jaw abnormalities?

MR. CHERR: You know, that's something I'll ask John (Shenker) about. He did make some measurements on that, but only in controls to investigate variability; we never really followed up on that.

Toxicity Testing of *Atractoscion nobilis* (Ayres 1860)

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The white seabass is a member of the family Scianidae and occurs from Magdalena Bay, Baja, California, to Juneau, Alaska, with an isolated population occurring in the Gulf of California (Miller and Lea, 1972). Hart (1973) reports that this species is uncommon north of San Francisco, California.

White seabass occur from the surface to a depth of 400 feet. They spawn near kelp beds off the California coast from March to August (Hart, 1973). The eggs and larvae are pelagic. Individuals mature at 29.5 inches (75 cm) in length and may attain lengths of 4 - 5 feet (122 - 153 cm) and weigh 80 - 90 pounds (36 - 41 kg) (Hart, 1973; Miller and Lea, 1972). Adults feed mainly on fish (e.g., anchovies, pilchard, herring, and smelts) as well as crayfish and squid (Hart, 1973).

The following information is summarized from Moser et al. (1983). White seabass spawn single-pelagic spherical eggs with a diameter of 1.24 - 1.32 mm. The eggs are clear in color with melanophores on the embryonic axis (1-day post fertilization) and on the head and trunk of the embryo (pre-hatch). Each egg has an oil globule 0.30 - 0.36 mm in diameter. The larvae are undifferentiated at hatch, with a typical length of 2.8 mm TL. The yolk sac is oval (approximately 0.9 x 1.5 mm) with one oil globule. There is a melanistic sheath covering the head and trunk.

Work on the rearing and toxicity test potential of white seabass embryos and larvae began in 1987. Developing embryos were provided by the Hubbs Marine Research Center, Sea World Research Institute, San Diego, California. Two asynchronous breeding populations provided embryos throughout much of the year. Both static and recirculating incubation systems were used, with increased hatching success observed in the recirculating system.

*Presented by Gary Chapman.

Five rearing experiments were conducted involving 35 rearing chambers. The following table represents the mean percent survival of larvae for all chambers.

TABLE 1. MEAN PERCENT SURVIVAL OF LARVAE (ALL CHAMBERS)

Days Post-Hatch	Number(n) Of Trials	Mean % Survival	SD	Range (%)	n>70%
3 - 4	31	90.6	17.8	20 - 100	28
5 - 6	35	68.7	25.9	0 - 100	17
9 - 11	33	32.0	20.7	0 - 64	0
13 - 14	28	24.6	14.0	4 - 50	0
15 - 16	22	19.3	13.0	4 - 49	0
20 - 21	6	20.5	16.4	2 - 42	0

Overall, the data reflect acceptable larval survival through day 6 post-hatch, then high and steady mortality through day 21 post-hatch. Variability in survival between chambers per experiment was high. The best survival rates occurred in six chambers with a mean survival of 60.5 percent (SD = 2.8; range = 56.0 - 64.0 percent), with one of these chambers having the best overall survival through day 21 post-hatch of 42 percent. Rearing parameters for these chambers included: (1) 3.78 liter clear glass jar rearing chamber; (2) rearing medium of bay or oceanwater; (3) medium volume of 2,500 - 3,000 ml; (4) static-renewal system at 50 - 60 percent medium replacement every 48 - 72 hours; (5) larval stocking density of 8.3 - 10.0/liter; (6) rotifers provided as initial prey at 10/ml variably beginning on day 1, 2, or 3 of the experiment; and (7) *Artemia* (if provided) as secondary prey at 5/ml beginning day 8.

Eight toxicity tests (seven copper and one zinc) were performed, lasting from 48 - 288 hours. The copper tests were conducted with larvae (hatch, 3 days post-hatch, and 7 days post-hatch) and embryos (approximately 24 hours pre-hatch). The zinc test utilized embryos (also approximately 24 hours pre-hatch). A LC50, using the Trimmed Spearman-Karber Method (Hamilton et al., 1977), could be calculated for three of the eight tests as in the following table.

TABLE 2. LC50 FOR COPPER AND ZINC TOXICITY TESTS

Toxicant	Test #	Cohort	Duration	Life Stage	LC50 (μg/l)	95% Confidence Limits (μg/l)
Copper	3	2	96 hours	Larvae	20.8	12.4/ 34.9
Copper	7	4	48 hours	Embryo	59.5	47.9/ 73.9
Zinc	8	4	48 hours	Embryo	209.5	91.6/479.2

Median lethal concentrations were not calculated for five of the tests (tests 1, 2, 4, 5, and 6) due to high mortality in the controls and all test concentrations. Tests 1 and 2 utilized embryos and larvae from cohort 1, and tests 4, 5, and 6 used larvae from cohort 3.

The white seabass demonstrates promise as a toxicity test species. At present, embryos are available throughout the year from Hubbs Marine Research Center. In preliminary tests, embryos and larvae show relative sensitivity to copper and zinc. Problems associated with the use of this species include: (1) the cost, time and space required to set-up and manage brood stock for large adults; (2) potential reduction of embryo viability/cohort due to shipment-induced stress; and (3) high variability of larval survival among and between cohorts. Further work will be required to better determine shipping and rearing requirements, (e.g., appropriate shipping temperature and oxygen levels) as well as optimum nutritional requirements for developing larvae.

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DISCUSSION

MR. BAILEY: Were those 10 rotifers per larvae?

MR. CHAPMAN: Ten per ml. All those are densities on a per-ml basis.

MR. BAILEY: How often did you feed them?

MR. CHAPMAN: Once daily.

MR. BAILEY: That sounds similar to striped bass larvae -- maybe 30 percent to 15 percent survival. It turns out, for a lot of those fish, the digestive enzymes don't undergo immediate development. If you look at the fish larvae under a microscope, they are taking the rotifers and excreting them within 20 minutes; and they're actually excreting intact organisms so they're getting very little nutrition per rotifer. It's not until day 9 - 11, that you begin to see an amorphous fecal string being excreted, so that they are actually digesting the animal. We basically went to at least 30 per ml 3 times a day, getting them over this critical period, and we get better than 95 percent survival consistently. But there is that very, very critical period, and, either way you do it, they may start dying. There's no way to stop it. They are not getting anything.

MR. CHAPMAN: We think that relates back to the fact that either the same thing is happening in the real world or they're feeding on something less obvious like fungi or protozoa on the surface of the larger particles. Do you think, in the real world, they are actually utilizing the real little stuff that's on the surface of those food particles that they're eating or are they just getting three antennae from one organism that they ingest?

MR. BAILEY: That's what they're eating -- you know, basically, the bands in this thing, the rotifers -- I don't know the technical term -- but that's what's missing. Maybe the rotifer body is intact, the algae that are within the body are intact, and they're not getting anything if you feed them the omega-3s or anything; they're not getting any of that, it's all --

MR. CHAPMAN: Just eating cilia?

MR. BAILEY: Yes, that's exactly it; and there's not much to it. And so they need to increase the frequency of feeding to keep them in contact all the time. And some of the enzymes, I think, in the striped bass, don't come into full strength, like, for 30 days after a hatch.

MR. CHAPMAN: That's interesting. When I hear things like that, I always wonder if it really works that way in nature, too, or if it's an artifact of what we're doing in the lab.

MR. BAILEY: I think in the trawling of the striped bass, anyway, they find a lot of the larvae in the really dense clumps of zooplankton. They're doing real well using glycogen reserves; they are maintaining. If they can maintain past that period, they're doing all right.

MR. ANDERSON: I have a question for Howard Bailey. Could that high of an *Artemia* density be a problem in terms of water quality or adsorption of chemicals?

MR. BAILEY: We use rotifers and it works out; we don't have a problem. The problem was in scaling it so you could actually do a test. We're running 19 5-gallon carboys of algae which we inoculated with rotifers, literally, every 4 days. We try to have 2 weeks of algae in the holding just in case something crashes. It's not bad because these rotifers are fresh water organisms; so, in freshwater tests the excess stays alive in your test container and you can pull a sample and count it and say how much additional you need to inoculate if you're getting really low. You know, if you're getting, say, below 10/ml or something like that, then you may want to reinoculate. So it's kind of nice because it's unlike *Artemia* in the fathead minnow system in which they all go to the bottom and die and you have to go through and pick them up. The rotifers are sitting there all the time, so they're feeding on them all day long.Once again, on the larvae, how large an exposure container are you using?

MR. CHAPMAN: For all toxicity tests they used 1-gallon jars with 2.5 - 3 l of water. Rearing occurred in the same jars plus a few trials in 12 - 20° C water in small Fiberglass tanks.

MR. BAILEY: What we went to, just to avoid having to raise enough rotifers, was a 5-gallon container inside of which were 500-ml Tripor beakers

with a screen in the side with the larvae in the Tripor; we could put several replicates in there. Then you only had to have enough rotifers for the small volume to which the larvae are confined. Hold them in those for the first 4 or 5 days and by just raising and lowering it once a day, you get water exchanges through the screen. We could put 30 larvae in one of those Tripors without any problem.

MR. ANDERSON: Is anybody pursuing this species?

MR. CHAPMAN: Not at this time.

MR. ANDERSON: It seems like it has a lot of promise, in terms of its sensitivity, particularly if you can increase control survival.

MR. CHAPMAN: My main concern is if we work for years to develop a method and then Hubbs no longer maintains their adult spawning stock -- then you've got problems.

MR. ANDERSON: Does that look like a possibility?

MR. CHAPMAN: I don't know; you'd have to talk to the people at Hubbs. I talked to Don Kent about 4 years ago to arrange this research project. I think it's a feasible test species, it's just a question at this point of how sensitive the species is compared to other species. And we're going to hear a talk about sole and hope to hear Jo Ellen's talk a little later. And there are some other candidates, I think, that are as good that, perhaps, don't have the potential drawback of somebody pulling the plug on Hubbs' culturing procedure. If Hubbs' culturing procedure were for 4-foot diameter fiberglass tanks, I wouldn't worry about it; but their tanks are big. I don't know how big, I've never measured them, but those are really big fish and they're really big tanks.

MR. HUNT: The larvae seem to transport very well. It might be possible to supply a large number of labs from one culture facility, as Hubbs is doing now. That would eliminate in-house culture requirements.

MR. CHAPMAN: Yes, if there's enough money in it, you know somebody will do it. I don't think there's any question about that. The one reason we're giving up on it, frankly, is that there are a number of other species that we're trying to work on, with a very limited budget, of ourselves, with roughly, say, $100,000 a year of research money. And right now we're putting most of that into the West Coast mysids -- we just don't have any money to put into the fish work. And I decided there was a lot of interest in a number of fish species down here in

California and that, perhaps, I should just let the people down here continue doing the fine work that they're doing and they'll come up with something. I encourage anybody down here to talk to Hubbs, if you want to work with this species.

Summary of Toxicity testing for
English Sole (*Parophrys vetulus*)

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The Bodega Marine Laboratory has worked with English sole since eaily 1987. We determined that adult sole were easy to collect, to maintain in laboratory culture, and to induce to spawn. Newly hatched larvae were highly amenable for use in 96-hour toxicity bioassays. A manuscript describing some of our work is currently in press (Shenker and Cherr, Bull. Environ. Contam. Toxicol.)

All of our attempts at inducing the spawning of fish collected during December and January were successful. The key to this success was collecting fish that were entering their reproductive state, and using hormonal treatments to further stimulate gonadal development. Ripening females in the field were easily recognized by their swollen ovaries, while males typically extruded milt when their abdomens were gently squeezed. Once spawning began, females were capable of producing viable eggs daily for periods of time ranging from 7 - 19 days.

Embryos obtained from eggs/sperm squeezed from ripe fish were easy to incubate in a slowly flowing water bath. Daily decanting of viable floating embryos eliminated those embryos that had died and sunk. Although we have not quantified the overall proportion of viable eggs, we estimated that about 50 percent of the eggs survived to hatching.

Hatching occurred in 84 - 96 hours at 12° C. Larvae were easy to maintain during their early post-hatch stage. The 2 - 3 mm-long primitive yolk-sac larvae were essentially quiescent, and did not require feeding for 4 - 5 days. We conducted 96 hour toxicity experiments on these yolk-sac larvae, and thus did not have to include

*Presented by Gary Cherr.

feeding in the daily procedures. An attractive feature of this test was that larvae do very well in small volumes (25 larvae in 25 ml), so we could test small volumes of suspected toxicants derived through an effluent fractionation process.

Throughout all of our experiments (we ran about 25), we did not have a single experiment crash, and larval survival in the controls averaged 95 percent (range = 87.3 - 100 percent).

Our only unsuccessful efforts with this species were to induce accelerated reproductive maturation in adults collected in September and October. We did not put much effort into this and cannot determine if it is really feasible to induce early maturation. Field studies on the early life stages of English sole suggest wild fish may occasionally spawn in mid-fall under certain natural environmental conditions. With sufficient effort, it may well be possible to expand the reproductive season of the species under culture conditions.

Our toxicity work with this species focused on a metal (zinc) and a complex organic industrial effluent. The mean LC50 for newly hatched larvae exposed to zinc was 14.5 mg/l (95 percent C.I. = 10.6 - 19.7 mg/l). Larvae of three other fish species tested with zinc exhibited LC50s quite close to the sole LC50 (*Menidia mendia* = 3.6 mg/l; *Pseudopleuronectes americanus* = 9.5 mg/l; *Fundulus heteroclitus* = 70.6 mg/l).

Current Status of Culture:

The only significant mortality we have observed has been in the developing embryos, which exhibited about a 50 percent mortality rate. Transferring the very fragile larvae from the hatching chamber to exposure chambers also typically induced some mortality. We normally transferred 25 larvae per chamber. One or two hours after transfer (and before adding the toxicant), we would examine each chamber and remove dead larvae. These dead larvae generally had adhered to the surface film, and were easy to identify. On average, 1 - 2 larvae would die during transfer.

Adults began feeding 7 - 10 days after capture. They readily consumed diets of squid and fish chopped into small pieces. We successfully maintained larvae through the onset of feeding by providing them with *Isochrysis galbana*, and they survived for about 10 days on this diet. However, we were unable to provide them with a larger prey item (e.g., rotifers) when they outgrew the phytoplankton.

Collection of organisms requires access to a vessel and otter trawl for mid-winter sampling. We were successful at collecting specimens in 50 - 60 m of water off Bodega Bay (a site identified by NMFS as having very clean conditions, and a population of English sole with very low body-burdens of toxicants, compared to urban embayments).

Calendar of availability is the major problem with this species. We were able to work on the larvae of this species only in December, January, and February. The spawning seasons may change from year-to-year, and effort in culturing may also extend the available spawning season.

We have run as many as 100 test chambers per week without undue strain, and this can be expanded by utilizing additional personnel. It is certainly possible to generate far larger numbers of larvae than can potentially be used. The most difficult and tedious step is loading larvae into the test chambers -- larvae are small and fragile, and this step requires patience and practice.

We worked on these larvae for a maximum of 8 weeks per year. We increased the relative period of time for toxicity testing by lyophilizing effluent samples during other times of the year, storing them until larvae became available, then reconstituting the lyophilized material (additional testing demonstrated that lyophilization of samples normally did not affect their toxicity).

We did not experience any test failures with this species. One person can set up 50 - 60 test chambers in about 3 hours, and enumerate mortality in those chambers in about 30 minutes. The primary problem with the use of this species is its limited seasonal availability.

Toxicity testing of *Menidia beryllina* (Silverside Minnow)

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We have no in-house culturing facility for *Menidia*. Consequently, we obtain larvae from one of two East Coast suppliers (Cultured Aquatics and Aquatic Indicators). Fish arrive at least 1 day prior to testing to allow for salinity acclimation and sorting. Generally, we see no more than a 5 percent die-off resulting from shipment. Fish are between the ages of 5 and 8 days old when they arrive and are already feeding on *Artemia* nauplii.

We have run three toxicity tests using copper sulfate as the reference toxicant. NOECs and precision for the reference toxicant data are presently being calculated and will be presented at a later date. In comparison to other species we used in the ambient bioassays, *Menidia* seldom showed a significant response. Of the seven ambient surveys in which at least one species tested showed a significant toxic response, the *Menidia* indicated significant toxicity only twice. Data from the San Francisco Bay Effluent Toxicity Characterization Program, however, indicate that the test is no less sensitive than other subchronic assays.

We normally design a *Menidia* bioassay to include six ambient, five reference toxicant, and two control treatments. To conduct one such test per week requires one person spending half of each day taking water chemistry measurements and preparing new samples and the other half doing the water change and record keeping. Since this test is not limited by organism availability, one could run as many tests per week as laboratory space and personnel allowed.

The year-round availability of *Menidia* and the excellent control survival and growth observed make it a very reliable test organism. In all eight of the our ambient surveys, we've had average control survival greater than 85 percent and average

control weights greater than 0.5 mg per larvae. Likewise, in bioassays run in the Effluent Program, approximately 90 percent of all testing met required control survival and weight. Generally, we see no drawbacks to using this species.

Note: To supplement our limited experience with these animals, we include information from laboratories participating in the Effluent Program.

DISCUSSION

MR. ANDERSON: I have a question. When using brine, did you adjust the pH of the brine before you used it? We have found that the pH of newly made brines tends to be high.

MS. HOFFMAN: We monitor the pH after adding the artificial sea salts (rather than using a brine).

MR. ANDERSON: It was added direct then?

MS. HOFFMAN: We use 40 Fathoms brand crystals. So we add that and have a pretty low stir rate and let it go for a little while, maybe half an hour, and then measure the pH. We had also measured the pH initially. We'd make sure that the change wasn't more than maybe 0.2 or 0.3 of the pH unit. As long as that change hadn't occurred, we'd go on. And we've never seen a case where we'd have to adjust the samples back down. But, for ammonia testing, when you are pretty sure that ammonia toxicity was occurring, that kind of difference might be substantial.

MR. ANDERSON: Do you find in testing with *Menidia* that the growth and survival endpoints generally have similar sensitivities?

MS. HOFFMAN: In the ambient program we've found that they're comparable. And we've certainly seen that in the effluent toxicity data. I think there were a couple of times when we had a more clearly significant trend looking at the survival data, such as at Hayward. Along a gradient closest to the effluent outfall, there was a definite statistical significance using the growth endpoint. But, further downstream, where we'd still see significance in survival, we might not see a similar significance in growth, (it was patchy).

MR. DINNEL: Have you got any reports out showing the information on the relative species sensitivity?

MS. HOFFMAN: Do you mean from the effluent characterization program?

MR. DINNEL: Right.

MS. HOFFMAN: No. The species sensitivity data hasn't been written up. Unfortunately, that's more of Susan Anderson's area of experience. My understanding is that this is all going to be written up at some point; but I don't know if they're waiting until the end of the program to do that. I don't think it's in a report yet.

MR. BAILEY: How did you conclude that 8 g/kg was the lower level for testing?

MS. HOFFMAN: Well, actually, I was told that. I heard that the range for *Menidia* is 6 to 33 g/kg. We'll adjust to 8 g/kg to account for any error in our measurement of the salinity because we use a refractometer and that's got an error range on it of about plus or minus 2 g/kg. We feel that it is safe to adjust it to 8 g/kg. But I didn't conclude a lower limit of 8 g/kg from any testing that I did; that was what I was told.

MR. BAILEY: I asked that question of EPA people and they said the six had come about because that was as low as they'd tested. I was just curious because we run them as low as zero in fresh water with no problems.

MS. HOFFMAN: I've never actually tried that low.

MR. BAILEY: The only difference would be that our fish were obtained from Clear Lake stocks.

MS. HOFFMAN: As opposed to?

MR. BAILEY: As opposed to -- I don't know where.

MS. HOFFMAN: Do you know at what salinity they culture at Clear Lake?

MR. BAILEY: We culture in fresh water.

MS. HOFFMAN: Oh, so you cultured them?

MR. BAILEY: Right. But they would go the other way because the final was full-strength sea water. Because it would give you that range, you could use the same species.

MR. HALL: In general, are you following the EPA East Coast method for this test?

MS. HOFFMAN: There's only one EPA method that I know of that we're following, and I'm not entirely clear that that's the East Coast method.

MR. HALL: One of the things we wrestled with, in terms of the fathead minnow bioassay and these other fish bioassays, is the issue of starting this test with fish within 24 hours of hatching. How do you think that beginning the test with 7-day old fish might affect the sensitivity?

MS. HOFFMAN: Well, the *Menidia* test is a test that you're supposed to start with fish between 7 and 9 days old; and that's the protocol. I wasn't involved in the development of the protocol. However, my understanding is that the fathead test is started with less than 24-hour old fish.

MR. HALL: Right.

MS. HOFFMAN: So that's a substantial difference between the tests. And, *a priori*, I would imagine that could conceivably be a more sensitive test given that you're starting with younger fish. But that's a guess.

MR. HALL: We do it, using a fathead minnow, within 24 hours and I thought that EPA protocol for silversides was within 24 hours.

MS. HOFFMAN: Maybe Gary Chapman could speculate on the difference. I don't know why the *Menidia* is run on 7-day old fish as opposed to a 24-hour old fish.

MR. BAILEY: It's supposedly more sensitive at this stage.

MR. CHAPMAN: I think that probably -- and I emphasize probably -- the reason is they wanted to use growth as an endpoint. And, if they used 7-day olds, then they were actually using actively feeding organisms rather than utilizing the yolk stage as a portion of the 7-day test.

MS. HOFFMAN: Although, you know, with the fathead minnow, I'm told that they're not actually actively feeding for the first 2 days of the test; and you still use a growth endpoint on that.

MR. CHAPMAN: That may be. I'm guessing. By the way, on the topic of *Menidia* and salinity -- and, again, I'll have to check on this -- I think I agree with Howard Bailey that they'll survive low salinity. And, to my recollection, when we did the San Francisco Bay study several years ago, we ran a test -- or, that is, Steve Schimmel and the people from Narragansett ran a test -- to look at the

effect of salinity on the toxicity of one of the effluents. I believe they went down to 5 g/kg with no problem at all, and the test organisms had been reared for a high salinity test. And they didn't have any problem.

MS. HOFFMAN: I think that I'm being conservative using a change of 5 g/kg per day. We do it just to maintain a consistency between the tests to the extent that we can. But it sounds like you really can change them by a lot more.

MR. HUNT: I've got a question I meant to ask Susan Anderson yesterday. We're in the middle of some interlaboratory studies and I am amazed at the low coefficients of variation between all the different laboratories testing at San Francisco Bay.

MS. HOFFMAN: Susan believes it's the best data in the country which, in part, is because it's the only data in the country.

MR. HUNT: Can you give us some advice on test precision between laboratories? I'm impressed that 20 dischargers using 11 species can get such similar results. Are they using different dilution waters?

MS. HOFFMAN: Well, remember that the CV data that I presented aren't for 20 discharges, rather they are for 6 or 7 discharges. On one of the columns, it was the number of labs and I think on that slide there were seven.

MR. HUNT: And those were the ones that used the *Menidia*?

MS. HOFFMAN: Right. It's confusing. I mean, I get confused in trying to understand the different parts of the effluent characterization program. But the species sensitivity phase is different from the QA round, which is the data for which you saw the IC50 CVs. That was from 7 laboratories, not from 20 laboratories.

MR. HUNT: Right. Are there some steps you've taken to try and standardize the laboratories?

MS. HOFFMAN: I think Susan Anderson would really be the best person to answer that. But I've had some experience with contracting laboratories; some of whom are represented here and might want to comment. I don't know what kind of input Susan has given the labs in terms of trying to coordinate them. But I think that one point that she might make, if she were here, is that the nature of these tests, allows for some flexibility. So different labs will employ somewhat different techniques which you would expect, being in different

places and being different people running them. But you still have a consistency with the tests. I don't think there was any particularly special coordination that was happening such that identical waters were being used or anything like that. Do any of the labs have any comments on that?

MS. HEJTMANEK: I believe we all used different dilution waters and we all pretty much followed the standard protocols as much as we could during the QA run.

MS. GARCIA: Last fall, Susan did come up with several questions that addressed the testing procedures for all the types of species used. This questionnaire was designed to bring all the laboratories together to get their opinions on the tests that are going on. And, as Erika Hofman was saying with *Menidia*, say with the ambient water in some of our sites, it comes in at 23 g/kg. Are you going to make the test at 20 or 25? So there's those kinds of questions. And there's dilution water and there's also ambient water being tested at all the sites.

MR. HUNT: What do you use for dilution water in the reference toxicant tests?

MS. GARCIA: That was for the reference toxicant test.

MR. HUNT: Was there one standard dilution water?

MS. HOFFMAN: I don't think they were all using the Bodega Bay control.

MS. GARCIA: We use the Bodega Bay sea water control and we also use, for different testing, Pyramid Spring Water or Arrowhead Spring Water; but for sea water control, we use Bodega Bay water.

MS. HOFFMAN: The thing that may help out, for those of you who might not know what Ms. Garcia was alluding to, was a questionnaire that Susan Anderson sent out probably 6 months ago -- and that addressed some of the issues that she'd either heard because of phone calls or indirectly from the laboratories; and then the different labs were given a period of time in which they were to respond to the questions. Then she had a meeting of the labs so she could tell them her decisions on some changes that she made on things that were particular to the Bay Area and the protocols. I think that was a really helpful way to coordinate people.

MR. HUNT: That was afterwards, though, wasn't it? Wasn't that after this run that you've shown the data for?

MS. HOFFMAN: Yes, actually, that was probably after the QA round; it was before the effluent variability run.

Toxicity Testing of *Menidia beryllina*

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We have been working with this species in a general sense since 1986 and have been using it for effluent toxicity testing since 1988. We have spawned the organisms year around in brood tanks (freshwater) and cultured the young in both fresh and saltwater. Currently, we use commercial vendors to supply larvae of an appropriate age for our tests to avoid the space and labor requirements associated with maintaining the broodstock cultures. The adults do very well on a diet of frozen adult brine shrimp, fresh tubificid worms, and formulated flake diet. The young require rotifers (*Brachionus* sp.) for several days until their mouths are large enough to accommodate *Artemia* nauplii.

Over the past year, we have conducted approximately 35 bioassays with this species and have had no "failures." For example, we have been evaluating effluent variability for several clients over the past 8 months. During this period, control survival has ranged between 83 and 100 percent (mean = 97 percent; SD = 5.4 percent; n = 22), with the biggest problem having been the larvae jumping out of the water and getting stuck to the sides of the beakers where they dry out. Average dry weight for the controls has been between 0.54 and 2.25 mg per fish with an average of 1.05 mg per fish (SD = 0.38; n = 22). LC50 values for the reference toxicant (anhydrous copper sulfate) ranged between 62.5 and 250 μg/l with a mean and standard deviation of 187 and 74 μg/l, respectively (n = 11).

With our current staff and lab configuration, one person can set-up and maintain three of these tests on a daily basis. Typically, this means two effluents and one reference toxicant, with the tests being initiated simultaneously. Thus, two people could maintain a total of six tests, at which point we would be limited by space in the environmental room.

The only comment I have regarding disadvantages is that the test is not particularly sensitive, at least to the effluents we have tested. In addition, given the variability between replicates and the fact that only three replicates are used, quite often reductions in growth and survival of 20 to 30 percent are not statistically significant. However, increasing the number of replicates would dramatically increase the amount of work and space required.

DISCUSSION

MR. HUNT: Howard, excuse me. That graph is showing NOECs?

MR. BAILEY: Yes.

MR. HUNT: From different tests on different dates?

MR. BAILEY: These are the dates on the bottom here. So it would be different groups of animals.

MR. HUNT: So it's getting identical NOECs every time?

MR. BAILEY: Exactly.

MR. CHAPMAN: Basically, the top line says there is no NOEC.

MR. BAILEY: There is no effect.

MR. HUNT: That's no effect at your highest concentration.

MR. BAILEY: Right.

MS. HOFFMAN: Do you have data that compares a reference toxicant series -- one that did show effect?

MR. BAILEY: Yes, but not here, it's in the reports.

MS. HOFFMAN: Was there a similar conclusion?

MR. BAILEY: Yes.

MS. HOFFMAN: But doesn't NOEC and LOEC really depend on what your variation is?

MR. BAILEY: Exactly, that is what I'm saying. We only have three replicates and the variation is such that we can't statistically call these effects, but there appears to be a trend there. If you ran a regression, and the regression was significant, and you took some percent weight as being acceptable, whatever it is, the percent weight loss, you would probably be at a much lower number.

MR. CHAPMAN: That's true. A lot, though, also depends on whether this is really general scatter or, if you have just a few very small organisms that are lowering your average weights. For example, if you have one small organism at 4.2 percent concentration, two small at 8.4, three small at 16.8, maybe the average dry weight is not the best thing to use; maybe it's the number of organisms that are above some critical size. I agree with you, and we discussed this yesterday a little bit; NOECs sometimes hide behind statistics.

MR. BAILEY: Sure.

MR. CHAPMAN: A critical issue in making biological decisions is whether, for example, a 10 percent growth reduction is really significant and if we should really be using regression. Normally, I would think, when you get a nice response like that, you would get more statistical significance.

MR. BAILEY: Well, this Dunnett's test is less sensitive than Williams' for something like this.

MR. ANDERSON: Is it that much more work to increase the number of replicates from three to five or six?

MR. BAILEY: It would double the work. You're going to be able to tend to half a test per day. I mean, you're siphoning out every individual beaker; you're cleaning and counting.

MR. CHERR: Would it be useful before one would even consider making that jump that you, go in and weigh each fish within a replicate? I am not talking about doing it in a test, but to determine ahead of time how much variability you have among fish within a replicate chamber because you may have six replicates and you're still going to have a lot of variability.

MR. BAILEY: Sure. And we could although I didn't get a chance to do it, determine how big an experiment would have to be to detect some level of effect.

MR. CHERR: I mean, you're just taking all the fish from one chamber --

MR. BAILEY: Yes, you take all the fish from one tank and you dry them, and weigh them together to get an average. So, basically, for each test concentration there are three replicates and three pooled weight estimates.

MR. CHAPMAN: I was thinking of individual fish weights when I was looking at this data and I was surprised that you didn't get significance -- that's the reason I was thinking that it was some sort of a weird result. No, I still think the bottom line is that, the way we need to analyze this type of test, is with regression. We may have to use data transformations before we can get good curve fits, and then we have to decide what we're going to call an effect level, whether it's 10 percent or 5 percent or 1 percent or 0.1 percent, or whatever.

MR. CHAPMAN: Then we have to realize there are confidence limits around those estimates and maybe, deal with the confidence limits, then fight like heck not to have to use either the upper or lower limits for our best estimates.

MR. BAILEY: Right.

MR. BAILEY: Okay, that pretty much covers any and all points I was going to make.

MR. CHAPMAN: I have one more question. If you were to have plotted your LC50s on your reference toxicants rather than your NOECs, what would the variability have looked like?

MR. BAILEY: With this test, most times it's the weight that's most sensitive, at least in the reference toxicants.

MR. CHAPMAN: But I would think the mortality data would have been tighter.

MR. BAILEY: Yes, it would have been a little bit cleaner. It definitely would have been.

MR. CHAPMAN: Because you normally look at, for reference toxicant response, an LC50, although you look at NOECs, too. But obviously the LC50 is generally a little bit better indicator of the test itself than this particular endpoint.

MR. CHERR: Well, you are saying, or what I've been hearing, is that the LC50 mortality may be equivalent or similar to the growth test.

MR. BAILEY: In this test, it's not as sensitive as the growth test. But the actual dose/response linc might be flatter, is what I'm saying. My sense of the data, going back and looking at it, is that mortality would be less affected by differences within the test.

MR. HUNT: I have some comments on statistics. The State of California is in the process of developing toxicity test acceptability criteria based on the number of replicates and the between-replicate variability. Tests that are to be used in California must specify the number of replicates based on power analysis, and must specify a statistic, such as the ANOVA error mean square, that sets a criterion for between-replicate variability. This guards against the potential loss of test sensitivity from increased variability due to poor quality control. This is especially important where compliance is based on NOECs, which are dependent on the level of variability in the test data. In the case of the *Menidia* test, I was wondering if using only three replicates limited the power of the test. The State is now in the process of adopting seven toxicity tests for use in California, and I will be interested to see how these tests, which have been developed by different people, will be standardized in terms of statistical quality assurance. I mention this because we were discussing the differences between NOECs and LC50s, especially the fact that the NOECs are dependent on the amount of replication and between-replicate variability for their statistical power and test sensitivity. As Gary Chapman mentioned, there may be a change at some point from NOECs to a number based on regression, such as an EC10 or EC1. In the meantime, this idea of specifying power and acceptable levels of variability will be very important in determining the NOEC numbers that are actually used for compliance by dischargers.

MR. ANDERSON: Another thing you can do is increase the number of replicates you use in your reference toxicant tests and use a smaller number of test concentrations to bracket your typical reference test NOEC.

MR. BAILEY: With the replicates, just to give you an idea, if you want to reduce the variance by half, if you have three replicates now, just going to four would not gain much at all. It's not a linear relationship. Basically, Erika Hoffmans's comments are correct; it's a good test. I mean, it's a test that I think most people can run without any problems and it can be run in a wide range of salinities. And our coefficients of variation for the number of tests we run are basically very similar to yours -- actually, low 30s. So I think it's reproducible in that sense. The things I'm talking about are basically refinements.

Toxicity Testing of *Anoplarchus purpurescens* (Gill 1861)

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The high cockscomb is a common winter spawning stichaeid occupying rocky intertidal shorelines of Pacific Coast estuaries from Point Buchon, California, to Attu Island in the Aleutians (Hart, 1973). Typical habitats are rock groins, jetties, and naturally occurring rock strewn outcroppings underlain by a sand-mud substrate, or a substrate composed of pebbles and stones, shells, and shell fragments (Schultz and Delacy, 1932).

High cockscomb females lay demersal egg masses with a mean number of eggs per mass of 1613 (Schultz and Delacy, 1932) to 2,192 (present study). Females guard the egg mass until hatching. Hart (1973) reported an egg incubation period of approximately 3 weeks. Egg diameter ranges from 1.367 - 1.780 mm. The eggs are spherical, white to gray in color, with one to three oil globules (Schultz and Delacy, 1932; present study) and no pigmentation. Larval length at hatch is 6.20 - 8.05 mm TL (present study). The yolk sac is oval with one oil globule. Larval pigmentation occurs: (1) on the dorsal surface of the intestine (proximal end); (2) along the ventral edge of the intestine; and (3) as numerous, regularly spaced ventral tail melanophores.

Rearing experiments and toxicity tests were begun during February 1987. Egg masses were collected by over-turning rocks exposed at low tide (0.5 feet) and found in moist depressions beneath the rocks. The egg masses were not attached to the substrate and were lifted from their "nests" with a small aquarium fish net or by hand. After removal, egg masses were placed in a 5-gallon bucket approximately a quarter full of seawater and transported to a holding container in the laboratory. The egg masses were split into four to six smaller units and placed in either a static or flow-through incubation system. Incubation water temperature was 8 - 11° C, and salinity

*Not presented at workshop.

ranged from 28 - 30 g/kg. Eyed eggs hatched in the laboratory from within 12 - 24 hours after collection to 13 days post-collection. Non-eyed eggs hatched up to 22+ days after collection. The best survival of embryos to hatch resulted from collections of eyed eggs.

Six rearing experiments were performed involving 26 rearing chambers, including 3 starvation trials. The mean percent survival of larvae for all chambers is represented in the following table:

TABLE 1. *A. purpurescens* **LARVAE SURVIVAL**

Days Post-Hatch	Number(n) Of Trials	Mean % Survival	SD	Range (%)	n>70%
3 - 4	21	87.4	28.2	7 - 100	18
7	22	86.3	27.4	0 - 100	20
10 - 11	20	81.0	22.6	20 - 100	16
13 - 14	20	68.6	24.4	7 - 100	10
17 - 18	20	44.5	22.8	0 - 95	2
20 - 21	19	38.3	20.1	13 - 85	2

Larval survival remained relatively high (i.e., >68.0 percent) through day 14 post-hatch with increased larval mortality through day 21 post-hatch. Variability in percent survival between chambers was high for all days-post-hatch intervals. The best overall survival through day 21 post-hatch occurred in four chambers with a mean survival of 69.3 percent (SD = 13.6; range = 56 - 85 percent). Rearing parameters for the chamber with 85 percent survival included: (1) 3.78 1 clear glass jar rearing chamber; (2) seawater medium; (3) medium volume of 2,500 ml; (4) static-renewal with 60 percent medium replacement every 48 - 72 hours; (5) larval stocking density of 8 larvae/l (1 day post-hatch); (6) rotifers provided as initial prey at 10/ml beginning day 1 of the experiment; and (7) *Artemia* provided at 5/ml beginning day 6.

Seven copper and one zinc toxicity tests were performed. Copper tests lasted 120 - 456 hours and utilized embryos to 14 days post-hatch larvae. The zinc test lasted 216 hours and used embryos. The following table represents six of the eight tests for which a LC50, using the Trimmed Spearman-Karber Method (Hamilton et al., 1977), was calculated.

TABLE 2. LC50 FOR COPPER TOXICITY TESTS

Toxicant	Duration	Life Stage	LC50 (μg/l)	95% Confidence Limits (μg/l)
Copper	168 hours	Larvae	136.92	109.23/171.62
Copper	168 hours	Larvae	211.19	143.59/310.62
Copper	456 hours	Embryo	41.53	35.60/48.44
	overall	Embryo-larval	34.55	29.57/40.37
Copper	312 hours	Embryo	41.49	236.10/47.68
	overall	Embryo-larval	35.36	32.54/38.41

The first two tests, utilizing 4-day old larvae, reflect variable outcomes and moderate sensitivity to copper (LC50 = 136.92 - 211.19 μg/l). The four tests using embryos and embryo-larvae represent more consistent and more sensitive results. It was discovered that copper may interfere with embryo hatch such that embryos in affective concentrations, although still alive, do not hatch, and larvae that do hatch in affective concentrations are less viable.

Median lethal concentrations could not be calculated for the zinc test and one copper test due to excessive mortality in the controls and other test concentrations.

Toxicity tests utilizing high cockscomb embryos show this species to be at least moderately sensitive to copper. The egg masses are easily collected, but available for only a limited period of time during the winter. To date, this species has not been successfully bred in captivity, although a single captive female did lay an egg mass in a holding trough in our laboratory. Further work needs to be conducted on the successful captive reproduction of this species. The adults are held captive

with minimum effort and space, and larval survival appears to be adequate (i.e., >68 percent through day 14 post-hatch), yet continued experimentation is required to determine optimum nutritional requirements for enhanced levels of larval survival.

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Kelp bass (Serranidae: *Paralabrax clathratus*)

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Our lab in Redondo Beach worked intensively with kelp bass between 1985 and 1988; lack of funding has stopped all work since then. The first 2 years were spent identifying environmental cues which stimulate spawning and developing methods for artificially spawning the fish outside their normal breeding season. In the second 2 years, we performed bioassays with single contaminants and complex effluents. Coupled with these efforts was a project aimed at identifying possible reproductive impairment in kelp bass from highly contaminated areas of Southern California and correlating reproductive effects with chemical contaminants. The biological data demonstrate that fish from contaminated areas should not be used as brood stock for mariculture or toxicity testing. The preliminary chemical data are intriguing and suggest a threshold for body burdens of organochlorine pesticides; we anticipate the definitive analyses to be completed soon. In Southern California, we suggest that broodstock be collected from Dana Point or the Channel Islands.

Eggs can be obtained in two ways - from natural spawns occurring during the breeding season of May/June through September or from spawns induced artificially following acclimation to photoperiod and temperatures simulating the natural breeding conditions. We have maintained two each with about four females and two males (all > 250 mm in length) continuously for the 4 years. Fish are fed *ad libitum* daily with squid and chopped smelt. The tanks are 5 feet in diameter, covered with plywood, and they receive a once-through supply of ambient seawater. Fish begin to spawn when water temperatures reach 17° C and 14-hour light. Typically in the first one or two spawns, the eggs are not fertilized. Spawning continues daily or every other day until early September as long as water temperatures exceed 17° C. If water temperatures drop, we add heaters to the tank.

*Not presented at workshop.

Eggs are skimmed from the surface into a basket and readied for toxicity testing. Because of the limited availability of eggs, we have developed a condensed spring-summer photo period/temperature cycle to facilitate spawning during the winter months. Further experimentation with the inducing hormone (LHRHa) is warranted because of the variable hatching rates of the embryos produced (from 0 percent to 79 percent). We feel that, given 2 or 3 years of study, this problem can be overcome and staggered batches of conditioned fish could produce satisfactory eggs year-round.

A distinct problem in using kelp bass eggs is the poor viability of eggs following transport; we therefore suggest that testing be performed at the site where the brood stock is maintained.

The toxicity test utilizes a static exposure encompassing the 4-day period between gastrulation and yolk sac absorption, at which time hatching success, developmental abnormalities, and survival are assessed. The protocol itself is easy, can be performed in a small space, and does not require the culture of larval food organisms. Forty ml of test solution are added to each 100-mm diameter plastic Petri dish; each control and test concentration has five replicates. Twenty 20 - 24-hour old eggs are pipetted into each dish and the dish is almost totally covered with the top. Although most of our experiments utilized ambient seawater of 34 g/kg salinity, a dilution series showed no differences from control down to 24 g/kg. After a 72-hour incubation at 18° C, endpoints were analyzed. Number of unhatched eggs, live larvae, and dead larvae were counted using a dissecting microscope. Live larvae were then examined for eye, fin, and notochord malformations; any one malformation identified the larva as abnormal.

In future tests, I want to include some changes which might improve test sensitivity. We had been preserving the larvae for later examination of malformations when time was tight. However, I want to include cardiac malformations as one of the criteria and these must be assessed on live larvae. Rather than using a simple +/- score for abnormalities, I'd like to use the graduated numeric scale developed by Judy Weis and statistically analyze total scores for three or four malformation categories, each of which is rated on a scale between zero and three (or five). Quality control

standards should also be developed; I think that average control survival should be >75 percent with a minimum of 60 percent survival in individual control chambers and malformation rates should be <10 percent. These figures are slightly more rigorous than those proposed for prolarval tests using striped bass, another species with buoyant eggs. I also want to compare viable hatch as an endpoint to the three separate endpoints currently used. A further refinement should pinpoint the time for test termination when using temperatures of 16 - 17°C or 19 - 21°C.

Since 1986, nine toxicity tests have been performed using naturally spawned eggs. Given the above quality standards for controls, 7 (78 percent) of these have yielded acceptable results. We had one instance where Redondo Beach water proved toxic while survival was acceptable in the control dilution water. We have not experienced any failures due to technician error. Toxicity tests have not yet been performed using artificially conditioned eggs.

A comparison with the results of copper reference toxicant tests between kelp bass and other published fishes shows that kelp bass is more sensitive than fishes with demersal eggs. For kelp bass, NOECs (total copper) were 18 μg/l for hatching success and survival and 10 μg/l for developmental abnormalities. These values are about an order of magnitude lower than 7-day NOECs for the species used in standard EPA larval tests, *Menidia beryllina* (102 μg/l) and *Cyprinodon variegatus* (175 μg/l). They also compare favorably to results of embryo exposures obtained by the Marine Bioassay Project using another indigenous California species, the topsmelt, and to *Pimephales promelas* 4- and 28-day tests. Further, kelp bass results are only slightly higher than those of organisms highly sensitive to copper, bivalve mollusks (6 - 12 μg/l) and seaweed (8 μg/l). We have also tested phenol, zinc, and chlorine dioxide in addition to a series of two complex mixtures (chlorinated seawater and sea-surface microlayer).

Given the current status of kelp bass research, acceptable tests can only be performed during the summer months. Year-round maintenance of brood stock requires 1 hour per day. Because of the large number of eggs spawned at any one time, the number of tests which can be conducted is limited only by the available

person-power. A team of three could conduct three tests in a standard 40 hour week. Staggering those 3 people over 6 - 7 days (with three available on each day of test termination) would allow six tests to be performed. Egg harvesting and preparation requires about 3 hours and yields enough eggs for greater than three tests. A single test (initial set-up, exposure and water quality measurement) requires 4 hours and test termination (water quality, evaluations, and clean-up), needs 6 - 8 hours. This test has several advantages over EPA larval tests - multiple effluent samples, water changes, and culture of food organisms are unnecessary. Tests like this which encompass the period of yolk sac absorption assess sensitivity during a critical stage of action for bioaccumulative lipophilic contaminants, chemicals which infrequently demonstrate toxicity during short-term tests.

California Halibut (*Paralichthys californicus*)

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Although we've only performed a few toxicity tests with halibut eggs and larvae, its potential should be discussed since it is one of the few commercially important California fishes to successfully spawn in culture. The Halibut Hatchery at the Redondo Marine Lab has obtained natural spawns for about the last 5 years and has recently been able to artificially induce spawning. Brood stock are maintained in large (>10 feet in diameter) tanks in flowing ambient seawater. Natural spawns are obtained every 2 - 3 days between May and September. Fish have been successfully conditioned to spawn outside of the normal breeding season by acclimation to photoperiod and temperature (16 - 18°C). For timed spawns, ripe females can be injected with a synthetic pituitary hormone analog (LHRHa). Spawning occurs 2 - 3 days later. Between 100,000 and 3 million eggs are released from each female.

The toxicity testing protocol is based upon that used for kelp bass. One-day old eggs are exposed to 40 ml toxicant in Petri dishes and incubated for 6 days at 18°C. Percent hatching success, developmental abnormalities, and survival are determined. The three categories for abnormalities are identical to those for kelp bass. For the two bioassays performed, greater than 75 percent control survival was obtained in all individual replicates and no control abnormalities were observed. NOECs for total copper were 180 μg/l for hatching and survival and less than 100 μg/l (the lowest concentration tested) for malformations. We feel that these results would be more consistent with those of kelp bass if we lengthened the test for 1 more day since yolk sac absorption was not complete at 7 days post-fertilization. One of the two phenol tests was terminated due to bacterial growth on the eggs.

*Not presented at workshop.

This species is promising because of the excellent viability of hormone-spawned eggs. Also, the eggs appear less sensitive to handling stress than kelp bass eggs and the halibut's longer development period would allow time for eggs to be transported. Older larvae are easier to work with than are kelp bass and could be used in kelp bass and could be used in bioassays focusing on growth effects. The Halibut Hatchery personnel is interested in utilizing their larvae for toxicity testing. The laboratory technicians are Jim Rounds and Thane Caro; their number is (213) 379-8559.

6. GENERAL DISCUSSION

MR. HUNT: I've got one more general question. Who and when, do you think, will decide these statistical issues? Gary (Chapman), do you have any idea what EPA's policy on that is?

MR. CHAPMAN: I wanted to get up and discuss a couple of these things. And I will tell you what I'd like to see happen and I don't know exactly where it should happen. Certainly, we should be active in it. But sometimes, it takes a grain of irritant to start a pearl growing; and I don't know where that irritant really should come from. I've been thinking about reference toxicants for a long time, first from the standpoint of using a reference toxicant response to adjust a test on a quantitative basis. And most people I've talked to, especially the statisticians will say, "no, it's a pass or fail test." That suggests, then, that there's no quantitative relationship between response to one toxicant and the response to another toxicant as a result of the condition of the test organism and/or changes in dilution water. I can certainly buy that for dilution water; I would like to think there was a better relationship as far as the organisms are concerned. To get a handle on that I was going the same direction Brian Anderson was talking about, and that is not running five or six reference toxicant concentrations, but running only one or two, either right at your best estimate of the LC50 or bracketing it with something like an LC20 and an LC80. If you know anything about the slope of your line, you can run a single point, like the estimated LC50. If the concentration that you think should be the LC50 is outside some confidence limit, based on your historic reference toxicant use, that's a fail. But what would be really interesting, is to run three different reference toxicants and see if they respond similarly or not. That would begin to tell us a lot more about what some of these reference toxicants really mean. And so I would encourage the multitoxicant, low number of concentrations approach. Another benefit of this approach is you can actually run fewer exposure tubes or bottles or aquaria or whatever or you can run more replicates. Because, one normal reference toxicant test in triplicate, would consist of 6 concentrations or 18 containers. Using 18 containers, in triplicate, you could run 6 reference toxicants at your best estimate of the LC50 and see how each one of them shifted. So there's a lot of work that can be done in that direction, I think, to make our tests more robust. Another thing I think we need

to begin to look at strongly is the question of the power of the statistical test. I know ASTM is going that way in developing their chronic test endpoints. Eventually, I think ASTM will come out with some guidelines; but, it sometimes takes a long time to get ASTM standards out. I personally would like to see people go more towards regression for chronic test analysis and make estimates of EC 1, 5, 10, or whatever. I think the only reason we've been comfortable with statistics in the past is that, with most of the tests that we have been running, we could detect a 20 percent difference and it was statistically significant. And, if a 10 percent difference wasn't statistically significant, that's probably not that big a deal anyway. I put a specific caveat in a recent EPA draft test protocol that NOECs can be statistically significant but biologically not important, or statistically insignificant but biologically very important. People need to understand that when you see NOEC, it doesn't really mean there wasn't much of an effect. In some cases, it can be a lot of effect. I think these revisions should start right here in the State of California because you are developing your program; you've got more action going at this point than any place else I know. Certainly, there is a lot of activity on the East Coast, but they are generally adopting existing EPA methodology. On the West Coast, we don't have existing methods so we're just beginning to develop all these protocols. So now's the time, if not to put these modifications in place, at least to make sure that they're addressed in these developments. And certainly things like the power of the statistical test should go into new programs because that's a very, very important concept. Because basically you are going to say, "We ran this test and we didn't get any effect at this effluent concentration, but we wouldn't have been able to detect a 20 percent effect," and it's important to be able to say that.

MR. ANDERSON: I have another general question. I was wondering what is going to be an acceptable level of precision for reference tests? The CVs seem to be anywhere from 25 to 60 percent; who will decide what level of precision is too high?

MR. CHAPMAN: There has been some analysis of test CVs; and in one analysis paper which I understand came out of ERL-Duluth, they compared some of their tests like the fathead minnow and *Ceriodaphnia* test CVs with CVs obtained on chemical analyses. Their analysis was that the better biological tests should have CVs like 20 or 30 percent; and they said that is comparable to what

people achieve on chemical analyses. We certainly think that 30 or 40 percent is probably acceptable. And we perhaps begin to get uncomfortable at 50 percent, but it's hard to say. Another thing that nobody really looks at very much, and it's come up in the past with respect to *Daphnia*, are people saying that selenium deficiency makes *Daphnia* more sensitive and that you have to have enough vanadium for them or they're more sensitive. And the question I asked was how much variability exists in natural populations. You see a coefficient of variation in a lab population and you say, "Hey, this isn't any good." But natural populations may go through very similar variation. On a temporal basis, you have no way of knowing whether natural variation is consistent with what you see in the lab. Your effluent is going to the real world at a time when your test organisms are very sensitive, that doesn't necessarily mean that things out in the real world are also more sensitive. Some probably are and some are not; they're going back and forth. But I think that coefficient of variation is merely an indication of biological variation; and I don't think it's fair to blame the laboratory or the organisms as being unacceptable because results are variable. People are ignoring the fact that those organisms out in the real world are variable, too.

MR. HALL: There is some discussion about the issue of bioassay versus chemical precision in EPA's technical support document for water quality-based control of toxics; it is just going through it's second revision and they've expanded that discussion to address those kinds of questions.

MR. CHAPMAN: That's good. I'm glad somebody has.

MR. HUNT: This may be an obvious comment on coefficients of variation, but the more sensitive the test, the higher the coefficient of variation is going to be. If you have a NOEC of 10 and it varies by 1, that's the same coefficient of variation as having an NOEC of 1,000 that varies by 100.

MR. CHAPMAN: That's correct.

MR. HUNT: So, if you have a very insensitive test, it's probably going to look better, precision-wise, if you're just looking at CVs.

MR. CHAPMAN: Well, I think we were discussing that the first night here before the meeting. And I said I thought -- just looking at other people's coefficients of variation and standard deviations, the variance around their test, it appears that CVs are calculated based on the concentration. And I said that I really

thought that they should be done using the log of concentration because, if the response is log-linear, then, for example, half of an LC50 and twice an LC50 would really tend to bracket the response. And you can get that, I think, if you were to calculate CVs based on the log of the concentration rather than the concentration themselves.

MR. CHERR: I am just wondering how useful CV information is with respect to NOEC data, particularly with what Howard was just saying. I mean that you can have a tremendous CV just because of the design with the replicates and the concentrations. Maybe LC50 data is much more appropriate.

MR. CHAPMAN: Yes, I think we need to talk about LC50s or EC10s.

MR. ANDERSON: We have looked at EC50s from our kelp reference toxicant data, and compared these to EC10s and EC25s. The CVs were around 45 percent for EC50s, 80 percent for EC25s, and 43 percent for EC10s. Try to explain that!

MR. CHAPMAN: That is amazing.

MR. BAILEY: I think some of these problems may be solved by just looking at the types of endpoints we look at in the tests and whether they are very discrete; whether we are measuring the problems in a specific stage, which only occurs at that stage, whether it's shell development of the mollusc test or something like that. A lot of the problems that I discussed don't occur so much in mortality data but in growth data where we are trying to compress what used to be a 28-day test into a 7-day test. And typically, your variability does come in the intermediate concentrations, which is what you would expect. But, with a 50 percent dilution factor in 28 days, those things separate out very nicely, which, again, is what you'd expect. So it may be that, in our haste to put more sublethal endpoints in a short-term test, in effect, we're clouding the interpretation and we may want to look at it more.

MR. CHAPMAN: That's true. When a nice short-term test for abalone or *Menidia* or anything else is developed, the idea is to pick a test that is as sensitive as possible compared to a full life cycle test. In the validation of this short-cut method, you should be running a full life cycle test, if possible, to compare the sensitivity. And, in a lot of these tests, perhaps we can't do that, but we can certainly do better than we've done to date. Of course, there's no reason to run a full life

cycle test if you can't first come up with the shortcuts, but eventually we need to do some of this validation work just to show that, if you use numbers from short-cut tests, you're going to achieve chronic protection.

7. WORKSHOP SUMMARY

Each of the tests described at this workshop represents a tool to be used in detecting or predicting toxic levels of pollutants in the marine environment. Like all tools, they can be improved through use; use providing experience and leading to acceptance and improvement. The most available, most simple, and most precise tool is the most useful. No single test, like no single tool, can suffice to complete a complex task; some tests (some species) are sensitive to one toxic material, some to another. An adequate toxicity testing "tool-box" needs to contain a variety of tools; two good hammers are not as valuable as one good hammer and one mediocre saw.

Of all the test species described at this workshop, only a few of the fish species appear to lack current applicability in toxicity tests, and this is perhaps due to inherent high natural mortality. All the other tests are good tools, but some are not always available, are not always simple, or lack documented precision. Best described and documented are the tests for mollusc embryos/larvae and for growth and survival of *Menidia beryllina*. Improvements for the former probably should include a longer test period for West Coast *Mytilus*, but this represents only a minor modification. Other tests that appear to be of broad current utility are the *Macrocystis* spore germination test, the abalone embryo/larval test, and the sea urchin embryo/larval test; each of these tests would gain better acceptance once exported successfully to additional laboratories.

A general willingness to participate in interlaboratory comparisons with most of these species was expressed by those at the workshop. Such comparisons would be most valuable with *Macrocystis* and with embryo/larval tests with mussels, abalone, and sea urchins. It is recommended that these tests be pursued as soon as an adequate experimental design is established.

More developmental work is necessary before tests with *Laminaria* (kelp gametophytes) and West Coast mysids or fish are ready for interlaboratory comparison. Of these tests, those for mysids are most important, because there is no reasonable alternative test with West Coast crustaceans, and crustaceans are often extremely sensitive to materials such as pesticides.

The relative sensitivities of the test species reported in this meeting are summarized in Appendix A. It must be noted that sensitivity to copper or zinc does not necessarily reflect sensitivity to other metals, and often has absolutely no relationship to sensitivity to other materials such as organic chemicals.

APPENDIX A

COMPARATIVE SENSITIVITY OF WEST COAST MARINE TEST SPECIES TO COPPER AND ZINC (EXCEPT AS NOTED ALL REFERENCED DATA REFER TO DATA PRESENTED IN THIS REPORT)

Species/Life Stage	Copper (μg/l)		Zinc (mg/l)	
	NOEC	EC/LC50	NOEC	EC/LC50
KELP				
Laminaria				
egg fertilization[1]	<50			
Macrocystis				
spore germination[2]	10-56		1.8-5.6	
spore germination[3]	52			
germ-tube length[2]	<10-32		<0.56	
germ-tube length[3]	23			
INVERTEBRATES				
Holmesimysis costata[4]	<11	27	0.058	0.078
Metamysidopsis elongata				
96-hour neonate survival[5]			<0.5	
44-day neonate survival[5]			<0.25	
23-day neonate growth[5]			<0.05	
Mysidopsis intii				
96-hour neonate survival[5]			<0.5	
25-day neonate survival[5]		<0.25		
7-day neonate growth[5]			<0.05	
Strongylocentrotus purpuratus				
embryo/larval[6]			0.012	<0.05
embryo/larval[7]	6.3			0.023
Haliotis rufescens				
embryo/larval[4]	<6	9	0.028	0.063
Mytilus edulis				
embryo/larval develop.[7]		21-35		0.019
Crassostrea gigas				
embryo/larval survival[8]		15->20		
embryo/larval develop.[8]		12.6		
embryo/larval develop.[7]		6.1		2.065
FISH				
Atherinops affinis				
96-hour survival (16-day old)[9]		138		
sperm/embryo[2]		94		
embryo hatching[2]		147		
96-hour survival (10-day old)[2]		264		
Paralabrax clathratus				
embryo hatch/survival[10]	18			

COMPARATIVE SENSITIVITY OF WEST COAST MARINE TEST SPECIES TO COPPER AND ZINC (EXCEPT AS NOTED ALL REFERENCED DATA REFER TO DATA PRESENTED IN THIS REPORT)

Species/Life Stage	Copper (μg/l) NOEC	Copper (μg/l) EC/LC50	Zinc (mg/l) NOEC	Zinc (mg/l) EC/LC50
Atractoscion nobilis				
embryo hatch[9]		60		0.209
96-hour survival (3-day old)[9]		21		
Parophrys vetulus				
96-hour survival (1-day old)[11]				14.5
Paralichthys californicus				
embryo hatch/survival[10]	180			
Menidia sp.				
96-hour LC50[12]		67-217		2.1-5.6
7-day growth/survival[13]	102			
Anoplarchus purpurescens				
7-day survival (1-day old)[9]		136-211		
embryo hatch/survival[9]		42		

[1]Steele

[2]B. Anderson, et al.

[3]Dean

[4]Hunt, et al.

[5]Langdon

[6]Bay

[7]Dinnel

[8]E. Hoffman and S. Anderson

[9]R. Hoffman

[10]Hose

[11]Shenker

[12]USEPA Water Quality Criteria Documents, EPA 440/5-84-031 and EPA 440/5-87-003

[13]USEPA Guidance Manual for Rapid Chronic Toxicity Tests on Effluents and Receiving Waters with Larval Inland Silversides (Menidia beryllina)

APPENDIX B

ROSTER OF WORKSHOP PARTICIPANTS
WORKSHOP ON WEST COAST SPECIES CULTURE AND TOXICITY TESTING
February 1990

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APPENDIX C

GLOSSARY OF TAXONOMIC AND COMMON NAMES

Macroalgae (Kelp)

Laminaria saccharina . . . . Sugar Wrack
Macrocystis pyrifera . . . . Giant Kelp

Mysids (Oppossum Shrimp)

Holmesimysis costata (formerly *Acanthomysis sculpta*)
Metamysidopsis elongata
Mysidopsis intii

Molluscs (Clams, Oysters, Abalone)

Haliotis rufescens . . . . Red Abalone
Crassostrea gigas . . . . Pacific Oyster
Mytilus edulis . . . . Blue Mussel
Mytilus californianus . . . . California Mussel

Echinoderms (Sea Urchins, Sand Dollars)

Strongylocentrotus droebachiensis . . . . Green Urchin
Strongylocentrotus franciscanus . . . . Red Urchin
Strongylocentrotus purpuratus . . . . Purple Urchin
Dendraster excentricus . . . . Sand Dollar

Fish

Atherinops affinis . . . . Topsmelt
Atherinopsis californiensis . . . . Jacksmelt
Attractoscion nobilis . . . . White Seabass
Parophrys vetulus . . . . English Sole
Menidia beryllina . . . . Inland Silverside
Anoplarchus purpurescens . . . . High Cockscomb
Paralabrax clathratus . . . . Kelp Bass
Paralichthys californicus . . . . California Halibut

APPENDIX D

ROSTER OF WORKSHOP ATTENDEES

WORKSHOP ON
WEST COAST SPECIES AND
TOXICITY TESTING
TUESDAY, FEBRUARY 27, 1990
(Invertebrates)

The meeting was convened, pursuant to notice, at 8:30 a.m., in the El Camino Room, Red Lion Motor Inn, Sacramento, California, Gary A. Chapman, Chairman, presiding.

ATTENDEES:

BRIAN ANDERSON	University of California, Santa Cruz, Institute of Marine Sciences
SUSAN ANDERSON	Lawrence Berkeley Laboratory
HOWARD BAILEY	University of California, Davis
STEVE BAY	Southern California Coastal Water Research Project
BOB BERGER	East Bay Municipal Utility District Wastewater Treatment Plant
DICK CALDWELL	Northwestern Aquatic Sciences
GARY CHERR	Bodega Marine Laboratory
THOMAS DEAN	Coastal Research Associates
PAUL DINNEL	University of Washington, Fisheries Research Institute
TIM HALL	National Council of the Paper Industry for Air and Stream Improvement

ROSTER OF WORKSHOP ATTENDEES

WORKSHOP ON
WEST COAST SPECIES AND
TOXICITY TESTING
TUESDAY, FEBRUARY 27, 1990
(Invertebrates)

BRIDGETT HEJTMANEK	MEC Analytical Systems
ERIKA HOFFMAN	Lawrence Berkeley Laboratory
JOHN HUNT	University of California, Santa Cruz, Institute of Marine Sciences
LISA KRIEGER	MEC Analytical Systems
A. KUBO	MEC Analytical Systems
CHRIS LANGDON	Oregon State University, Hatfield Marine Science Center
PHIL OSHIDA	U.S. EPA Region 9
LAURA PHILLIPS	U.S. EPA Headquarters
MARK SLATTERY	E.A. Engineering, Science and Technology
RICHARD STEELE	U.S. EPA Environmental Research Laboratory
LAURA TARGART	MEC Analytical Systems
LAURA TOM	U.S. EPA Region 9

ROSTER OF WORKSHOP ATTENDEES

MEETING
OF THE
MARINE TOXICITY WORKSHOP
WEDNESDAY, FEBRUARY 28, 1990
(Fish)

The meeting was convened, pursuant to adjournment, at 8:30 a.m., in the El Camino Room, Red Lion Motor Inn, Sacramento, California, Gary A. Chapman, Chairman, presiding.

ATTENDEES:

BRIAN ANDERSON	University of California, Santa Cruz, Institute of Marine Sciences
CAROL BACH	East Bay Municipal Utility District Wastewater Treatment Plant
HOWARD BAILEY	University of California, Davis
STEVE BAY	Southern California Coastal Water Research Project
GARY CHERR	Bodega Marine Laboratory
PAUL DINNEL	University of Washington, Fisheries Research Institute
MARY ELLEN GARCIA	EA Engineering Science and Technology
TIM HALL	National Council of the Paper Industry for Air and Stream Improvement
BRIDGETT HEJTMANEK	MEC Analytical Systems
ERIKA HOFFMAN	Lawrence Berkeley Laboratory
JOHN HUNT	University of California, Santa Cruz, Institute of Marine Sciences

ROSTER OF WORKSHOP ATTENDEES

MEETING
OF THE
MARINE TOXICITY WORKSHOP
WEDNESDAY, FEBRUARY 28, 1990
(Fish)

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A. KUBO   MEC Analytical Systems

LAURA PHILLIPS   U.S. EPA Headquarters

RICHARD STEELE   U.S. EPA Environmental Research Laboratory

LAURA TARGART   MEC Analytical Systems