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DEVELOPMENT OF THE ECHINODERM SPERM BIOASSAY FOR TESTING TOXIC SUBSTANCES

by

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FOREWORD

ABSTRACT

Preliminary procedures for conducting a sperm bioassay using gametes from green sea urchins (Strongylocentrotus droebachiensis) and sand dollars (Dendraster excentricus) are outlined. Sperm bioassays were generally conducted by exposing the sperm cells to a set of conditions (or toxicant) for 15-60 minutes before addition of the eggs for fertilization. Declining fertilization success was used as an indication of compromise to sperm viability.

Sperm bioassays of physical water quality parameters indicated that fertilization was generally successful at temperatures from 2 to 22°C, salinities > 24 ppt and pH values of 7.5, 8.0 and 8.5. Green sea urchin sperm appear to be slightly more sensitive to environmental variables than sand dollar sperm. Fertilization was generally successful in artificial seawater at salinities ≥ 30 ppt.

Sea urchin sperm bioassays of silver in natural seawater yielded 15-, 30-, 60- and 90-min EC50 concentrations of 590, 171, 83 and 52 μg/l silver by probit analysis. Silver concentrations of < 40 μg/l in natural seawater appeared to be stimulatory as percent fertilization in these concentrations was greater than control fertilization. Sea urchin sperm were more sensitive to silver in artificial seawater than in natural seawater with 30- and 60-min EC50's of 51 and < 20 μg/l silver.

Sand dollar sperm bioassays of silver in natural seawater yielded 15-, 30-, 60- and 90-min EC50 concentrations of 201, 124, 47 and 25 μg/l silver. Sand dollar sperm were also more sensitive to silver in artificial seawater than in natural seawater with 30- and 60-min EC50's of 56 and 25 μg/l.

Sea urchin bioassays of the organochlorine pesticide endosulfan in natural seawater yielded 30-, 60-, 90- and 120-min EC50's of 353, 143, < 66 and 195 μg/l in natural seawater. Endosulfan proved more toxic in artificial seawater with 30- and 60-min EC50's of 189 and < 101 μg/l.

Sand dollar sperm bioassays of endosulfan in natural seawater produced 30-, 60-, 90- and 120-min EC50's of 157, 151, 113 and 80 μg/l endosulfan. Endosulfan proved less toxic in artificial seawater with 30- and 60-min EC50's of 193 and 184 μg/l.

In comparative tests, both sea urchin and sand dollar sperm proved more sensitive to silver and endosulfan than the eggs. Temperature and salinity interactions with silver and endosulfan were assessed in matrix design bioassays and summarized using response surface plotting. Additionally, the interactive toxicity of various concentrations of silver and/or

endosulfan was investigated in one test with sand dollar sperm. This test found that the toxicity of silver and endosulfan in combination was additive.

In comparative 96-h sea urchin embryo bioassays of silver and endosulfan in natural seawater retardation of development was first observed in approximately 50 µg/l silver and 253 µg/l endosulfan. Similar 72-h sand dollar embryo bioassays of silver and endosulfan in natural seawater showed retardation of development in concentrations as low as 25 µg/l silver and 158 µg/l endosulfan.

There was a high degree of similarity between sperm sensitivity in the short-term bioassays and the 72- or 96-h embryo tests. Results from 30- to 90-min sperm bioassays are in the range of sensitivity for many 48- to 96-h bioassays previously reported for aquatic animals.

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SECTION I

CONCLUSIONS

Preliminary procedures for conducting a sperm bioassay have been defined using gametes from green sea urchins (Strongylocentrotus droebachiensis) and sand dollars (Dendraster excentricus).

The sperm bioassay is a static test which exposes sperm cells to test and control solutions for short periods of time (typically 15-120 minutes). Eggs are added to the test containers after the sperm exposure period has ended. Samples are then tabulated for fertilization success and the results analyzed by standard bioassay statistical methods.

The optimal environmental conditions for conducting sperm bioassays are: temperature 2-22°C; pH - 7.5-8.5; and salinities > 24 ppt. These conditions vary slightly depending on species. Sand dollar sperm appear to be slightly less sensitive to environmental variables (temperature, salinity and pH) than green sea urchin sperm.

Green sea urchin and sand dollar sperm are approximately as sensitive to silver and endosulfan in seawater as many other animals with the possible exception of fish in long-term tests and some invertebrate larvae.

We conclude from these tests that the sperm bioassay, coupled with proper chemical analytical techniques, has great potential for quick and sensitive assessments and monitoring of the toxicity of chemical substances in the marine environment. This test, used in conjunction with other short-term screening tests (*e.g.* the Ames microbial mutagen test), could help to establish indices of toxicity for a wide array of chemicals which would be used in setting priorities for further research, routine biomonitoring of receiving waters and regulatory action.

SECTION II

RECOMMENDATIONS

1. Additional research is needed to refine and optimize the sperm bioassay procedures for use both in standard laboratory toxicity screening tests and for biomonitoring of receiving water quality.

2. Additional testing is needed to define and control optimum sperm/egg ratios and number of replicates to reduce between-test variance.

3. Streamline the test protocol by investigating the use of disposable test tubes.

4. Definition of the chemical interactions (reaction rates and products) of toxic substances is needed to relate the corresponding biological responses to toxicants in natural seawater. Testing of a greater number of chemical compounds is needed.

5. The relationship between toxicity and dissolved organics in seawater require further testing. The use of artificial seawater may aid these investigations.

6. Testing should be extended to include two additional species of sea urchins (the purple urchin, *Strongylocentrotus purpuratus* and the red urchin, *S. franciscanus*) to determine if sperm from a variety of echinoids are of equal sensitivity to toxic chemicals.

7. Include in the test program a non-echinoderm marine invertebrate (like the oyster, *Crassostrea gigas* or mussel, *Mytilus edulis*) to determine between-phyla sperm sensitivities.

8. Comparative 96-h acute fish and invertebrate flow-through bioassays are needed to validate the sensitivity of the sperm bioassay to aid application to water quality criteria and regulatory action.

SECTION III

INTRODUCTION

GENERAL

Scientists charged with the responsibility of assessing, monitoring and regulating the use of toxic substances are being increasingly challenged by the proliferation of chemicals for a wide range of societal applications. Biological screening techniques which can be conducted rapidly at least cost and which retain maximum sensitivity are needed in conjunction with sensitive analytical chemical techniques. Various aspects of biological systems must be considered, including toxicity, mutagenicity, carcinogenicity and teratogenicity. The currently popular Ames microbial mutagenesis test (Ames *et al.* 1975, McCann and Ames 1976) is one example of a refined and powerful short-term bioassay. This test uses single-cell bacteria to predict the mutagenicity (and, to a large extent, carcinogenicity) of many chemicals to higher animals. This test is not entirely precise or absolute, but it does provide a valuable screening test that is quick and practical and has the additional benefit of providing valuable insight into basic biological functions and relationships.

Short-term tests in relation to toxicity that are as powerful as the Ames test are presently lacking. Life-cycle and larval-stage bioassays are possible alternatives, but these are very time-consuming and difficult to conduct and interpret. Enzyme bioassays are somewhat quicker and very sensitive, but discrete biochemical reactions are being analyzed rather than a functioning biological system.

The sperm cell is a functioning biological unit which has been the object of extensive research, especially in relation to sea urchins (Boolootian 1966, Tyler and Tyler 1966, Czihak 1975, Giudice 1973). Sperm cells have been successfully bioassayed with a variety of toxicants including metals (Lillie 1921, Hoadley 1923, Young and Nelson 1974, McIntyre 1973), petroleum (Allen 1971, Lönning and Hagström 1975), organic compounds (Hagström and Lönning 1973) and municipal wastewaters (Muchmore and Epel 1973, Stober *et al.* 1977, Stober *et al.* 1978). These studies have shown sperm cells to be sensitive to a variety of toxicants in short-term tests (*i.e.* minutes) at or below concentrations causing mortality in standard 96-h fish bioassays and often approaching the sensitivity of larval and life-cycle bioassays.

Based on this information, we are suggesting that a standardized bioassay procedure using echinoderm sperm cells be developed and used to provide a screening technique for the establishment of toxicity indices for a wide array of chemical toxicants. Toxicity indices would be useful as general biological baseline data, for setting priorities for further research, biomonitoring of receiving waters and regulatory action.

The purpose of this research is to define a set of standard conditions for conducting bioassays with sea urchin and sand dollar sperm and to utilize this procedure to determine the toxicity of two distinctly different reference toxicants: a metal (silver) and an organochlorine pesticide (endosulfan).

CHEMISTRY

Silver

Silver occurs naturally in seawater at reported concentrations of 0.15 (Bryan 1971) and 0.28 (Waldichuk 1974) µg/l. Silver discharges to the oceans approximate 5 x 10^3 metric tons/year naturally and 7 x 10^3 metric tons/year from mining (Waldichuk 1974).

Silver nitrate is highly soluble in cold freshwater (1,200 g/l (Weast and Selby 1967). The solubility of silver nitrate in seawater was not located in our review of the literature. However, Bryan (1971) indicates that when silver is added to seawater, silver chloride is the probable product and has a solubility of approximately 2.0-2.5 mg/l.

Endosulfan

Endosulfan ($C_9H_6O_3Cl_6S$) is the common name of the insecticide 6, 7, 8, 9, 10, 10-hexachloro-1, 5, 5a, 6, 9, 9a-hexahydro-6, 9-methano-2, 4, 3-benzodioxathiepine-3-oxide, or α, β-1, 2, 3, 4, 7, 7-hexachloro-bicyclo-(2, 2, 1)-heptene-(2)-bis-hydroxymethylene-(5, 6)-sulfite, or 5-norbornene-2, 3-dimethanol-1, 4, 5, 6, 7, 7-hexachlorocyclic sulfite. It is formed by the Diels-Alder addition of hexachlorocyclopenadiene and cis-butene-1, 4-diol, followed by reaction of the addition product with thionyl chloride. Technical endosulfan is approximately a 7:3 mixture of two stereoisomers, α (alpha) and β (beta) endosulfan (α endosulfan is synonymous with endosulfan 2, A, I and Thiodan A or I. β endosulfan is synonymous with endosulfan B, II or Thiodan B or II). The melting point for the technical material is 80-90°C (Maier-Bode 1968). The reported solubility at 22°C and pH 7.2 in tap water is 0.15 mg/l for α endosulfan and 0.06 mg/l for β endosulfan (Braun and Frank 1973). At 20°C the solubility in acetone is 33 percent, in chloroform 50 percent, in ethanol 5 percent and in methanol 11 percent (Maier-Bode 1968).

The principal degradation products of endosulfan are endosulfan sulfate and endosulfan diol. Other products include endosulfan ether, endosulfan hydroxy ether and endosulfan lactone (Maier-Bode 1968).

Endosulfan is marketed under the trade name Thiodan and its potent insecticidal properties are directly primarily against the Colorado potato beetle, flea beetle, imported cabbageworm, peach tree borer, tarnished plant bug as well as various species of aphids and leafhoppers.

AQUATIC TOXICOLOGY

Silver

Silver is one of the most toxic metals to microbes and aquatic animals, yet it is relatively non-toxic to man and terrestrial vertebrates. Silver has been shown to the the most toxic heavy metal to bacteria (Albright *et al.* 1972), fish and invertebrates (Amiard 1976, Jones 1964), and usually followed closely by mercury in toxicity.

Silver is unusual among metals in combining very low solubility for most of its compounds with exceedingly high toxicity of the soluble fraction. For silver to be toxic in low doses, the ionic form (Ag^+) must come into direct contact with metabolically active sites such as cell membranes or gas exchange surfaces. The biological action of silver apparently involves reversible chemical bonding with enzymes and other active molecules at cell surfaces. Binding of the -SH groups seems to be the principal mechanism of enzyme inhibition by formation of silver mercaptides (Cooper and Jolly 1970). The biological action of silver has been found to be strongly dependent on temperature, oxygen, pH and hardness or salinity.

Silver has been found to be toxic to bacteria in 30-min exposures at a concentration of 1 μg/l (Albright *et al.* 1972) and affects biological oxygen demand values by 50 percent at 0.3 mg/l (Sheets 1957).

Silver concentrations toxic to crustaceans have been determined for the crab, Carcinus maenas (adult 96-h LC50 = 1-2 mg/l; zoea I = 0.01-0.1mg/l) and the shrimp, Palaemon serratus (adult 96-h LC50 = 10 mg/l; zoea I = 0.2-5 mg/l) (Amiard 1976). Daphnia magna suffers immobilization in 5.1μg/l as $AgNO_3$ (Anderson 1948), while Clarke (1947) found 0.2 mg/l (as Ag_2SO_4) toxic to the barnacle, Balanus balanoides in 5-day tests.

Molluscs appear to be especially sensitive to silver. Embryo 48-h LC50's for the oyster, Crassostrea virginica and the clam Mercenaria mercenaria are reported as 5.8 and 21.0 μg/l silver in static tests, respectively, and the scallop (Argopecten irradians) embryo 96-h LC50 was 33 μg/l (Calabrese *et al.* 1977). Snails (Taphius glabratus) react to 100 μg/l silver by retracting the foot (Harry and Aldrich 1963), while the mud snail, Nassarius obsoletus, shows distressed behavior and reduced oxygen consumption at 500 μg/l silver (MacInnes and Thurberg 1973).

Fish also show a high sensitivity to silver. Ninety-six hour LC50's for rainbow trout, Salmo gairdneri, are reported by Hale (1977) as 28.8 μg/l and by Davies *et al.* (1978) as 6.5 (soft water) and 13.0 (hard water) μg/l. Goettle and Davies (1978) have recorded 96-h LC50's for fathead minnows, speckled dace and mottled sculpin as 3.9, 4.9 and 5.3 μg/l in soft water and 4.8, 13.6 and 13.6 μg/l in hard water, respectively. Silver concentrations of 70 μg/l are acutely toxic to bass in 24-h (Coleman 1974). Long-term exposures (10 months) of rainbow trout have yielded a "no effect" level between 0.18-0.40 μg/l Ag (Goettle and Davies 1978), while Jones (1939) defines the chronic threshold level for sticklebacks, Gasterosteus aculeatus, as 3 μg/l.

Endosulfan

Aquatic organisms differ considerably in their sensitivity to endosulfan and its metabolites. Fish appear to be the most sensitive group of organisms, generally having short-term LC50's in the range of 1 to 25 μg/l, although a 24-h LC50 as low as 0.02 μg/l has been reported for the harlequin fish (Rasbora heteromorpha) (Alabaster 1969). Knauf and Schulze (1973) tested several groups of organisms and found fish to be 10,000 times more susceptible to the toxic action of endosulfan and its metabolites than worms or snails. The increasing order of sensitivity was worms < snails < crabs < insects < fish. This order of sensitivity was similar for the two isomers α and β endosulfan and the sulfate. However, all groups were equally sensitive to the non-sulfur containing metabolites of endosulfan. The 48-h LC50's calculated for these metabolites were a minimum of 10^3 times greater than those obtained with the sulfur compounds.

The reported toxicities of endosulfan to crustacea range from 24-h LC50's of 1 μg/l for Carinoganunarus rocsellii to 300 μg/l for Daphnia magna (Oeser *et al.* 1971). Insects, while generally less susceptible than fish, do have a few members within the group that are affected by low levels of endosulfan. Sanders and Cope (1968) determined the 96-h LC50 for stonefly naiads (Pteronarcys californica) to be as low as 2.3 μg/l though later instars of the naiads were more resistant. Annelida and mollusca 48-h LC50 values have been reported to be in the order of several mg/l (Oeser *et al.* 1971).

Aquatic plants appear relatively resistant to endosulfan and its metabolites. Knauf and Schulze (1973) have shown that the metabolism of algae was unaffected during a 5-day continuous exposure to 1-2 mg/l endosulfan.

Several water quality and biotic factors are known to influence endosulfan toxicity. Macek *et al.* (1969) point out the importance of temperature in evaluating toxicity. Exposure of rainbow trout (Salmo gairdneri) to endosulfan at 1.6, 7.2 and 12.7°C resulted in 24-h LC50's of 13, 6.1 and 3.2 μg/l, respectively, indicating an increased susceptibility at higher temperatures. Schoettger (1970) found similar temperature effect with rainbow trout and western white sucker (Catostomus commersoni). Changes in salinity have increased the toxicity to guppies (Lebistes reticulatus) (Greve and Verschuuren 1971); however, when guppies were first adapted to seawater the toxicity of endosulfan in seawater and freshwater was not appreciably different (Oeser *et al.* 1971). Additionally, Knauf and Schulze (1973) found no significant difference in endosulfan toxicity to the golden orfe (Idus melanotus) in freshwater or seawater. Schoettger (1970) considered pH the most important water quality parameter affecting toxicity. In studies involving the western white sucker, the aging of alkaline (pH 8.4) solutions of endosulfan for 48-h significantly reduced the toxicity compared with solutions of a similar concentration of neutral or slightly acidic pH's.

Schoettger (1970) also investigated the influence of biotic factors on toxicity and found older and heavier fish to be more resistant to endosulfan. However, concentrations of endosulfan of up to 50 mg/l in 30- or 120-min exposures did not affect the hatchability of rainbow trout eggs nor subsequent survival of the fry.

SECTION IV

MATERIALS AND METHODS

GENERAL

All bioassays were conducted at the Fisheries Research Institute's Mobile Bioassay Laboratory located at the West Point Sewage Treatment Plant, Seattle, Washington.

Test Animals

Green sea urchins (*Strongylocentrotus droebachiensis*) were collected from a rocky breakwater in Elliott Bay, central Puget Sound, at a depth of 1-5 m below mean lower low water. Animals for general bioassay use were held in ambient flowing seawater while a second group was held in cooled flowing seawater to assess the effects of lower temperatures on gamete availability both early and late in the spawning cycle. Both groups were fed algae free-choice.

Sand dollars (*Dendraster excentricus*) were collected from a sandy beach in southern Puget Sound from approximately mean lower low water level and maintained in ambient flowing seawater on sand substrate. Plankton and detritus in the ambient seawater and sand substrate provided the only food supply.

Spawning and Gamete Quantification

The test animals were spawned separately by Tyler's (1949) KCl method (0.5-1.0 ml KCl injected through the peristomal membrane into the coelomic cavity). Sperm were collected in 100 ml fresh seawater and kept in this concentrated condition until just prior to use. Eggs were collected in 100-250 ml fresh seawater and washed 2-3 times before use. All gametes were kept cool in an ambient water bath (see Appendix Table and Figure 1).

Subsamples of sperm were counted by compound microscope and hemacytometer at 400X after fixing in 10 percent acetic acid in seawater. Subsamples of eggs were counted using a dissecting microscope at 10-20X.

Unless noted otherwise, sperm were diluted to a final concentration of 50×10^6/ml and eggs diluted to 5×10^3/ml. Most bioassays were conducted using 0.1 ml aliquots of sperm solution in 25 ml of test solution with the subsequent addition of 1 ml aliquots of egg solution. This combination produced a final sperm/egg ratio of 1000/1 and dilution of the test solutions of 0.4 percent upon sperm dosing and 4.0 percent during egg addition.

Sperm Bioassay Procedure

Most sperm bioassays were conducted by adding 0.1 ml aliquots of sperm solution to 25 ml replicate test and control solutions contained in 25 x 150 mm glass test tubes held in a circulating water bath of desired temperature. After an appropriate incubation period (typically 15-60 min) 1.0 ml of eggs was added to the sperm-toxicant solution and fertilization allowed to proceed for 15 min. The test tubes were then carefully decanted and the settled eggs poured into labeled vials containing 10 percent formalin in seawater for sample preservation and storage. The term "sperm exposure time" as used in this report is defined as the time between sperm addition to the toxicant and subsequent addition of the eggs. The sperm (and eggs) are exposed to the toxicant an additional period of time while fertilization (if any) takes place. This "extra" period of exposure is presently of undefined duration but should remain constant between bioassays using the same sperm-egg contact time interval.

Each egg sample was later tabulated for percent fertilization by resuspending the eggs in each vial, pouring the contents onto a grid-lined glass slide and scoring 100 eggs for presence or absence of the fertilization membrane. Partially-formed membranes were tabulated as unfertilized eggs. Occasional broken membranes which otherwise appeared complete were counted as fertilized (Appendix Figure 1).

Embryo Bioassays

Embryo bioassays were conducted in a series of 250-ml glass beakers each containing 100 ml of test solution or control dilution water. Each concentration was duplicated. The bioassays were initiated by inoculating each container with approximately 5,000 freshly fertilized eggs. Development was allowed to proceed for 72 or 96 hours. Temperature control was maintained by an ambient water bath. At 72 or 96 hours the samples were fixed and stored in 10 percent formalin in seawater. Stirring or aeration of the test containers was not required. Mortality was assessed by comparing triplicate subsample counts of embryos from each container at 72 or 96 hours with triplicate subsample counts from two extra initial control samples.

Stages of embryonic development and abnormalities were assessed in subsamples of 100 embryos using a compound microscope at 40X and decriptions by Hagström and Lönning (1973), Kobayashi (1971), Okubo and Okubo (1962) and Rulon (1956).

Water Quality Control

The natural seawater used in most bioassays was pumped from Puget Sound from a depth of approximately 10 m by a 7.5 hp cast-iron pump. All supply and distribution lines were PVC plastic (conditioned in seawater for 3+ years). The laboratory head tank was fiberglass. Prior to use in bioassays, all natural seawater was filtered through a 5μm cellulose filter cartridge and an activated carbon cartridge.

Salinity was measured ± 0.1 ppt with a Beckman RS-5 salinometer or hydrometer. Lower salinity solutions were prepared by dilution with fresh tap water dechlorinated with activated carbon or deionized water.

Temperature was measured with a thermometer graduated to 0.1° Celsius and controlled during bioassays with either an ambient temperature water bath or a heating/cooling circulating water bath.

Monitoring of pH was with an Orion specific ion/pH meter and pH electrode. Any required pH adjustments of the bioassay test or dilution water were made with gradual additions of either reagent grade HCl or NaOH to the desired pH.

Artificial seawater used in several bioassays was a commercial mixture called Marine Environment. This solution was filtered (0.45 μm) and adjusted to the appropriate salinity and pH before use.

Acclimation and conditioning of green sea urchins to cooler than ambient water was accomplished with 1.0 hp Frigid Unit water chiller in a slow flow-through system.

SILVER BIOASSAYS

Silver test solutions were freshly prepared by appropriate additions of 100 or 1000 mg/l stock solutions of reagent grade $AgNO_3$ in distilled water to a measured volume of seawater. Each test solution was then split into replicate 25 ml bioassay samples and a sample for chemical analysis. This procedure was repeated until all test concentrations were prepared with the desired number of replicates. Simultaneously, other test solutions were prepared in precisely the same manner using a certified commercial silver reference solution (1 ml = 1 mg as silver). Samples of these solutions were retained for chemical analysis in conjunction with the $AgNO_3$ bioassay test solutions to provide a standard curve for comparison of the bioassay test concentrations by least squares regression analysis. Sperm or embryo bioassays were then conducted as outlined above.

The chemical samples were transported to the Fisheries Research Institute's Water Quality Laboratory at the University of Washington, where they were analyzed by flame atomic absorption spectrophotometry within six hours of sample preparation. Details of the instrumentation and methods are as follows:

Instrument:	Perkin-Elmer 303 Atomic Absorption Spectrophotometer with DCR1 Concentration Readout Assembly and Silver Lamp
Lamp Current:	10 milliamps
Slit:	4
Wave Length:	328.1
Oxidant:	Air
Fuel:	Acetylene

Other analyses of silver in freshwater or seawater under various laboratory conditions were conducted using the same instrument and methodology outlined above

ENDOSULFAN BIOASSAYS

Stock solutions were prepared by adding technical grade endosulfan (obtained from U.S. EPA Laboratory, Corvallis) in excess of its reported solubility (*ca* 250 μg/l) to prefiltered (0.45 μm) natural or artificial seawater. The stock solutions were filtered again and then diluted to form test solutions of desired concentrations which in turn were divided into replicate 25 ml test volumes and added to the bioassay tubes. The test solution from one of the tubes at each concentration was used only in the determination of the actual concentration of endosulfan at the initiation of each bioassay. Similar procedures were followed for α endosulfan, β endosulfan, endosulfan ether and endosulfan sulfate (obtained from FMC Corporation, Middleport, N.Y., and EPA, HERL, Research Triangle Park, N.C.).

Chemical analyses of endosulfan test solutions were performed as follows: the 25 ml water sample and approximately 10 ml of nanograde hexane were added to a 60 ml separating funnel equipped with a Teflon stopcock. The funnel was shaken vigorously and after phase separation the hexane was drained into a 50 ml volumetric flask. The water sample was extracted a second time with an additional 10 ml hexane, which was added to the first portion. The flask was brought to volume (which represented a 1:2 dilution) and approximately 3-4 g of anhydrous sodium sulfate was added. A portion of the extracted pesticide in hexane was transferred to glass tubes equipped with aluminum foil lined caps and stored until analyzed by gas chromatography.

The gas chromatographic operating conditions were as follows:

Instrument:	Perkin-Elmer Model Sigma 1
Detector:	10 mC ^{63}Ni electron capture
Column:	6 ft x 2 mm I.D. glass column packed with 100/120 mesh Supelcoport coated with 3 percent SP-2100
Column flow:	40 cc 95 percent argon — 5 percent methane/minute
Detector flow:	65 cc 95 percent argon — 5 percent methane/minute
Temperature:	
Injector:	245°C
Column:	230°C
Detector:	300°C

Analytical reference standards of endosulfan (99.8 percent purity), α endosulfan (98 percent purity), β endosulfan (99 percent purity), endosulfan ether (100 percent purity) and endosulfan sulfate (100 percent purity) (obtained from EPA, HERL, Research Triangle Park, N.C. and FMC Corporation, Middleport, N.Y.) were used in constructing standard curves and monitoring the performance of the gas chromatograph.

SPERM BIOASSAY DATA ANALYSIS

Generally, each sperm bioassay conducted under a specific set of conditions was replicated four times using sperm from four separate males. This experimental design yielded four EC50 estimates per test condition, which took into consideration any variability due to differences in sperm quality and the counting-dilution error relating to sperm dosing.

All sperm bioassay responses (percent eggs fertilized) to be used for EC50 calculations were corrected for natural responsiveness of the controls by Abbott's formula (Step 12, Appendix Table 1) (Finney 1971). The corrected responses were used for the EC50 calculations using the BMD03S computer program for probit analysis with an attached FORTRAN program to calculate 95 percent fiducial limits for each EC50 by the methods of Litchfield and Wilcoxon (1949). Average EC50's were then calculated for each set of replicate EC50's by computing the mean and the 95 percent confidence limits based on the variability of the individual replicate EC50's. The mean EC50 ± 95 percent confidence limits was then used as the basis for comparison of toxic effects between test conditions.

It should be noted here that 95 percent fiducial limits about an LC50 (or EC50) constitute a statement of probability that there is one chance in twenty that an individual LC50 does not fall within specified limits. Fiducial limits are not confidence limits which can be used for comparison of between-test treatments. Fiducial limits are the result of applying a transformation (usually the probit transformation) to percent mortality to create a linear relationship between mortality and log concentration using non-normal distributed data from bioassays. Calculation of confidence limits requires that the data approximate a normal distribution. A population of individual estimates of an LC50 should fit a normal distribution. Thus, 95 percent confidence limits may be calculated for the mean of the individual EC50's. The methods and discussion presented above closely follow the recommendations of Hodson *et al.* (1976).

SECTION V

CHEMISTRY

SILVER

Solubility

Tests were conducted to determine the solubility of silver (as $AgNO_3$) in seawater and its efficiency of measurement by flame atomic absorption spectrophotometry (AAS) as compared with freshwater. Test results of silver added to freshwater and seawater samples (12°C) show that the recovery of silver is linear up to the highest concentration of 2.5 mg/l in freshwater. Recovery of silver is somewhat less linear and begins to decline above 2.0 mg/l in seawater (Figure 1). Filtered (0.45 µm) silver-freshwater solutions again show a linear concentration-absorbance relationship with only a slight loss of silver due to filtration. Recovery of silver from 0.45 µm filtered silver-seawater samples is linear up to approximately 1.0 mg/l silver, after which silver measurements remain constant at approximately 165 absorbance units ($\sim$ 1.0 mg/l silver). Thus, the apparent solubility of silver in seawater at 12°C is approximately 1.0 mg/l.

Effect of Filtration Pore Size

Filtration of silver-spiked seawater with a series of small-pore-size filters produced interesting results. Filtration of 1.0 mg/l silver solutions with filters ranging from 0.1 to 5.0 µm pore size did not change the recovery of silver by AAS (Figure 2). Hence, silver, when added to seawater at concentrations $\leq$ 1.0 mg/l, is probably all in solution or in a colloidal state too fine to filter. Additions of silver $\geq$ 1.5 mg/l show essentially constant recovery of silver with filtration at 1, 3 and 5 µm pore sizes, and decreasing recovery of silver as pore size decreases below 1 µm. This indicates that there is a continuum of particle size from approximately 1.0 µm to very-fine colloidal. The 3.0 mg/l silver concentration (Figure 2) was interesting because the recovery of silver after 0.1 µm filtration was less than would be expected to be in silver solutions of 1.0 mg/l. Evidently, the relatively large colloidal fraction scavenged some of the silver which would normally be in solution under these test conditions (Temp. = 12°C, pH = 7.7, salinity = 29.8 ppt). Also of interest is that filtration of samples to be used in biological assays with the standard 0.45 µm filter would leave a large component of colloidal silver in samples dosed with > 1.0 mg/l, which may not exert a direct toxic effect in the assay, but still be measured (in part) by AAS.

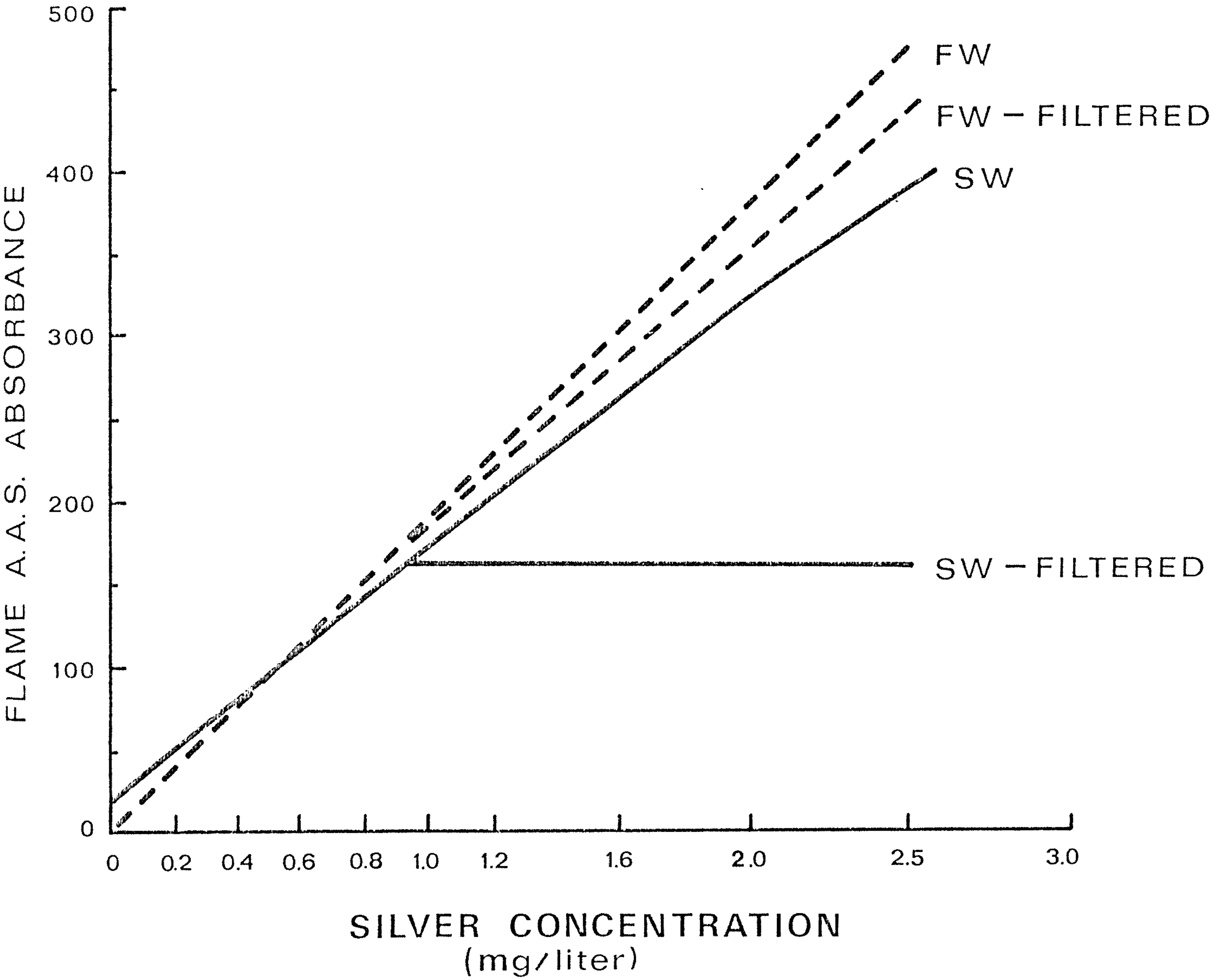


Figure 1. AAS recovery of silver concentrations in freshwater (FW) and natural seawater (SW) before and after passage through a 0.45 μm filter.

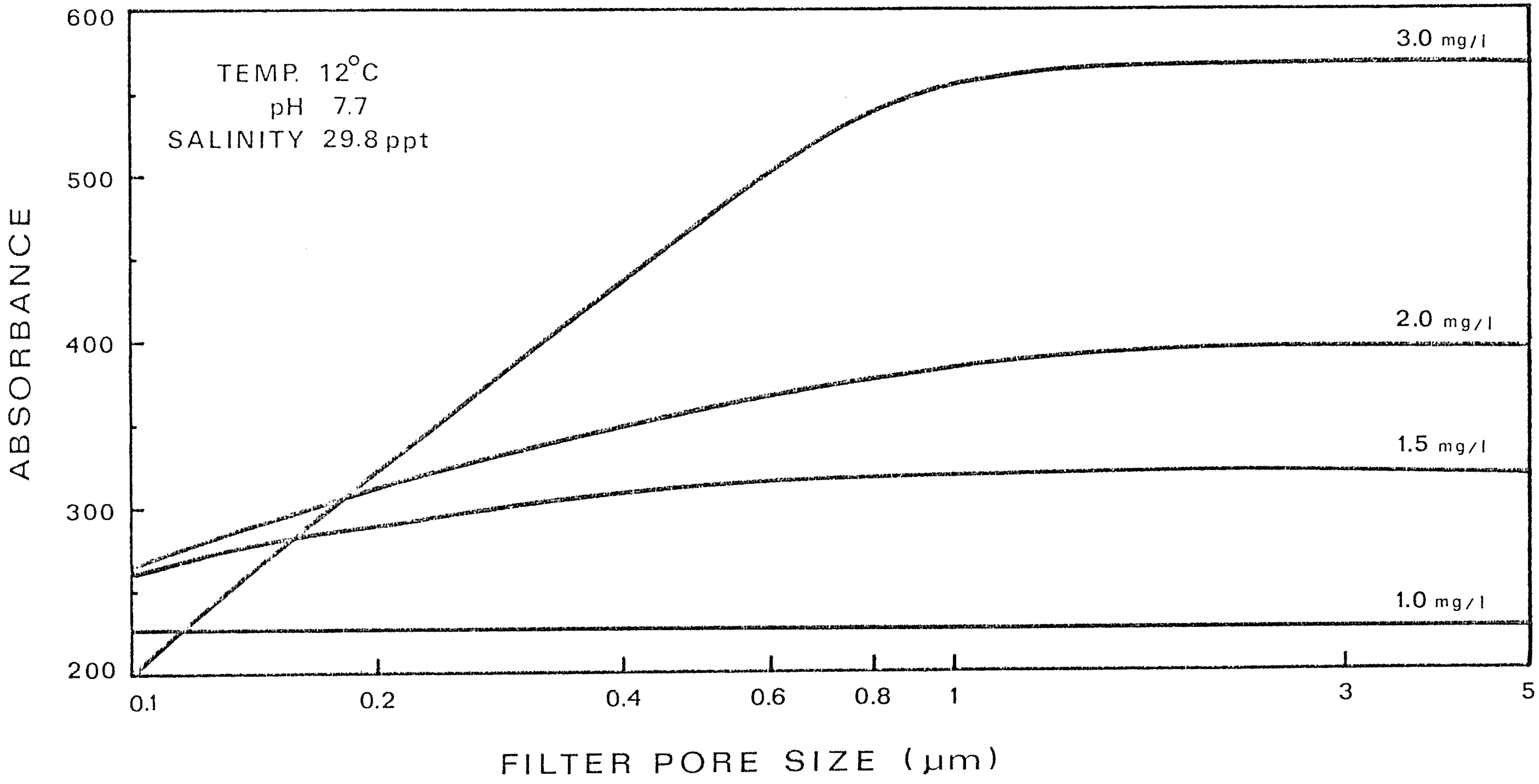


Figure 2. AAS recovery of silver concentrations of 1.0, 1.5, 2.0 and 3.0 mg/l in natural seawater after passage through filters with 0.1 to 5.0 μm pore sizes.

Effect of Temperature

The apparent limits of solubility (as determined by filtration at 0.45 μm) of silver in seawater were determined for four temperatures. The solubility of silver was approximately 1.1, 1.2, 1.5 and 2.0 mg/l at 7, 12, 17 and 22°C, respectively (Figure 3).

Effect of Salinity

The effect of various salinities on the recovery of silver by AAS was not entirely predictable. Measurements of silver added to distilled water (= 0 ppt) was linear and essentially complete (Figure 4). Recovery of silver was greater at 30 ppt salinity than at 20 ppt. Recovery of silver at 10 ppt fluctuated with dose but was similar to silver measurements at 20 ppt. Measurements were not made at salinities between 0 and 10 ppt, but it would be expected that recovery would gradually increase as salinity decreased until the measurements converge with the curve for distilled water.

Silver Concentrations in Biological Assays

Most sperm and embryo bioassays were conducted at silver concentrations < 500 μg/l. Hence, all silver in the bioassays should be in solution or in a colloidal state that would not be filtered by a 0.45 μm membrane filter. Occasional tests at or above the apparent saturation level of silver in seawater will be discussed later.

ENDOSULFAN

The absolute retention times of endosulfan ether, α and β endosulfan and endosulfan sulfate were approximately 1.1, 2.2, 2.8 and 3.4 minutes, respectively. Representative gas chromatograms of endosulfan standards and seawater extracts are presented in Figure 5. Solutions of endosulfan at pH 8.5 apparently degraded at a faster rate than those at pH values of 7.5 and 8.0. The appearance of a peak eluting prior to α endosulfan suggests the possible formation of endosulfan alcohol (Figure 6). However, only concentrations derived from α and β endosulfan peak areas were used in calculating the EC50 values for all bioassays.

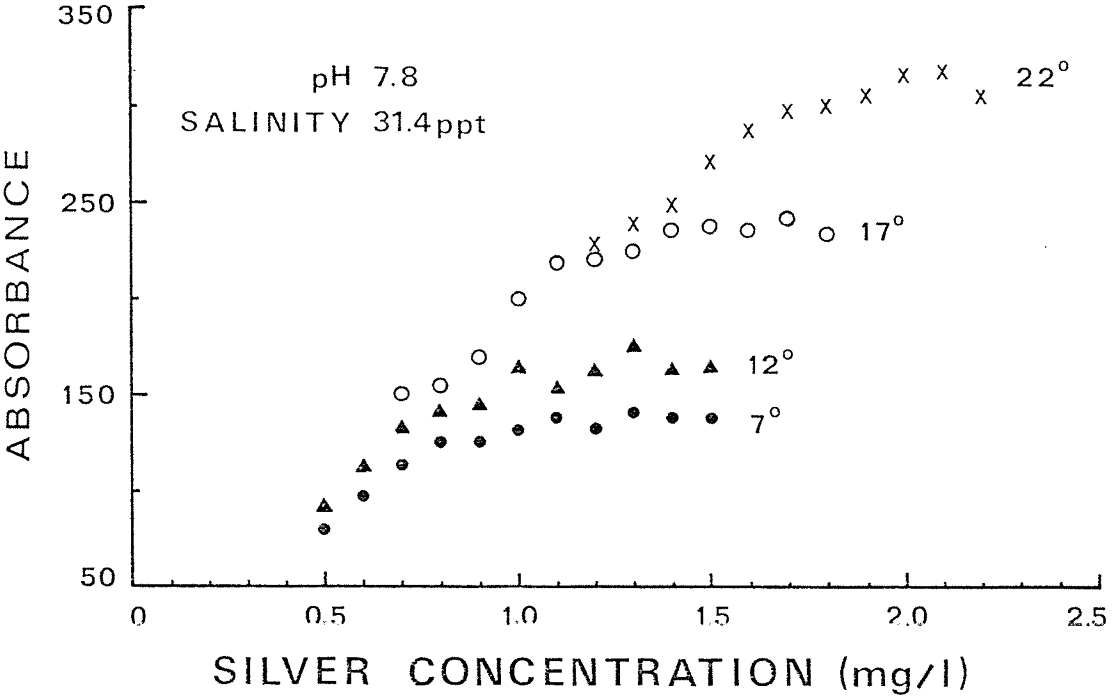


Figure 3. AAS recovery of silver in seawater at 7, 12, 17 and 22°C.

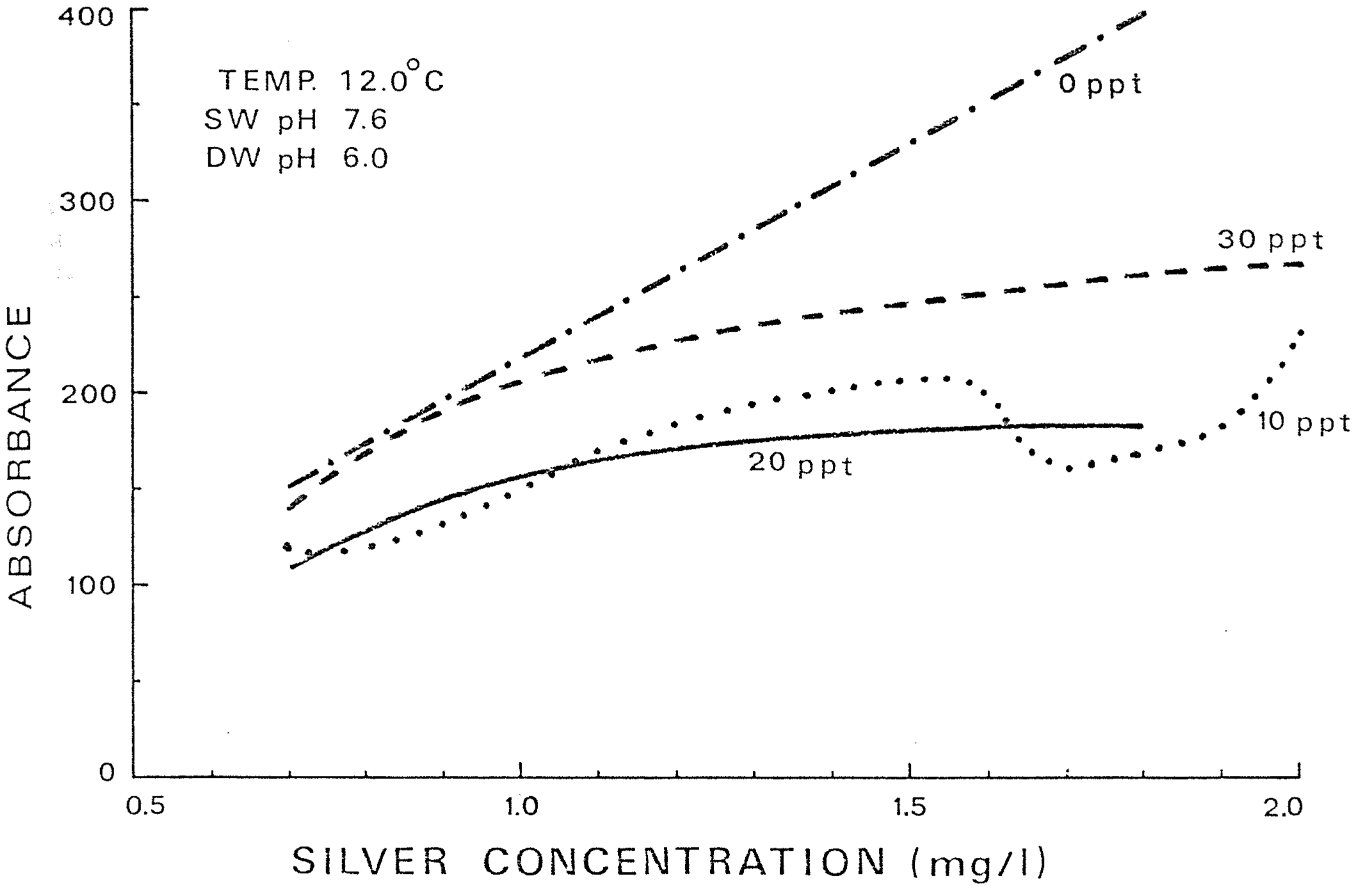
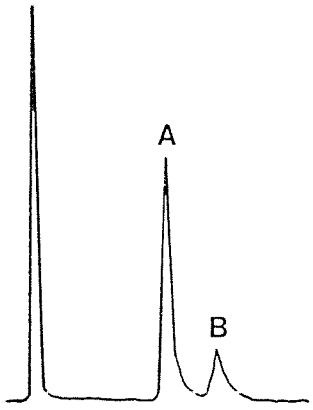
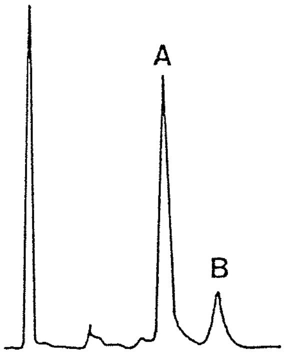


Figure 4. AAS recovery of silver in natural seawater at salinities of 0, 10, 20 and 30 ppt.



Reference Standard



Seawater Extract

Figure 5. Gas chromatograms of endosulfan reference standard and seawater extract. A and B isomers are indicated. Conditions: 3% SP-2100 on 100/120 Supelcoport, 6 ft. x 1/4" glass, column temperature 230°C, flow rate 40 cc/min., 95% Ar/5% CH_4, detector: ECD.

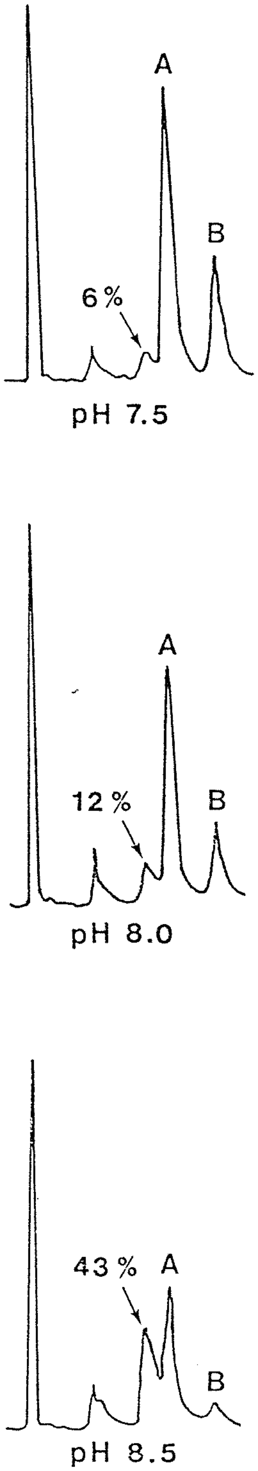


Figure 6. Gas chromatograms of endosulfan extracts from natural seawater at pH 7.5, 8.0 and 8.5. The A and B forms are labeled as well as the percent composition of an unknown endosulfan by-product. Conditions: same as for Figure 5.

SECTION VI

GREEN SEA URCHIN BIOASSAYS

SPERM BIOASSAYS

Physical Parameters

Spawning--

Initiation of spawning activity in green sea urchins from the natural population in Elliott Bay and individuals conditioned in cooled seawater were monitored and compared. The first detectable spawning activity occurred on November 22 and December 12, 1978 for ambient and cooled sea urchins, respectively (Table 1). From November 5, 1978 to January 3, 1979 only males responded to the KCl injections. First female spawning occurred January 5, 1979. Essentially all animals were spawning by February 2, 1979. Animals which had been conditioned since October 25, 1978 did not spawn earlier than the natural population and lagged slightly behind them.

Egg production by KCl-injected females gradually increased from an average of 14,800 eggs/female on January 5, 1979 to 894,000 on February 12, 1979 (Table 2). Likewise, average percent fertilization of the eggs increased from low levels in January to 91.9 percent on February 12, 1979. Low fertilization success early in the spawning season was primarily due to a high percentage of immature eggs (indicated by the presence of a distinct nucleus) in the samples.

Eggs from females conditioned in cooled seawater did not show a distinct increase in either egg production or viability over the natural population. Equivalent samples on January 19, 1979 yielded an average egg production and fertilization success of 5,500 eggs/female and 18.2 percent fertilization for the natural population versus 23,000 eggs/female and 44.6 percent fertilization for the conditioned animals. Both groups fell short of the egg production and viability of the natural population recorded for February 2 and 12, 1979. Thus, it appears that a 2-3-month period of conditioning with cooled seawater was not successful in stimulating early gamete production in the sea urchins.

Termination of the active spawning period was apparently prolonged by the cool-water conditioning. Sea urchins maintained in ambient seawater showed the first signs of natural spawning on March 27, 1979. On April 2, 1979 90 percent of the individuals from this same group failed to respond to KCl injection. First signs of natural spawning in the conditioned animals was detected on April 11, 1979. However, a large proportion of these animals continued spawning for 4-8 weeks longer, with 50 percent of the

Table 1. Pattern of early spawning of green sea urchins (induced with 1 ml KCl) from ambient seawater and cooled seawater.

Date	#Spawned/# Injected with KCl	
	Ambient Seawater	Cooled Seawater
5 Nov 1978	0/4	
22 Nov	1/4 Male only	
5 Dec	2/4 Male only	0/4
12 Dec		1/4 Male only
18 Dec	4/6 Male only	0/4
3 Jan 1979	3/6 Male only	1/6 Male only
5 Jan	6/9 Male and Female	
19 Jan	6/7 Male and Female	6/10 Male and Female
2 Feb	6/6 Male and Female	

Table 2. Average number of eggs and their viability as obtained from green sea urchins early in the spawning season.

Date	Sample Size	Average Number of Eggs/Female	% Fertilization	
			Mean	Standard Deviation
5 Jan 1979	3	14,800	36.7	36.8
19 Jan	3	5,500	18.2	13.6
2 Feb	3	233,300	78.9	19.4
12 Feb	10	894,000	91.9	6.1

conditioned animals still spawning on June 8, 1979. Many of these animals had been spawned with KCl injection earlier in the season. This, in conjunction with the cooled water, may have helped to prolong spawning activity. The weekly averages of ambient temperatures and salinities and the conditioning water temperatures are presented in Figure 7.

Sperm and Egg Quantification--

Standardization of sperm and egg densities added to each bioassay test container is essential to reduce within- and between-test variability. Within-test variability is minimized by using equal aliquots of the same sperm and egg samples for all test containers in a bioassay test series. Between-test variability, however, is partially dependent on the technician's ability to reproduce the same concentrations of sperm and eggs from each set of animals throughout the spawning season.

Quantification of sperm and egg samples was normally done by microscopic counts of one or two subsamples of the concentrated gametes followed by dilution with a calculated amount of seawater (Appendix Table 1). To assess the accuracy of this procedure, five samples each of sperm and eggs were subjected to 10 replicate counts and the means, 95 percent confidence limits and ranges were recorded (Table 2). The 95 percent confidence limits show that the sample population means (n = 10) for sperm and egg counts should fall within ± 15 percent and ± 11 percent, respectively. However, since the population means of each sperm and egg batch are normally estimated by only one or two samples, the variation of individual counts becomes important. The range values (Table 3) show deviations from the sample means by as much as 46 percent for sperm and 41 percent for egg counts. Thus, using a target sperm/egg ratio of 5,000,000/5,000 (= 1000/1) for each bioassay container, the worst case counting-dilution error could produce a sperm/egg ratio of 2,700,00/7,050 (= 383/1) for a single subsample count. Based on sperm/egg ratio fertilization tests (see next section) a 383/1 sperm/egg ratio would be unsatisfactory for optimal control fertilization. Methods must be investigated for reducing or eliminating this large source of between-test variability to enhance the precision of the sperm bioassay.

Effect of Sperm/Egg Ratio--

Previous tests with sand dollars and sea urchins had suggested that a sperm/egg ratio of 1000/1 would generally produce > 90 percent fertilization in control samples for 5 to 15-min "exposure" ("exposure" = sperm time in dilution seawater prior to addition of the eggs) periods without decreasing the sensitivity of the test. To further clarify this relationship, sets of bioassays were conducted using 15-, 30- and 60-min sperm "exposure" periods and varying sperm/egg ratios in control seawater. The results of these tests suggest that the 1000/1 ratio may be satisfactory for 15-min sperm "exposure" periods, but that 30- and 60-min "exposure" periods may require a higher sperm/egg ratio to yield consistently high control fertilization (Figure 8). The 15-min "exposure" tests yielded > 90 percent fertilization down to a 640/1 ratio. Fertilization in the 30- and 60-min tests was < 90 percent below a ratio of 2560/1. The values plotted in Figure 8 represent averages of several tests. There was a rather high variability in the results of replicate tests at the same "exposure" time. Several 30- and 60-min "exposure" tests showed > 90 percent fertilization at a ratio of

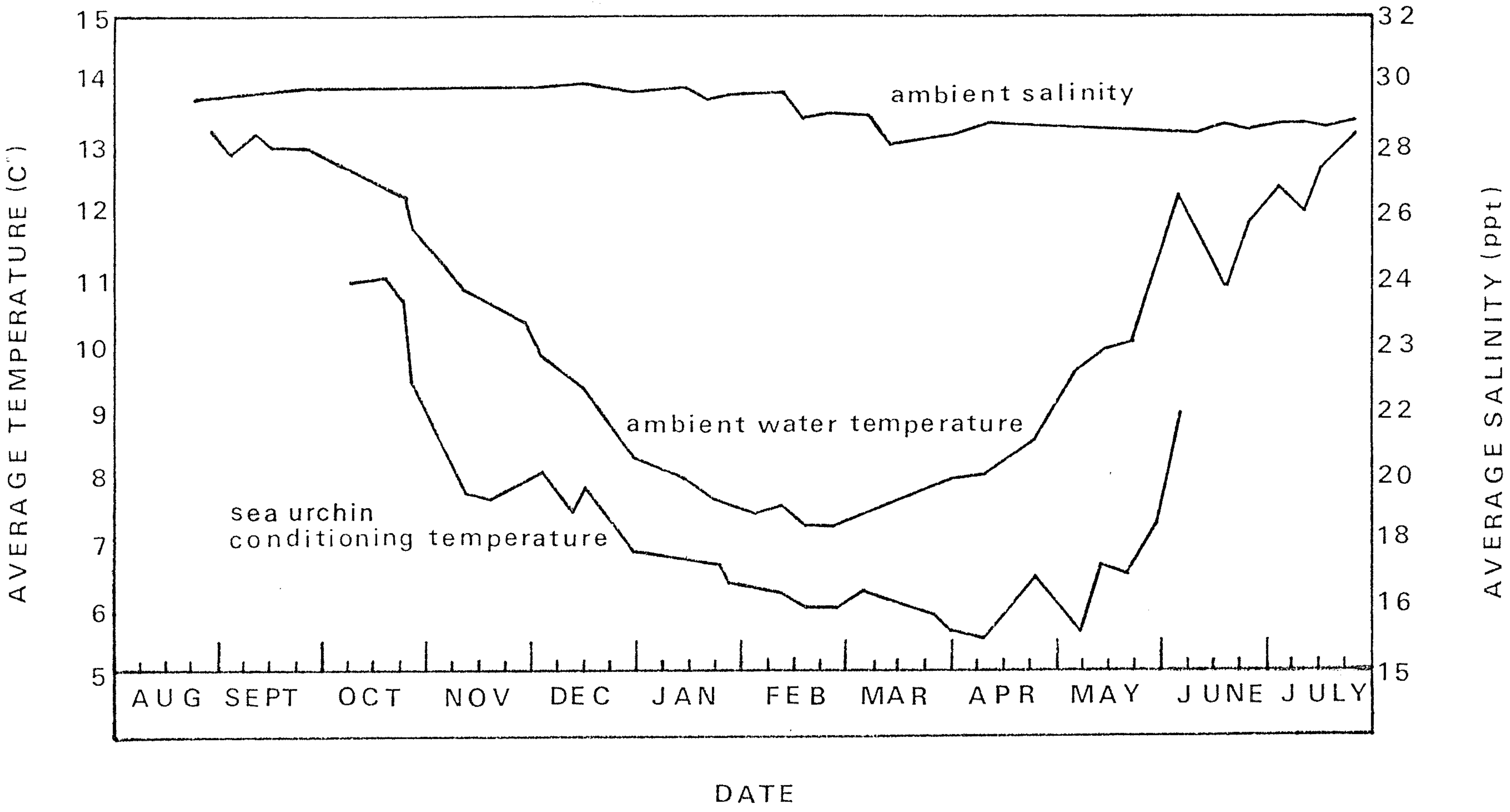


Figure 7. Weekly average seawater temperatures (ambient and from the sea urchin conditioning tank) and salinity measured at the West Point Laboratory.

Table 3. Mean, 95% confidence limits and range values/ml of 10 replicate counts of 5 different sea urchin sperm and egg solutions.

Sample	Mean ± 95% Confidence Interval	Range
	Sperm ($\times 10^8$)	
1	2.64 ± 0.25	1.85 - 3.25
2	0.22 ± 0.03	0.12 - 0.32
3	5.09 ± 0.57	3.50 - 6.80
4	29.40 ± 1.20	26.00 - 32.00
	24.60 ± 2.00	21.00 - 30.00
	Eggs	
1	793.0 ± 42.2	680 - 890
2	5620.0 ± 339.5	4500 - 6300
3	6420.0 ± 733.4	3800 - 7700
4	5290.0 ± 438.3	4200 - 6500
5	8030.0 ± 339.5	7000 - 8700

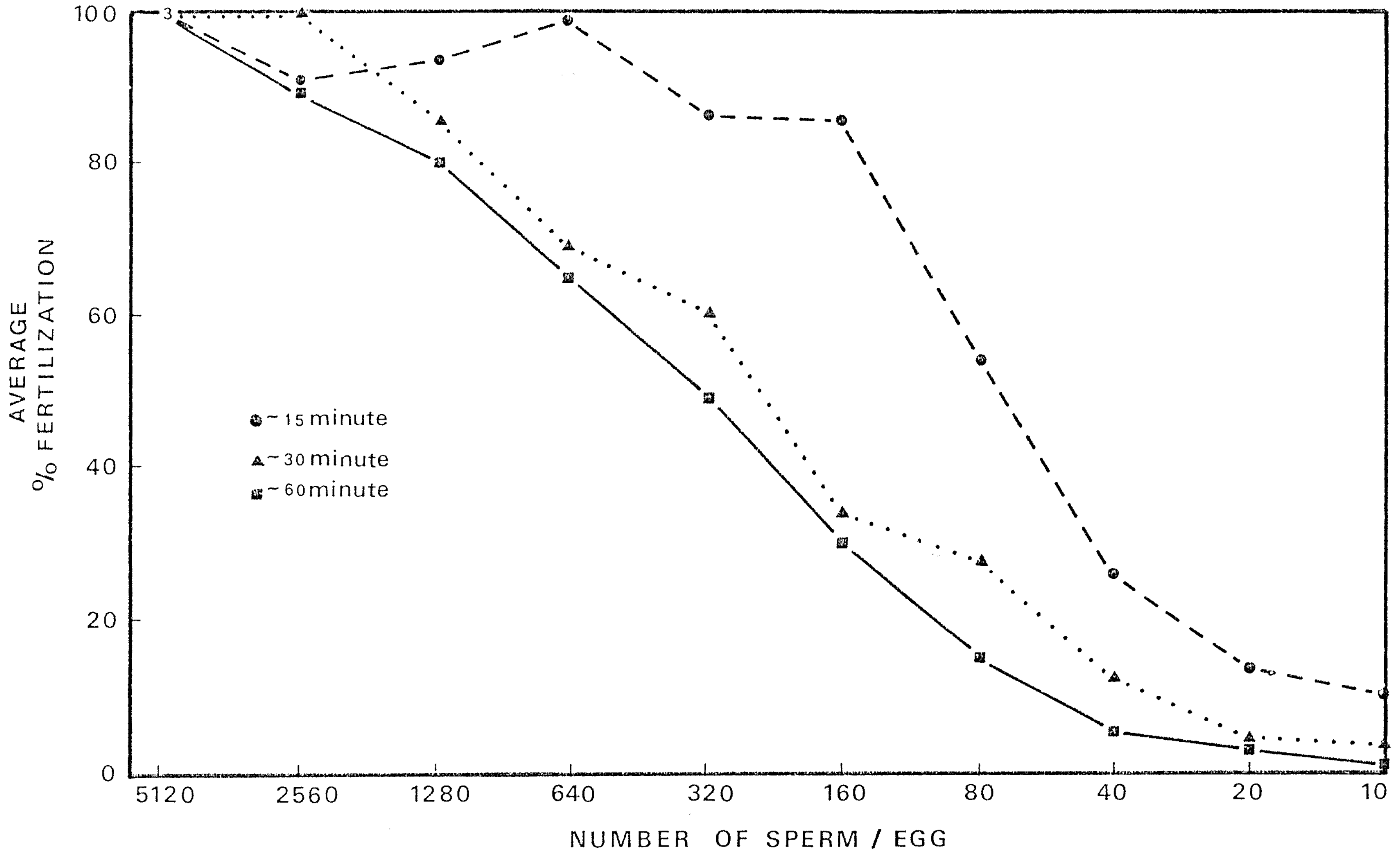


Figure 8. Average fertilization success after 15-, 30- and 60-min sperm "exposures" to natural seawater at sperm/egg ratios of 10-5120/1.

1280/1 while other replicates were substantially < 90 percent. The reasons for this variability are not totally known but may include: 1) counting-dilution error, 2) gamete quality, 3) slow activation of the sperm and 4) inhibition of sperm by egg secretions (egg water).

Subsequent tests with silver and endosulfan were conducted at a sperm/egg ratio of 1000/1 so that these tests could be related to those previously conducted at this ratio, and to maintain a base level of sensitivity for the test. The results of those tests which did not exhibit optimal control fertilization were related to the control fertilization by adjusting all results to the percent of control fertilization (i.e. control = 100 percent). This was necessary with the data in Figure 8 (5120/1 = 100 percent) as the presence of 10-20 percent immature eggs reduced the maximum possible fertilization to < 90 percent.

Sources of Variability--

As mentioned in previous sections, there are several factors that may affect the within- and between-test variability. Three sets of tests were conducted to assess variability associated with difference combinations of eggs and sperm and to test the uniformity of replicate samples within tests.

One test series used sperm from 10 different males to fertilize eight replicate samples each of eggs from a single female. The mean percent fertilization and standard deviation for each set of replicates is summarized in Table 4. The average fertilization for all 10 tests was 94.7 percent with a narrow average standard deviation of 2.1. A second test series using eggs from 10 different females fertilized by sperm from only one male produced essentially the same results with an average fertilization of 94.1 percent and an average standard deviation of 2.6 (Table 4). In both of these tests the individual replicate standard deviations fell between 1.1 and 4.8 indicating a fairly uniform response in each replicate sample.

A third set of 10 tests conducted over a longer period of time matched 10 males with 10 females on a one-to-one basis to approximate the normal between-test control pattern. This test series produced a lower fertilization success (average = 77.3 percent) and higher replicate variability (average standard deviation = 6.1) (Table 4). The bulk of these tests were conducted early in the spawning season when some immature eggs were present in the samples, thus causing a reduction in fertilization success. However, the high individual standard deviations suggest other factors may also be important. The counting-dilution error discussed in a previous section would be greatest in this third test since all gametes of one sex or the other came from a single batch in the first two tests, thus reducing this type error by approximately 1/2. Another factor causing higher variability is that the fertilization success in the third test series was closer to the mid-point of the response curve. As one approaches the limits of the response curve (i.e. 0 or 100 percent) variability would be expected to decrease since the situation is changing from a dose-response effect to an all-or-none response.

These tests reinforce the need for optimal control fertilization (which minimizes the control variability) so that toxicant dose-response curves

Table 4. Average percent fertilization ($\bar{x}$) and standard deviations (σ) of three test series using three different combinations of male and female sea urchins. Each test entry represents eight replicate samples.

	10 M	1 F		1 M	10 F		10 M	10 F
M	$\bar{X}$	σ	F	$\bar{X}$	σ	M-F Pair	$\bar{X}$	σ
1	94.5	1.9	1	96.1	1.7	1	89.7	3.5
2	90.4	3.2	2	95.9	2.4	2	86.5	5.1
3	91.5	2.2	3	97.5	1.4	3	93.1	2.9
4	98.1	1.2	4	92.8	3.5	4	70.9	12.0
5	97.0	1.7	5	97.1	1.3	5	44.0	9.8
6	95.6	2.9	6	93.1	3.0	6	76.8	6.7
7	95.1	2.0	7	96.5	1.8	7	89.6	3.7
8	95.9	1.7	8	95.0	3.3	8	86.2	5.1
9	96.2	1.1	9	83.1	4.8	9	88.6	1.7
10	92.6	2.9	10	93.9	3.3	10	47.2	10.4
Average	94.7	2.1		94.1	2.6		77.3	6.1

can be analyzed by standard probit analysis without compromising data transformations. Factors which affect the control fertilization should be further investigated to enhance the reproducibility of the sperm bioassay.

Sperm and Egg Quality Through Time--

Optimal control fertilization depends on the availability of healthy, viable gametes. Early in the spawning season fertilization of the eggs may be less than optimal due to the presence of immature eggs. Late-season gametes may be less viable due to resorption of the gonadal tissue after normal spawn-out time. Gamete viability is also a function of time after initiation of spawning. Sperm are "activated" by dilution into seawater during spawning, the amount of activation being partially dependent on the amount of dilution and various water quality parameters (i.e. salinity, temperature, trace metals, etc.).

Two tests were conducted to measure the length of time that the gametes remained viable after induced spawning with KCl. Tests conducted on February 12 and May 2, 1979, utilized replicate samples of 10 and 8 male-female pairs, respectively. Water temperature was 7.7°C, pH 8.1 and salinity was 29-30 ppt during each test series. The sperm and eggs were maintained at normal test concentrations (sperm = 5,000,000/ml; eggs = 5000/ml) during the 72-h test period. Sperm "exposure" to control seawater prior to egg addition was 15 min.

Both tests showed approximately 90+ percent fertilization up to 24 hours (the February 12 tests showed high variability with 9.0- and 15.0-h tests yielding only 88 and 79 percent fertilization, respectively) (Figure 9). Surprisingly, fertilization was still partially successful at 48 and 72 hours, although < 90 percent. The May 2 test series suggests that sperm may not be fully activated under the above test conditions for several hours as average fertilization success gradually increases from 92.3 percent at 1.5 hours to 98.0 percent at 7 hours.

Average fertilization in the May 2 test series was consistently higher and less variable than in the February 12 tests. This again suggests that mid- to late-season gametes are more dependable test "organisms" than early-season gametes. Egg maturity has already been mentioned as one factor affecting early-season viability. Other possible factors affecting early viability were not specifically investigated but may include physical water quality parameters, food supply and possible endogenous biochemical factors of the gametes themselves.

Temperature, Salinity and pH--

Sets of bioassays were conducted in natural seawater to define the ranges of temperature, salinity and pH which would normally allow optimal fertilization in the absence of toxicants.

Temperature bioassays conducted at 28 ppt salinity and pH 8.0 show that fertilization is generally successful from 2-17°C for 30-min sperm "exposures". Fertilization dropped to only 7.5 percent at 22°C (Figure 10). (Sand dollar and sea urchin data are shown in Figures 10, 11 and 12 to facilitate comparison, but sand dollar results will be discussed in a

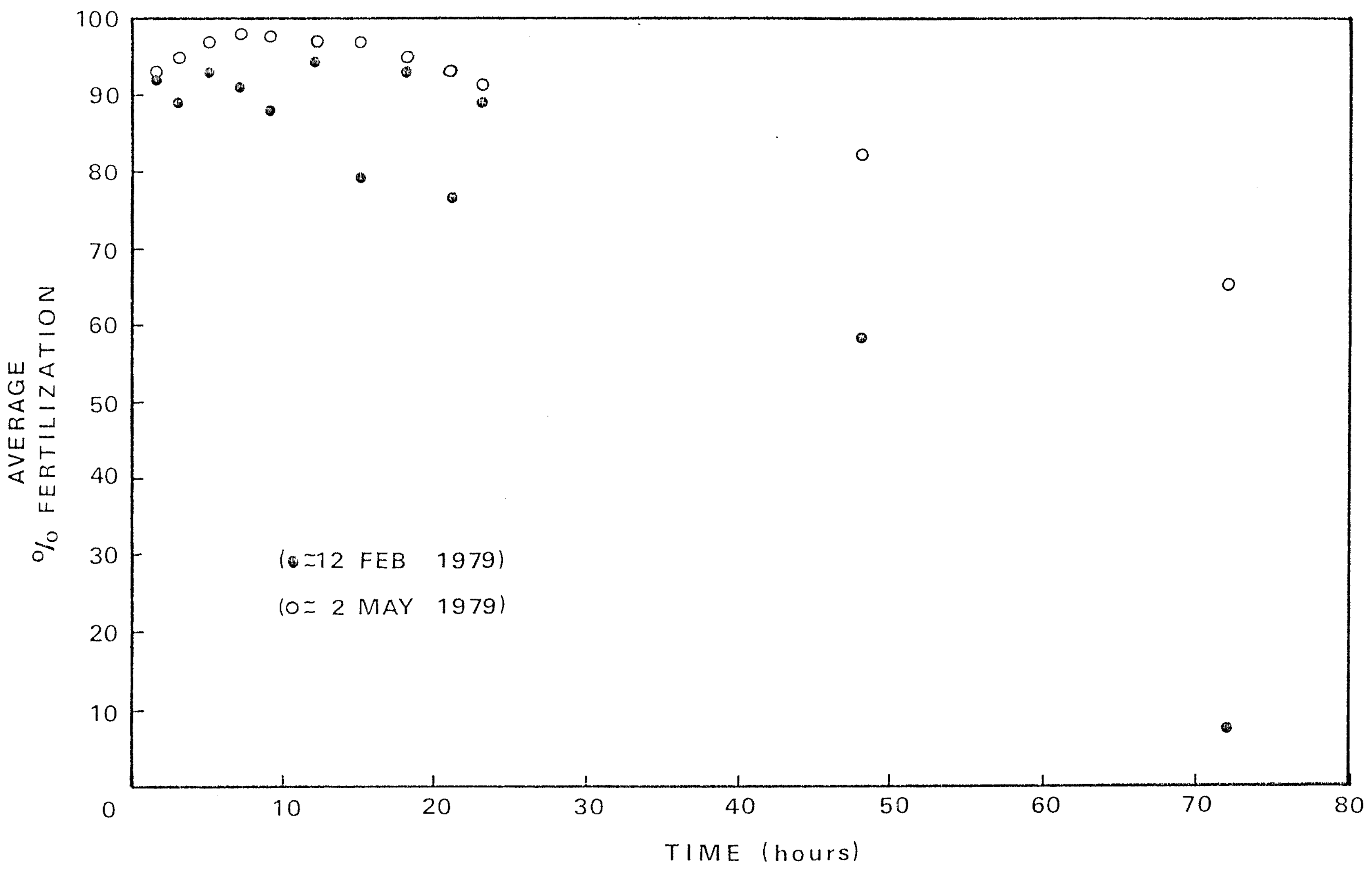


Figure 9. Long-term viability of sea urchin sperm held at concentrations of 50×10^6/ml of natural seawater as indicated by average fertilization success over a 72-h time period.

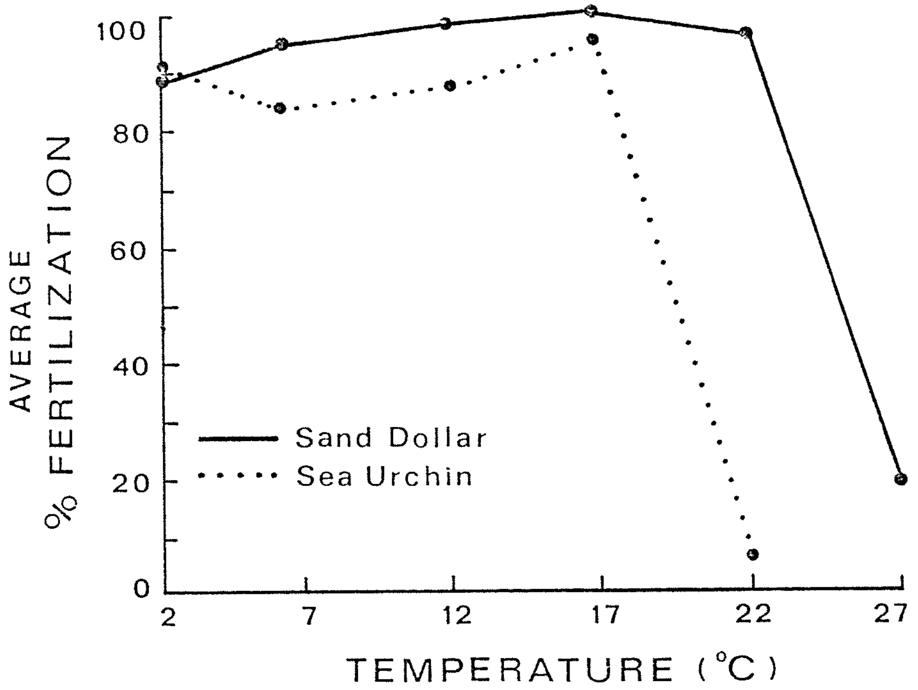


Figure 10. Average percent fertilization after 15-min "exposures" of sand dollar and sea urchin sperm to natural seawater at 2, 7, 12, 17, 22 and 27°C.

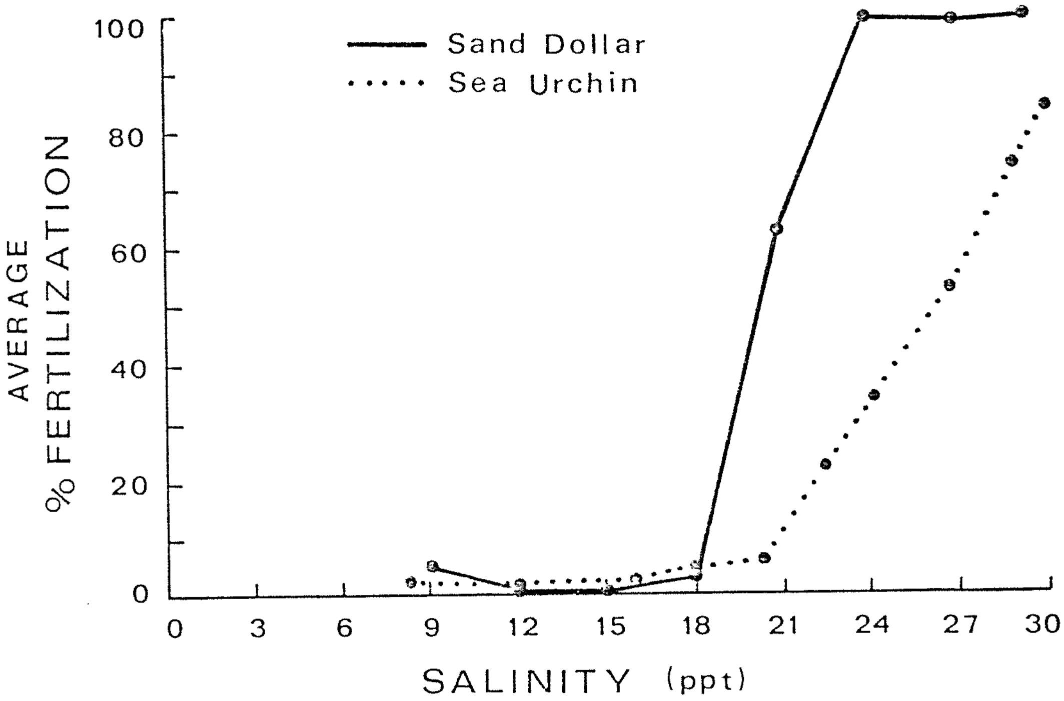


Figure 11. Average percent fertilization after 15-min "exposures" of sand dollar and sea urchin sperm to natural seawater at salinities from 0-30 ppt.

following section.)

Salinity bioassays at 12°C and pH 8.0 produced essentially no fertilization success at salinities < 20 ppt with increasing fertilization from 22-29 ppt for 15-min sperm "exposures" (Figure 11). The apparently linear relationship between salinity and fertilization and the less than optimal fertilization at 29 ppt (85.5 percent) in this test series suggests that sea urchin gametes may require salinities > 29 ppt. Ambient salinities at the West Point Laboratory were typically in the 27-29 ppt range during most sea urchin sperm bioassays. Thus, some of the variability and sub-optimal fertilization in tests controls may have been due to salinity stress. This situation can possibly be corrected in future tests by obtaining higher-salinity natural seawater from another location or by using artificial seawater of ≥ 30 ppt.

Bioassays of varying pH at 12.0°C and 29 ppt salinity showed generally poor fertilization at pH values ≤ 7.0 and ≥ 9.0. Fertilization was sub-optimal at pH 7.5 and most successful at 8.0 and 8.5 for 15-min sperm "exposures" (Figure 12).

To assess the interactive relationship between salinity, temperature, pH and fertilization success, a matrix of tests at 4 salinities, 5 temperatures and 4 pH values was conducted in properly adjusted natural seawater. Generally, all tests at pH 7.0 showed negligible fertilization; tests at pH 7.5 reflected poor fertilization, and tests at 8.0 and 8.5 showed generally successful fertilization at the higher salinities (Figure 14).

Fertilization success showed a gradual decline in relation to salinity at pH values of 7.5, 8.0 and 8.5. This reinforces the above suggestion that optimal sea urchin fertilization requires salinities > 29 ppt.

Responses to temperature were inconsistent. Figure 14 shows a drop in fertilization success at 7°C in each plot. Whether this was a "real" response or an artifact of between-test variability is not known. Other than this inconsistency, fertilization was generally good from 2-17°C at the higher salinities and pH values. Fertilization was totally unsuccessful at 22°C.

Artificial Seawater--

Since fertilization did not appear to be optimal at ambient seawater salinities, one bioassay series was conducted comparing fertilization success in artificial seawater and natural seawater. With the exception of one test point (30-min "exposure" at 27.0 ppt salinity) fertilization was > 90 percent in artificial seawater in salinities of 25.2 to 36.2 ppt for 15- and 30-min sperm "exposures" (temperature = 8.5°C, pH = 7.9) (Figure 15). However, 60-min "exposure" to artificial seawater provided > 90 percent fertilization only at salinities ≥ 30.6 ppt. An equivalent test of sperm in natural seawater for 60 minutes showed similar fertilization responses at 28.8 and 27.0 ppt, but fertilization success in natural seawater was less than artificial seawater at salinities ≤ 25.2 ppt. These data reinforce the indication that salinities < 29 ppt are stressful to green sea urchin sperm and indicate that artificial seawater (≥ 30 ppt salinity) may

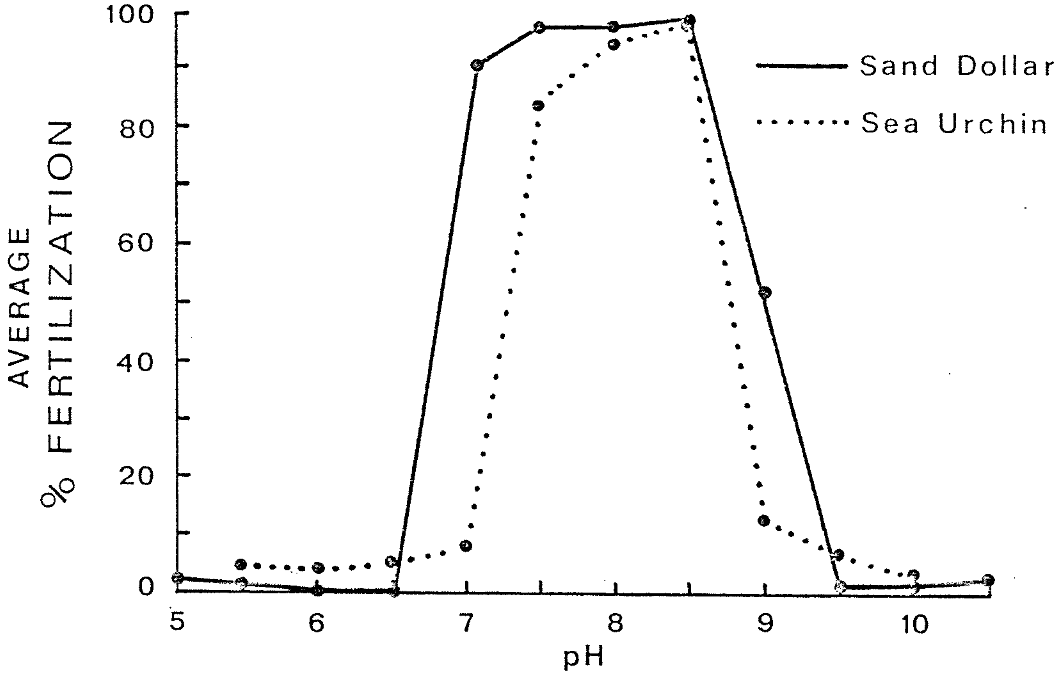


Figure 12. Average percent fertilization after 15-min "exposures" of sand dollar and sea urchin sperm to natural seawater at pH's from 5.0 - 10.5.

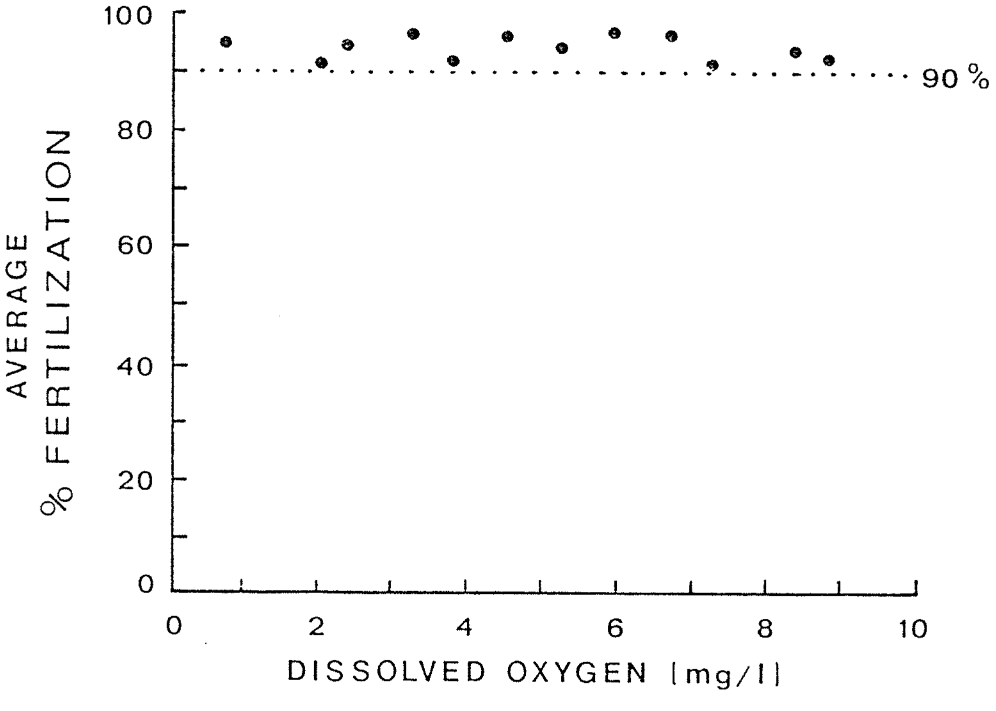


Figure 13. Average percent fertilization after 15-min "exposures" of sand dollar sperm to natural seawater with dissolved oxygen levels from approximately 1-9 mg/l.

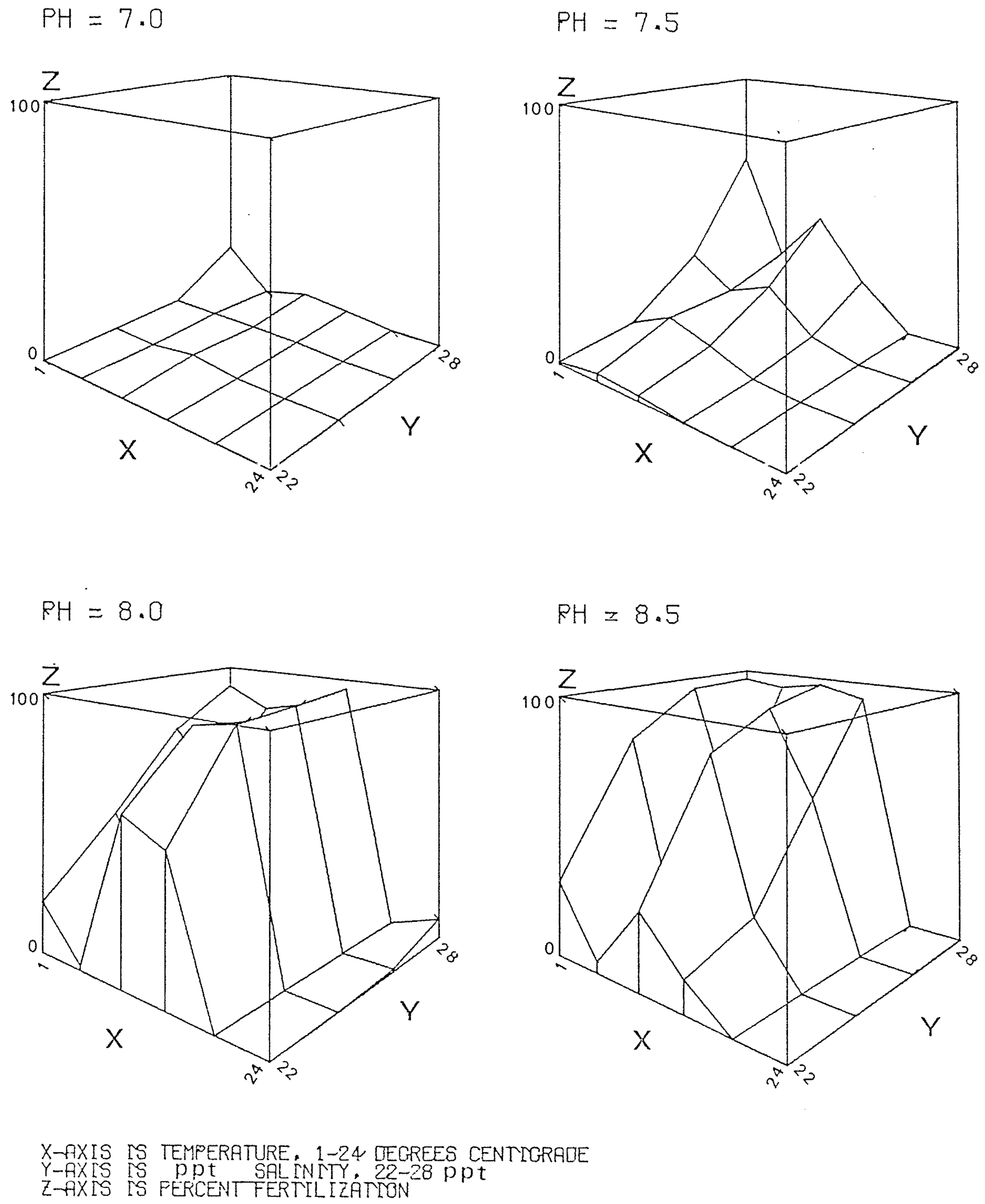


Figure 14. Response surface plots of average percent fertilization in a matrix design bioassay series at temperatures of 2, 7, 12, 17 and 22°C; salinities of 22, 24, 26 and 28 ppt; and pH's of 7.0, 7.5, 8.0 and 8.5 (sea urchin).

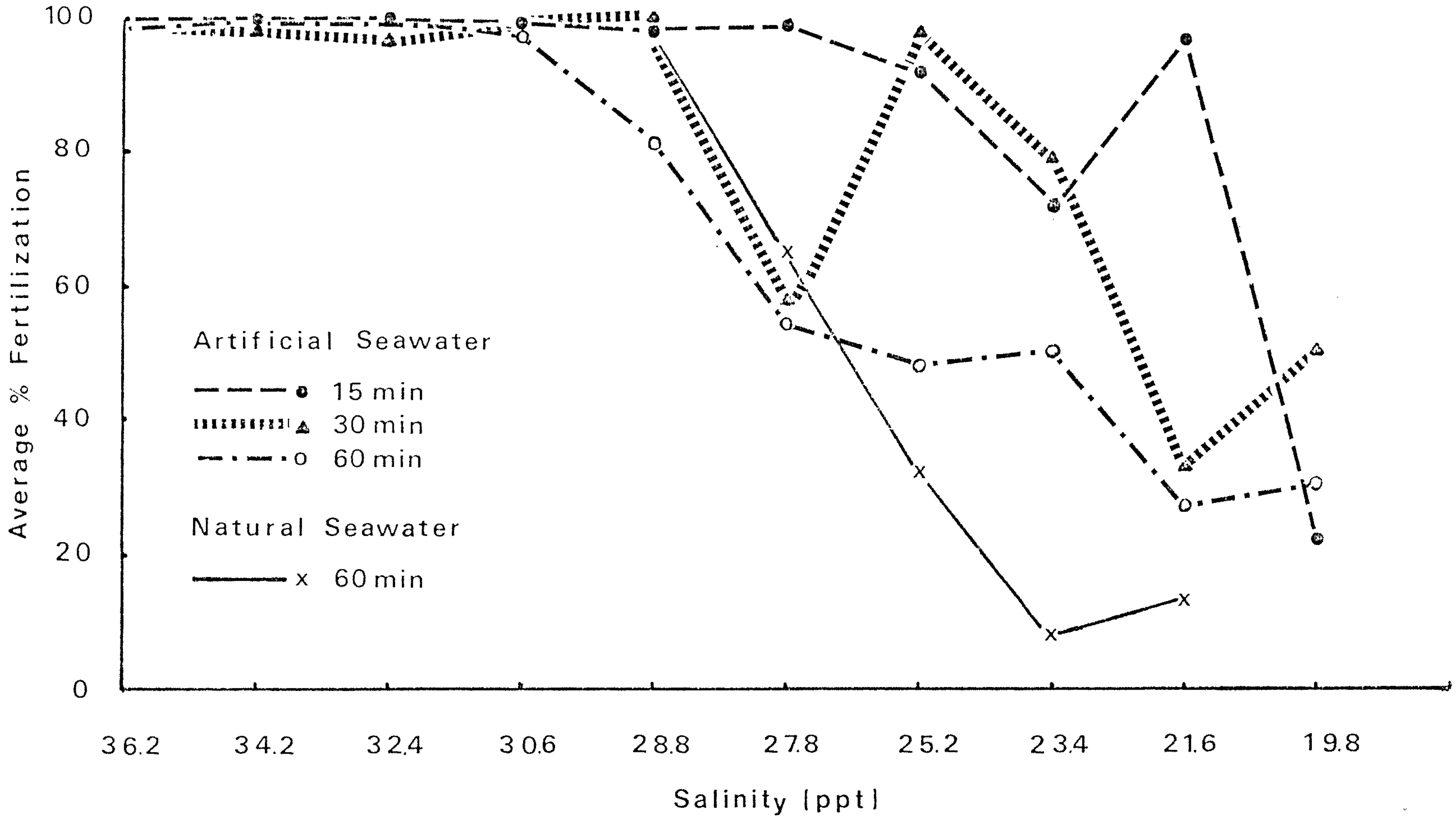


Figure 15. Fertilization success after 15-, 30- and 60-min sea urchin sperm "exposures" to artificial seawater and 60-min "exposures" to natural seawater at salinities from 19.8 - 36.2 ppt.

provide an excellent test medium for the fertilization bioassay, especially when dilution water of a known standard composition is desirable.

Silver

Time-Concentration--

Sperm bioassays of silver in natural seawater were conducted with 20 silver concentrations and sperm exposure times of 15, 30, 60 and 90 minutes. Each test was replicated two to four times.

The EC50's decreased with each increase in exposure time. The calculated EC50's and 95 percent confidence limits at 15, 30, 60 and 90 minutes were 590 ± 1861, 171 ± 13, 83 ± 19 and 52 ± 37 μg/l silver, respectively.

One factor which was not described by the EC50 analyses above is the apparent stimulatory effect of silver at concentrations less than the EC50 concentrations. The average plotted values for 30-, 60-, 90- and 120-min exposure tests clearly show higher than control fertilization for each concentration-response curve (Figure 16). This same effect was evident in most of the other sperm bioassays conducted with silver. The possible reasons for this low-level stimulation will be discussed later.

Sperm Versus Egg Sensitivity--

Tests were conducted to determine the relative sensitivity between sperm and eggs exposed to silver in seawater. Sperm exposed to silver for 30 minutes showed successful (> 96 percent) fertilization in concentrations of silver of up to 150 μg/l and declining fertilization success in ≥ 175 μg/l (Figure 17). When eggs only were exposed for 30 minutes, fertilization was successful in up to 200 μg/l silver. Sperm and eggs exposed simultaneously for 30 minutes were generally more sensitive to silver than either sperm or eggs alone. Concentrations of silver as low as 50 μg/l reduced fertilization success by approximately 50 percent.

A second test series focused on egg sensitivity to high concentrations of silver. This test showed that eggs remained viable for fertilization (≥ 95 percent) in silver concentrations as high as 8.0 mg/l. Eggs in 16.0 mg/l silver showed a reduction in fertilization to 38.5 percent, but "control" solutions of silver which were dosed with sperm and eggs simultaneously showed a similar reduction in fertilization at 16 mg/l. Thus, silver in seawater appears to affect primarily the viability of the sperm.

Effects of pH--

The toxicity of silver in seawater at pH 7.5, 8.0 and 8.5 was tested in one test series using sperm and eggs from one sea urchin pair and dosing duplicate samples for each silver concentration and pH. The EC50's and 95 percent confidence limits were essentially the same for each pH (Table 5). Thus, varying the pH of silver-seawater test solutions between 7.5 and 8.5 should not significantly alter the toxicity of silver.

Effects of Temperature and Salinity--

A matrix of bioassays was conducted using a range of silver concentra-

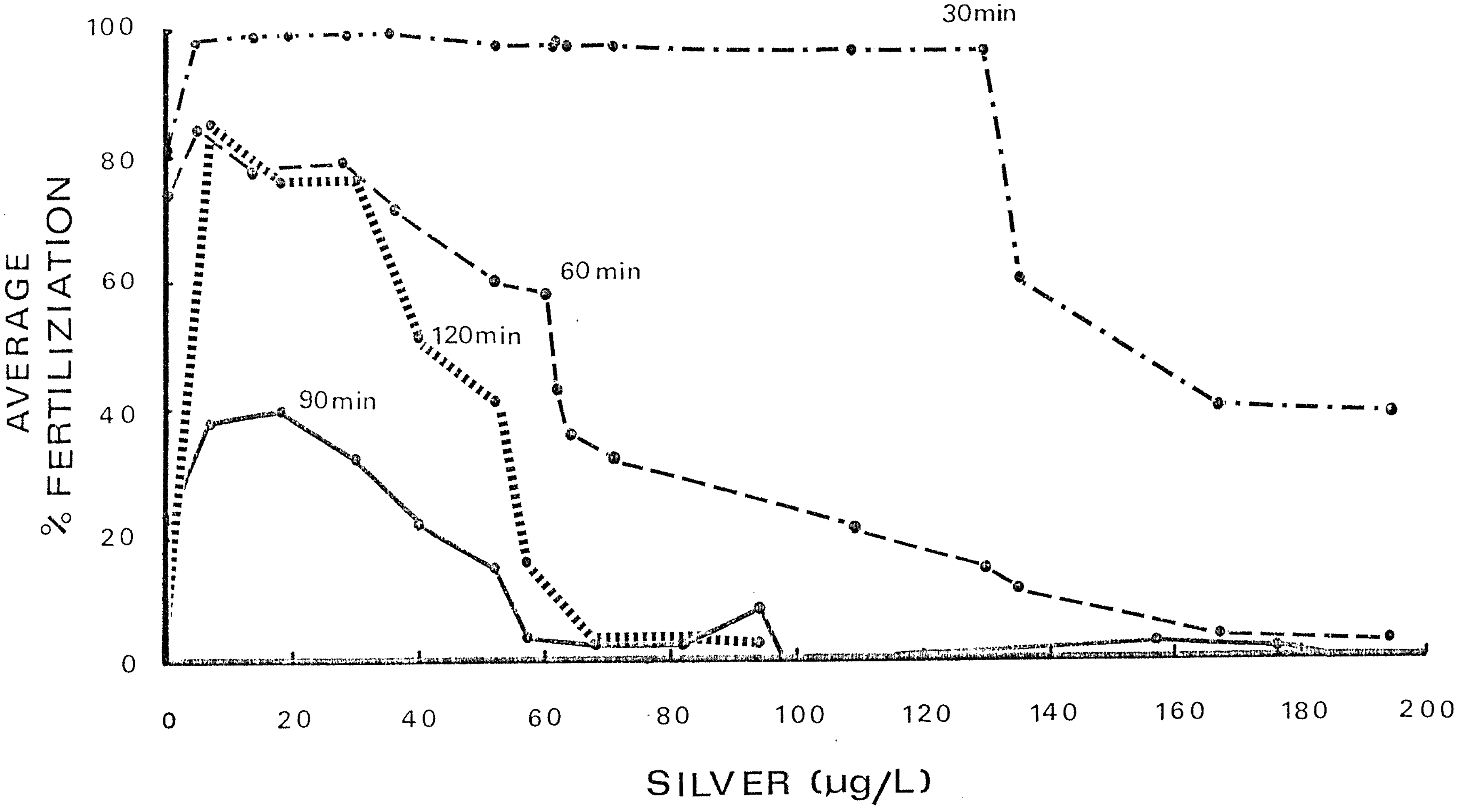


Figure 16. Average fertilization success after 30-, 60-, 90- and 120-min exposures of sea urchin sperm to silver in natural seawater.

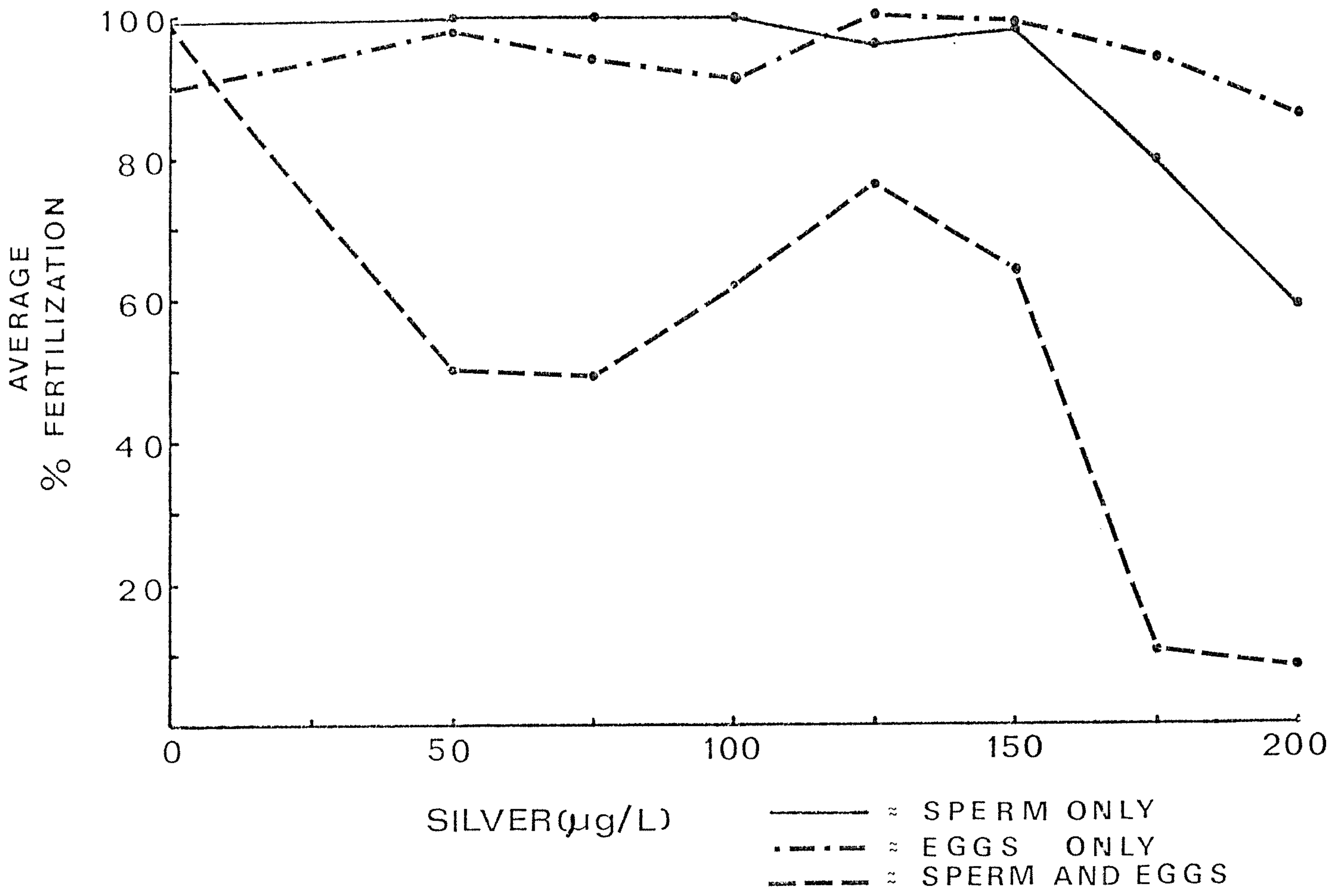


Figure 17. Average fertilization success after 30-min exposures of sea urchin sperm and/or eggs to silver in natural seawater at 27.9 ppt salinity, pH 8.0 and 7.7°C.

Table 5. Fifty % effective concentrations (EC50) and 95% confidence limits of silver test solutions at pH 7.5, 8.0 and 8.5.

pH	Average EC50 (μg/1)	95% Confidence Limits (μg/1)
7.5	261	20 - 502
8.0	252	0 - 608
8.5	241	185 - 307

Table 6. EC50's and 95% confidence limits of silver bioassays conducted at 3 different temperatures and salinities (pH = 8.0).

Temperature (°C)	Salinity (ppt)	Average EC50 (μg/1)	95% Confidence Limits (μg/1)
7	28	227	204 - 250
7	26	71	0 - 140
7	24	41	35 - 47
12	28	113	97 - 129
12	26	170	151 - 189
12	24	110	98 - 122
17	28	38	18 - 58
17	26	31	25 - 37
17	24	<19	—

tions in seawater at 7, 12 and 17°C; 24, 26 and 28 ppt salinity; and pH 8.0. Response surface plotting shows an obvious decrease in fertilization success with increasing silver concentration (Figure 18). Fertilization also generally declines with decreasing salinity for a given silver concentration. As noted earlier, green sea urchin sperm are probably stressed by any salinities below about 29 ppt. The effect of temperature is not quite as obvious. There appears to be a greater sensitivity to silver at the higher temperature range at each salinity and at the low temperature range at 24 ppt salinity. These same trends are shown by the individual test EC50's listed in Table 6.

Artificial Seawater--

Thirty- and 60-min sperm bioassays of silver in artificial seawater suggest that silver may be more toxic in artificial seawater than in natural seawater. The average EC50 and 95 percent confidence limits for the 30- and 60-min tests were 51 ± 197 and < 20 μg/l silver. These EC50 values are substantially less for equivalent EC50's in natural seawater (171 and 83 μg/l for 30- and 60-min EC50's).

Endosulfan

Time-Concentration Responses--

Sperm bioassays of endosulfan in natural seawater were conducted with nine endosulfan concentrations and sperm exposure times of 30, 60, 90 and 120 minutes. Each test was replicated four times.

The EC50 and 95 percent confidence limits of tests for 30-, 60-, 90- and 120-min exposure times were 353 ± 97, 143 ± 160, < 66 and 195 ± 102 μg/l endosulfan. The EC50 calculations for the 90-min exposure times were highly variable or not possible to calculate. The percent fertilization for all concentrations from 65.9 to 291.5 μg/l endosulfan in these tests was generally < 30 percent, making it difficult to calculate a regression equation.

The 90- and 120-min sperm bioassays utilized sperm/egg ratios of 2000/1 and 4000/1, respectively, in an attempt to maintain control fertilization at ≥ 90 percent. However, fertilization success in natural seawater controls for 90- and 120-min tests only averaged 10.5 and 53.0 percent, respectively. Reasons for poor control fertilization at the longer exposure periods are presently not known. Sperm senescence is one possibility. Even though concentrated batches of sperm maintain some viability for as long as 72 hours (Figure 9), it may be that activation is greatly enhanced (and thus, longevity reduced) when the sperm are diluted to 2×10^5/ml in the test tubes. Another possibility is that the sperm have "sticky heads" which cause them to "plate out" with time on the sides of the test tubes, thus reducing the number of sperm available for fertilization.

The reasons for the lower toxicity response seen in the 120-min tests are not clear. Some variable factor (possibly dissolved organic material) in natural seawater which has a capacity for binding dissolved endosulfan may be responsible for the fluctuating toxicities seen in many of the endosulfan bioassays.

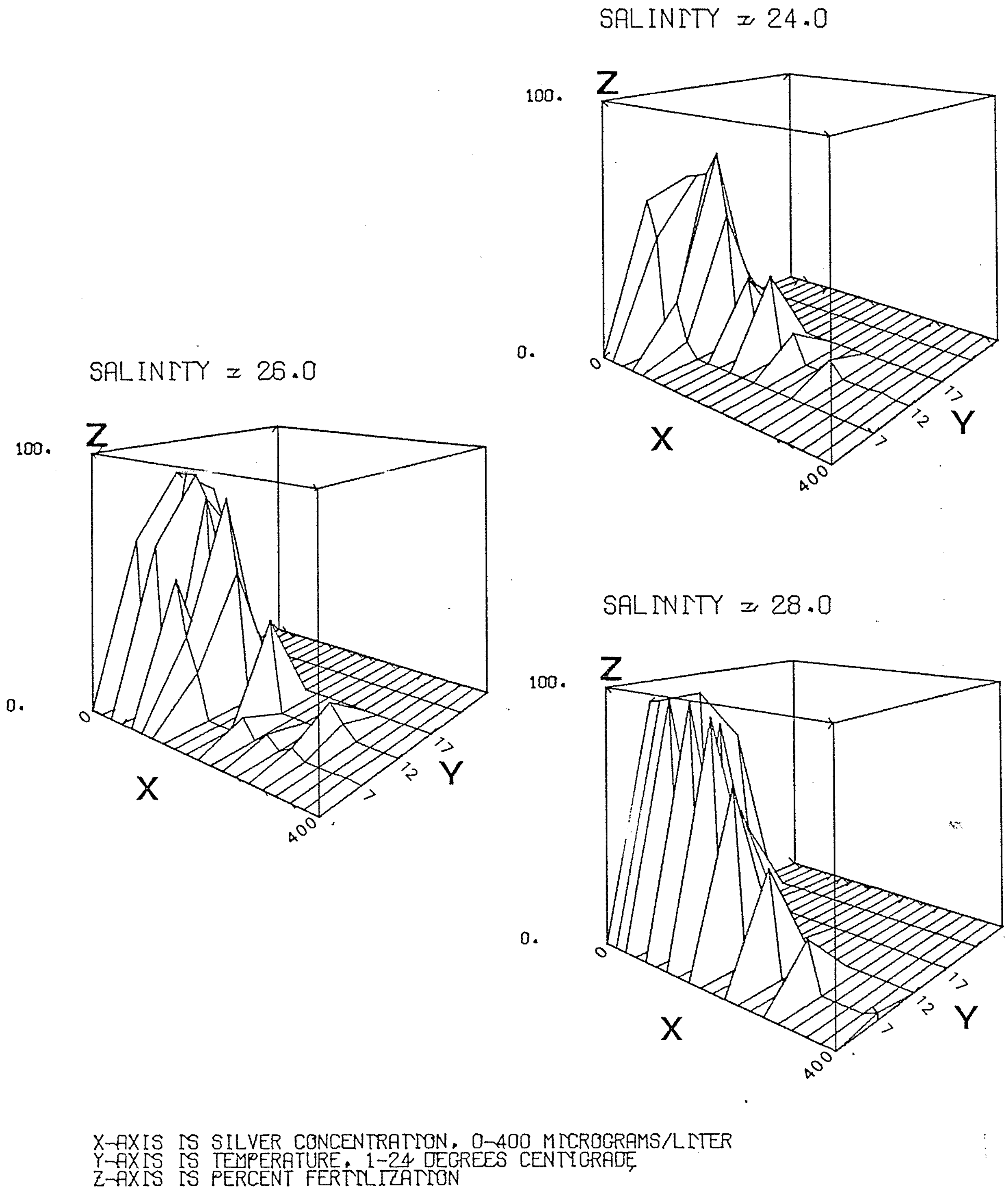


Figure 18. Response surface plots of average fertilization success after 30-min sea urchin sperm exposures to a matrix design bioassay series of 0-400 µg/l silver in natural seawater at 7, 12 and 17°C; 24, 26 and 28 ppt salinity; and pH 8.0.

One other factor which may have affected the results of the time-concentration bioassays was the presence of a small amount of acetone (approximately 20 µl/l) which was used as a solvent for the endosulfan in this test series. Fertilization in acetone-seawater controls was the same as in the normal seawater controls for the 30-min exposure tests and substantially greater for the 60-, 90- and 120-min bioassays. Acetone at a concentration of 20 µl/l in seawater may have a stimulatory effect on the sperm and eggs.

A bioassay test series of acetone in natural seawater showed > 90 percent fertilization in acetone concentrations up to 50 µl/l for up to 90-min bioassays. Fertilization was < 90 percent in acetone concentrations ≥ 100 µl/l.

All other endosulfan bioassays were conducted without the use of acetone (or other carrier solvent) to eliminate any possibilities of acetone-induced toxicity or stimulatory effect.

Sperm Versus Egg Sensitivity--

Eggs exposed to endosulfan in natural seawater for 60 minutes showed successful fertilization in concentrations of up to 750.5 µg/l, which was the limit of solubility of endosulfan in the batch of seawater used for this test series. The primary toxicity of endosulfan is to the sperm and not the eggs.

Effects of pH--

The effect of varying seawater pH on toxicity of endosulfan was determined by testing duplicate sets of samples at pH 7.5, 8.0 and 8.5 with the same sperm and egg solution. Thirty-min sperm exposures yielded EC50's and 95 percent confidence limits of 401 ± 51 and 460 ± 61 µg/l endosulfan for the pH 8.0 and 8.5 tests. No EC50 estimate was possible for pH 7.5 due to poor control fertilization. The present data do not lend themselves to comparisons of toxicity except to say that the toxicity is not substantially different between pH 8.0 and 8.5.

The EC50's determined for the pH tests are dependent on the forms of endosulfan that are used to calculate the amount of endosulfan in the samples. Normally, only endosulfan α and β isomers are used for the toxicity calculations. However, a third, presently unidentified form or degradation by-product (possibly endosulfan diol), is present in most bioassay samples analyzed by gas chromatography (GC). This unidentified form appears to vary with pH, being most prevalent at high pH values. In the endosulfan-pH test discussed above this third GC peak represents approximately 6, 12 and 43 percent of the total endosulfan at pH values of 7.5, 8.0 and 8.5 (Figure 6). Inclusion of this peak in the concentration calculations has the effect of increasing any individual EC50's by approximately 6, 12 and 43 percent at pH 7.5, 8.0 and 8.5, respectively. While any EC50 increases would be relatively minor at pH 7.5 and 8.0, the 43 percent increase at pH 8.5 produces a new EC50 of 582 versus 460 µg/l. Hence, the conversion of endosulfan α and β to this third form may decrease the toxicity of total endosulfan to sea urchin sperm.

Effects of Temperature and Salinity--

A matrix of bioassays was conducted using a range of endosulfan concentrations in seawater at 7, 12 and 17°C; 24, 26 and 28 ppt salinity and pH 8.0. Response surface plotting shows a decrease in fertilization success in both controls (0 µg/l) and endosulfan at 24.0 ppt salinity (Figure 19). Fertilization success was good in controls and low concentrations of endosulfan in 26.0 ppt salinity but decreased in the higher endosulfan concentrations (as compared with the response at 28.0 ppt salinity). Temperature interactions seemed to be an important factor only at 24.0 ppt salinity, where fertilization was nil in both controls and endosulfan at 17°C and in the endosulfan concentrations only at 12°C (Figure 19). The negative effect of increasing endosulfan concentration on fertilization is most clearly seen in the 26.0 ppt salinity plot. Fertilization was generally good in all but the highest concentrations at 28.0 ppt salinity. The average EC50's and 95 percent confidence limits of the endosulfan matrix bioassays are listed in Table 7.

Artificial Seawater--

Thirty- and 60-min sperm exposure tests were conducted with endosulfan dissolved in artificial seawater. The 30- and 60-min EC50's and 95 percent confidence limits of four replicate samples were 189 ± 78 and < 101 µg/l endosulfan, respectively. These values are somewhat less than the equivalent EC50's in natural seawater (353 and 143 µg/l).

Reasons for the possible increase of endosulfan toxicity in artificial seawater are not presently known. Degradation rates and products of endosulfan may be different between artificial and natural seawater. Unlike natural seawater, artificial seawater contains few dissolved organics which might complex with endosulfan. Further testing with natural and artificial seawater will be necessary to resolve the differences.

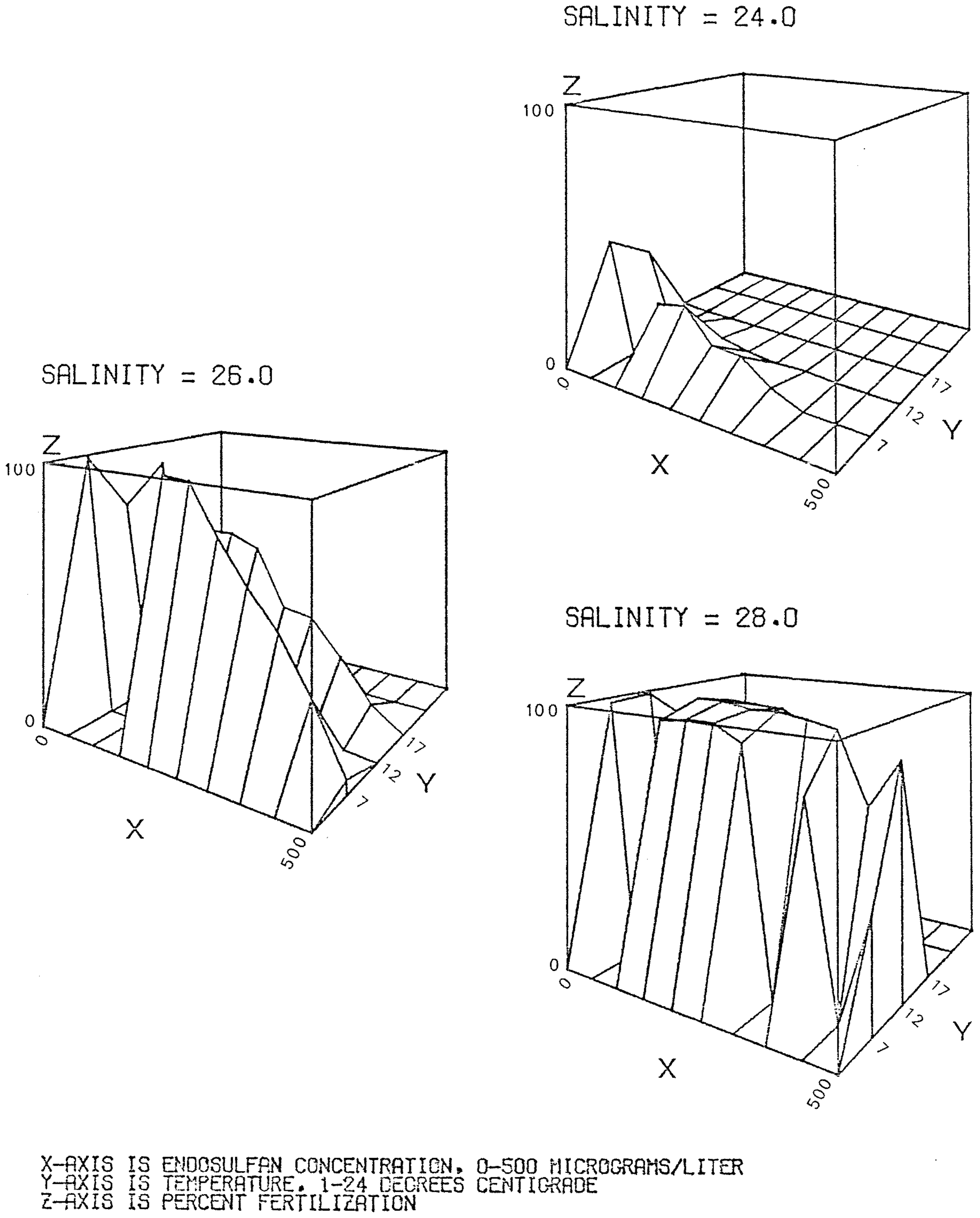
Summary of Sea Urchin Sperm Bioassay Data--

Individual and mean EC50's, fiducial limits, confidence limits and test conditions of silver and endosulfan bioassays are summarized in Appendix Tables 2 and 3.

EMBRYO BIOASSAYS

Silver

A 96-h bioassay of sea urchin embryos was conducted to determine the relative toxicity of silver to sperm and developing embryos. The results show that there was no evident effect on development at initial calculated silver concentrations of 3.12, 6.25, 12.5 and 25.0 µg/l (Figure 20). Retardation of development is evident at 50 and 100 µg/l. All embryos in 200 µg/l silver were killed at the post-hatch blastula stage and were undergoing cytolysis. Embryos in 400 µg/l silver were arrested in the pre-hatch 32-128 cell stages, but were still alive. Evidently, the embryos in the 400 µg/l silver were partially protected from its toxic effects by the fertilization membrane which remains intact until hatching. Embryos in the 200 µg/l silver were probably killed when they hatched out of this protective membrane.


SALINITY = 24.0

SALINITY = 26.0

SALINITY = 28.0

Figure 19. Response surface plots of average fertilization success after 30-min sea urchin sperm exposures to 0-500 µg/l endosulfan in natural seawater at 7, 12 and 17°C; 24, 26 and 28 ppt salinity; and pH 8.0

TABLE 7. AVERAGE EC50's AND 95% CONFIDENCE LIMITS OF ENDOSULFAN BIOASSAYS CONDUCTED AT 3 DIFFERENT TEMPERATURES AND SALINITIES (pH = 8.0).

Temperature (°C)	Salinity (ppt)	EC50 (µg/l)	95% CONFIDENCE LIMITS (µg/l)
7	28	524	496-554
7	26	381	363-398
7	24	< 88	—
12	28	569	525-613
12	26	86	0-172
12	24	< 83	—
17	28	405	355-455
17	26	216	82-350
17	24	< 84	—

TABLE 8. DEVELOPMENT PATTERNS OF SEA URCHIN EMBRYOS DURING A 72-HOUR EXPOSURE TO SILVER IN NATURAL SEAWATER.

Silver (µg/l)	% Composition		
	Blastula	Gastrula	Prism
0	0	97	3
5	4	75	21
10	3	86	11
20	5	83	12
40	8	87	5
80	32	68	0

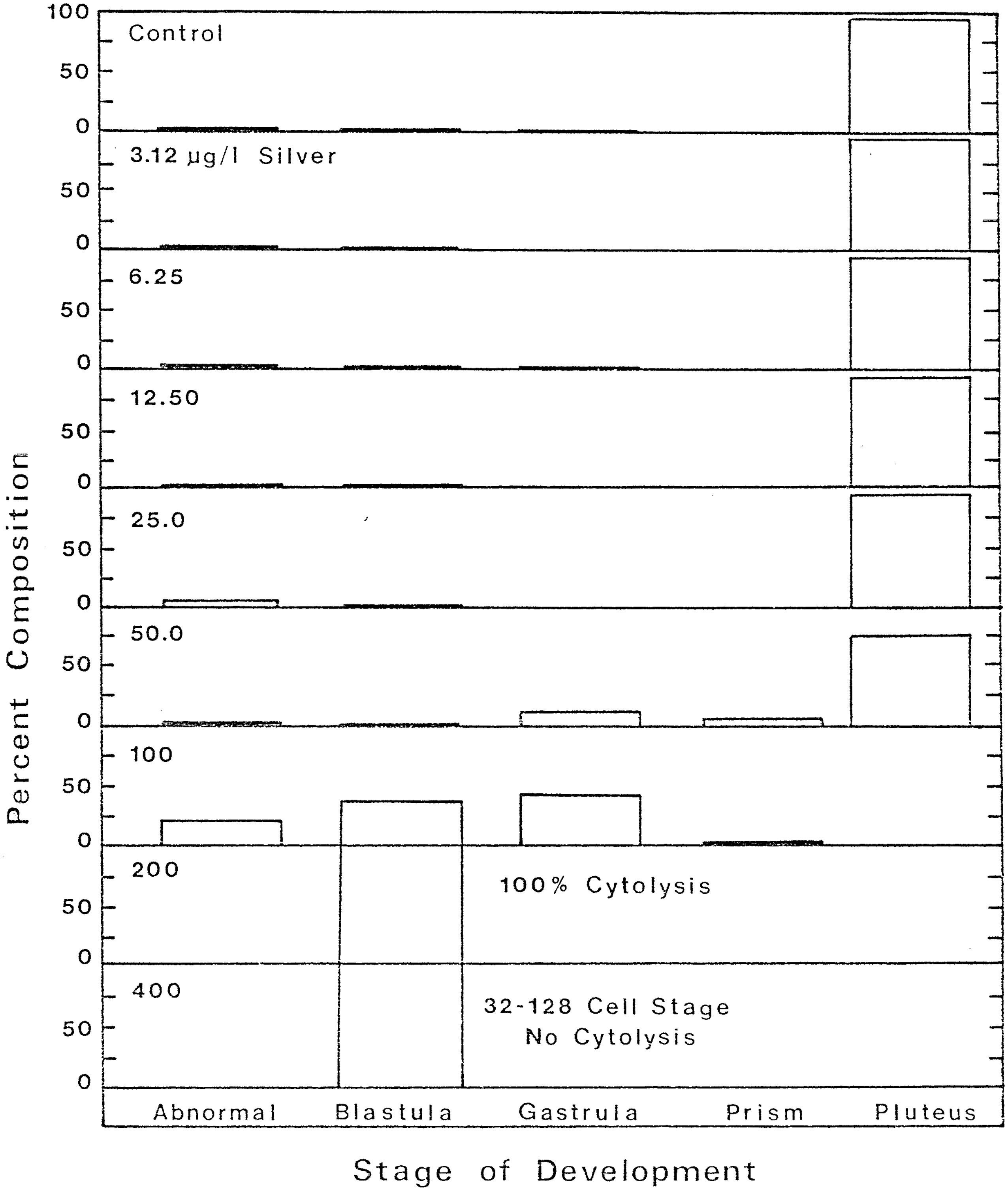


Figure 20. Percent composition of the stages of development of sea urchin embryos after 96-h exposures to 0-400 µg/l silver in natural seawater.

The retardation in development of embryos at silver concentrations of 50-100 µg/l compares favorably with the 60- and 90-min sperm test EC50's of 83 and 52 µg/l. Sea urching sperm appear to be as sensitive to low concentrations of silver in seawater in 60- and 90-min exposures as the developing embryos are in 96-h exposures.

The similarity in sensitivity between sperm and embryos is reinforced by an earlier preliminary 72-h embryo test with silver which was terminated before the embryos reached the pluteus stage. The results of this test showed accelerated development of embryos (i.e. more have reached the prism stage) in 5, 10 and 20 µg/l silver; a normal development rate in 40 µg/l; and retardation of development in 80 µg/l (Table 8). This same pattern of low-level silver stimulation was illustrated for the sperm bioassays in Figure 16.

Endosulfan

A 96-h sea urchin embryo bioassay of endosulfan in seawater resulted in only minor effects to the embryos over the range of concentrations tested. The embryos showed essentially the same pattern of development as controls in initial concentrations of endosulfan of 63.0 - 231.6 µg/l. Development was slightly retarded in 264.9 and 253.1 µg/l (Figure 21). Final concentrations of endosulfan measured in the test beakers after 96 h were an average of 67.2 percent lower than initial values. Hence, it is difficult to define an exact concentration of endosulfan which may have produced the retardation in the two highest concentrations. However, it is evident that some concentration of endosulfan between 67.5 and 264.9 µg/l did produce an embryo response. These values are roughly equivalent to the sperm bioassay EC50's of 143 and < 66 µg/l endosulfan for 60- and 90-min sperm exposures. The short-duration sperm bioassay is of approximately the same sensitivity to endosulfan as the 96-h embryo bioassays.

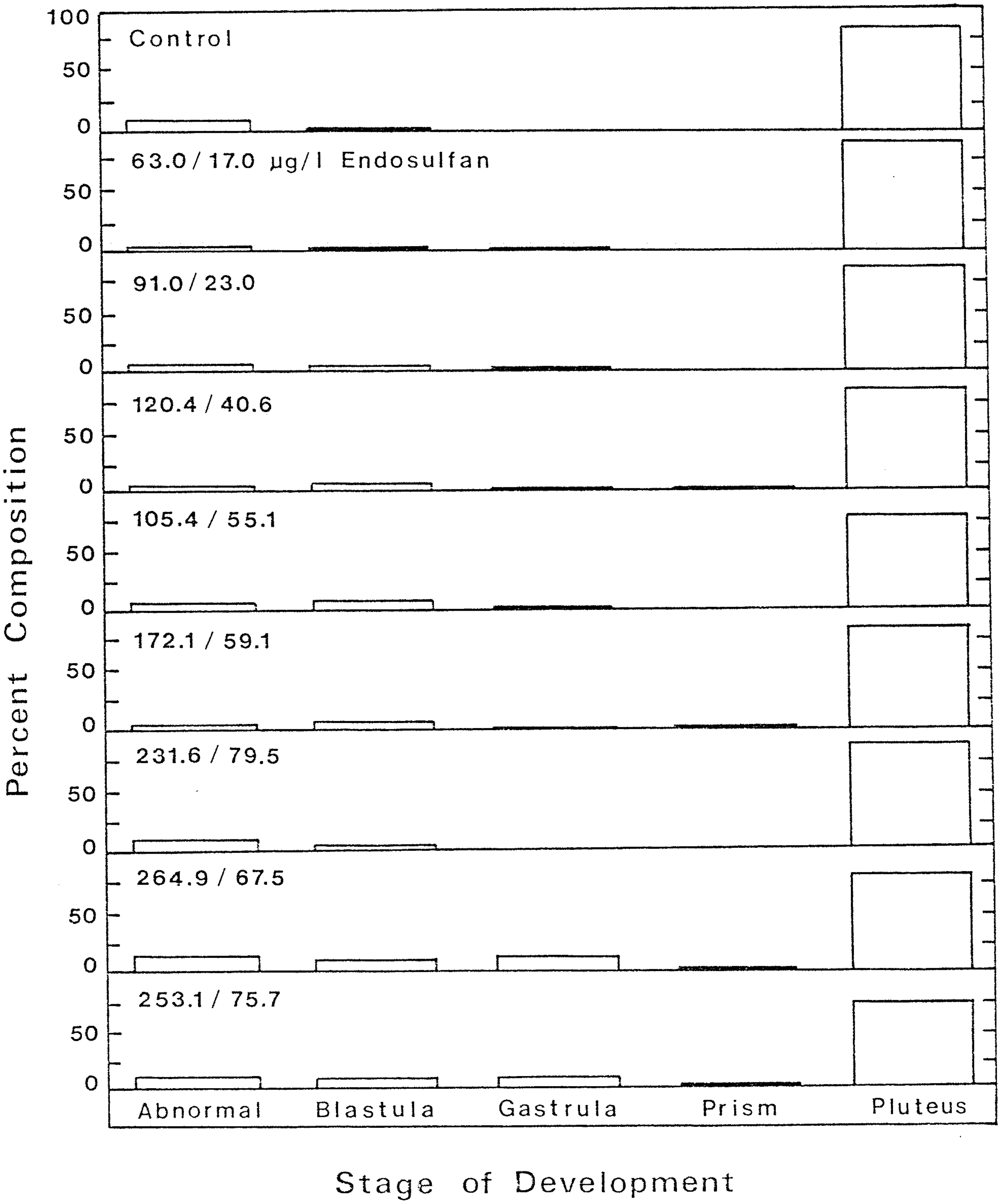


Figure 21. Percent composition of the stages of development of sea urchin embryos after 96-h exposures to 0 - 264.9 (initial) ug/l endosulfan in natural seawater. Endosulfan concentrations at the end of 96-h are also indicated (i.e. initial/final).

SECTION VII

SAND DOLLAR BIOASSAYS

SPERM BIOASSAYS

Physical Parameters

Spawning--

Initiation of spawning activity of sand dollars was monitored during the spring of 1979. Approximately half of the sand dollars injected with 0.5 ml of 0.5 M KCl spawned slightly from April 4 to June 29, 1979 (Table 9). Spawning was substantially improved on July 5 (6/8 spawned) with males producing satisfactory quantities of sperm. Egg quantity and maturity was assessed from July 5 - July 27, 1979. Females showed a gradual increase from minimal egg production on July 5 and 12 to an average of 553,880 eggs per female on July 27 (Table 10). Maturity of the eggs was evaluated by adding an excess of sperm for fertilization and scoring the presence or absence of the fertilization membrane in a subsample of 100 eggs. Fertilization success gradually increased from an average of 66.6 percent on July 5 to 98.6 percent on July 27 (Table 10).

Termination of spawning was observed during the fall of 1978 and 1979. The fall 1978 spawn-out occurred approximately the last week of September, as the sand dollars were difficult to spawn with KCl on September 26, 1978. This date closely corresponded to the time when ambient water temperatures began the winter decline (Figure 7). The spawn-out pattern was similar for fall 1979 with 8/10 of the animals spawning on September 26; 4/10 spawning on October 6 and only 1/10 spawning on October 16, 1979.

Sand dollars were not held in thermally conditioned seawater toward the end of their spawning cycle. However, successful extension of the sea urchin spawning cycle suggests that sand dollars can also be held in a ripe condition past normal spawn-out time by holding at a constant temperature of approximately 13-15°C.

Sperm and Egg Quantification--

The density of sperm and eggs in the initial spawning beakers were microscopically assessed by one or two counts before diluting to a standard density (see Appendix Table 1). To assess the accuracy of these counts for determining the dilution factor, 8 samples of sperm and 10 samples of eggs were subjected to 10 replicate counts and the means, 95 percent confidence limits and ranges were recorded (Table 11). The 95 percent confidence limits indicate that the population means should fall within ± 21.5 and

Table 9. Pattern of early spawning of sand dollars (induced with approximately 0.5 ml KCl) held in ambient temperature seawater.

Date	# Spawned/# Injected with KCl	
4 April 1979	4/4 (Slightly)	
8 June	5/10 (Slightly)	All male
15 June	5/8 (Slightly)	1 male, 4 female
29 June	5/8 (Slightly)	3 male, 2 female
5 July	6/8	3 male, 3 female

Table 10. Average number of eggs and their viability as obtained from sand dollars early in the spawning season.

			% Fertilization	
Date	Sample size	Average number of eggs/female	Mean	Standard deviation
5 July 1979	5	Minimal	66.6	10.6
12 July	5	Minimal	87.2	2.6
20 July	5	474,200	94.0	3.5
27 July	5	553,880	98.6	0.8

Table 11. Mean, 95% confidence limits and range values /ml of 10 replicate counts of different sand dollar sperm and egg solutions

SAMPLE	MEAN ± 95% CONFIDENCE LIMITS	RANGE
	Sperm (x10^7)	
1	5.3 ± 1.1	3 - 6
2	18.0 ± 2.5	14 - 26
3	12.7 ± 2.1	7 - 18
4	25.0 ± 3.2	20 - 34
5	25.2 ± 2.3	21 - 30
6	18.3 ± 2.7	12 - 23
7	4.9 ± 0.9	3 - 6
8	42.2 ± 4.0	34 - 50
	Eggs	
1	263.0 ± 37.4	220 - 350
2	123.0 ± 18.5	90 - 180
3	553.0 ± 42.4	450 - 650
4	446.0 ± 43.2	340 - 550
5	236.0 ± 29.8	160 - 290
6	662.0 ± 58.6	540 - 800
7	673.0 ± 53.0	530 - 800
8	319.0 ± 25.8	260 - 370
9	413.0 ± 44.7	330 - 540
10	438.0 ± 54.9	310 - 540

± 15.0 percent of the sample means for sperm and egg counts, respectively. However, as pointed out in Section IV for the sea urchins, the variation between the individual counts is important since the dilution factor was normally determined by only one of two counts per sample. The range values (Table 11) show deviations from the means by as much as 44.8 percent for sperm counts and 46.3 percent for egg counts. Using a target sperm/egg ratio of 5,000,000/5,000 (1,000/1) for each bioassay container, the worst case counting-dilution error could produce sperm/egg ratios as low as 2,760,000/7,315 (= 377/1) or as high as 7,240,000/2,685 (= 2,696/1) for single subsample counts. Based on the sperm/egg ratio tests (see below) for sand dollars, both of these sperm/egg ratios should produce > 90 percent control fertilization. However, the overall sensitivity of the sperm bioassay is partially dependent on the sperm/egg ratio used in the test. Thus, reduction of the counting-dilution error would help to refine the precision of the test.

Effect of Sperm/Egg Ratio--

Three replicated tests were conducted during the 1978 spawning season to determine the minimum sperm/egg ratio required for successful fertilization in control seawater. Average fertilization was > 90 percent at sperm/egg ratios of 500/1 and above for 15-min "exposures" to natural seawater. Ratios ≤ 100/1 produced < 90 percent fertilization (Figure 22). Additional sperm/egg ratio tests were conducted for 30-, 60-, 90- and 120-min sperm "exposure" times during the 1979 spawning cycle. Results of four tests at each "exposure" time generally indicated successful fertilization at sperm/egg ratios ≥ 250/1. Average fertilization success began to decline below 90 percent at sperm/egg ratios ≤ 125/1 (Figure 24). Hence, the usual dosing level of 1000 sperm to 1 egg should normally provide adequate sperm for good control fertilization for sperm "exposure" times up to 120 minutes if the counting-dilution errors are minimized.

All subsequent sperm bioassays with silver and endosulfan were conducted at a sperm/egg ratio of 1000/1 so that the results could be related to the sea urchin bioassays described in Section VI.

Sperm and Egg Viability Through Time--

A preliminary set of six tests investigated the length of time which sand dollar sperm remained viable. Average fertilization of five out of six of the tests showed > 95 percent fertilization for at least 390 minutes and a decrease to 18 percent in 24 hours (Figure 23). One test out of the six showed a marked reduction in sperm viability after only 60 minutes with a decline to 0 percent fertilization in 240 minutes. Thus, monitoring of the control fertilization during tests is important to ensure that an occasional "weak" batch of sperm is weeded out. Figure 23 also shows an increase in fertilization success between 30 and 60 minutes. This effect was also seen for sea urchins (Figure 9) and may indicate that sperm are not fully activated for 1-2 hours after dilution with seawater.

Temperature, Salinity and pH--

To assess the interactive relationships between salinity, temperature, pH and sand dollar fertilization success, a matrix of tests at six salinities, five temperatures and four pH values was conducted using tap water dechlorin-

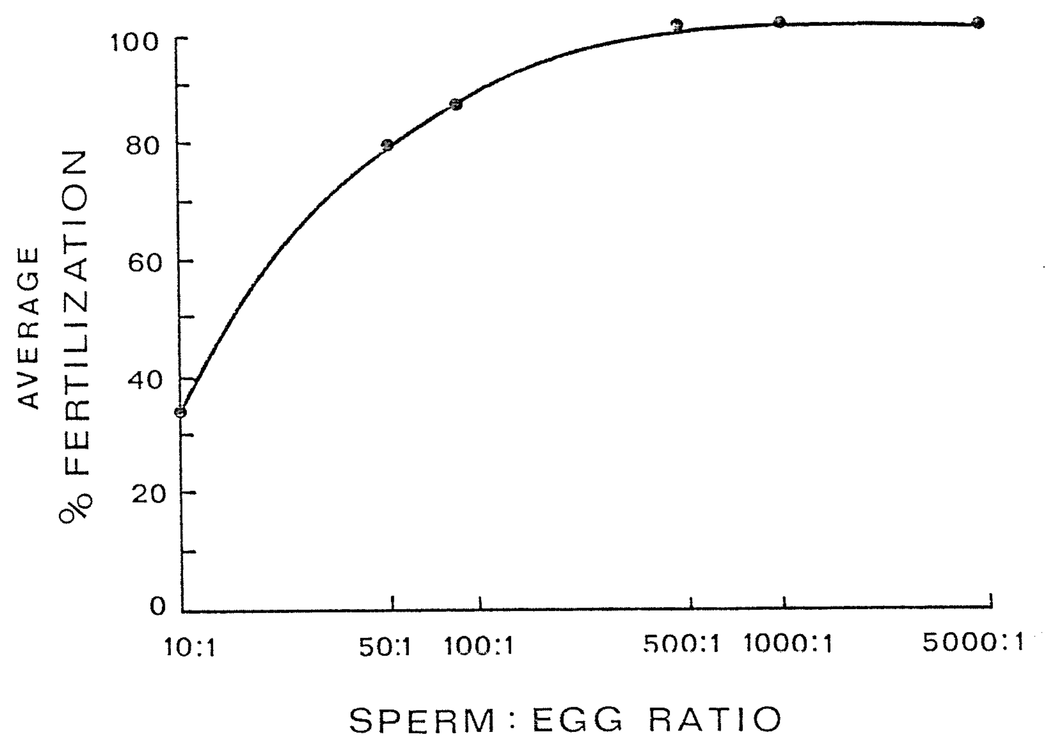


Figure 22. Average percent fertilization after 15-min sand dollar sperm "exposures" to natural seawater at sperm/egg ratios of 10-5000/1.

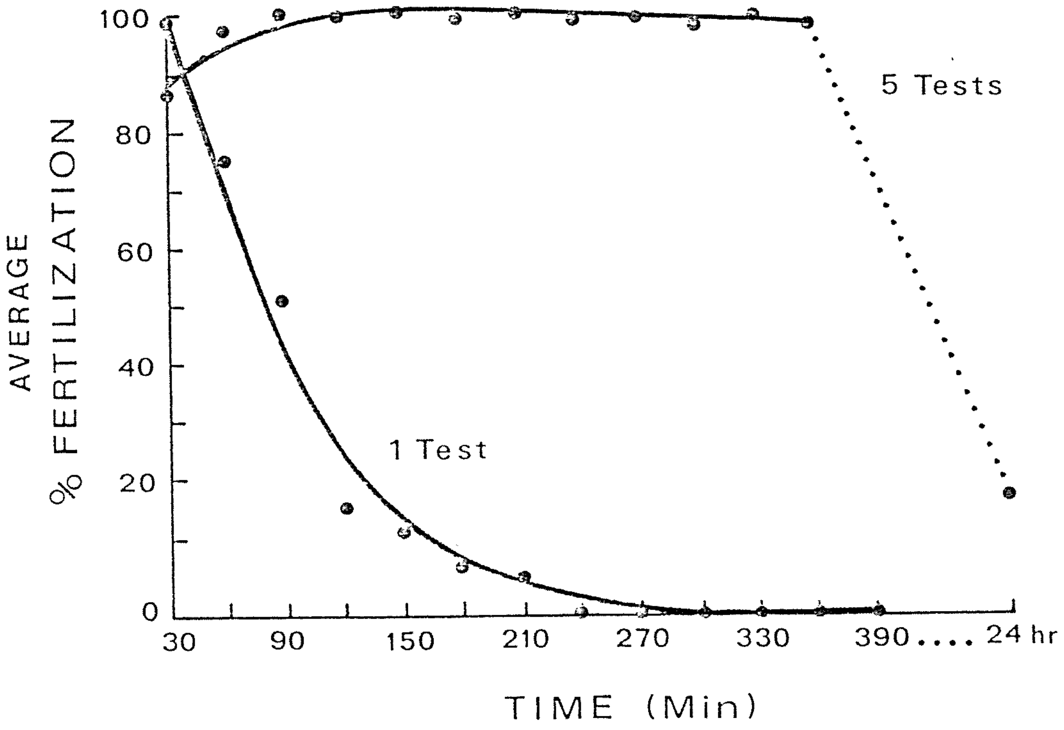


Figure 23. Long-term viability of sand dollar sperm held at concentrations of 50 x 10^6/ml of natural seawater as indicated by average fertilization success over a 24-h time period.

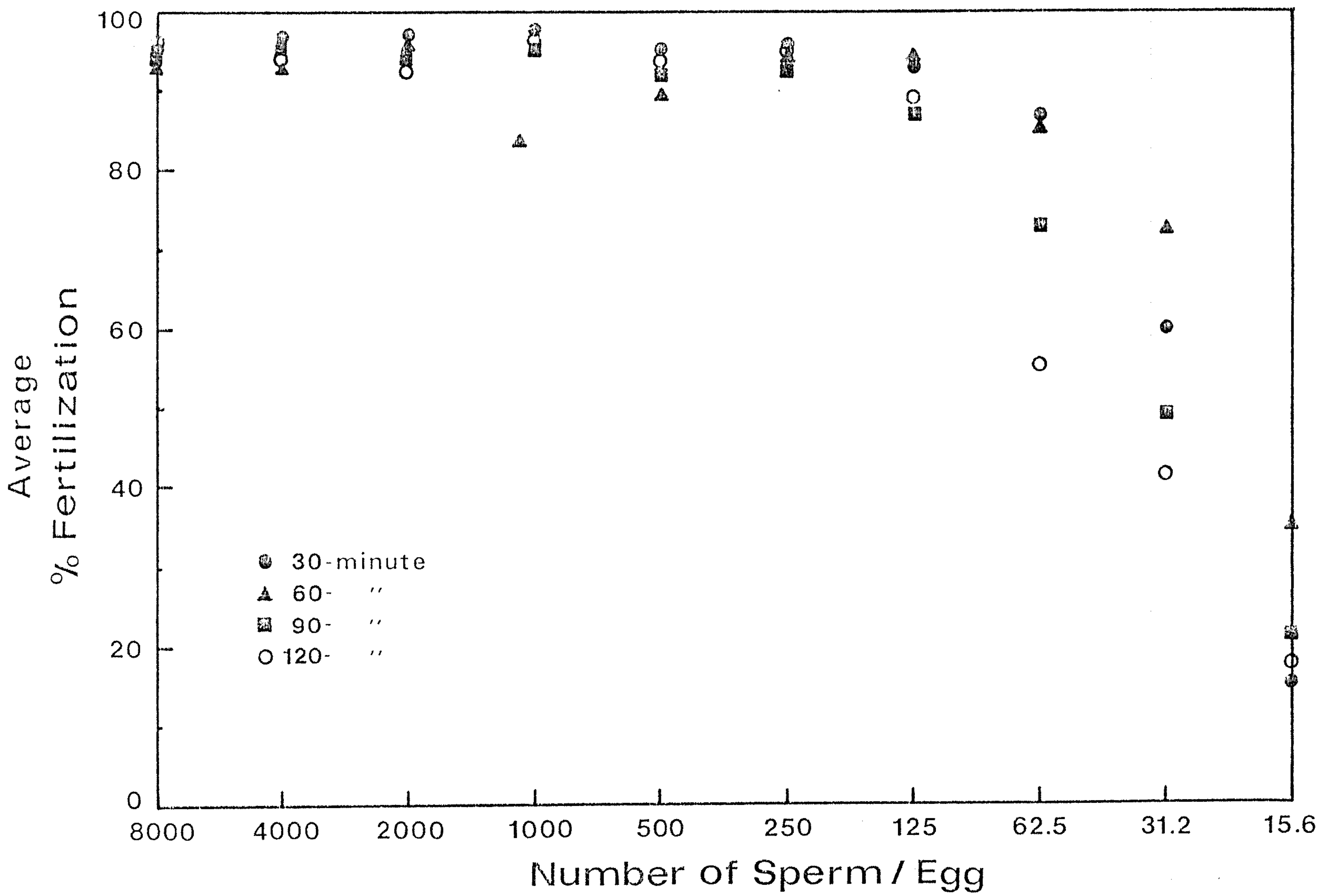


Figure 24. Average percent fertilization after 30-, 60-, 90- and 120-min sperm "exposures" to natural seawater at sperm/egg ratios of 15.6 - 8000/1.

ated with an activated carbon cartridge as the dilution water for salinity reduction. Response surface plots of these tests (Figure 25) indicate that fertilization success was generally best at pH 8.0, 28 ppt salinity and temperatures of 7-17°C. Fertilization was generally poor at all salinities < 28 ppt, especially at pH values < 8.0.

Subsequent matrix tests with silver and endosulfan suggested that the fertilization success of the tests decribed above was less than should have been expected. The dilution water in the toxicant tests was deionized water from the Fisheries Water Quality Laboratory instead of West Point dechlorinated tap water. We hypothesized that the tap water produced a toxic response not related to the simple osmotic stress of the lowered salinity. To test this hypothesis, the interactive test matrix of salinity, pH and temperature was repeated using deionized water. The results of this second set of tests showed marked increases in fertilization success throughout the entire set of tests (Figure 26). This set of tests showed generally successful fertilization at salinities of 24, 26 and 28 ppt, at the median temperatures of 7, 12 and 17°C, and at pH values of 7.0, 7.5 and 8.0. Temperatures of 2, 22 and 27°C; salinities < 24 ppt and a pH of 8.5 appear to be relatively stressful conditions for sperm functioning.

These two sets of tests emphasize the sensitivity of sperm cells to poor water quality and underscore the need for quality control in all sperm bioassays. Dilution water must be of high quality and bioassay test tubes and spawning containers must be carefully chosen and cleaned. The spawning containers and other laboratory test supplies that may come into contact with the eggs or sperm must be considered as possible sources of toxicity. Glass is generally acceptable, while some plastics have proven to be toxic to the sensitive stages of marine organisms (Bernhard and Zattera, 1970). Certain detergents have also been shown by Bernhard and Zattera to interfere with successful cultivation of marine organisms.

Dissolved Oxygen--

Bioassays of dissolved oxygen content (reduced with nitrogen gas) showed that sperm remained viable during 15-min exposures to levels of dissolved oxygen as low as 0.8 mg/l (Figure 13). The use of sperm bioassay in waters low in dissolved oxygen should pose no problem.

Silver

Time-Concentration Responses--

Sand dollar sperm bioassays of silver in natural seawater were conducted with nine silver concentrations and sperm exposure times of 15, 30, 60 and 90 minutes. Each test was replicated four times. The average EC50's and 95 percent confidence limits for 15-, 30-, 60- and 90-min exposure times were 201 ± 105, 124 ± 27, 47 ± 11 and 25 ± 12 μg/l silver, respectively.

One interesting characteristic of silver bioassays is that there typically is an increase in fertilization above 0.8 mg/l, especially in the 0.8-1.2 mg/l range (Figure 27). The reasons for this are not entirely understood, however, the 0.8-1.2 mg/l range of silver in seawater represents the concentrations which are at the limits of solubility (at 12°C). Super-

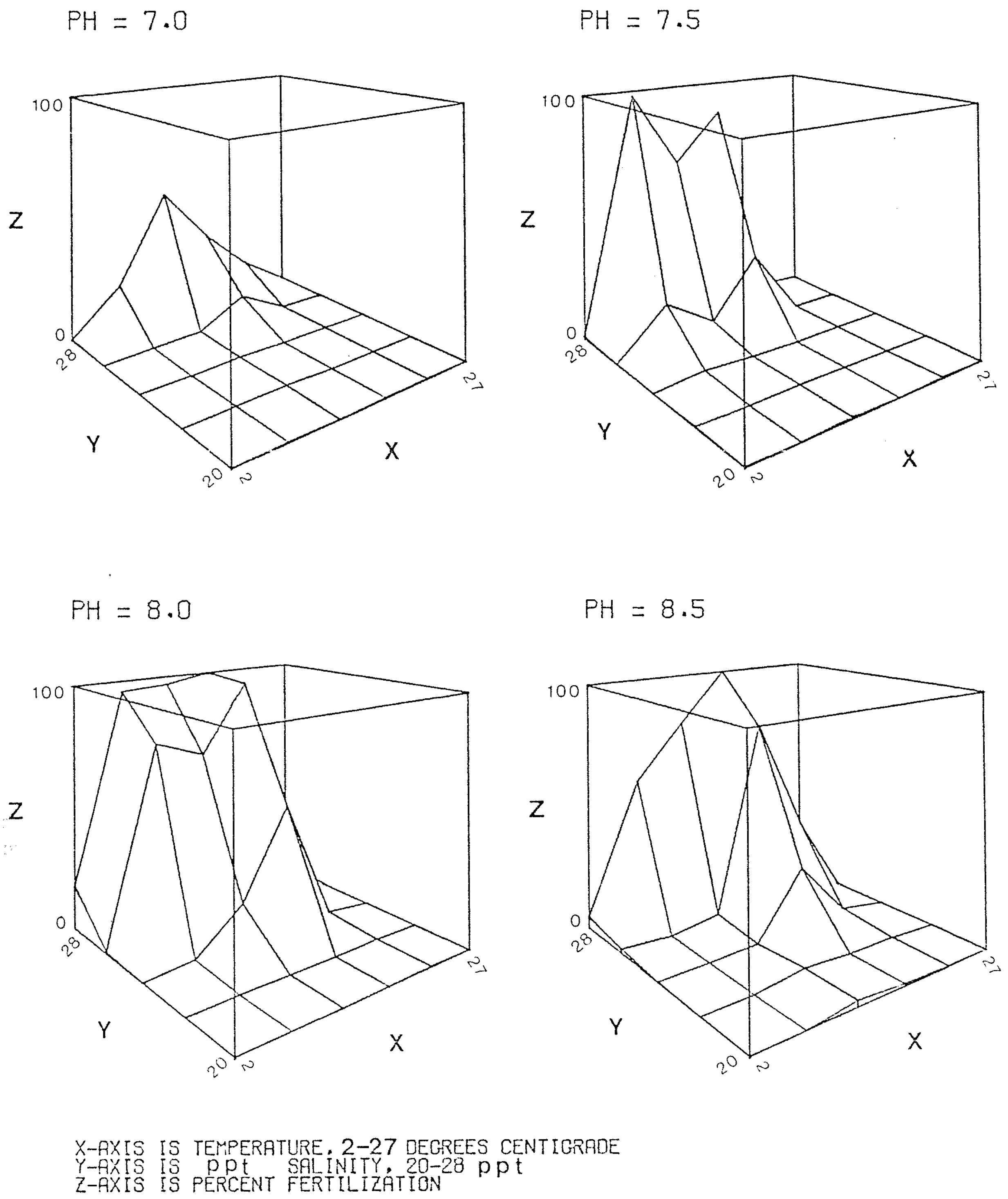


Figure 25. Response surface plots of average fertilization success after 30-min sand dollar sperm "exposures" to natural seawater at 2, 7, 12, 17, 22 and 27°C; 20, 22, 24, 26 and 28 ppt salinities; and pH's 7.0, 7.5, 8.0 and 8.5. Tap water dechlorinated with activated carbon was the dilution water.

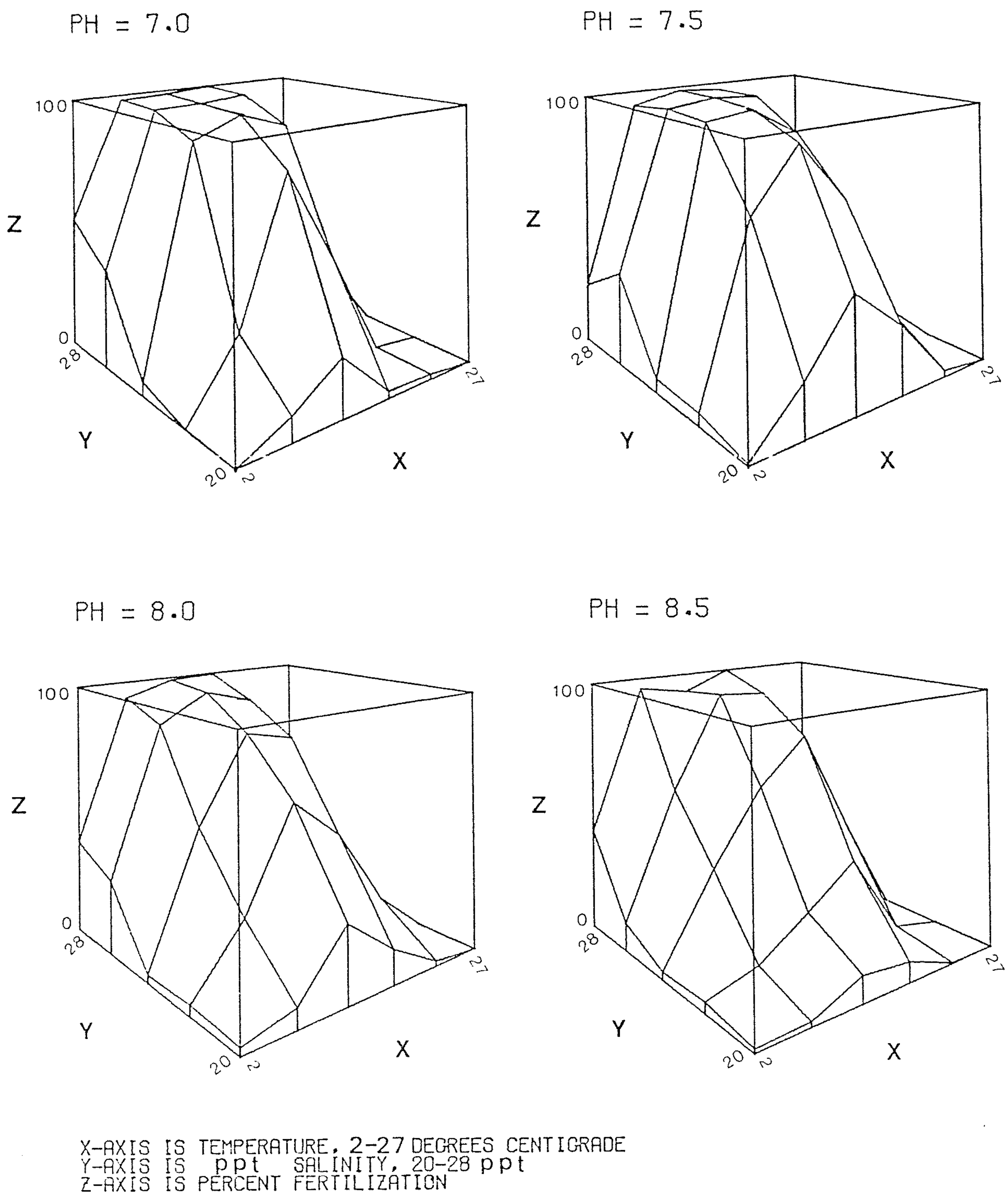


Figure 26. Response surface plots of average fertilization success after 30-min sand dollar sperm exposures to natural seawater at 2, 7, 12, 17, 22 and 27°C; 20, 22, 24, 26 and 28 ppt salinities; and pH's 7.0, 7.5, 8.0 and 8.5. Deionized water was used as the dilution water.

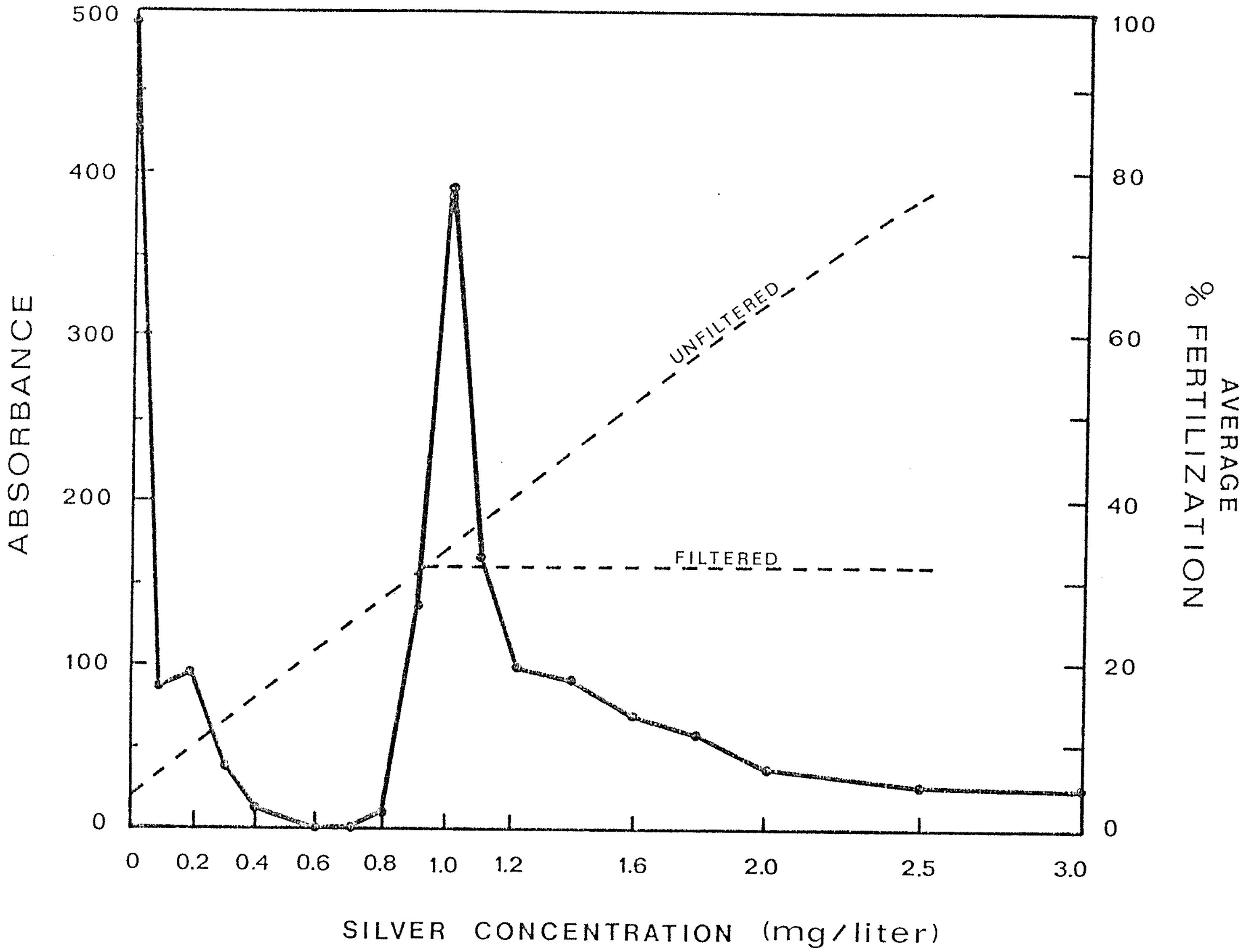


Figure 27. Average percent fertilization after 15-min exposures of sand dollar sperm to 0-3.0 mg/l silver in natural seawater. Also shown in relation to the fertilization curve is the relationship between silver recovery by AAS before and after filtration at 0.45 µm.

imposed on Figure 27 are lines showing the expected recovery of silver by AAS for filtered and unfiltered samples of silver in seawater. These lines suggest that the dramatic increase in fertilization may be due to a change in form (i.e. decrease in ionic silver) or the physical availability of silver related to the saturation point of silver in seawater. Additional work will be required to substantiate this hypothesis.

Sperm Versus Egg Sensitivity--

Sand dollar eggs exposed to silver in seawater (average EC50 > 250 μg/l) proved to be much less sensitive than sperm exposed to the same silver concentrations (average EC50 = 48 μg/l) for 60 minutes (Figure 28). The sensitivity of both sperm and eggs exposed simultaneously for 60 minutes (exposed in separate tubes and then added together at 60 minutes) was essentially the same as sperm exposed alone (EC50 = 54 μg/l). These results indicate the action of silver is primarily on the sperm cell and that exposure of the eggs does not produce an additive toxic response.

Effect of pH--

Sand dollar sperm bioassays of silver were conducted in natural seawater adjusted to pH 7.5, 8.0 or 8.5 with HCl or NaOH. The EC50's and 95 percent confidence limits for tests at pH 7.5, 8.0 and 8.5 were 53 ± 8, 50 ± 7 and 41 ± 4 μg/l silver, respectively. The EC50's are essentially the same for pH values of 7.5 and 8.0 indicating no significant difference in silver toxicity. A slight reduction of the EC50 at pH 8.5 was observed. However, response surface plots of sperm bioassays of natural seawater at various pH values, temperatures and salinities (Figures 25 and 26) indicate slightly reduced fertilization as pH increases from 8.0 to 8.5. The slight increase of toxicity of the silver bioassay at pH 8.5 may be due to the increased pH rather than an increase in the toxic effects of silver.

Effects of Temperature and Salinity--

A matrix of bioassays was conducted using a range of silver concentrations in natural seawater at 7, 12 and 17°C; 24, 26 and 28 ppt salinity and pH 8.0. Response surface plotting of the results (Figure 29) shows generally increasing toxicity with increasing silver concentration and decreasing salinity. Toxicity was also greatest at the highest temperature (17°C). The average EC50's (Table 12) for each set of tests within the matrix design support the patterns of toxicity observed above for the response surface plots. It is evident that stresses due to reduced salinity and high temperatures can alter the sensitivity of the sperm bioassay to toxicants such as silver.

Artificial Seawater--

Thirty- and 60-min sperm bioassays of silver in artificial seawater suggested that silver may be more toxic in this matrix than in natural seawater. The average EC50's and 95 percent confidence limits for the 30- and 60-min tests were 56 ± 6 and 25 ± 20 μg/l silver, respectively. These values are substantially less than equivalent 30- and 60-min EC50's for silver bioassays in natural seawater (124 and 47 μg/l silver, respectively).

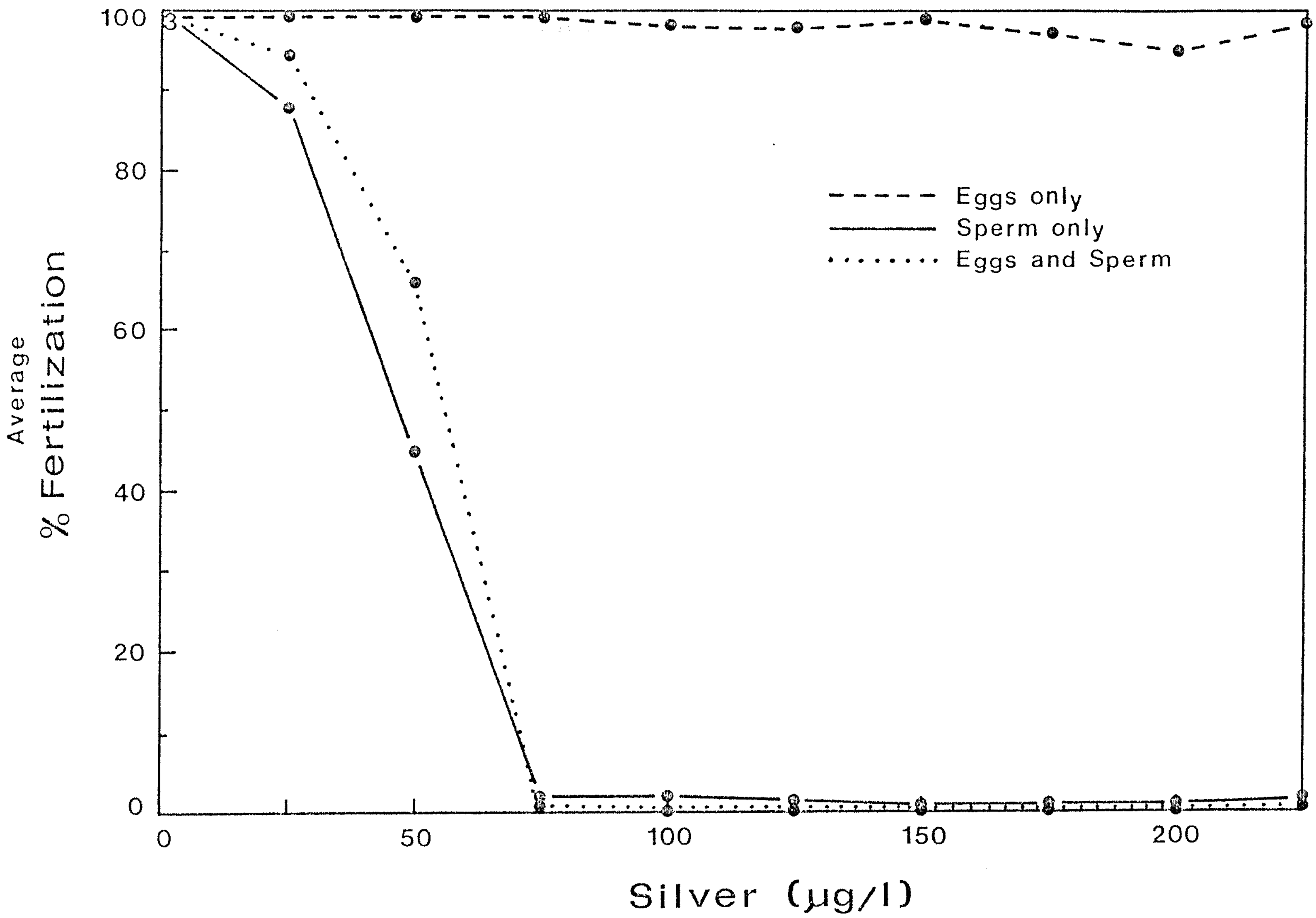


Figure 28. Average fertilization success after 60-min sand dollar sperm, egg,or sperm and egg exposures to silver in natural seawater.

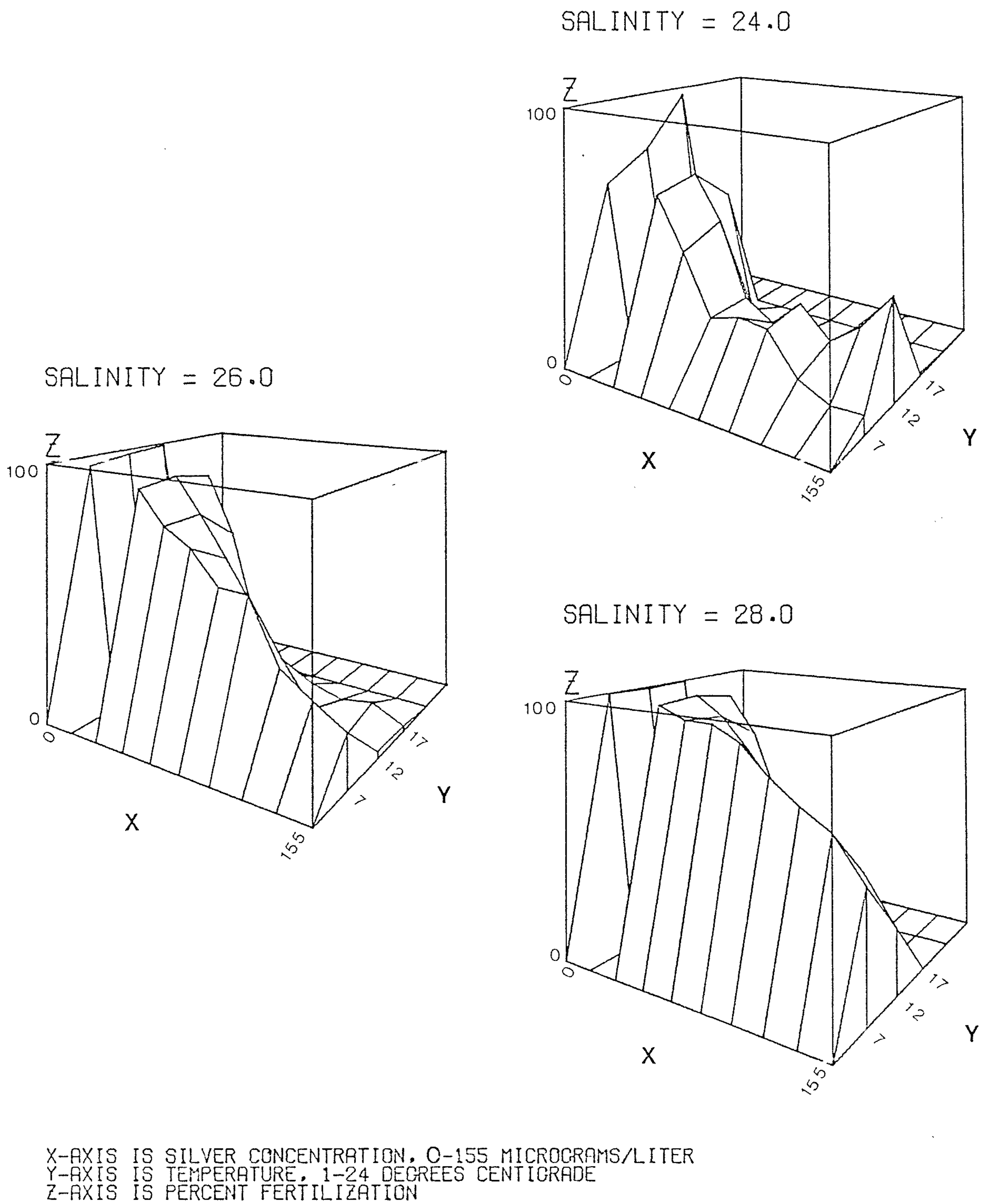


Figure 29. Response surface plots of fertilization success following 30-min sand dollar sperm exposures to silver in natural seawater in a matrix array of three temperatures and three salinities at pH 8.0.

TABLE 12. AVERAGE EC50'S AND 95% CONFIDENCE LIMITS OF SILVER BIOASSAYS CONDUCTED AT THREE TEMPERATURES AND THREE SALINITIES AT pH 8.0

Temperature (°C)	Salinity (ppt)	Average EC50 (μg/l)	95% Confidence Limits (μg/l)
7	24	49	0-105
7	26	111	82-140
7	28	134	117-151
12	24	85	2-168
12	26	83	70- 96
12	28	96	60-132
17	24	45	39- 51
17	26	66	62- 70
17	28	72	0-218

TABLE 13. AVERAGE EC50'S AND 95% CONFIDENCE LIMITS OF ENDOSULFAN BIOASSAYS CONDUCTED AT THREE TEMPERATURES AND THREE SALINITIES AT pH 8.0.

Temperature (°C)	Salinity (ppt)	Average EC50 (μg/l)	95% Confidence Limits (μg/l)
7	24	105	83-128
7	26	<85	—
7	28	168	109-217
12	24	144	106-182
12	26	127	87-167
12	28	239	61-417
17	24	204	174-234
17	26	155	114-196
17	28	238	162-319

Endosulfan

Time-Concentration Responses

Sperm bioassays of endosulfan in natural seawater were conducted with nine endosulfan concentrations and sperm exposure times of 30, 60, 90 and 120 minutes. Each test was replicated four times. The average EC50's and 95 percent confidence limits for 30-, 60-, 90- and 120-min exposure times were 157 ± 42, 151 ± 11, 113 ± 27 and 80 ± 18 µg/l endosulfan, respectively. Sperm sensitivity to endosulfan increased with increased exposure time.

Sperm Versus Egg Sensitivity--

Endosulfan bioassays using 60-min exposures of sperm only, eggs only and sperm plus eggs (simultaneous exposure in separate containers) were conducted to assess the relative sensitivity between eggs and sperm. The eggs generally proved to be more resistant to endosulfan toxicity than sperm (egg and sperm average EC50's = 356 ± 57 and 286 ± 82 µg/l, respectively) (Figure 30). Sperm and eggs exposed simultaneously were generally more sensitive to endosulfan than either exposed singly (sperm plus egg average EC50 = 224 ± 470 µg/l). The toxic effects of endosulfan may be at least partially additive when both sets of gametes are exposed simultaneously.

Effects of pH--

Sand dollar sperm bioassays of endosulfan were conducted with natural seawater adjusted to pH 7.5, 8.0 and 8.5 with HCl or NaOH. The toxicity of endosulfan was essentially the same in all three tests. Average EC50's and 95 percent confidence limits for tests at pH 7.5, 8.0 and 8.5 were 231 ± 33, 271 ± 20 and 229 ± 39 µg/l endosulfan, respectively.

Effects of Temperature and Salinity--

A matrix of sand dollar sperm bioassays was conducted using a range of endosulfan concentrations in natural seawater at 7, 12 and 17°C; 24, 26 and 28 ppt salinity and pH 8.0. Response surface plots of the results show generally decreasing fertilization success with increasing endosulfan (Figure 31). Fertilization success is also generally less at salinities < 28 ppt and at 7°C. The higher toxic response at 7°C is in contrast to the silver matrix tests (Figure 29) where a greater toxic response occurred at 17°C instead of at 7°C. The responses graphed in Figure 31 are reinforced by the average EC50's calculated for each set of tests at a specific temperature and salinity (Table 13).

Artificial Seawater--

Endosulfan in artificial seawater did not prove to be more toxic to sand dollar sperm than endosulfan in natural seawater. Thirty- and 60-min EC50's for endosulfan in artificial seawater were 193 ± 62 and 184 ± 47 µg/l versus 157 ± 42 and 151 ± 11 µg/l for natural seawater. These results are different than those using sea urchin sperm where endosulfan was substantially more toxic in artificial seawater. Additional tests will be required to establish a relationship between chemical toxicity and type of seawater.

Chemical Form of Endosulfan--

Sand dollar sperm bioassays of four chemical forms of endosulfan (α, β,

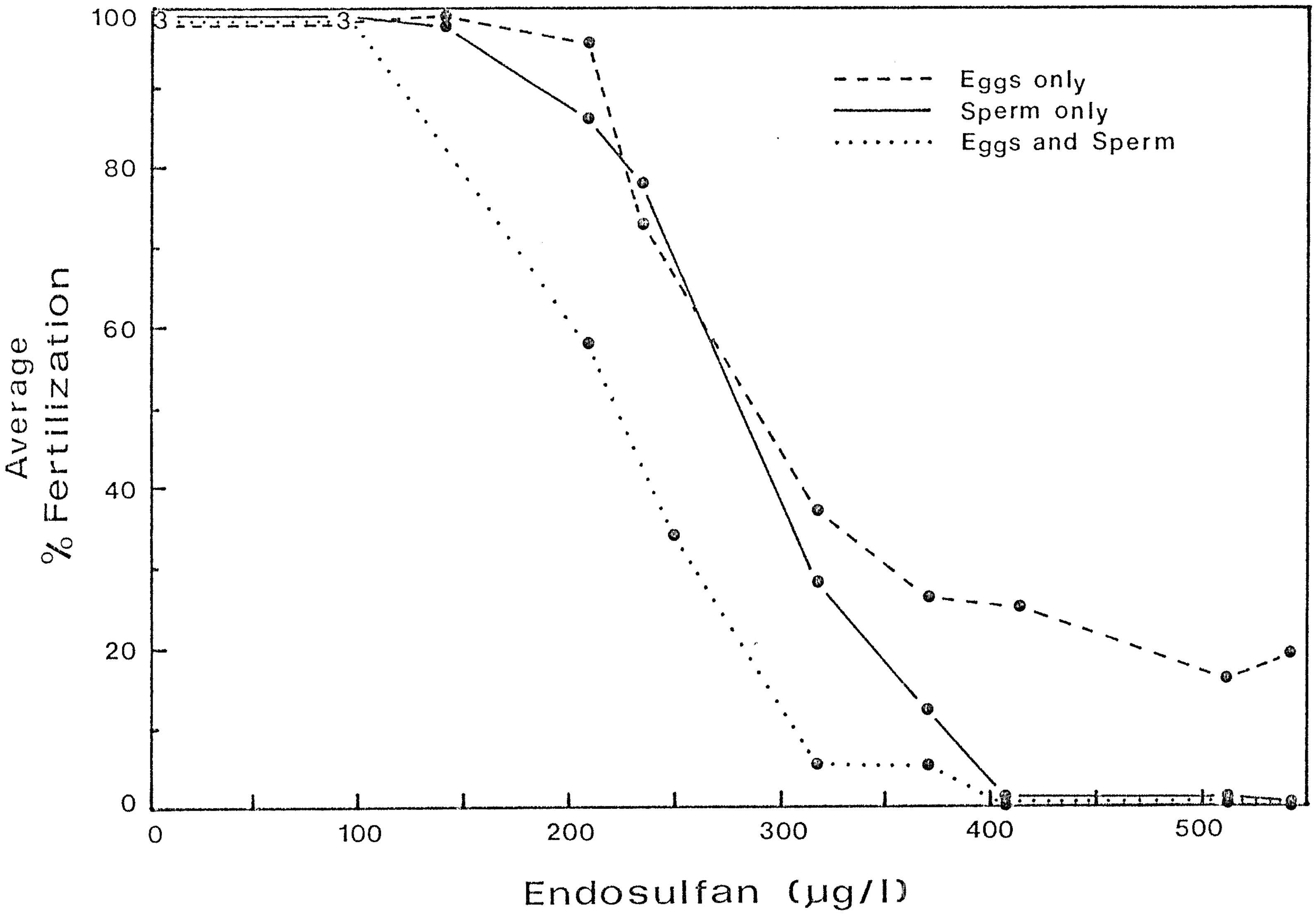


Figure 30. Average fertilization success following 60-min sand dollar sperm, egg, or sperm and egg exposures to endosulfan in natural seawater.

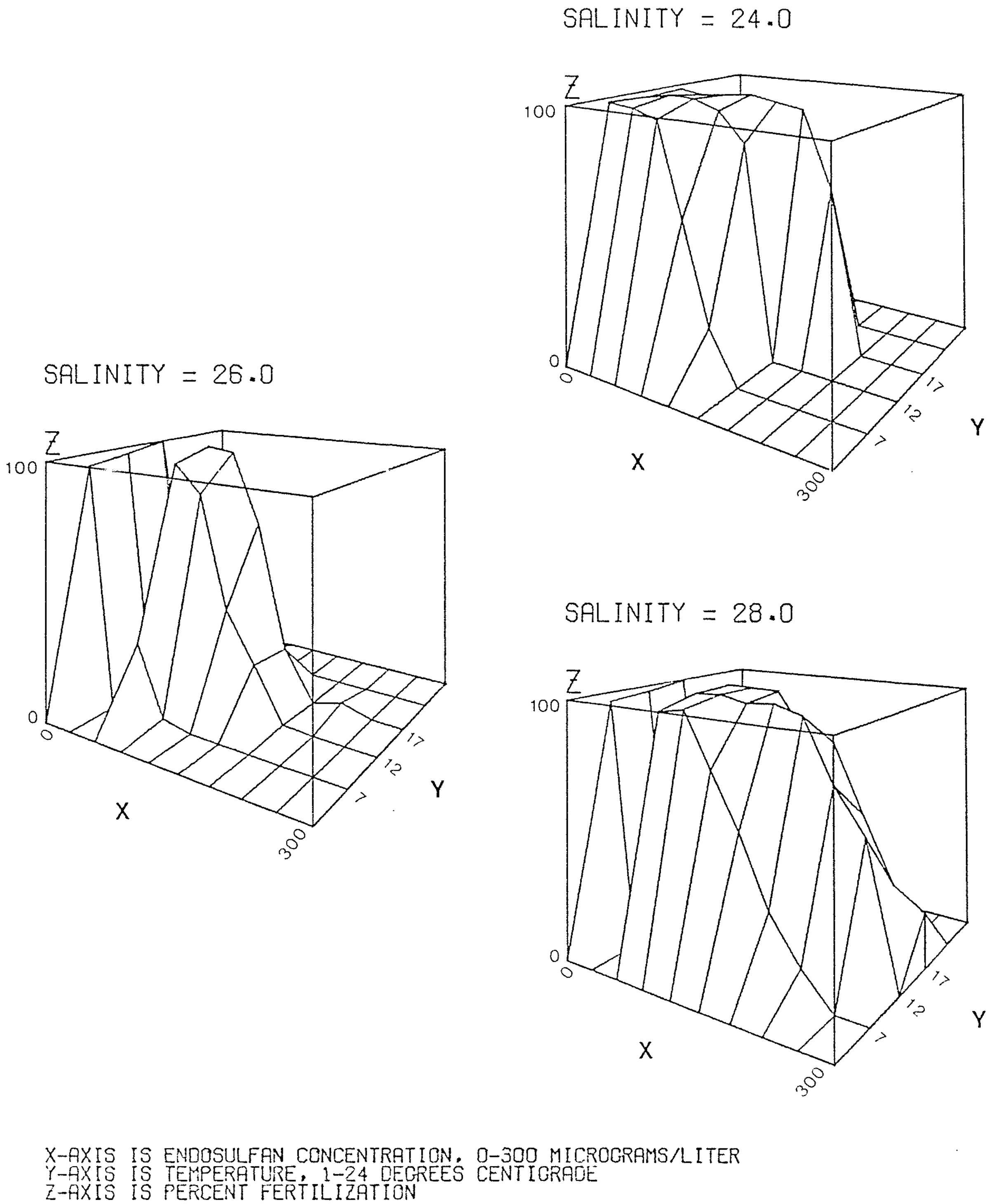


Figure 31. Response surface plots of average fertilization following 30-min sand dollar sperm exposures to endosulfan in a matrix array of three temperatures and three salinities at pH 8.0.

sulfate and ether) were conducted to assess the relative toxicity between these forms in natural seawater. The resulting 60-min EC50's and 95 percent confidence limits of endosulfan α, β, sulfate and ether were 262 ± 43, > 48 < 74, 208 ± 64 and 533 ± 622 μg/l, respectively. The relative toxicity of these four forms as determined by sperm bioassay was β > sulfate > α > ether.

Interactive Toxicity of Silver and Endosulfan--

To test for possible synergistic, antagonistic or additive toxicity of combinations of silver and endosulfan, a matrix design of silver and/or endosulfan concentrations in natural seawater was tested with 30-min exposures of sand dollar sperm. Silver was added in calculated concentrations of 0, 25, 50, 75, 100 and 125 μg/l. Endosulfan was tested at measured concentrations of 0, 58, 105, 147 and 194 μg/l (Table 14).

The resulting pattern of egg fertilization showed increasing toxicity of silver (≥ 25 μg/l) and endosulfan (≥ 147 μg/l) alone as well as generally additive toxicity of combinations of these two toxicants (Table 15).

Summary of Sand Dollar Sperm Bioassay Data--

Individual and mean EC50's, fiducial limits, confidence limits and test conditions of silver and endosulfan bioassays are summarized in Appendix Tables 4 and 5.

EMBRYO BIOASSAYS

Silver

A 72-h bioassay of sand dollar embryos was conducted to determine the relative toxicity of silver to sperm and developing embryos. The results show no evident effect of silver on development at calculated concentrations of 3.12, 6.25 and 12.5 μg/l (Table 16). Slight retardation of development was observed in 25 μg/l with progressively more retardation from 50 to 1000 μg/l. Embryos in 200 μg/l suffered high mortality and cytolysis while embryos in 400 and 1000 μg/l showed less mortality and cytolysis. The 200 μg/l embryos were at the post-hatch stage while embryos in 400 and 1000 μg/l silver were retarded to the pre-hatch stage. The fertilization membrane evidently afforded an extra margin of protection to the pre-hatch embryos.

The results of the 72-h embryo bioassays of silver closely correspond to the toxicity of silver determined by sperm bioassays (15-, 30- 60- and 90-min sperm EC50's = 201, 124, 47 and 25 μg/l silver, respectively). The lowest levels of toxicity determined by the 72-h embryo bioassay were successfully predicted by 60- to 90-minute sperm bioassays with less subjectivity and time.

Endosulfan

A 72-h bioassay of sand dollar embryos was conducted to determine the relative toxicity of endosulfan to sperm and developing embryos. The results show no effect of endosulfan on development at initial measured concentrations of 48.2, 81.9 and 98.0 μg/l. Slight retardation of embryo

Table 14. Matrix design of silver and/or endosulfan concentrations used to test the interactive toxicity of silver and endosulfan in natural seawater.

SILVER / ENDOSULFAN

KEY

0 / 0	25 / 0	50 / 0	75 / 0	100 / 0	125 / 0
0 / 58	25 / 58	50 / 58	75 / 58	100 / 58	125 / 58
0 / 105	25 / 105	50 / 105	75 / 105	100 / 105	125 / 105
0 / 147	25 / 147	50 / 147	75 / 147	100 / 147	125 / 147
0 / 194	25 / 194	50 / 194	75 / 194	100 / 194	125 / 194

Table 15. Fertilization success following 30-min exposures of sand dollar sperm to silver and/or endosulfan concentrations as outlined in Table 14. The small numbers are fertilization success in each of four replicates. The large numbers are average fertilization.

91 92 94 93 92	79 89 53 89 78	54 79 73 68 68	33 58 42 25 40	31 29 16 2 20	47 10 2 0 15
85 98 96 93 93	86 81 83 87 84	61 65 58 32 54	39 16 55 7 29	18 0 6 1 6	16 0 0 0 4
94 98 86 93 93	71 83 81 74 78	78 39 67 58 60	48 4 35 0 22	33 0 3 0 9	11 0 0 1 3
80 84 88 93 86	82 24 57 21 46	58 5 54 4 30	38 0 4 0 10	19 1 3 1 6	5 0 0 5 2
54 33 82 47 54	57 3 3 2 16	41 3 4 3 13	14 0 1 0 4	1 0 1 0 0	0 0 1 0 0

TABLE 16. SURVIVAL AND DEVELOPMENT PATTERN OF SAND DOLLAR EMBRYOS EXPOSED TO 3.12-1000 µg/l SILVER IN A 72-HOUR STATIC BIOASSAY.

SILVER		STAGE OF DEVELOPMENT (%)				72-HOUR SURVIVAL
(µg/l)		ABNORMAL	BLASTULA	GASTRULA	PLUTEUS	EMBRYOS/ml
0	A	5			95	45.7
	B	11			89	48.0
3.12	A	7			93	59.0
	B	6			94	42.3
6.25	A	8			92	51.0
	B	5			95	44.3
12.5	A	3			97	41.0
	B	12			88	46.0
25	A	9		(Early Pluteus)	91	48.7
	B	15			85	48.0
50	A	Most Late Gastrula — Early Pluteus; Few Blastula				44.7
	B					55.0
100	A	All Blastula, No Cytolysis				53.7
	B					47.3
200	A	All Blastula, Most Undergoing Cytolysis				12.0
	B					15.0
400	A	Pre-Blastula (64-128 Cell Stage), Only Slight Cytolysis				34.3
	B					36.0
1000	A	8-32 Cell Stage, No Cytolysis				50.3
	B					53.3

development was observed in 157.6, 187.8, 226.9 and 256.6 μg/l endosulfan and more pronounced retardation from 326.1 - 423.2 μg/l (Table 17). Additionally, many of the embryos were poorly formed in endosulfan concentrations ≥ 187.8 μg/l. Survival was essentially the same in controls (0 μg/l) and all endosulfan concentrations.

There was an average decrease of endosulfan in the test containers of 47.1 percent during the 72-h exposure period. Thus, the concentrations of endosulfan affecting development as noted above are conservative estimates of toxicity.

Sand dollar sperm bioassays appear to be more sensitive indicators of endosulfan toxicity than 72-h embryo bioassays (30-, 60-, 90- and 120-min sperm EC50's = 157, 151, 113 and 80 μg/l endosulfan, repsectively. The lowest level of toxicity detected by embryo bioassay was 157.6 μg/l, which is essentially the same as the 30-min EC50 for the sperm bioassay. Sperm exposure times > 30 minutes further increased the sensitivity of the sperm bioassay to endosulfan.

TABLE 17. SURVIVAL AND DEVELOPMENT PATTERN OF SAND DOLLAR EMBRYOS EXPOSED TO 48.2 - 423.2 μg/l ENDOSULFAN IN A 72-HOUR STATIC BIOASSAY.

ENDOSULFAN (μg/l) (INITIAL/FINAL)		STAGE OF DEVELOPMENT (%)				72-HOUR SURVIVAL
		ABNORMAL	BLASTULA	GASTRULA	PLUTEUS	EMBRYOS/ml
0	A	14			86	53.7
	B	9			91	51.3
48.2/34.1	A	9			91	47.3
	B	7			93	51.3
81.9/57.8	A	11			89	47.7
	B	11			89	55.7
98.0/68.1	A	8			92	52.3
	B	11			89	49.3
157.6/84.8	A	9	(Some Early Pluteus)		91	49.3
	B	7			93	54.7
187.8/106.2	A		Most Early Pluteus,			45.0
	B		Many Poorly Formed			46.3
26.9/127.5	A		Most Early Pluteus,			62.3
	B		Many Poorly Formed			53.7
256.6/137.3	A		Most Early Pluteus,			49.7
	B		Many Poorly Formed			44.0
326.1/160.9	A		Most Late Gastrula — Early Pluteus,			60.3
	B		Many Poorly Formed			50.0
356.0/186.4	A		Few Blastula, Most Gastrula — Early			47.7
	B		Pluteus, Many Poorly Formed			55.7
423.2/212.5	A		Most Blastula — Gastrula, Few			45.0
	B		Pluteus, Many Poorly Formed			49.7

SECTION VIII

DISCUSSION

The sperm bioassay is proving to be a very quick and sensitive test for many toxicants in marine waters. Sea urchin sperm sensitivity to silver and endosulfan generally agree with the levels of toxicity reported for other aquatic animals with these substances (see Section III C). Exceptions to this may be the greater sensitivity of fish, especially in long-term tests, and some invertebrate larval stages.

The sperm bioassay has most of the advantages of both static and flow-through bioassays with few of the disadvantages. Being a static test, it utilizes single batches of dilution water with constant, closely defined water quality characteristics. Temperature fluctuations are avoided by using a constant-temperature water bath. The short duration of the sperm assay minimizes loss or degradation of the toxicant in the test containers and facilitates toxicant analysis. The test response (percent eggs fertilized) is easily quantified in an objective manner and the results easily analyzed by accepted standard bioassay statistical methodology. Adult animals are easily obtained in coastal marine waters and maintained in the laboratory. The adult animals are rarely harmed by the KCl spawning treatment allowing them to be used repeatedly (2 to 3 times with about 30-day resting intervals) as sources of gametes or returned unharmed to their natural environment.

This test should prove to be especially useful for monitoring the variable toxicity of chemicals which change form or degrade rapidly through time after introduction into seawater, or complex wastewater samples which normally receive extensive chemical characterization but no biological assessment. The sperm bioassay should also prove to be a valuable biomonitoring procedure for receiving water quality as well as a standardized laboratory procedure. Its use in a matrix arrangement with multiple bioassay parameters has been illustrated in this report. The synergistic or antagonistic interactions of combinations of toxicants can also be analyzed in a similar matrix design.

The sperm bioassay described in this report needs further refinements to optimize its usefulness and repeatability. The tests reported here used a ratio of 1000 sperm/egg for both species. However, the results suggest that a 1000 sperm/egg ratio may not be high enough for green sea urchin sperm while 250 sperm/egg may be a satisfactory ratio for sand dollars. Optimum sperm/egg ratios should be determined for each species. The optimum ratio is that which yields consistent control fertilization of $\geq$ 90 percent but which is not so high as to decrease the sensitivity of the test to toxicants.

Between-test variability should be reduced to narrow the confidence limits of the mean EC50's and facilitate comparisons between treatments or species. Variability can be reduced by more accurate enumeration of initial sperm and egg densities so that the final dilutions to pre-determined standard densities will yield minimal variability between individual sperm and egg samples. Fiducial and confidence limits around an EC50 may be tightened by increasing the number of test replicates per treatment; the optimum number probably being a compromise between the number required for a solid statistical base on the one hand and those realistically possible based on time and funds on the other.

Disposable test tubes (with caps) should help to refine the sperm bioassay be reducing the time and effort involved in washing test tubes and storage vials. The eggs would be preserved by adding formalin to each test tube after the egg fertilization period and subsequently capping each tube until the eggs could be counted. This procedure would also eliminate any possibility of contamination from previous toxicant bioassays or from cleaning agents.

We hypothesize that sperm from many species of sea urchins (and sand dollars) are of essentially equal sensitivity to toxic substances. This report has provided an overview of the development and use of a sperm bioassay for testing toxic substances. The sensitivity of sperm from two echinoderm species was compared using two reference toxicants. We found that green sea urchin sperm and sand dollar sperm are roughly of equal sensitivity to both silver and endosulfan based on generally overlapping 95 percent confidence intervals of the mean EC50's of both species for the time-concentration tests (Figures 32 and 33). These comparisons are admittedly rough (and non-statistical), as the test methods were continually undergoing refinement and the optimum sperm/egg ratio may be different for each species. Additional replicates (*i.e.* > 4) may be necessary to yield reasonably tight estimates of a mean EC50. Even then, valid statistical comparisons between species and/or treatments may not be completely possible. Mean EC50's and 95 percent confidence limits based on the variation within a population of individual replicate EC50's do not take into consideration the variation (*i.e.* fiducial limits) around each individual EC50. Thus, methods of comparisons of EC50's must be refined along with the testing methodology.

Improved availability of ripe test animals is desirable. Presently, by maintaining green sea urchins in cooled seawater during their normal spawn-out period, gametes can be obtained from approximately February to June. Sand dollars appear to be normally ripe from June to October and possibly through November if thermally conditioned. December and January may represent periods of time when gametes may not be available from these two species. Testing should be extended to other species of sea urchins to close this gap. The purple sea urchin, Strongylocentrotus purpuratus, has been widely used for experimental biology purposes along the U.S. Pacific Coast. This species is plentiful on the open coast and has a prolonged

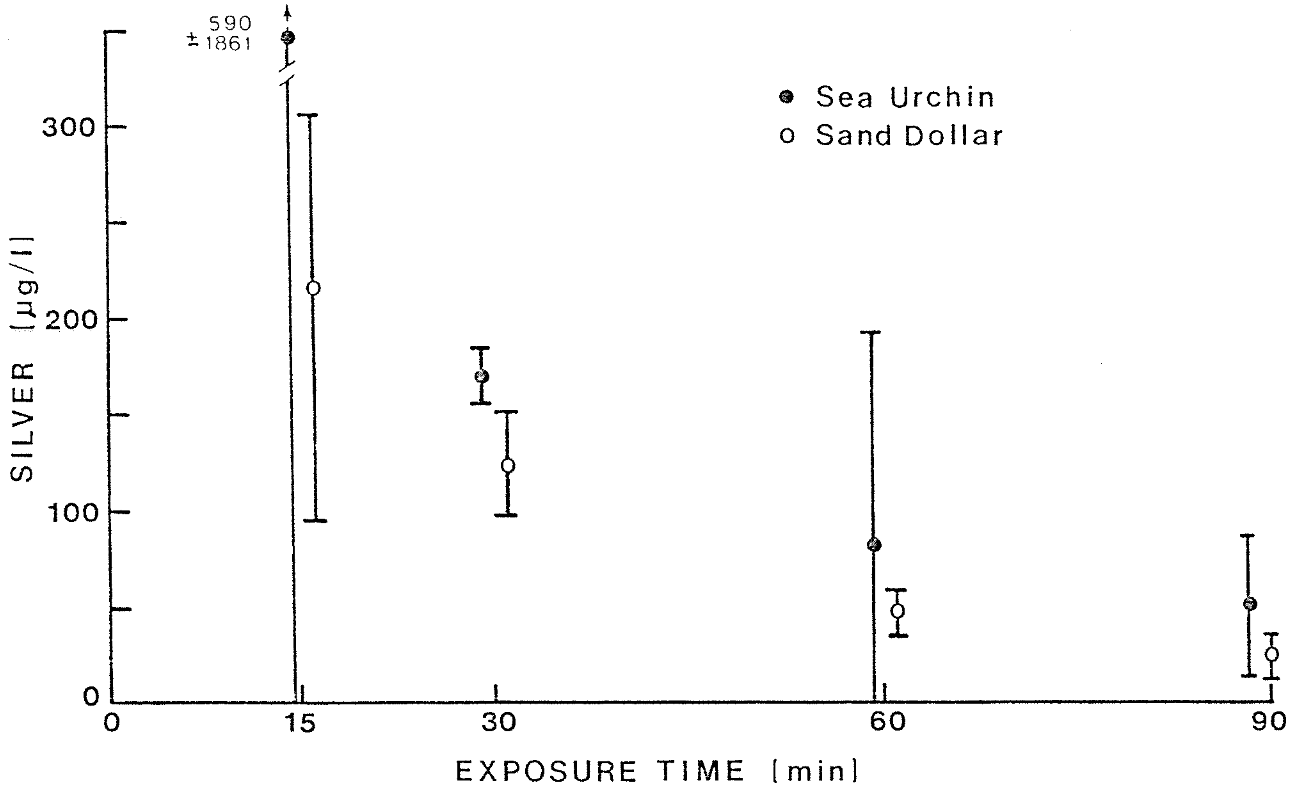


Figure 32. Comparison of sea urchin and sand dollar EC50's and 95% confidence intervals for 15-, 30-, 60- and 90-min sperm exposures to silver.

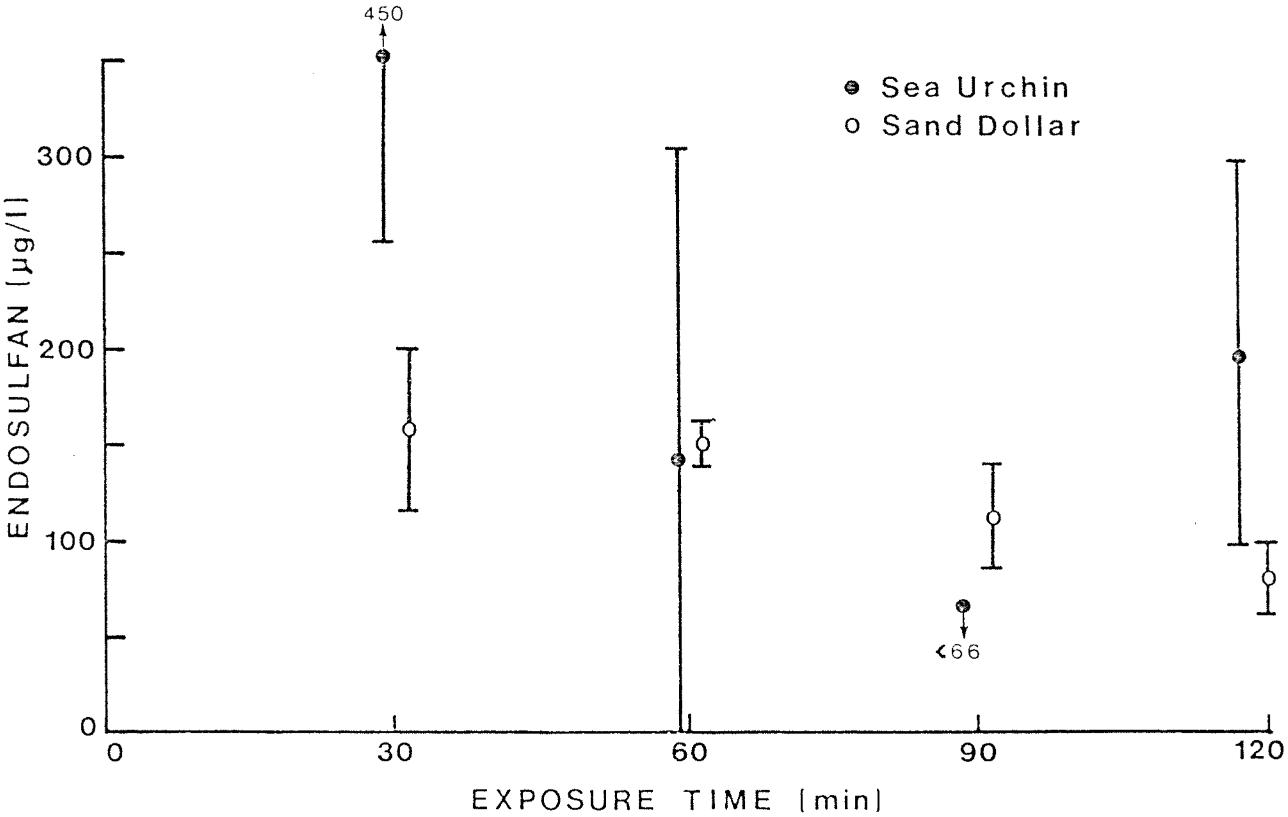


Figure 33. Comparison of sea urchin and sand dollar EC50's and 95% confidence intervals for 30-, 60-, 90- and 120-min sperm exposures to endosulfan.

spawning cycle compared to S. droebachiensis (Himmelman 1978) with active spawning beginning in December (Cochran and Engelman 1975). The red urchin, S. franciscanus, is also abundant on the Pacific Coast. Should sperm of these species all prove equally sensitive to various toxicants, it would then be interesting to compare our results with similar testing of other U.S. and foreign species.

An extended corollary of this hypothesis is that sperm cells from many other marine animals exhibiting external fertilization may show the same sensitivity in this test procedure as sea urchin sperm. Oysters (Crassostrea gigas) should provide a valuable comparison since they can be thermally conditioned to spawn out of season and the embryos serve as the basis of the Washington State receiving water quality bioassay (Woelke 1972).

The use and application of the sperm bioassay can be further extended by conducting comparative 96-h bioassays with selected species of marine fish and invertebrates. Such comparisons will relate the sensitivity of the sperm bioassay to existing toxicity data and help validate this test for use in refining water quality criteria and for assessing potential ecological impacts.

SECTION IX

REFERENCES

Alabaster, J. S. 1969. Survival of fish in 164 herbicides, insecticides, wetting agents and miscellaneous substances. Int. Pest Control 11:29-35.

Albright, L. J., J. W. Wentworth and E. M. Wilson. 1972. Technique for measuring metallic salt effects upon the indigenous heterotrophic microflora of a natural water. Wat. Res. 6:1589-1596.

Allen, H. 1971. Effects of petroleum fractions on the early development of a sea urchin. Marine Poll. Bull. 2:138-140.

Ames, B. N., J. McCann and E. Yamasaki. 1975. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutatenicity test. Mutation Res. 31:347-364.

Amiard, J. C. 1976. Étude expérimentale de la toxicité aigue de sels de cobalt d'atimoine, de strontium et d'argent chez quelques custacés et leurs larves et chez quelques téléostéens. Rev. Intern. Océanogr. Méd. 43:79-95.

Anderson, B. G. 1948. The apparent thresholds of toxicity of Daphnia magna for chlorides of various metals when added to Lake Erie water. Trans. Amer. Fish. Soc. 78:96-113.

Bernhard, M. and A. Zattera. 1970. The importance of avoiding chemical contamination for a successful cultivation of marine organisms. Helgoländer Wiss. Meeresunters 20:655-675.

Boolootian, R. A. 1966. Reproductive physiology. *In:* Physiology of echinoderms. R. A. Boolootian, Ed. John Wiley and Sons, New York. pp. 561-614.

Braun, H. E. and K. Frank. 1973. Unpublished data. H. E. Braun, Ontario Ministry of Agriculture and Food, Guelph, Ontario.

Bryan, G. W. 1971. The effects of heavy metals (other than mercury) on marine and estuarine organisms. Proc. Roy. Soc. London B. 177:389-410.

Calabrese, A., F. P. Thurberg and E. Gould. 1977. Effects of cadmium, mercury and silver on marine animals. Marine Fish. Review 39(4):5-11.

MFR Paper 1244.

Clarke, G. L. 1947. Poisoning and recovery in barnacles and mussels. Biol. Bull. 92(1):73-91.

Cochran, R. C., and F. Engelmann. 1975. Environmental regulation of the annual reproductive season of Strongylocentrotus purpuratus (Stimpson). Biol. Bull. 148:393-401.

Coleman, R. L. and J. E. Cearley. 1974. Silver toxicity and accumulation in largemouth bass and bluegill. Bull. Environ. Contam. and Toxicol. 12(1):53-61.

Cooper, C. F., and W. C. Jolly. 1970. Ecological effects of silver iodide and other weather modification agents: a review. Wat. Resources Res. 6(1):88-98.

Czihak, G. 1975. The sea urchin embryo. *In:* Biochemistry and morphogenesis. G. Czihak, Ed. Springer-Verlag, New York. 700 pp.

Davies, P. H., J. P. Goettl, and J. R. Sinley. 1978. Toxicity of silver to rainbow trout (Salmo gairdneri). Wat. Res. 12(2):113-117.

Finney, D. J. 1971. Probit analysis. 3rd Ed. Cambridge Univ. Press, Cambridge, Mass. 333 pp.

Giudice, G. 1973. Developmental biology of the sea urchin embryo. Academic Press, New York. 469 pp.

Goettl, J. P., Jr. and P. H. Davies. 1978. Water pollution studies. *In:* Colorado Fisheries Research Review 1976-1977. O. B. Cope, Ed. Colo. State Publ. Code DOW-R-R-F76-77. pp. 42-46.

Greve, P. A., and H. G. Verschuren. 1971. Die Toxizitaet von Endosulfan fuer Fische in Oberflaechengewaessern. Schriftenr. Ver. Wasserboden-Lufthyg. 34:63-67. *Cited in:* National Research Council Associate Committee on Scientific Criteria for Environmental Quality, 1975. Endosulfan: its effects on environmental quality. NRC Assoc. Comm. on Sci. Criteria for Environ. Quality Rept. 11. 100 pp.

Hagström, B. E., and S. Lönning. 1973. The sea urchin egg as a testing object in toxicology. Acta Pharmacologica et Toxicologica. 32(supp. 1):1-49.

Hale, J. G. 1977. Toxicity of metal mining wastes. Bull. Environ. Contam. and Toxicol. 17(1):66-72.

Harry, H. W., and D. V. Aldrich. 1963. The distress syndrome in Taphius glabratus (Say) as a reaction in toxic concentrations of inorganic ions. Malacologia 1:283-289.

Himmelman, J. H. 1978. Reproductive cycle of the green sea urchin, Strongylocentrotus droebachiensis. Can. J. Zool. 56:1828-1836.

Hoadley, L. 1923. Certain effects of the salts of the heavy metals on the fertilization reaction in Arbacia punctulata. Biol. Bull. 44(6): 255-280.

Hodson, P. V., C. W. Ross, A. J. Niimi and D. J. Spry. 1977. Proc. 3rd Aquatic Toxicity Workshop. Surveillance Rpt. EPS-5-AR-77-1, Environ. Protect. Service, Halifax, Nova Scotia, Can.:15-31.

Jones, J. R. E. 1939. The relation between the electrolytic solution pressures of the metals and their toxicity to the stickleback (Gasterosteus aculeatus L.). J. Exp. Biol. 16:425-437.

Jones, J. R. E. 1964. The metals as salts. *In:* Fish and river pollution. Butterworths, London. pp. 66-82.

Knauf, W., and E. F. Schulze. 1973. New findings on the toxicity of endosulfan and its metabolites to aquatic organisms. *In:* Studies of the impact of endosulfan on the environment. Tab. 12. Submitted in fulfillment of Pesticide Registration Notice 70-15. *Cited in:* National Research Council Associate Committee on Scientific Criteria for Environmental Quality, 1975. Endosulfan: its effects on environmental quality. NRC Assoc. Comm. of Sci. Criteria for Environ. Quality. Rep. 11 100 pp.

Kobayashi, N. 1971. Fertilized sea urchin eggs as an indicatory material for marine pollution bioassay, preliminary experiments. Publ. Seto Mar. Biol. Lab. 18(6):379-406.

Lillie, F. R. 1921. Studies of fertilization, X. The effects of copper salts on the fertilization reaction in Arbacia and a comparison of mercury effects. Biol. Bull. 41:125-143.

Litchfield, J. T., Jr. and F. Wilcoxon. 1949. A simplified method of evaluating dose-effect experiments. J. Pharm. and Exp. Therapeutics. 96(2):99-113.

Lönning, S., and B. E. Hagström. 1975. The effects of crude oils and the dispersant Corexit 8666 on sea urchin gametes and embryos. Norw. J. Zool. 23:121-129.

Macek, K. J., C. Hutchinson and O. B. Cope. 1969. The effects of temperature on the susceptibility of bluegills and rainbow trout of selected pesticides. Bull. Environ. Contam. Toxicol. 3:174-183.

MacInnes, J. R., and F. P. Thurberg. 1973. Effects of metals on the behavior and oxygen consumption of the mud snail. Mar. Poll. Bull. 4:185-186.

Maier-Bode, H. 1968. Properties, effect, residues and analytics of the insecticide endosulfan. Residue Rev. 22:1-44.

McCann, J., and B. N. Ames. 1976. Detection of carcinogens as mutagens in the Salmonella/microsome test: Assay of 300 chemicals: Discussion. Proc. Nat. Acad. Sci. USA 73(3):950-954.

McIntyre, J. D. 1973. Toxicity of methylmercury for steelhead trout sperm. Bull. Environ. Contam. and Toxicol. 9:98-99.

Muchmore, D., and D. Epel. 1973. The effects of chlorination of wastewater in some marine invertebrates. Marine Biol. 19:93-95.

Oeser, H., S. G. Gorbach and W. Knauf. 1971. Endosulfan and the environment. "Giornate Fitopathologie" (Workshop on Phytopathology), Udine, Italy, May, 1971. *Translation in:* Studies of the impact of endosulfan on the environment. Tab. 11. Submitted in fulfillment of Pesticide Registration Notice 70-15. *Cited in:* National Research Council Associate Committee on Scientific Criteria for Environmental Quality, 1975. Endosulfan: its effects on environmental quality. NRC Assoc. Comm. on Sci. Criteria for Environ. Quality Rep. 11. 100 pp.

Okubo, K., and T. Okubo. 1962. Study on the bio-assay method for the evaluation of water pollution-II. Use of fertilized eggs of sea urchins and bivalves. Bull. Tokai Reg. Fish. Res. Lab. 32:121-140.

Rulon, O. 1956. Effects of cobaltous chloride on development in the sand dollar. Phyiol. Zool 29:51-63.

Sanders, H. O., and O. B. Cope. 1968. The relative toxicities of several pesticides to naiads of three species of stone flies. Limnol. Oceaogr. 13:112-117.

Schoettger, R. A. 1970. Toxicology of Thiodan in several fish and aquatic invertebrates. U.S.D.I., Bur. Sport Fish. Wildlife Investigations in Fish Control 35:1-31.

Sheets, W. D. 1957. Toxicity studies of metal-finishing wastes. Sew. and Indust. Wastes 29(12):1380-1384.

Stober, Q. J., P. A. Dinnel, M. A. Wert, D. H. DiJulio and R. E. Nakatani. 1977. Toxicity of West Point Effluent to Marine Indicator Organisms, Part II. FRI-UW-7737. Final Report to Municipality of Metropolitan Seattle, Fisheries Research Institute, College of Fisheries, University of Washington, Seattle. 82 pp.

Stober, Q. J., P. A. Dinnel, E. F. Hurlburt, D. H. DiJulio, S. P. Felton and R. E. Nakatani. 1978. Effects of Seawater Chlorination on Marine Organisms. Technical Report UW-NRC-9 to U.S. Nuclear Regulatory Commission, College of Fisheries, University of Washington, Seattle. 134 pp.

Tyler, A. 1949. A simple, non-injurious, method for inducing repeated spawning of sea urchins and sand dollars. Collect. Net. 19:19-20.

Tyler, A., and B. S. Tyler. 1966. The gametes, some procedures and properties. *In:* Physiology of echinodermata. R. A. Boolootian, Ed. John Wiley and Sons, New York. pp. 639-682.

Waldichuk, M. 1974. Some biological concerns in heavy metal pollution. *In:* Pollution and physiology of marine organisms. F. J. Vernberg and W. B. Vernberg, Ed. Academic Press, New York. pp. 1-57.

Weast, R. C., and S. M. Selby, Ed. 1967. Handbook of chemistry and physics. 48th Edition. The Chemical Rubber Co., Cleveland, Ohio.

Woelke, C. E. 1972. Development of a receiving water quality bioassay criterion based on the 48-h Pacific oyster (Crassostrea gigas) embryo. Wash. Depart. of Fish. Tech. Rept. No. 9. 93 pp.

Young, L. G., and L. Nelson. 1974. The effects of heavy metal ions on the mortality of sea urchin spermatozoa. Biol. Bull. 147:236-246.

SECTION X

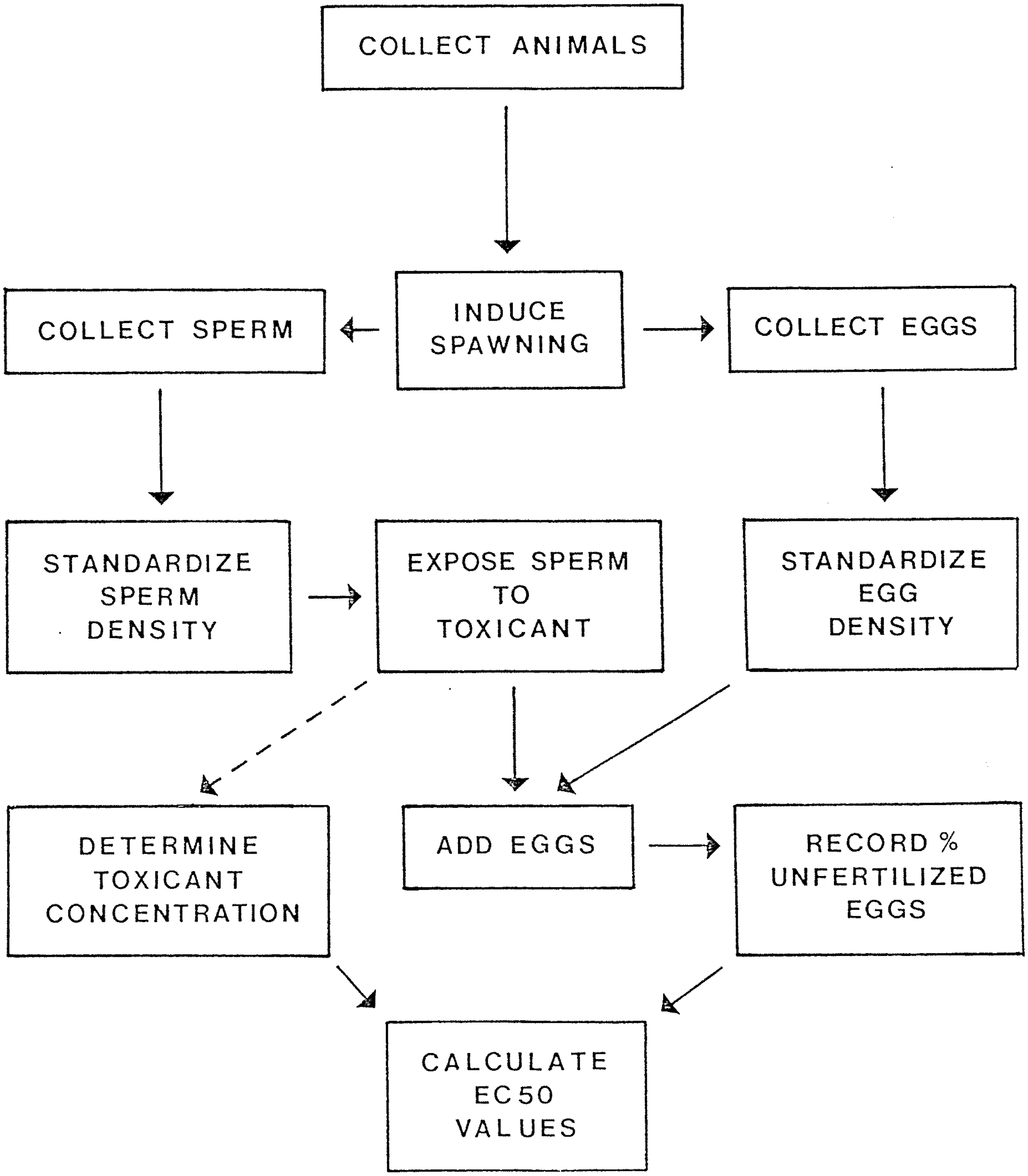
APPENDIX

APPENDIX TABLE 1. PRELIMINARY ECHINODERM SPERM BIOASSAY PROTOCOL.

STEP	PROCEDURE
1	Collect test animals from sites not subject to compromise by municipal or industrial discharges. Hold animals in the laboratory in flowing seawater filtered through gravel and sand filters and activated carbon. Feeding is unnecessary for animals not held for extended periods of time.
2	Spawn animals by inverting over a 100-ml beaker full of seawater. Inject approximately 0.5 ml (for sand dollars) or 1.0 ml (for sea urchins) of 0.5 M potassium chloride (KCl) into celomic cavity through the peristomal membrane or oral opening (Tyler 1949). Allow to spawn 10-20 minutes.
3	Decant some seawater from the 100-ml beakers containing sperm and mix thoroughly. Decant eggs (from several females, if necessary) into a larger beaker and wash with fresh seawater two to three times. Let settle between washes.
4	Determine sperm density in concentrated samples by adding 0.1-1.0 ml subsample to 10 ml glacial acetic acid (to kill sperm) and seawater adjusted to 100 ml final volume. Add 1 drop of diluted sperm solution to a hemacytometer counting chamber. Let sperm settle 15-20 minutes. Count sperm as per standard method for red blood cell counts. Determine sperm density in the concentrated solution by the following formula: $\#\ \text{Sperm/ml} = \frac{(\text{Dilution factor})(\#\ \text{of sperm counted})(4{,}000)}{\text{Number of squares counted}}\ (1{,}000)$
5	Standardize sperm density to 50×10^6/ml by the following formula: $\text{Dilution factor} = \frac{\text{Number of sperm in concentrated solution}}{50 \times 10^6}$
6	Determine egg density by a subsample (0.1-1.0 ml) counted directly under a dissecting microscope at 10-20X.

APPENDIX TABLE 1. (continued)

STEP	PROCEDURE
7	Standardize egg density to 5×10^3/ml by the following formula: $\text{Dilution factor} = \frac{\text{Number of eggs in concentrated solution}}{5 \times 10^3}$
8	Add 0.1 ml standardized sperm solution to each test tube containing 25.0 ml total volume of toxicant/seawater or seawater (control). Mix gently and allow to incubate for desired sperm exposure time (usually 15-60 minutes).
9	Add 1.0 ml standardized egg solution to each test tube following the desired sperm exposure period. Mix gently and allow 20 minutes for eggs to fertilize and settle.
10	Decant most solution from test tubes and pour the settled eggs into vials containing 10-15% formalin in seawater. Cap vials and store until analyzed.
11	To analyze, mix samples and pour a subsample onto a microscope slide etched with a counting grid. Determine percent unfertilized eggs by scoring presence or absence of the fertilization membrane of 100 eggs. Eggs with only partial membrane formation are counted as unfertilized. Damaged eggs are not counted.
12	Repeat tests that exhibit less than 90% control fertilization. Adjust test responses for natural responsiveness of controls (when $\geq$ 90% and < 100%) by Abbott's formula (Finney 1971): $\text{Adjusted test response} = \frac{\text{\% Test response} - \text{\% Control response}}{100 - \text{\% Control response}} (100)$
13	Determine test EC50's and 95% fiducial limits by probit analysis (Finney 1971), using adjusted test responses and toxicant concentration data from split sample chemical analyses or from calculated concentrations.



Appendix Figure 1. Simplified flow diagram of the echinoderm sperm bioassay procedures.

APPENDIX TABLE 2. SUMMARY OF THE SEA URCHIN SPERM BIOASSAY TEST DATA AND TEST CONDITIONS USING SILVER AS THE TOXICANT.

TEST NO.	EC50 (µg/l)	95% FIDUCIAL LIMITS	95% CONFIDENCE LIMITS	TEST CONDITIONS Temp. (°C)	Salinity (ppt)	pH	Exposure time (min)*
			Silver Time-Dose				
80A	59	45- 78	—	7.5	28.0	8.0	60
80B	134	119-152	—	7.5	28.0	8.0	60
80C	—	—	—	7.5	28.0	8.0	60
80D	56	41- 75	—	7.5	28.0	8.0	60
80ABD	$\bar{x}$ = 83	—	0-193	7.5	28.0	8.0	60
81A	165	132-208	—	7.5	28.0	8.0	30
81B	168	116-244	—	7.5	28.0	8.0	30
81C	168	129-219	—	7.5	28.0	8.0	30
81D	183	140-239	—	7.5	28.0	8.0	30
81ABCD	$\bar{x}$ = 171	—	158-184	7.5	28.0	8.0	30
129A	443	370-529	—	12.0	28.1	7.8	15
129B	736	637-850	—	12.0	28.1	7.8	15
129AB	$\bar{x}$ = 590	—	0-2451	12.0	28.1	7,8	15
85A	35	20- 61	—	7.6	27.7	8.0	90
85B	56	34- 93	—	7.6	27.7	8.0	90
85C	—	—	—	7.6	27.7	8.0	90
85D	64	40-103	—	7.6	27.7	8.0	90
85ABC	$\bar{x}$ = 52	—	15- 89	7.6	27.7	8.0	90
			Silver Sperm-Egg Sensitivity				
82A	225-250	—	—	7.7	27.9	8.0	30
82B	225-250	—	—	7.7	27.9	8.0	30
83A	8000-16,000	—	—	7.7	27.9	8.0	30^1
83B	8000-16,000	—	—	7.7	27.9	8.0	30^1
84A	—	—	—	7.7	27.9	8.0	30^2
84B	—	—	—	7.7	27.9	8.0	30^2

TABLE 2. (continued)

				TEST CONDITIONS			
TEST NO.	EC50	95% FIDUCIAL LIMITS	95% CONFIDENCE LIMITS	Temp. (°C)	Salinity (ppt)	pH	Exposure time (min)*
			Silver — pH				
119A	242	210-280	—	8.1	29.1	7.5	30
119B	280	227-345	—	8.1	29.1	7.5	30
119AB	$\bar{x}$ = 261	—	20-502	8.1	29.1	7.5	30
119C	224	183-275	—	8.1	29.1	8.0	30
119D	280	235-334	—	8.1	29.1	8.0	30
119CD	$\bar{x}$ = 252	—	0-608	8.1	29.1	8.0	30
119E	245	173-349	—	8.1	29.1	8.5	30
119F	236	129-430	—	8.1	29.1	8.5	30
119EF	$\bar{x}$ = 241	—	185-307	8.1	29.1	8.5	30
			Silver — Matrix				
70A	234	175-313	—	7.0	28.0	8.0	30
70B	207	170-251	—	7.0	28.0	8.0	30
70C	240	187-308	—	7.0	28.0	8.0	30
70D	227	200-258	—	7.0	28.0	8.0	30
70ABCD	$\bar{x}$ = 227	—	204-250	7.0	28.0	8.0	30
71A	106	51-217	—	7.0	26.0	8.0	30
71B	63	46- 85	—	7.0	26.0	8.0	30
71C	—	—	—	7.0	26.0	8.0	30
71D	44	36-53	—	7.0	26.0	8.0	30
71ABD	$\bar{x}$ = 71	—	0-140	7.0	26.0	8.0	30
73A	40	12-133	—	7.0	24.0	8.0	30
73B	--	—	—	7.0	24.0	8.0	30
73C	—	—	—	7.0	24.0	8.0	30
73D	41	35- 48	—	7.0	24.0	8.0	30
73AD	$\bar{x}$ = 41	—	35- 47	7.0	24.0	8.0	30

TABLE 2. (continued)

TEST NO.	EC50	95% FIDUCIAL LIMITS	95% CONFIDENCE LIMITS	TEST CONDITIONS Temp. (°C)	Salinity (ppt)	pH	Exposure time (min)*
74A	122	70-210	—	12.0	28.0	8.0	30
74B	117	82-166	—	12.0	28.0	8.0	30
74C	99	59-168	—	12.0	28.0	8.0	30
74D	113	72-179	—	12.0	28.0	8.0	30
74ABCD	$\bar{x}$ = 113	—	97-129	12.0	28.0	8.0	30
75A	176	136-228	136-228	12.0	26.0	8.0	30
75B	177	124-253	—	12.0	26.0	8.0	30
75C	173	128-234	—	12.0	26.0	8.0	30
75D	152	112-207	—	12.0	26.0	8.0	30
75ABCD	$\bar{x}$ = 170	—	151-189	12.0	26.0	8.0	30
76A	114	61-213	—	12.0	24.0	8.0	30
76B	99	32-300	—	12.0	24.0	8.0	30
76C	113	18-707	—	12.0	24.0	8.0	30
76D	114	69-189	—	12.0	24.0	8.0	30
76ABCD	$\bar{x}$ = 110	—	98-122	12.0	24.0	8.0	30
77A	36	7-183	—	17.0	28.0	8.0	30
77B	31	3-280	—	17.0	28.0	8.0	30
77C	47	14-152	—	17.0	28.0	8.0	30
77D	—	—	—	17.0	28.0	8.0	30
77ABC	$\bar{x}$ = 38	—	18- 58	17.0	28.0	8.0	30
78A	36	29- 44	—	17.0	26.0	8.0	30
78B	28	19- 41	—	17.0	26.0	8.0	30
78C	30	24- 37	—	17.0	26.0	8.0	30
78D	30	22- 42	—	17.0	26.0	8.0	30
78ABCD	$\bar{x}$ = 31	—	25- 37	17.0	26.0	8.0	30

TABLE 2. (continued)

TEST NO.	EC50	95% FIDUCIAL LIMITS	95% CONFIDENCE LIMITS	TEST CONDITIONS Temp. (°C)	Salinity (ppt)	pH	Exposure time (min)*
79A	<19	—	—	17.0	24.0	8.0	30
79B	<19	—	—	17.0	24.0	8.0	30
79C	<19	—	—	17.0	24.0	8.0	30
79D	<19	—	—	17.0	24.0	8.0	30
			Silver — Artificial Seawater				
137A	—	—	—	9.1	30.0	8.0	30
137B	35	29- 43	—	9.1	30.0	8.0	30
137C	—	—	—	9.1	30.0	8.0	30
137D	66	49- 88	—	9.1	30.0	8.0	30
137CD	$\bar{x}$ = 51	—	0-248	9.1	30.0	8.0	30
138A	<20	—	—	9.1	30.0	8.0	60
138B	<20	—	—	9.1	30.0	8.0	60
138C	<20	—	—	9.1	30.0	8.0	60
138D	<20	—	—	9.1	30.0	8.0	60

* Exposure time to sperm unless otherwise noted

[1] Exposure time to eggs only

[2] Exposure time to eggs and sperm

APPENDIX TABLE 3. SUMMARY OF THE SEA URCHIN SPERM BIOASSAY TEST DATA AND TEST CONDITIONS USING ENDOSULFAN AS THE TOXICANT.

TEST NO.	EC50 (µg/l)	95% FIDUCIAL LIMITS	95% CONFIDENCE LIMITS	TEST CONDITIONS Temp. (°C)	Salinity (ppt)	pH	Exposure time (min)*
			Endosulfan Time-Dose				
97A	397	362-437	—	7.9	28.8	8.0	30
97B	412	393-432	—	7.9	28.8	8.0	30
97C	315	282-352	—	7.9	28.8	8.0	30
97D	288	250-331	—	7.9	28.8	8.0	30
97ABCD	$\bar{x}$ = 353	—	256-450	7.9	28.8	8.0	30
98A	170	114-253	—	7.9	28.8	8.0	60
98B	70	68- 71	—	7.9	28.8	8.0	60
98C	190	157-229	—	7.9	28.8	8.0	60
98D	--	—	—	7.9	28.8	8.0	60
98ABC	$\bar{x}$ = 143	—	0-303	7.9	28.8	8.0	60
100A	<66	—	—	7.7	28.0	8.0	90[1]
100B	<66	—	—	7.7	28.0	8.0	90[1]
100C	<66	—	—	7.7	28.0	8.0	90[1]
100D	<66	—	—	7.7	28.0	8.0	90[1]
101A	280	185-423	—	7.7	28.0	8.0	120[2]
101B	202	69-588	—	7.7	28.0	8.0	120[2]
101C	146	104-207	—	7.7	28.0	8.0	120[2]
101D	153	91-259	—	7.7	28.0	8.0	120[2]
101ABCD	$\bar{x}$ = 195	--	97-297	7.7	28.0	8.0	120[2]

TABLE 3 (continued)

TEST NO.	EC50 (µg/l)	95% FIDUCIAL LIMITS	95% CONFIDENCE LIMITS	TEST CONDITIONS Temp. (°C)	Salinity (ppt)	pH	Exposure time (min)*
			Endosulfan Sperm-Egg Sensitivity				
105A	>751	—	--	12.0	28.0	8.0	60^3
105B	>751	—	—	12.0	28.0	8.0	60^3
105C	>751	—	—	12.0	28.0	8.0	60^4
105D	>751	—	—	12.0	28.0	8.0	60^4
			Endosulfan pH				
128A	--	—	—	8.5	28.0	7.5	30
128B	—	—	—	8.5	28.0	7.5	30
128C	397	354-444	—	8.5	28.0	8.0	30
128D	405	316-519	—	8.5	28.0	8.0	30
128CD	$\bar{x}$ = 401	--	350-452	8.5	28.0	8.0	30
128E	353	257-486	—	8.5	28.0	8.5	30
128F	567	506-636	—	8.5	28.0	8.5	30
128EF	$\bar{x}$ = 460	—	0-1820	8.5	28.0	8.5	30
115A	552	518-588	—	7.0	28.0	8.0	30
115B	512	445-589	--	7.0	28.0	8.0	30
115C	519	436-617	—	7.0	28.0	8.0	30
115D	512	432-607	—	7.0	28.0	8.0	30
115ABCD	$\bar{x}$ = 524	—	496-554	7.0	28.0	8.0	30
116A	620	415-925	—	12.0	28.0	8.0	30
116B	543	431-684	—	12.0	28.0	8.0	30
116C	--	—	—	12.0	28.0	8.0	30
116D	545	512-581	—	12.0	28.0	8.0	30
116ABD	$\bar{x}$ = 569	—	525-613	12.0	28.0	8.0	30

TABLE 3 (continued)

				TEST CONDITIONS			
TEST NO.	EC50 (μg/l)	95% FIDUCIAL LIMITS	95% CONFIDENCE LIMITS	Temp. (°C)	Salinity (ppt)	pH	Exposure time (min)*
117A	361	308-423	—	17.0	28.0	8.0	30
117B	435	394-479	—	17.0	28.0	8.0	30
117C	415	379-454	—	17.0	28.0	8.0	30
117D	410	382-441	—	17.0	28.0	8.0	30
117ABCD	$\bar{x}$ = 405	—	355-455	17.0	28.0	8.0	30
120A	384	329-448	—	7.0	26.0	8.0	30
120B	374	335-416	—	7.0	26.0	8.0	30
120C	370	342-400	—	7.0	26.0	8.0	30
120D	394	358-433	—	7.0	26.0	8.0	30
120ABCD	$\bar{x}$ = 381	—	363-398	7.0	26.0	8.0	30
121A	<134	—	—	12.0	26.0	8.0	30
121B	52	47- 57	—	12.0	26.0	8.0	30
121C	140	52-378	—	12.0	26.0	8.0	30
121D	67	65- 69	—	12.0	26.0	8.0	30
121BCD	$\bar{x}$ = 86	—	0-172	12.0	26.0	8.0	30
122A	90	61-133	—	17.0	26.0	8.0	30
122B	248	199-308	—	17.0	26.0	8.0	30
122C	255	206-314	—	17.0	26.0	8.0	30
122D	269	183-396	—	17.0	26.0	8.0	30
122ABCD	$\bar{x}$ = 216	—	82-350	17.0	26.0	8.0	30
124A	< 88	—	—	7.0	24.0	8.0	30
124B	< 88	—	—	7.0	24.0	8.0	30
124C	58	54- 62	—	7.0	24.0	8.0	30
124D	< 88	—	—	7.0	24.0	8.0	30

TABLE 3 (continued)

TEST NO.	EC50 (μg/1)	95% FIDUCIAL LIMITS	95% CONFIDENCE LIMITS	TEST CONDITIONS Temp. (C°)	Salinity (ppt)	pH	Exposure time (min)*
125A	< 83	—	—	12.0	24.0	8.0	30
125B	< 83	—	—	12.0	24.0	8.0	30
125C	< 83	—	—	12.0	24.0	8.0	30
125D	< 83	—	—	12.0	24.0	8.0	30
126A	< 84	—	—	17.0	24.0	8.0	30
126B	< 84	—	—	17.0	24.0	8.0	30
126C	< 84	—	—	17.0	24.0	8.0	30
126D	< 84	—	—	17.0	24.0	8.0	30
Endosulfan — Artificial Seawater							
139A	122	29-520	—	8.9	30.0	8.0	30
139B	181	138-238	—	8.9	30.0	8.0	30
139C	222	178-277	—	8.9	30.0	8.0	30
139D	229	200-262	—	8.9	30.0	8.0	30
139ABCD	$\bar{x}$ = 189	—	111-277	8.9	30.0	8.0	30
140A	<101	—	—	8.9	30.0	8.0	60
140B	<101	—	—	8.9	30.0	8.0	60
140C	<101	—	—	8.9	30.0	8.0	60
140D	<101	—	—	8.9	30.0	8.0	60

* Exposure time to sperm unless otherwise noted

[1] 2000 sperm/egg ratio

[2] 4000 sperm/egg ratio

[3] Exposure time to eggs only

[4] Exposure time to eggs and sperm

APPENDIX TABLE 4. SUMMARY OF THE SAND DOLLAR SPERM BIOASSAY TEST DATA AND TEST CONDITIONS USING SILVER AS THE TOXICANT.

TEST NO.	EC50 (μg/l)	95% FIDUCIAL LIMITS	95% CONFIDENCE LIMITS	TEST CONDITIONS Temp. (°C)	Salinity (ppt)	pH	Exposure time (min)*
			Silver Time-Dose				
109A	179	157-197	—	13.2	28.0	8.0	15
109B	283	237-338	—	13.2	28.0	8.0	15
109C	126	109-146	—	13.2	28.0	8.0	15
109D	216	91-516	—	13.2	28.0	8.0	15
109ABCD	$\bar{x}$ = 201	—	96-306	13.2	28.0	8.0	15
110A	115	101-131	—	13.0	28.0	8.0	30
110B	111	102-120	—	13.0	28.0	8.0	30
110C	123	107-141	—	13.0	28.0	8.0	30
110D	148	126-173	—	13.0	28.0	8.0	30
110ABCD	$\bar{x}$ = 124	—	97-151	13.0	28.0	8.0	30
111A	42	36- 51	—	13.2	28.0	8.0	60
111B	56	47- 67	—	13.2	28.0	8.0	60
111C	41	28- 61	—	13.2	28.0	8.0	60
111D	49	45- 54	—	13.2	28.0	8.0	60
111ABCD	$\bar{x}$ = 47	—	36- 58	13.2	28.0	8.0	60
112A	21	19- 22	—	13.2	28.0	8.0	90
112B	32	17- 60	—	13.2	28.0	8.0	90
112C	16	0-771	—	13.2	28.0	8.0	90
112D	30	22- 42	—	13.2	28.0	8.0	90
112ABCD	$\bar{x}$ = 25	—	13- 37	13.2	28.0	8.0	90

TABLE 4. (continued)

TEST NO.	EC50	95% FIDUCIAL LIMITS	95% CONFIDENCE LIMITS	TEST CONDITIONS Temp. (°C)	Salinity (ppt)	pH	Exposure time (min)*
			Silver Sperm-Egg Sensitivity				
125A	49	25- 94	—	12.8	28.0	8.0	60
125B	43	5-357	—	12.8	28.0	8.0	60
125C	44	22- 88	—	12.8	28.0	8.0	60
125D	54	15-195	—	12.8	28.0	8.0	60
125ABCD	$\bar{x}$ = 48	—	40- 56	12.8	28.0	8.0	60
126A	>225	—	—	12.8	28.0	8.0	60[1]
126B	>225	—	—	12.8	28.0	8.0	60[1]
126C	>225	—	—	12.8	28.0	8.0	60[1]
126D	>225	—	—	12.8	28.0	8.0	60[1]
127C	54	39- 73	—	12.8	28.0	8.0	60[2]
127D	53	40- 70	—	12.8	28.0	8.0	60[2]
127CD	$\bar{x}$ = 54	—	48- 60	12.8	28.0	8.0	60[2]
			Silver — pH				
122A	53	44- 65	—	12.8	28.0	7.5	60
122B	58	50- 68	—	12.8	28.0	7.5	60
122C	46	35- 61	—	12.8	28.0	7.5	60
122D	53	45- 62	—	12.8	28.0	7.5	60
122ABCD	$\bar{x}$ = 53	—	45- 61	12.8	28.0	7.5	60
123A	45	40- 51	—	12.8	28.0	8.0	60
123B	51	45- 57	—	12.8	28.0	8.0	60
123C	47	37- 60	—	12.8	28.0	8.0	60
123D	55	47- 65	—	12.8	28.0	8.0	60
123ABCD	$\bar{x}$ = 50	43- 57					

TABLE 4. (continued)

				TEST CONDITIONS			
TEST NO.	EC50	95% FIDUCIAL LIMITS	95% CONFIDENCE LIMITS	Temp. (°C)	Salinity (ppt)	pH	Exposure time (min)*
124A	42	32- 56	—	12.8	28.0	8.5	60
124B	43	37- 49	—	12.8	28.0	8.5	60
124C	38	15- 94	—	12.8	28.0	8.5	60
124D	42	28- 64	—	12.8	28.0	8.5	60
124ABCD	$\bar{x}$ = 41	—	37- 45	12.8	28.0	8.5	60
			Silver Matrix				
113A	7	2- 22	—	7.0	24.0	8.0	30
113B	77	59-102	—	7.0	24.0	8.0	30
113C	79	64- 98	—	7.0	24.0	8.0	30
113D	34	28- 41	—	7.0	24.0	8.0	30
113ABCD	$\bar{x}$ = 49	—	0-105	7.0	24.0	8.0	30
114A	—	—	—	12.0	24.0	8.0	30
114B	91	56-146	—	12.0	24.0	8.0	30
114C	78	50-122	—	12.0	24.0	8.0	30
114D	—	—	—	12.0	24.0	8.0	30
114BC	$\bar{x}$ = 85	—	2-168	12.0	24.0	8.0	30
115A	<30	—	—	17.0	24.0	8.0	30
115B	44	5-362	—	17.0	24.0	8.0	30
115C	45	16-121	—	17.0	24.0	8.0	30
115D	<30	—	—	17.0	24.0	8.0	30
115BC	$\bar{x}$ = 45	—	39- 51	17.0	24.0	8.0	30

TABLE 4. (continued)

				TEST CONDITIONS			
TEST NO.	EC50	95% FIDUCIAL LIMITS	95% CONFIDENCE LIMITS	Temp. (°C)	Salinity (ppt)	pH	Exposure time (min)*
116A	127	55-293	—	7.0	26.0	8.0	30
116B	103	93-114	—	7.0	26.0	8.0	30
116C	125	113-137	—	7.0	26.0	8.0	30
116D	89	82- 96	—	7.0	26.0	8.0	30
116ABCD	$\bar{x}$ = 111	—	82-140	7.0	26.0	8.0	30
117A	78	68- 90	—	12.0	26.0	8.0	30
117B	75	66- 87	—	12.0	26.0	8.0	30
117C	85	78- 93	—	12.0	26.0	8.0	30
117D	94	84-106	—	12.0	26.0	8.0	30
117ABCD	$\bar{x}$ = 83	—	70- 96	12.0	26.0	8.0	30
118A	67	51- 87	—	17.0	26.0	8.0	30
118B	69	53- 90	—	17.0	26.0	8.0	30
118C	65	24-174	—	17.0	26.0	8.0	30
118D	64	49- 84	—	17.0	26.0	8.0	30
118ABCD	$\bar{x}$ = 66	—	62- 70	17.0	26.0	8.0	30
119A	150	132-170	—	7.0	28.0	8.0	30
119B	>150	—	—	7.0	28.0	8.0	30
119C	100	96-105	—	7.9	28.0	8.0	30
119D	152	134-173	—	7.0	28.0	8.0	30
119ACD	$\bar{x}$ = 134	—	117-151	7.0	28.0	8.0	30
120A	112	98-127	—	12.0	28.0	8.0	30
120B	>150	—	—	12.0	28.0	8.0	30
120C	84	75- 94	—	12.0	28.0	8.0	30
120D	93	84-104	—	12.0	28.0	8.0	30
120ACD	$\bar{x}$ = 96	—	60-132	12.0	28.0	8.0	30

TABLE 4. (continued)

				TEST CONDITIONS			
TEST NO.	EC50	95% FIDUCIAL LIMITS	95% CONFIDENCE LIMITS	Temp. (°C)	Salinity (ppt)	pH	Exposure time (min)*
121A	60	53- 68	—	17.0	28.0	8.0	30
121B	>90 <105	—	—	17.0	28.0	8.0	30
121C	>60 < 75	—	—	17.0	28.0	8.0	30
121D	83	79- 86	—	17.0	28.0	8.0	30
121AD	$\bar{x}$ = 72	—	0-218	17.0	28.0	8.0	30
			Silver — Artificial Seawater				
154A	55	40- 76	—	13.2	28.0	8.0	30
154B	56	52- 62	—	13.2	28.0	8.0	30
154C	—	—	—	13.2	28.0	8.0	30
154D	—	—	—	13.2	28.0	8.0	30
154AB	$\bar{x}$ = 56	—	50- 62	13.2	28.0	8.0	30
155A	31	29- 33	—	13.2	28.0	8.0	60
155B	29	25- 33	—	13.2	28.0	8.0	60
155C	16	6- 46	—	13.2	28.0	8.0	60
155D	<15	—	—	13.2	28.0	8.0	60
155ABC	$\bar{x}$ = 25	—	5- 45	13.2	28.0	8.0	60

* Exposure time to sperm unless otherwise noted.

[1] Exposure time to eggs only.

[2] Exposure time to eggs and sperm.

APPENDIX TABLE 5. SUMMARY OF THE SAND DOLLAR SPERM BIOASSAY TEST DATA AND TEST CONDITIONS USING ENDOSULFAN AS THE TOXICANT.

				TEST CONDITIONS			
TEST NO.	EC50 (μg/l)	95% FIDUCIAL LIMITS	95% CONFIDENCE LIMITS	Temp. (°C)	Salinity (ppt)	pH	Exposure time (min)*
			Endosulfan Time-Dose				
49A	152	125-171	—	13.3	28.0	8.0	30
49B	162	135-195	—	13.3	28.0	8.0	30
49C	188	158-224	—	13.3	28.0	8.0	30
49D	124	61-253	—	13.3	28.0	8.0	30
49ABCD	$\bar{x}$ = 157	—	115-199	13.3	28.0	8.0	30
50A	147	123-173	—	13.3	28.0	8.0	60
50B	150	130-168	—	13.3	28.0	8.0	60
50C	161	153-168	—	13.3	28.0	8.0	60
50D	146	118-182	—	13.3	28.0	8.0	60
50ABCD	$\bar{x}$ = 151	—	140-162	13.3	28.0	8.0	60
51A	135	98-186	—	13.3	28.0	8.0	90
51B	109	85-140	—	13.3	28.0	8.0	90
51C	114	61-213	—	13.3	28.0	8.0	90
51D	94	57-154	—	13.3	28.0	8.0	90
51ABCD	$\bar{x}$ = 113	—	86-140	13.3	28.0	8.0	90
52A	88	84- 92	—	13.3	28.0	8.0	120
52B	79	65- 96	—	13.3	28.0	8.0	120
52C	74	60- 92	—	13.3	28.0	8.0	120
52D	>49 <82	—	—	13.3	28.0	8.0	120
52ABCD	$\bar{x}$ = 80	—	62- 98	13.3	28.0	8.0	120

TABLE 5. (continued)

				TEST CONDITIONS			
TEST NO.	EC50	95% FIDUCIAL LIMITS	95% CONFIDENCE LIMITS	Temp. (°C)	Salinity (ppt)	pH	Exposure time (min)*
			Endosulfan Sperm-Egg Sensitivity				
73A	229	49-1077	—	13.0	28.0	8.0	60
73B	283	242- 332	—	13.0	28.0	8.0	60
73C	279	76-1021	—	13.0	28.0	8.0	60
73D	354	346- 363	—	13.0	28.0	8.0	60
73ABCD	$\bar{x}$ = 286	—	204-368	13.0	28.0	8.0	60
73E	377	286-495	—	13.0	28.0	8.0	60^1
73F	320	269-382	—	13.0	28.0	8.0	60^1
73G	395	220-707	—	13.0	28.0	8.0	60^1
73H	333	270-410	—	13.0	28.0	8.0	60^1
73EFGH	$\bar{x}$ = 356	—	299-413	13.0	28.0	8.0	60^1
73I	261	166-409	—	13.0	28.0	8.0	60^2
73J	187	109-323	—	13.0	28.0	8.0	60^2
73IJ	$\bar{x}$ = 224	—	0-694	13.0	28.0	8.0	60^2
			Endosulfan — pH				
67A	235	207-267	—	12.6	28.0	7.5	30
67B	212	172-261	—	12.6	28.0	7.5	30
67C	258	238-280	—	12.6	28.0	7.5	30
67D	217	175-269	—	12.6	28.0	7.5	30
67ABCD	$\bar{x}$ = 231	—	198-264	12.6	28.0	7.5	30
68A	285	265-306	—	12.6	28.0	8.0	30
68B	257	250-264	—	12.6	28.0	8.0	30
68C	265	242-289	—	12.6	28.0	8.0	30
68D	276	245-312	—	12.6	28.0	8.0	30
68ABCD	$\bar{x}$ = 271	—	251-291	12.6	28.0	8.0	30
69A	247	225-272	—	12.6	28.0	8.5	30
69B	201	99-408	—	12.6	28.0	8.5	30
69C	239	233-245	—	12.6	28.0	8.5	30
69D	276	236-332	—	12.6	28.0	8.5	30
69ABCD	$\bar{x}$ = 229	—	190-268	12.6	28.0	8.5	30

TABLE 5. (continued)

				TEST CONDITIONS			
TEST NO.	EC50	95% FIDUCIAL LIMITS	95% CONFIDENCE LIMITS	Temp. (°C)	Salinity (ppt)	pH	Exposure time (min)*
			Endosulfan Matrix				
53A	174	169- 179	—	7.0	28.0	8.0	30
53B	211	177- 252	—	7.0	28.0	8.0	30
53C	122	97- 154	—	7.0	28.0	8.0	30
53D	163	137- 193	—	7.0	28.0	8.0	30
53ABCD	$\bar{x}$ = 168	—	109-217	7.0	28.0	8.0	30
54A	<262	—	—	12.0	28.0	8.0	30
54B	<262	—	—	12.0	28.0	8.0	30
54C	253	215- 299	—	12.0	28.0	8.0	30
54D	225	165- 308	—	12.0	28.0	8.0	30
54 CD	$\bar{x}$ = 239	—	61-417	12.0	28.0	8.0	30
55A	282	76-1048	—	17.0	28.0	8.0	30
55B	267	225- 317	—	17.0	28.0	8.0	30
55C	228	187- 278	—	17.0	28.0	8.0	30
55D	176	128- 242	—	17.0	28.0	8.0	30
55ABCD	$\bar{x}$ = 238	—	162-319	17.0	28.0	8.0	30
56A	65	60- 70	—	7.0	26.0	8.0	30
56B	<68	—	—	7.0	26.0	8.0	30
56C	<68	—	—	7.0	26.0	8.0	30
56D	>68 <85	—	—	7.0	26.0	8.0	30
57A	158	129- 193	—	12.0	26.0	8.0	30
57B	130	107-158	—	12.0	26.0	8.0	30
57C	97	93-101	—	12.0	26.0	8.0	30
57D	121	109-134	—	12.0	26.0	8.0	30
57ABCD	$\bar{x}$ = 127	—	87-167	12.0	26.0	8.0	30

TABLE 5. (continued)

TEST NO.	EC50	95% FIDUCIAL LIMITS	95% CONFIDENCE LIMITS	TEST CONDITIONS Temp. (°C)	Salinity (ppt)	pH	Exposure time (min)*
58A	192	182-202	—	17.0	26.0	8.0	30
58B	146	132-160	—	17.0	26.0	8.0	30
58C	147	120-181	—	17.0	26.0	8.0	30
58D	133	108-163	—	17.0	26.0	8.0	30
58ABCD	$\bar{x}$ = 155	—	114-196	17.0	26.0	8.0	30
59A	110	105-114	—	7.0	24.0	8.0	30
59B	84	71- 98	—	7.0	24.0	8.0	30
59C	106	102-110	—	7.0	24.0	8.0	30
59D	118	115-121	—	7.0	24.0	8.0	30
59ABCD	$\bar{x}$ = 105	—	83-128	7.0	24.0	8.0	30
60A	147	141-153	—	12.0	24.0	8.0	30
60B	141	108-184	—	12.0	24.0	8.0	30
60C	>134	—	—	12.0	24.0	8.0	30
60D	>134	—	—	12.0	24.0	8,0	30
60AB	$\bar{x}$ = 144	—	106-182	12.0	24.0	8.0	30
61A	202	192-213	—	17.0	24.0	8.0	30
61B	193	166-223	—	17.0	24.0	8.0	30
61C	>195	—	—	17.0	24.0	8.0	30
61D	217	203-223	—	17.0	24.0	8.0	30
61ABD	$\bar{x}$ = 204	—	174-234	17.0	24.0	8.0	30
Endosulfan — Artificial Seawater							
71A	152	131-176	—	13.0	28.0	8.0	30
71B	190	166-217	—	13.0	28.0	8.0	30
71C	186	176-197	—	13.0	28.0	8.0	30
71D	246	198-305	—	13.0	28.0	8.0	30
71ABCD	$\bar{x}$ = 193	—	131-255	13.0	28.0	8.0	30

TABLE 5. (continued)

					TEST CONDITIONS		
TEST NO.	EC50	95% FIDUCIAL LIMITS	95% CONFIDENCE LIMITS	Temp. (°C)	Salinity (ppt)	pH	Exposure time (min)*
72A	155	151-159	--	13.0	28.0	8.0	60
72B	190	. 172-210	—	13.0	28.0	8.0	60
72C	169	146-195	--	13.0	28.0	8.0	60
72D	223	125-395	--	13.0	28.0	8.0	60
72ABCD	$\bar{x}$ = 184	—	137-231	13.0	28.0	8.0	60
			Endosulfan β				
75A	>48 <74	—	—	13.5	28.0	8.0	60
75B	>48 <74	—	—	13.5	28.0	8.0	60
75C	65	44- 94	—	13.5	28.0	8.0	60
75D	>48 <74	—	—	13.5	28.0	8.0	60
			Endosulfan α				
76A	269	214-339	—	13.5	28.0	8.0	60
76B	239	217-264	—	13.5	28.0	8.0	60
76C	243	234-253	—	13.5	28.0	8.0	60
76D	297	291-303	—	13.5	28.0	8.0	60
76ABCD	$\bar{x}$ = 262	—	219-305	13.5	28.0	8.0	60
			Endosulfan Ether				
77A	>530 <678	—	—	13.5	28.0	8.0	60
77B	>457 <531	—	—	13.5	28.0	8.0	60
77C	484	328-715	—	13.5	28.0	8.0	60
77D	582	25-13595	—	13.5	28.0	8.0	60
77CD	$\bar{x}$ = 533	—	0-1155	13.5	28.0	8.0	60

TABLE 5. (continued)

				TEST CONDITIONS			
TEST NO.	EC50	95% FIDUCIAL LIMITS	95% CONFIDENCE LIMITS	Temp. (°C)	Salinity (ppt)	pH	Exposure time (min)*
			Endosulfan Sulfate				
79A	>288 <351	—	—	13.5	28.0	8.0	60
79B	193	144-260	—	13.5	28.0	8.0	60
79C	194	73-517	—	13.5	28.0	8.0	60
79D	238	229-247	—	13.5	28.0	8.0	60
79BCD	$\bar{x}$ = 208	—	146-272	13.5	28.0	8.0	60

* Exposure time to sperm unless otherwise noted

[1] Exposure time to eggs only

[2] Exposure time to eggs and sperm