SECTION 15

PURPLE URCHIN, *Strongylocentrotus purpuratus* AND SAND DOLLAR, *Dendraster excentricus* LARVAL DEVELOPMENT TEST METHOD

Adapted from a method developed by
Steven Bay and Darrin Greenstein
Southern California Coastal Water Research Project
Westminster, CA 92683

TABLE OF CONTENTS

SECTION 15

SEA URCHIN, *Strongylocentrotus purpuratus* AND SAND DOLLAR, *Dendraster excentricus* LARVAL DEVELOPMENT TEST

15.1 SCOPE AND APPLICATION

15.1.1 This method estimates the chronic toxicity of effluents and receiving waters to the developing embryos of the purple sea urchin, *Strongylocentrotus purpuratus*, and the sand dollar, *Dendraster excentricus*, during a 72-h static non-renewal exposure. The effects include the synergistic, antangonistic, and additive effects of all chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms.

15.1.2 Detection limits of the toxicity of an effluent or chemical substance are organism dependent.

15.1.3 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling and because the test chambers are not sealed, highly volatile and highly degradable toxicants in the source may not be detected in the test.

15.1.4 This test is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

15.1.5 This method should be restricted to use by, or under the supervision of, professionals experienced in aquatic toxicity testing. Specific experience with any toxicity test is usually needed before acceptable results become routine.

15.2 SUMMARY OF METHOD

15.2.1 The method provides the step-by-step instructions for

performing a 72-h static non-renewal test using the early development of the purple sea urchin, *Strongylocentrotus purpuratus*, and the sand dollar, *Dendraster excentricus*, to determine the toxicity of substances in marine and estuarine

waters. The test endpoint is normal larval development and may include mortality if modified for total counts at test initiation and termination.

15.3 **INTERFERENCES**

15.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities, Equipment, and Supplies).

15.3.2 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling and Sample Handling, and Sample Preparation for Toxicity Tests).

15.4 **SAFETY**

15.4.1 See Section 3, Health and Safety

15.5 **APPARATUS AND EQUIPMENT**

15.5.1 Tanks, trays, or aquaria -- for holding and acclimating adult sea urchins and sand dollars, e.g., standard salt water aquarium or Instant Ocean Aquarium (capable of maintaining seawater at 10-20EC), with appropriate filtration and aeration system.

15.5.2 Air pump, air lines, and air stones -- for aerating water containing broodstock or for supplying air to test solutions with low dissolved oxygen.

15.5.3 Constant temperature chambers or water baths -- for maintaining test solution temperature and keeping dilution water supply, gametes, and embryo stock suspensions at test temperature (15EC) prior to the test.

15.5.4 Water purification system -- Millipore Super-Q, Deionized water (DI) or equivalent.

15.5.5 Refractometer -- for determining salinity.

15.5.6 Hydrometer(s) -- for calibrating refractometer.

15.5.7 Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

15.5.8 Thermometer, National Bureau of Standards Certified (see USEPA METHOD l70.l, USEPA, l979) -- to calibrate laboratory thermometers.

15.5.9 pH and DO meters -- for routine physical and chemical measurements.

15.5.10 Standard or micro-Winkler apparatus -- for determining DO (optional) and calibrating the DO meter.

15.5.11 Winkler bottles -- for dissolved oxygen determinations.

15.5.12 Balance -- Analytical, capable of accurately weighing to 0.0001 g.

15.5.13 Fume hood -- to protect the analyst from effluent or formaldehyde fumes.

15.5.14 Glass stirring rods -- for mixing test solutions.

15.5.15 Graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 50-l000 mL for making test solutions. (Note: not to be used interchangeably for gametes or embryos and test solutions).

15.5.16 Volumetric flasks -- Class A, borosilicate glass or non-toxic plastic labware, 100-1000 mL for making test solutions.

15.5.17 Pipets, automatic -- adjustable, to cover a range of delivery volumes from 0.010 to 1.000 mL.

15.5.18 Pipet bulbs and fillers -- PROPIPET® or equivalent.

15.5.19 Wash bottles -- for reagent water, for topping off graduated cylinders, for rinsing small glassware and instrument electrodes and probes.

15.5.20 Wash bottles -- for dilution water.

15.5.21 20-liter cubitainers or polycarbonate water cooler jugs -- for making hypersaline brine.

15.5.22 Cubitainers, beakers, or similar chambers of non-toxic composition for holding, mixing, and dispensing dilution water and other general non-effluent, non-toxicant contact uses. These should be clearly labeled and not used for other purposes.

15.5.23 Beakers, 5-10 mL borosilicate glass -- for collecting sperm from sand dollars.

15.5.24 Beakers, 250 mL borosilicate glass -- for preparation of test solutions.

15.5.25 Beakers, 100 mL borosilicate glass -- for spawning; to support sea urchins and to collect sea urchin and sand dollar eggs.

15.5.26 Beakers, 1,000 mL borosilicate glass -- for rinsing and settling sea urchin eggs.

16.5.27 Vortex mixer -- to mix sea urchin semen in tubes prior to sampling.

15.5.28 Compound microscope -- for examining gametes, counting sperm cells (200-400x), eggs and embryos and (100x), and examining larvae. Dissecting scopes are sometimes used to count eggs at a lower magnification. One piece of equipment worthy of a special mention is an inverted microscope. The use of an inverted scope is not required, but recommended. Its use reduces the exposure of workers to hazardous fumes (formalin or glutaraldehyde) during the counting of larvae and reduces sample examination time. Alternatively, a Sedgewick-Rafter cell may be used on a regular compound scope.

15.5.29 Counter, two unit, 0-999 -- for recording sperm, egg, embryo, and larval counts.

15.5.30 Sedgwick-Rafter counting chamber -- for counting egg and embryo stock and examining larval development at the end of the test.

15.5.31 Centrifuge tubes, test tubes, or vials -- for holding semen.

15.5.32 Hemacytometers, Neubauer -- for counting sperm.

15.5.33 Siphon hose (3 mm i.d.) -- for removing wash water from settled eggs.

15.5.34 Perforated plunger -- for maintaining homogeneous distribution of eggs and embryos during sampling and distribution to test chambers.

15.5.35 Enamel or plastic tray -- for optional spawning platform.

15.5.36 Nitex® screening (0.5mm mesh) -- cleaning egg solutions.

15.5.37 60 µm NITEX® filter -- for filtering receiving waters.

15.6 **REAGENTS AND SUPPLIES**

15.6.1 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests).

15.6.2 Data sheets (one set per test) -- for data recording (see Figures 1-4).

15.6.3 Tape, colored -- for labelling test chambers and containers.

15.6.4 Markers, water-proof -- for marking containers, etc.

15.6.5 Parafilm -- to cover graduated cylinders and vessels containing gametes and embryos.

15.6.6 Gloves, disposable -- for personal protection from contamination.

15.6.7 Pipets, serological -- l-l0 mL, graduated.

15.6.8 Pipet tips -- for automatic pipets. Note: pipet tips for handling semen should be cut off to produce an opening about 1 mm in diameter; pipet tips for handling eggs should be cut off to produce an opening about 2 mm in diameter. This is necessary to provide smooth flow of the viscous semen, accurate sampling of eggs, and to prevent injury to eggs passing through a restricted opening. A clean razor blade can be used to trim pipet tips.

15.6.9 Coverslips -- for microscope slides.

15.6.10 Lens paper -- for cleaning microscope optics.

15.6.11 Laboratory tissue wipes -- for cleaning and drying electrodes, microscope slides, etc.

15.6.12 Disposable countertop covering -- for protection of work surfaces and minimizing spills and contamination.

15.6.13 pH buffers 4, 7, and l0 (or as per instructions of instrument manufacturer) -- for standards and calibration check (see USEPA Method l50.l, USEPA, l979).

15.6.14 Membranes and filling solutions -- for dissolved oxygen probe (see USEPA Method 360.l, USEPA, l979), or reagents for modified Winkler analysis.

15.6.15 Laboratory quality assurance samples and standards -- for the above methods.

15.6.16 Test chambers -- 30-mL glass scintillation vials with polypropylene caps, four chambers per concentration.

15.6.17 Formaldehyde, 10%, in seawater -- for preserving larvae. Note: formaldehyde has been identified as a carcinogen and is irritating to skin and mucous membranes. It should not be used at a concentration higher than necessary to achieve morphological preservation of larvae for counting and only under conditions of maximal ventilation and minimal opportunity for volatilization into room air.

15.6.18 Glutaraldehyde, 1% in seawater -- for preserving larvae.

Figure 1. Sample experiment set-up sheet.

SEA URCHIN EMBRYO DEVELOPMENT TEST

EXPERIMENT DESCRIPTION

Bioassay No._____ Start Date_____ Start time_____

Investigator_____ End Date_____ End Time_____

Sample Numbers	Sample	Concentration	Other Information

Figure 2. Sample worksheet for urchin spawning information.

SEA URCHIN DEVELOPMENT TEST

SPAWNING WORKSHEET

Bioassay no._____ Date _____

Spawning

No.	Injection time	Sex	Accepted? (Comments)
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			

Sperm density

#sperm counted=_____
_____
_____ mean=_____
_____

(mean)_____ x (5 x 10^6)=_____ sperm/mL

Egg dilution

eggs counted=_____
_____ mean=_____
_____

(mean)_____ x 100=_____ eggs/mL in stock

eggs/mL in stock ÷ 1,000= _____ Egg dilution factor

Figure 3. Sample worksheet for sea urchin fertilization information.

SEA URCHIN DEVELOPMENT TEST
FERTILIZATION WORKSHEET

Bioassay No. _____
Date_____

_____mL eggs used _____mL dilution water used

Fertilization and initiation

_____mL in egg dilution x 1,000 eggs/mL
= _____eggs in dilution

_____eggs in dilution x 500 sperm/egg
= _____sperm needed

_____sperm needed ÷ _____sperm/mL in sperm dilution=_____mL sperm dilution needed

Percent fertilized after 10 min _____

Time of inoculation_____

Figure 4. Sample data sheet for embryo microscopic examination.

SEA URCHIN DEVELOPMENT TEST: RESULTS

Bioassay No._____ Date_____

Counter_____

Number	Sample	Abnormal	Normal	%Normal	Notes

15.6.19 Acetic acid, 10%, reagent grade, in filtered (10F) seawater -- for preparing killed sperm dilutions for sperm counts.

15.6.20 Haemo-Sol or equivalent cleaner -- for cleaning hemacytometer and cover slips.

15.6.21 0.5 M KCl solution -- for inducing spawning.

15.6.22 Syringe, disposable, 3 or 5 mL -- for injecting KCl into sea urchins and sand dollars to induce spawning.

15.6.23 Needles, 25 gauge -- for injecting KCl.

15.6.24 Pasteur pipets and bulbs -- for sampling eggs from spawning beakers.

15.6.25 Hematocrit capillary tubes -- for sampling sperm for examination and for loading hemacytometers.

15.6.26 Microscope well-slides -- for pre-test assessment of sperm activity and egg condition.

15.6.27 Reference toxicant solutions (see Section 15.10.2.4 and Section 4, Quality Assurance).

15.6.28 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms (see Section 5, Facilities, Equipment, and Supplies).

15.6.29 Effluent and receiving water -- see Section 8, Effluent and Surface Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests.

15.6.30 Dilution water and hypersaline brine -- see Section 7, Dilution Water and Section 15.6.24, Hypersaline Brines. The dilution water should be uncontaminated 1-µm-filtered natural seawater. Hypersaline brine should be prepared from dilution water.

15.6.31 HYPERSALINE BRINES

15.6.31.1 Most industrial and sewage treatment effluents

entering marine and estuarine systems have little measurable salinity. Exposure of larvae to these effluents will usually require increasing the salinity of the test solutions. It is important to maintain an essentially constant salinity across all treatments. In some applications it may be desirable to match the test salinity with that of the receiving water (See Section 7.1). Two salt sources are available to adjust salinities -- artificial sea salts and hypersaline brine (HSB) derived from natural seawater. Use of artificial sea salts is necessary only when high effluent concentrations preclude salinity adjustment by HSB alone.

15.6.31.2 Hypersaline brine (HSB) can be made by concentrating natural seawater by freezing or evaporation. HSB should be made from high quality, filtered seawater, and can be added to the effluent or to reagent water to increase salinity. HSB has several desirable characteristics for use in effluent toxicity testing. Brine derived from natural seawater contains the necessary trace metals, biogenic colloids, and some of the microbial components necessary for adequate growth, survival, and/or reproduction of marine and estuarine organisms, and it can be stored for prolonged periods without any apparent degradation. However, even if the maximum salinity HSB (100‰) is used as a diluent, the maximum concentration of effluent (0‰) that can be tested is 66% effluent at 34‰ salinity (see Table 1).

15.6.31.3 High quality (and preferably high salinity) seawater should be filtered to at least 10 µm before placing into the freezer or the brine generator. Water should be collected on an incoming tide to minimize the possibility of contamination.

15.6.31.4 Freeze Preparation of Brine

15.6.31.4.1 A convenient container for making HSB by freezing is one that has a bottom drain. One liter of brine can be made from four liters of seawater. Brine may be collected by partially freezing seawater at -10 to -20EC until the remaining liquid has reached the target salinity. Freeze for approximately six hours, then separate the ice (composed mainly of fresh water) from the remaining liquid (which has now become hypersaline).

TABLE 1. MAXIMUM EFFLUENT CONCENTRATION (%) THAT CAN BE TESTED AT 34‰ WITHOUT THE ADDITION OF DRY SALTS GIVEN THE INDICATED EFFLUENT AND BRINE SALINITIES.

Effluent Salinity ‰	Brine 60 ‰	Brine 70 ‰	Brine 80 ‰	Brine 90 ‰	Brine 100 ‰
0	43.33	51.43	57.50	62.22	66.00
1	44.07	52.17	58.23	62.92	66.67
2	44.83	52.94	58.97	63.64	67.35
3	45.61	53.73	59.74	64.37	68.04
4	46.43	54.55	60.53	65.12	68.75
5	47.27	55.38	61.33	65.88	69.47
10	52.00	60.00	65.71	70.00	73.33
15	57.78	65.45	70.77	74.67	77.65
20	65.00	72.00	76.67	80.00	82.50
25	74.29	80.00	83.64	86.15	88.00

15.6.31.4.2 It is preferable to monitor the water until the target salinity is achieved rather than allowing total freezing followed by partial thawing. Brine salinity should never exceed 100‰. It is advisable not to exceed about 70‰ brine salinity unless it is necessary to test effluent concentrations greater than 50%.

15.6.31.4.3 After the required salinity is attained, the HSB should be filtered through a 1 µm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4EC (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

15.6.31.5 Heat Preparation of Brine

15.6.31.5.1 The ideal container for making brine using heat-assisted evaporation of natural seawater is one that (1) has a high surface to volume ratio, (2) is made of a non-corrosive material, and (3) is easily cleaned (fiberglass containers are ideal). Special care should be used to prevent any toxic materials from coming in contact with the seawater being used to generate the brine. If a heater is immersed directly into the seawater, ensure that the heater materials do not corrode or leach any substances that would contaminate the brine. One successful method is to use a thermostatically controlled heat exchanger made from fiberglass. If aeration is needed, use only oil-free air compressors to prevent contamination.

15.6.31.5.2 Before adding seawater to the brine generator, thoroughly clean the generator, aeration supply tube, heater, and any other materials that will be in direct contact with the brine. A good quality biodegradable detergent should be used, followed by several (at least three) thorough reagent water rinses.

15.6.31.5.3 Seawater should be filtered to at least 10 µm before being put into the brine generator. The temperature of the seawater is increased slowly to 40EC. The water should be aerated to prevent temperature stratification and to increase water evaporation. The brine should be checked daily (depending on the volume being generated) to ensure that the salinity does not exceed 100‰ and that the temperature does not exceed 40EC. Additional seawater may be added to the brine to obtain the volume of brine required.

15.6.31.5.4 After the required salinity is attained, the HSB should be filtered through a 1 µm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4EC (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

15.6.31.6 Artificial Sea Salts

15.6.31.6.1 No data from sea urchin or sand dollar larval tests using sea salts or artificial seawater (e.g., GP2) are available

for evaluation at this time, and their use must be considered provisional.

15.6.31.7 Dilution Water Preparation from Brine

15.6.31.7.1 Although salinity adjustment with brine is the preferred method, the use of high salinity brines and/or reagent water has sometimes been associated with discernible adverse effects on test organisms. For this reason, it is recommended that only the minimum necessary volume of brine and reagent water be used to offset the low salinity of the effluent, and that brine controls be included in the test. The remaining dilution water should be natural seawater. Salinity may be adjusted in one of two ways. First, the salinity of the highest effluent test concentration may be adjusted to an acceptable salinity, and then serially diluted. Alternatively, each effluent concentration can be prepared individually with appropriate volumes of effluent and brine.

15.6.31.7.2 When HSB and reagent water are used, thoroughly mix together the reagent water and HSB before mixing in the effluent. Divide the salinity of the HSB by the expected test salinity to determine the proportion of reagent water to brine. For example, if the salinity of the brine is 100‰ and the test is to be conducted at 34‰, 100‰ divided by 34‰ = 2.94. The proportion is 1 part brine plus 1.94 parts reagent water. To make 1 L of dilution water at 34‰ salinity from a HSB of 100‰, 340 mL of brine and 660 mL of reagent water are required. Verify the salinity of the resulting mixture using a refractometer.

15.6.31.8 Test Solution Salinity Adjustment

15.6.31.8.1 Table 2 illustrates the preparation of test solutions (up to 50% effluent) at 34‰ by combining effluent, HSB, and dilution water. Note: if the highest effluent concentration does not exceed 50% effluent, it is convenient to prepare brine so that the sum of the effluent salinity and brine salinity equals 68‰; the required brine volume is then always equal to the effluent volume needed for each effluent concentration as in the example in Table 2.

15.6.31.8.2 Check the pH of all test solutions and adjust to within 0.2 units of dilution water pH by adding, dropwise, dilute

hydrochloric acid or sodium hydroxide (see Section 8.8.9, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

15.6.31.8.3 To calculate the amount of brine to add to each effluent dilution, determine the following quantities: salinity of the brine (SB, in ‰), the salinity of the effluent (SE, in ‰), and volume of the effluent to be added (VE, in mL). Then use the following formula to calculate the volume of brine (VB, in mL) to be added:

$$VB = VE \times (34 - SE)/(SB - 34)$$

15.6.31.8.4 This calculation assumes that dilution water salinity is 34 ± 2‰.

15.6.31.9 Preparing Test Solutions

15.6.31.9.1 Ten mL of test solution are needed for each test chamber. To prepare test solutions at low effluent concentrations (<6%), effluents may be added directly to dilution water. For example, to prepare 1% effluent, add 1.0 mL of effluent to a 100-mL volumetric flask using a volumetric pipet or calibrated automatic pipet. Fill the volumetric flask to the 100-mL mark with dilution water, stopper it, and shake to mix. Pour into a (150-250 mL) beaker and stir. Distribute equal volumes into the replicate test chambers. The remaining test solution can be used for chemistry.

15.6.31.9.2 To prepare a test solution at higher effluent concentrations, hypersaline brine must usually be used. For example, to prepare 40% effluent, add 400 mL of effluent to a 1-liter volumetric flask. Then, assuming an effluent salinity of 2‰ and a brine salinity of 66‰, add 400 mL of brine (see equation above and Table 2) and top off the flask with dilution water. Stopper the flask and shake well. Pour into a (100-250 mL) beaker and stir. Distribute equal volumes into the replicate test chambers. The remaining test solution can be used for chemistry.

TABLE 2. EXAMPLES OF EFFLUENT DILUTION SHOWING VOLUMES OF EFFLUENT (AT X‰), BRINE, AND DILUTION WATER NEEDED FOR ONE LITER OF EACH TEST SOLUTION.

FIRST STEP: Combine brine with reagent water or natural seawater to achieve a brine of 68-x‰ and, unless natural seawater is used for dilution water, also a brine-based dilution water of 34‰.

SERIAL DILUTION:

Step 1. Prepare the highest effluent concentration to be tested by adding equal volumes of effluent and brine to the appropriate volume of dilution water. An example using 40% is shown.

Effluent Conc. (%)	Effluent x‰	Brine (68-x)‰	Dilution Water* 34‰
40	800 mL	800 mL	400 mL

Step 2. Make serial dilutions from the highest test concentration.

Effluent Conc. (%)	Effluent Source	Dilution Water* (34‰)
20	1000 mL of 40%	1000 mL
10	1000 mL of 20%	1000 mL
5	1000 mL of 10%	1000 mL
2.5	1000 mL of 5%	1000 mL
Control	none	1000 mL

INDIVIDUAL PREPARATION:

Effluent Conc. (%)	Effluent x‰	Brine (68-x)‰	Dilution Water* 34‰
40	400 mL	400 mL	200 mL
20	200 mL	200 mL	600 mL
10	100 mL	100 mL	800 mL
5	50 mL	50 mL	900 mL
2.5	25 mL	25 mL	950 mL
Control	none	none	1000 mL

*May be natural seawater or brine-reagent water equivalent.

15.6.31.10 Brine Controls

15.6.31.10.1 Use brine controls in all tests where brine is used. Brine controls contain the same volume of brine as does the highest effluent concentration using brine, plus the volume of reagent water needed to reproduce the hyposalinity of the effluent in the highest concentration, plus dilution water. Calculate the amount of reagent water to add to brine controls by rearranging the above equation, (See, 15.6.31.8.3) setting SE = 0, and solving for VE.

$$VE = VB \times (SB - 34)/(34 - SE)$$

If effluent salinity is essentially 0‰, the reagent water volume needed in the brine control will equal the effluent volume at the highest test concentration. However, as effluent salinity and effluent concentration increase, less reagent water volume is needed.

15.6.32 TEST ORGANISMS, PURPLE URCHINS

15.6.32.1 Sea Urchins, *Strongylocentrotus purpuratus* (approximately 6 of each sex per test).

15.6.32.2 Species Identification

15.6.32.2.1 Although identification of purple sea urchins, *Strogylocentrotus purpuratus*, is usually a simple matter of confirming general body color, size, and spine appearance, those unfamiliar with the species should seek confirmation from local experts.

15.6.32.3 Obtaining Broodstock

15.6.32.3.1 Adult sea urchins (*Strongylocentrotus purpuratus*) can be obtained from commercial suppliers or collected from uncontaminated intertidal areas. State collection permits are usually required for collection of sea urchins and collection is prohibited or restricted in some areas. The animals are best transported "dry," surrounded either by moist seaweed or paper towels dampened with seawater. Animals should be kept at approximately their collection or culture temperature to prevent thermal shock which can prematurely induce spawning.

15.6.32.4 Broodstock Culture and Handling

15.6.32.4.1 The adult sea urchins are maintained in glass aquaria or fiberglass tanks. The tanks are supplied continuously (approximately 5 L/min) with filtered natural seawater, or salt water prepared from commericial sea salts is recirculated. The animals are checked daily and any obviously unhealthy animals are discarded.

15.6.32.4.2 Although ambient temperature seawater is usually acceptable, maintaining sea urchins in spawning condition usually requires holding at a relatively constant temperature. The culture unit should be capable of maintaining a constant temperature between 10 and 14EC with a water temperature control device.

15.6.32.4.3 Food for sea urchins -- kelp, recommended, but not necessarily limited to, *Laminaria sp.*, *Hedophyllum sp.*, *Nereocystis sp.*, *Macrocystis sp.*, *Egregia sp.*, *Alaria sp.* or romaine lettuce. The kelp should be gathered from known uncontaminated zones or obtained from commerical supply houses whose kelp comes from known uncontaminated areas, or romaine lettuce. Fresh food is introduced into the tanks at least several times a week. Sun dried (12-24 hours) or oven dried (60EC overnight) kelp, stores well at room temperature or frozen, rehydrates well and is adequate to maintain sea urchins for long periods. Decaying food and fecal pellets are removed as necessary to prevent fouling.

15.6.32.4.4 Natural seawater (>30‰) is used to maintain the adult animals and ($32‰) as a control water in the tests.

15.6.32.4.5 Adult male and female (if sexes known) animals used in field studies are transported in separate or partitioned insulated boxes or coolers packed with wet kelp or paper toweling. Upon arrival at the field site, aquaria (or a single partitioned aquarium) are filled with control water, loosely covered with a styrofoam sheet and allowed to equilibrate to the holding temperature before animals are added. Healthy animals will attach to the kelp or aquarium within hours.

15.6.32.4.6 To successfully maintain about 25 adult animals for seven days at a field site, 40-L glass aquaria using aerated,

recirculating, clean saline water (32‰) and a gravel bed filtration system, are housed within a water bath, such as an INSTANT OCEAN® Aquarium. The sexes should be held separately if possible.

15.6.33 TEST ORGANISMS, SAND DOLLARS

15.6.33.1 Sand Dollars, *Dendraster excentricus*, (approximately 6 of each sex per test).

15.6.33.2 Species Identification

15.6.33.2.1 Although identification of sand dollars, *Dendraster excentricus*, is usually a simple matter of confirming general body appearance, those unfamiliar with the species should seek confirmation from local experts.

15.6.33.3 Obtaining Broodstock

15.6.33.3.1 Adult sand dollars (*Dendraster excentricus*) can be obtained from commercial suppliers or collected from subtidal zones (most areas) or from intertidal zones of some sheltered waters (e.g., Puget Sound). State collection permits may be required for collection of sand dollars and collection prohibited or restricted in some areas. The animals are best transported "dry," surrounded either by moist seaweed or paper towels dampened with seawater. Animals should be kept at approximately their collection or culture temperature to prevent thermal shock which can prematurely induce spawning.

15.6.33.4 Broodstock Culture and Handling

15.6.33.4.1 The adult sand dollars are maintained in glass aquaria or fiberglass tanks. The tanks are supplied continuously (approximately 5 L/min) with filtered natural seawater, or saltwater prepared from commercial sea salts is recirculated. The animals are checked daily and any obviously unhealthy animals are discarded. For longer periods than a few days, several centimeters or more of a sand substrate may be desirable.

15.6.33.4.2 Although ambient temperature seawater is usually acceptable, maintaining sand dollars in spawning condition usually requires holding at a relatively constant temperature.

The culture unit should be capable of maintaining a constant temperature between 8 and 12EC with a water temperature control device.

15.6.33.4.3 Sand dollars will feed on suspended or benthic materials such as phytoplankton, benthic diatoms, etc. No reports of laboratory populations being maintained in spawning condition over several years are known. It is probably most convenient to obtain sand dollars, use them, and then discard them after they cease to produce good quality gametes.

15.6.33.4.4 Natural seawater (>30‰) is used to maintain the adult animals and ($32‰) as a control water in the tests.

15.6.33.4.5 Adult male and female (if sexes known) animals used in field studies are transported in separate or partitioned insulated boxes or coolers packed with wet kelp or paper toweling. Upon arrival at the field site, trays or aquaria (or a single partitioned aquarium) are filled with control water, loosely covered with a styrofoam sheet and allowed to equilibrate to the holding temperature before animals are added.

15.6.33.4.6 To successfully maintain about 25 adult animals for seven days at a field site, 40-L glass aquaria using aerated, recirculating, clean saline water (>30‰) are housed within a water bath, such as an INSTANT OCEAN^{R} Aquarium. The sexes should be held separately if possible.

15.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION AND STORAGE

15.7.1 See Section 8, Effluent and Receiving Water Sampling and Sample Handling, and Sampling Preparation for Toxicity Tests.

15.8 CALIBRATION AND STANDARDIZATION

15.8.1 See Section 4, Quality Assurance.

15.9 QUALITY CONTROL

15.9.1 See Section 4, Quality Assurance.

15.10 TEST PROCEDURES

15.10.1 TEST DESIGN

15.10.1.1 The test consists of at least four replicates of five effluent concentrations plus a dilution water control. Tests that use brine to adjust salinity must also contain four replicates of a brine control.

15.10.1.2 Effluent concentrations are expressed as percent effluent.

15.10.2 TEST SOLUTIONS

15.10.2.1 Receiving waters

15.10.2.1.1 The sampling point is determined by the objectives of the test. At estuarine and marine sites, samples are usually collected at mid-depth. Receiving water toxicity is determined with samples used directly as collected or with samples passed through a 60 µm NITEX® filter and compared without dilution, against a control. Using four replicate chambers per test, each containing 10 mL, and 400 mL for chemical analysis, would require approximately 440 mL of sample per test.

15.10.2.2 Effluents

15.10.2.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of at least 0.5 is commonly used. A dilution factor of 0.5 provides hypothesis test discrimination of ± 100%, and testing of a 16 fold range of concentrations. Hypothesis test discrimination shows little improvement as dilution factors are increased beyond 0.5 and declines rapidly if smaller dilution factors are used. **USEPA recommends that one of the five effluent treatments must be a concentration of effluent mixed with dilution water which corresponds to the permittee's instream waste concentration (IWC).** At least two of the effluent treatments must be of lesser effluent concentration than the IWC, with one being at least one-half the concentration of the IWC. If 100‰ HSB is used as a diluent, the maximum concentration of effluent that can be tested will be 66% at 34‰ salinity.

15.10.2.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12% and 1.56%).

15.10.2.2.3 The volume in each test chamber is 10 mL.

15.10.2.2.4 Effluent dilutions should be prepared for all replicates in each treatment in one container to minimize variability among the replicates. Dispense into the appropriate effluent test chambers.

15.10.2.3 Dilution Water

15.10.2.3.1 Dilution water should be uncontaminated 1-µm-filtered natural seawater or hypersaline brine prepared from uncontaminated natural seawater plus reagent water (see Section 7, Dilution Water). Natural seawater may be uncontaminated receiving water. This water is used in all dilution steps and as the control water.

15.10.2.4 Reference Toxicant Test

15.10.2.4.1 Reference toxicant tests should be conducted as described in Quality Assurance (see Section 4.7).

15.10.2.4.2 The preferred reference toxicant for sea urchins and sand dollars is copper chloride ($CuCl_2 \circ H_2O$). Reference toxicant tests provide an indication of the sensitivity of the test organisms and the suitability of the testing laboratory (see Section 4 Quality Assurance). Another toxicant may be specified by the appropriate regulatory agency. Prepare a copper reference toxicant stock solution (2,000 mg/L) by adding 5.366 g of copper chloride ($CuCl_2 \circ 2H_2O$) to 1 liter of reagent water. For each reference toxicant test prepare a copper sub-stock of 3 mg/L by diluting 1.5 mL of stock to one liter with reagent water. Alternatively, certified standard solutions can be ordered from commercial companies.

15.10.2.4.3 Prepare a control (0 Fg/L) plus four replicates each of at least five consecutive copper reference toxicant solutions (e.g., from the series 3.0, 4.4, 6.5, 9.5, 13.9, 20.4, and 30.0 Fg/L, by adding 0.10, 0.15, 0.22, 0.32, 0.46, 0.68, and 1.00 mL of sub-stock solution, respectively, to 100-L volumetric flasks

and filling to 100-mL with dilution water). Alternatively, certified standard solutions can be ordered from commercial companies. Start with control solutions and progress to the highest concentration to minimize contamination.

15.10.2.4.4 If the effluent and reference toxicant tests are to be run concurrently, then the tests must use embryos from the same spawn. The tests must be handled in the same way and test solutions delivered to the test chambers at the same time. Reference toxicant tests must be conducted at 34 ± 2‰.

15.10.3 COLLECTION OF GAMETES FOR THE TEST

15.10.3.1 Spawning Induction

15.10.3.1.1 Pour seawater into 100 mL beakers and place in 15EC bath or room. Allow to come to temperature. Select a sufficient number of sea urchins or sand dollars (based upon recent or past spawning success) so that three of each sex are likely to provide gametes of acceptable quantity and quality for the test. During optimal spawning periods this may only require six animals, three of each sex, when the sexes are known from prior spawning. During other periods, especially if the sex is not known, many more animals may be required.

15.10.3.1.2 Care should be exercised when removing sea urchins from holding tanks so that damage to tube-feet is minimized. Following removal, sea urchins should be placed into a container lined with seawater-moistened paper towels to prevent reattachment.

15.10.3.1.3 Place each sand dollar, oral side up, on a 100 mL beaker filled with 15EC seawater or each sea urchin onto a clean tray covered with several layers of seawater moistened paper towels.

15.10.3.1.4 Handle sexes separately once known; this minimizes the chance of accidental egg fertilization. Throughout the test process, it is best if a different worker, different pipets, etc. are used for males (semen) and females (eggs). Frequent washing of hands is a good practice.

15.10.3.1.5 Fill a 3 or 5 mL syringe with 0.5 M KCl and inject

0.5 mL through the soft periostomal membrane of each sea urchin (See Figure 5) or into the oral opening each sand dollar. If sexes are known, use a separate needle for each sex. If sexes are not known, rinse the needle with hot tap water between each injection. This will avoid the accidental injection of sperm from males into females. Note the time of injection on the data sheet.

15.10.3.1.6 Spawning of sea urchins is sometimes induced by holding the injected sea urchin and gently shaking or swirling it for several seconds. This may provide an additional physical stimulus, or may aid in distributing the injected KCl.

15.10.3.1.7 Place the sea urchins onto the beakers or tray (oral side down). Place the sand dollars onto the beakers (oral side up). Females will release orange (sea urchins) or purple (sand dollars) eggs and males will release cream-colored semen.

15.10.3.1.8 As gametes begin to be shed, note the time on the data sheet and separate the sexes. Place male sand dollars with the oral side up atop a small (5-10 mL) glass beaker filled with 12EC seawater. Leave spawning sea urchin males on tray or beaker (oral side down) for semen collection. Female sand dollars and sea urchins are left to shed eggs into the 100-mL beakers.

15.10.3.1.9 If sufficient quantities of gametes are available, only collect gametes for the first 15 min after each animal starts releasing. This helps to insure good quality gametes. As a general guideline, do not collect gametes from any individual for more than 30 minutes after the first injection.

15.10.3.1.10 If no spawning occurs after 5 or 10 minutes, a second 0.5 mL injection may be tried. If animals do not produce sufficient gametes following injection of 1.0 mL of KCl, they should probably not be reinjected as this seldom results in acquisition of good quality gametes and may result in mortality of adult urchins.

15.10.3.1.11 Sections 15.10.4.2 and 15.10.6.4 describe collection and dilution of the sperm and eggs. While some of the gamete handling needs to be in a specific order, parts of the procedure can be done simultaneously while waiting for gametes to settle.

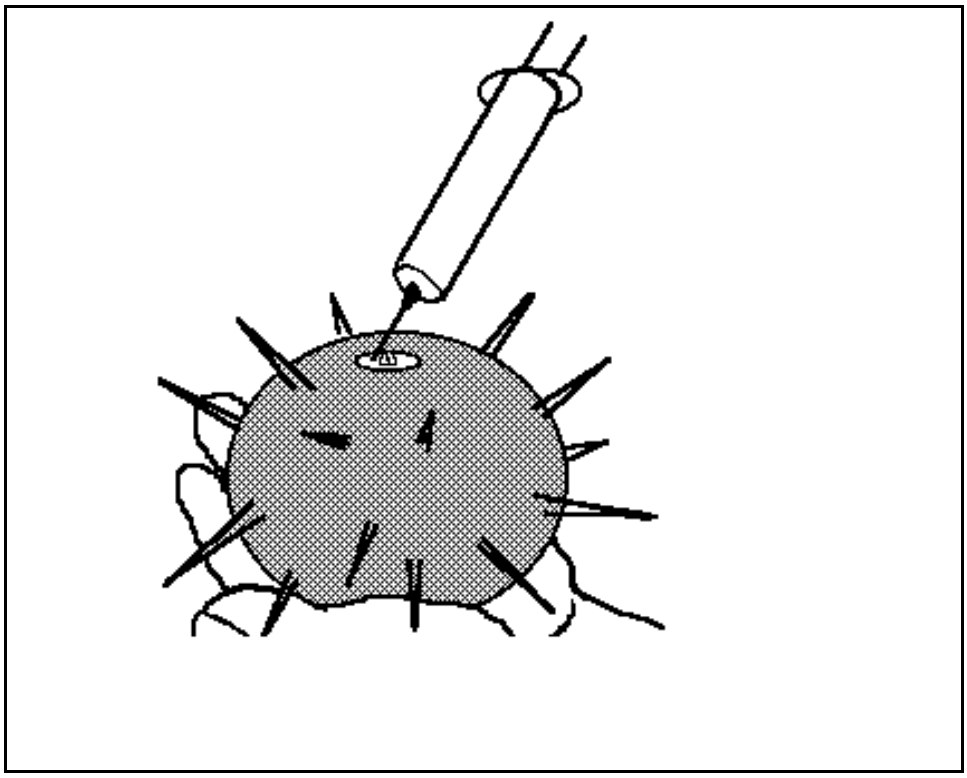


Figure 5. Showing the location and orientation used in the injection of KCl into sea urchins to stimulate spawning.

15.10.3.2 Collection of Sperm

15.10.3.2.1 Sea urchin semen should be collected dry (directly from the surface of the sea urchin), using either a Pasteur pipette or a 0.1 mL autopipette with the end of the tip cut off so that the opening is at least 2 mm. Pipette semen from each male into separate 1-15 mL conical test tubes, stored in an ice water bath.

15.10.3.3 Viability of Sperm

15.10.3.3.1 Early in the spawning process, place a very small amount of sperm from each male sea urchin or sand dollar into

dilution water on a microscope slide (well slides work nicely). Examine the sperm for motility; use sperm from males with high sperm motility.

15.10.3.4 Pooling of Sperm

15.10.3.4.1 Pool equal quantities of semen from each of the sea urchin males that has been deemed good. If possible, 0.025 mL should be pooled from each of those used and a total of at least 0.05 mL of pooled semen should be available. Sperm collected from good male sand dollars should be pooled after first decanting off the overlying water (the final sand dollar sperm density usually is between $2x10^{9}$ and $2x10^{10}$ sperm/mL).

15.10.3.5 Storage of Sperm

15.10.3.5.1 Cover each test tube or beaker with a cap or parafilm, as air exposure of semen may alter its pH through gas exchange and reduce the viability of the sperm. Keep sperm covered and on ice or refrigerated (<5EC). The sperm should be used within 4 h of collection.

15.10.4 PREPARATION OF SPERM DILUTION FOR USE IN THE TEST

15.10.4.1 Sperm Dilution

15.10.4.1.1 When ready to use sperm, mix by agitating the tube with a vortex mixer. Add about 0.025 mL of semen to a 100 mL beaker containing 50 mL of 15EC dilution water. Stir this solution thoroughly with a Pasteur pipette. A drop of egg solution from each female may be placed on a well slide and a small amount of sperm solution added to test fertilization. If no fertilization membrane forms on eggs from any female, then new gametes should be collected. Keep the sperm dilution covered and at 15EC until ready for use. This dilution should be used to fertilize the eggs within 1.5 hours of being made.

15.10.4.2 Sperm Density Determination

15.10.4.2.1 Take 0.5 mL subsample of the sperm solution and add it to 5 mL of 10% acetic acid in a 50 mL graduated cylinder, to kill the sperm. Bring the volume to 50 mL with dilution water. Mix by inversion and place one drop of the killed sperm solution

onto each side of a hemocytometer. Let sperm settle for about 15 minutes. Count the number of sperm in 80 small squares on each side of the hemocytometer. If the counts for each side are within 80% of one another, then take the mean of those two counts. If the counts are not that close, then refill the hemocytometer, recount and take the mean of the four counts. Use the following equations to determine sperm density and record the results on the spawning worksheet (Figure 2).

$$\#\text{sperm/mL} = \frac{(\text{dilution})(\text{count})(\text{hemacytometer conversion factor})(\text{mm3/mL})}{\text{number of squares counted}}$$

dilution=100
conversion factor=4000
mm^3/mL=1000
number of squares=80

Therefore:

$$\#\text{sperm/mL} = (\text{count})(5 \times 10^6) \qquad \text{(Equation 2A)}$$

15.10.5 PREPARATION OF EGG SUSPENSION FOR USE IN THE TEST

15.10.5.1 Acceptability of Eggs

15.10.5.1.1 Place a small sample of eggs from each female in the counting chamber and examine eggs with the microscope. Look for the presence of significant quantities of immature or abnormal appearing eggs (germinal vesicle present, unusually large or small or irregularly shaped). Do not use the eggs from females having more than 10% abnormal eggs or from females whose eggs did not fertilize during the test in Section 15.10.5.1.

15.10.5.2 Pooling of Eggs

15.10.5.2.1 Allow eggs to settle in the collection beakers. Decant some of the water from the collection beakers taking care not to pour off many eggs. Pour the remaining sea urchin eggs

through the Nitex® screen (to remove fecal material and other debris) into a 1 liter beaker. Repeat with each of the "good" females. Bring the volume up to about 600 mL with dilution water. Allow the eggs to settle to the bottom again. Siphon off about 400 mL of the overlying water and then bring back up to 600 mL with dilution water. Do not allow the temperature to rise above the 15EC test temperature; somewhat cooler temperatures for holding would be acceptable.

15.10.5.2.2 Pooled sand dollar eggs should be treated gently and no additional screening or rinsing step is recommended. Mix well once just before subsampling for egg stock calculations. This is best done in a large graduated cylinder appropriate for the number of eggs available. Cover with parafilm and invert gently several times.

15.10.5.3 Density of Eggs

15.10.5.3.1 Using a plunger, mix the sea urchin egg suspension well. While continuing to mix, remove a 10 mL sample and place in a 1 liter graduated cylinder. Bring the volume up to 1 liter with dilution water. Mix this dilution well and remove a 1 mL sample to a counting cell. Count all the eggs in the 1 mL sample. Repeat the process and take the mean of the two counts. Calculate the number of eggs per mL in the stock solution using Equation 3 and record the results.

# of eggs in count x 100= # eggs/mL in stock (Equation 3)

15.10.5.4 Dilution of Eggs

15.10.5.4.1 When using scintillation vials as the test chamber, the final concentration of eggs in the diluted stock must be 250 eggs/0.25 mL, which is equal to 1,000 eggs/mL. To calculate the dilution factor for the eggs, use Equation 4. (If larger test chambers are used, the total number of eggs used will be greater and the stock solution density may be adjusted, but the final concentration of eggs in the test solutions must remain 25 eggs/mL).

# of eggs/mL in stock ÷ 1,000= Dilution factor (Equation 4)

15.10.5.4.2 The dilution factor must be greater than one. If

not, concentrate the eggs and recount (starting at Section 15.4.5.3 The dilution factor minus 1 equals the number of parts of water that go with one part of eggs in the final dilution. For example: if the dilution factor were 5.3, then 4.3 parts of water would be used with 1 part eggs.

15.10.5.4.3 Make a dilution of the egg stock so that there is more than enough volume to perform the bioassay.

15.10.5.5 Fertilization of Eggs

15.10.5.5.1 The recommended initial sperm to egg ratio for fertilization of the eggs is 500:1. Calculate the volume of sperm dilution (Section 15.10.5.1) to add to the egg dilution, by using the following equations and record the results (Figure 3).

volume of egg dilution x 1,000 eggs/mL= total # of eggs in dilution (Equation 5A)

total # of eggs in dilution x 500 sperm/egg= # of sperm needed (Equation 5B)

# of sperm needed ÷ # sperm/mL in sperm dilution= mL of sperm solution (Equation 5C)

15.10.5.5.2 Add this volume of the sperm dilution to the egg dilution and mix gently with a plunger. Wait 10 min, then check for fertilization. If fertilization is not at least 90%, add a second volume of the sperm dilution. Wait 10 min and recheck. If fertilization is still not 90%, then the test must be restarted with different gametes.

15.10.5.5.3 The test should be initiated within 1 hour of fertilization being achieved.

15.10.6 START OF THE TEST

15.10.6.1 Prior to Beginning the Test

15.10.6.1.1 The test should begin as soon as possible, preferably within 24 h of sample collection. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity tests unless permission is granted by the permitting authority. In no case

should the sample be used in a test more than 72 h after sample collection (see Section 8 Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Test).

15.10.6.1.2 Just prior to test initiation (approximately 1 h), the temperature of a sufficient quantity of the sample to make the test solutions should be adjusted to the test temperature (15 ± 1EC) and maintained at that temperature during the addition of dilution water.

15.10.6.1.3 Increase the temperature of the water bath, room, or incubator to the required test temperature (15 ± 1EC).

15.10.6.1.4 Randomize the placement of test chambers in the temperature-controlled water bath, room, or incubator at the beginning of the test, using a position chart. Assign numbers for the position of each test chamber using a random numbers or similar process (see Appendix A, for an example of randomization). Maintain the chambers in this configuration throughout the test, using a position chart. Record these numbers on a separate data sheet together with the concentration and replicate numbers to which they correspond. Identify this sheet with the date, test organism, test number, laboratory, and investigator's name, and safely store it away until after the sea urchins or sand dollars have been examined at the end of the test.

15.10.6.1.5 Note: Loss of the randomization sheet would invalidate the test by making it impossible to analyze the data afterwards. Make a copy of the randomization sheet and store separately. Take care to follow the numbering system exactly while filling chambers with the test solutions.

15.10.6.1.6 Arrange the test chambers randomly in the water bath or controlled temperature room. Once chambers have been labeled randomly and filled with test solutions, they can be arranged in numerical order for convenience, since this will also ensure random placement of treatments.

15.10.6.1.6 If mortality is to be included as an endpoint, at least 5 extra control chambers should be set up and identified on the randomization sheet as initial count chambers.

15.10.6.2 Delivery of Fertilized Eggs

15.10.6.2.1 Gently mix the solution of fertilized eggs. Deliver 0.25 mL of egg solution to each vial, using an automatic pipette with the tip cut off to provide at least a 0.5 mm opening. Deliver the embryos into the test chambers directly from the pipette, taking care not to touch the pipette to the test solution. The egg solution temperature must be within 1EC of the test solutions. Keep the eggs well mixed during the delivery procedure.

15.10.6.3 Incubation

15.10.6.3.1 The embryos are incubated for 72 hours in the test chambers at 15 ± 1EC at ambient light level.

15.10.6.3.2 The optional extra control chambers for initial counts should be counted as soon as possible after test initiation. If they are sampled and counted in a non-destructive manner they may be returned to the test but used only as a check for larval developmental rate. They must not be used for for routine control counts at the end of the test.

15.10.7 LIGHT, PHOTOPERIOD, SALINITY AND TEMPERATURE

15.10.7.1 The light quality and intensity should be at ambient laboratory conditions. Light intensity should be 10-20 µE/m^2/s, or 50 to 100 foot candles (ft-c), with a 16 h light and 8 h dark cycle.

15.10.7.2 The water temperature in the test chambers should be maintained at 15 ± 1EC. If a water bath is used to maintain the test temperature, the water depth surrounding the test cups should be as deep as possible without floating the chambers.

15.10.7.3 The test salinity should be in the range of 34 ± 2‰. The salinity should vary by no more than ±2‰ among the chambers on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.

15.10.7.4 Rooms or incubators with high volume ventilation should be used with caution because the volatilization of the

test solutions and evaporation of dilution water may cause wide fluctuations in salinity. Covering the test chambers with clean polyethylene plastic may help prevent volatilization and evaporation of the test solutions.

15.10.8 DISSOLVED OXYGEN (DO) CONCENTRATION

15.10.8.1 Aeration may affect the toxicity of effluent and should be used only as a last resort to maintain a satisfactory DO. The DO concentration should be measured on new solutions at the start of the test (Day 0). The DO should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). If it is necessary to aerate, all treatments and the control should be aerated. The aeration rate should not exceed that necessary to maintain a minimum acceptable DO and under no circumstances should it exceed 100 bubbles/minute, using a pipet with a 1-2 mm orifice, such as a 1 mL KIMAX® serological pipet No. 37033, or equivalent.

15.10.9 OBSERVATIONS DURING THE TEST

15.10.9.1 Routine Chemical and Physical Observations

15.10.9.1.1 The DO should be measured in each test solution at the beginning of the exposure period.

15.10.9.1.2 The temperature, pH, and salinity should be measured in all each test solution at the beginning of the exposure period. Temperature should also be monitored continuously or observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be measured in a sufficient number of test chambers at the end of the test to determine temperature variation in the environmental chamber.

15.10.9.1.3 Record all the measurements on the data sheet.

15.10.9.2 Routine Biological Observations

15.10.9.2.1 Developing embryos do not need to be monitored during the test under normal circumstances.

15.10.10 TERMINATION OF THE TEST

15.10.10.1 Ending the Test

15.10.10.1.1 Record the time the test is terminated.

15.10.10.1.2 Temperature, pH, dissolved oxygen, and salinity are measured at the end of the exposure period in one test chamber at each concentration and in the control(s).

15.10.10.2 Sample Preservation

15.10.10.2.1 To terminate the test, add 1.0 mL of 37% (concentrated) buffered formalin to each sample to give a final formalin concentration of 4%. As an alternate fixative, 0.5 mL of 1.0% glutaraldehyde may be used, in each test chamber. Tightly cap and gently mix each chamber and store for later evaluation. (If the test is performed in larger chambers, a 10 mL subsample of well mixed test solution is to be taken from each chamber and preserved).

15.10.10.3 Counting

15.10.10.3.1 It is recommended that the embryos be examined within one week of preservation. Longer storage times may also be used, but run the risk of sample degradation due to improper preservation. Larvae can be counted directly in the scintillation vials using an inverted microscope. If an inverted scope is not available, then samples should be loaded into a Sedgewick-Rafter cell, as follows. The embryos should first be allowed to settle to the bottom of the sample chamber. All but about 1 mL of the overlying liquid should then be removed. All of the remaining liquid containing the embryos should then be transferred to the counting chamber. Whichever scope is used, the embryos should be examined at about 100x power. The first 100 embryos encountered are counted using a multi-unit handcounter to track normal versus abnormal larvae. Record the data by sample number on a data sheet (Figure 4).

15.10.10.3.2 Mortality can be determined only if: (1) all surviving larvae are counted (either in the test vials with an inverted microscope or by <u>total</u> transfer to a counting chamber); or (2) the test solution is stirred with a plunger and

quantitative subsampling is conducted followed by total larval counts on the subsample. The latter procedure requires homogeneous distribution of larvae in the test solution, quantitative transfer of larvae (without adherence to transfer hardware or test chambers), and accurate volume measurements. Mortality is most important to consider with point estimates (e.g., EC25) or when mortality occurs at the NOEC for normal development.

15.10.10.4 Endpoint

15.10.10.4.1 Normal Larvae

15.10.10.4.1.1 Normally developed pluteus larvae have several distinctive characteristics:

(1) The larvae should have a pyramid shape with a pair of skeletal rods that extend at least half the length of the long axis of the larvae (Figure 6D).

(2) The gut should be differentiated into three parts (Figure 6E). If the gut appears lobed and constricts distally in specimens with an obstructed view (e.g., Figure 6D), then normal gut development may be inferred.

(3) Development of post-oral arms has begun.

15.10.10.4.2 Abnormal Larvae

15.10.10.4.2.1 Larvae need only be scored as abnormal or normal to conduct the test, but the categories of abnormalities may be tracked as well. Abnormal larvae should fit into one of the following categories:

(1) Pathological prehatched: Embryos at the single or multi-cell stage with the fertilization membrane still visible.

(2) Pathological hatched: larvae that have no fertilization membrane and demonstrate an extensive

degree of malformation or necrosis. Most of these larvae appear as dark balls of cells or dissociated blobs of cells.

(3) Inhibited: larvae at the blastula or gastrula stage that have no gut differentiation or have no or underdeveloped skeleton. These larvae appear to be developing regularly, but are at a stage earlier than attained by control organisms (e.g., Figure 6A-C).

(4) Gut abnormalities: larvae whose overall appearance is normal, but have guts that are lacking, undifferentiated, abnormally shaped or project outside of the larvae (exogastrulated).

(5) Skeletal abnormalities: larvae whose overall appearance is normal, but have missing spicules, extraneous spicules or rods growing in abnormal directions. Note: Some larvae may exhibit a separation of the rods at the apex. This may be caused by preservation and should not be termed abnormal. Since the test is started with already fertilized eggs, any unfertilized eggs that are encountered should not be counted as either normal or abnormal, but should be ignored.

15.11 **SUMMARY OF TEST CONDITIONS**

15.11.1 A summary of test conditions and test acceptability criteria is listed in Table 3.

15.12 **ACCEPTABILITY OF TEST RESULTS**

15.12.1 Test results are acceptable only if all the following requirements are met:

(1) larval normality must be at least 80% in the controls.

(2) the minimum significant difference (%MSD) is #20% relative to the controls.

15.13 **DATA ANALYSIS**

15.13.1 GENERAL

15.13.1.1 Tabulate and summarize the data. Calculate the

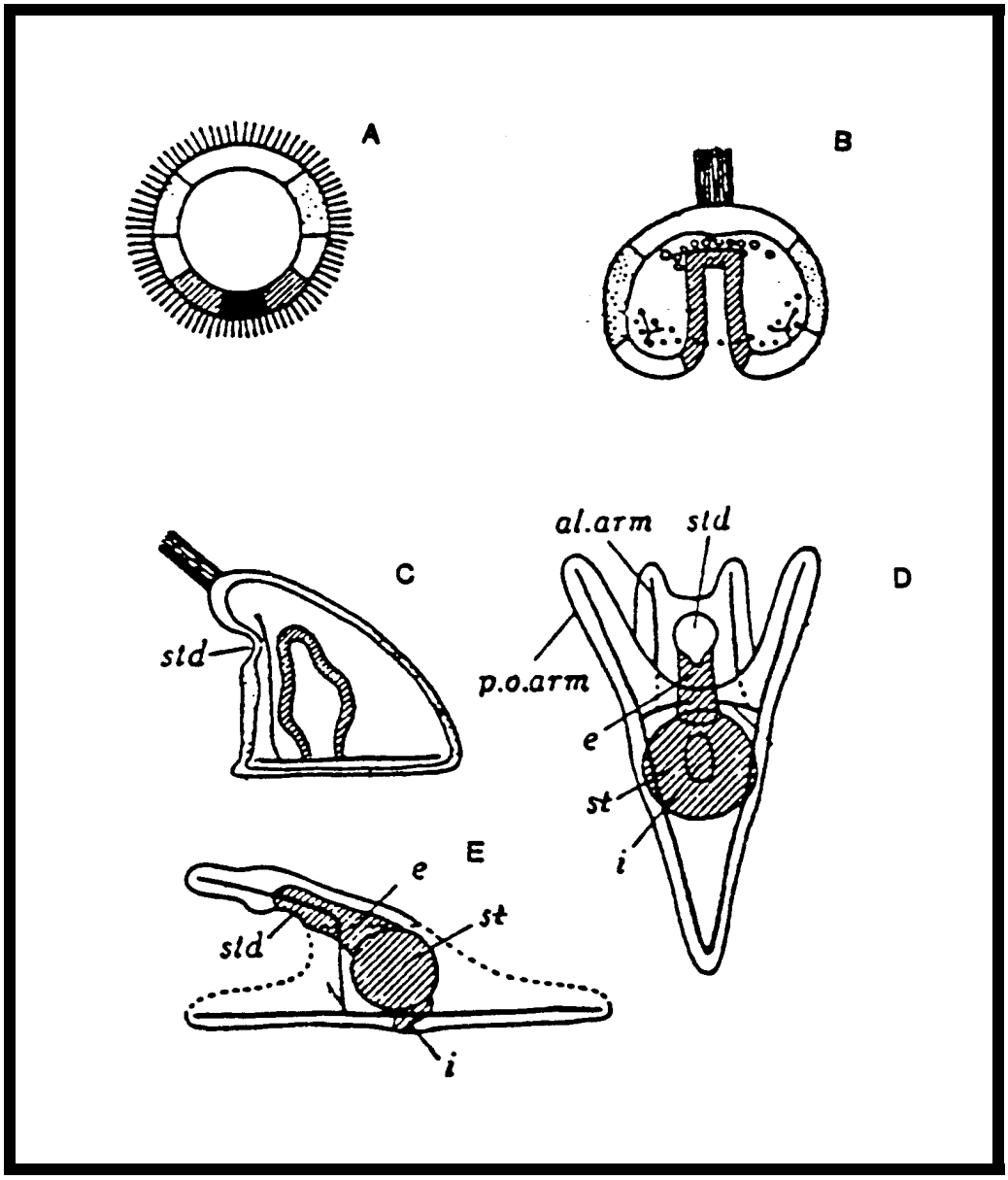


Figure 6. Stages of sea urchin embryo development (modified from Kume and Dan 1957). A. blastula; B. gastrula; C. prism; D. pluteus (frontal view); E. pluteus (lateral view). *al.arm*: anterior lateral arm, *e*: esophagus, *i*: intestine, *st*: stomach, *std*: stomodaeum.

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR THE PURPLE URCHIN, *STRONGYLOCENTROTUS PURPURATUS*, AND SAND DOLLAR, *DENDRASTER EXCENTRICUS* EMBRYO DEVELOPMENT TEST WITH EFFLUENTS AND RECEIVING WATERS

1. Test type:	Static non-renewal
2. Salinity:	34 ± 2‰
3. Temperature:	15 ± 1EC
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 µE/m_2/s (Ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	30 mL
8. Test solution volume:	10 mL
9. No. replicate chambers per concentration:	4
10. Dilution water:	Uncontaminated 1-µm-filtered natural seawater or hypersaline brine prepared from natural seawater
11. Test concentrations:	Effluents: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
12. Dilution factor:	Effluents: $0.5 Receiving waters: 100% receiving water and a control
13. Test duration:	72 ± 2 hr
14. Endpoint:	Normal development; mortality can be included
15. Test acceptability criteria:	$80% normal shell development in the controls; must achieve a %MSD of <25%

16. Sampling requirements:	One sample collected at test initiation, and preferably used within 24 h of the time it is removed from the sampling device (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests)
17. Sample volume required:	1 L per test

proportion of normally developed larvae for each replicate. A sample set of test data is listed in Table 4.

15.13.1.2 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

15.13.1.3 The endpoints of toxicity tests using the purple sea urchin are based on the reduction in proportion of normally developed larvae. The IC25 is calculated using the Linear Interpolation Method (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). LOEC and NOEC values for development are obtained using a hypothesis testing approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981) (see Section 9). Separate analyses are performed for the estimation of the LOEC and NOEC endpoints and for the estimation of the IC25. See the Appendices for examples of the manual computations, and examples of data input and program output.

15.13.2 EXAMPLE OF ANALYSIS OF PURPLE SEA URCHIN, *STRONGYLOCENTROTUS PURPURATUS*, DEVELOPMENT DATA

15.13.2.1 Formal statistical analysis of the larval development data is outlined in Figure 7. The response used in the analysis is the proportion of normally developed larvae in each test or control chamber. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the IC25 endpoint. Concentrations at which there are no normally developed larvae in any of the test chambers are excluded from statistical analysis of the NOEC and LOEC, but included in the estimation of the IC25.

TABLE 4. DATA FROM PURPLE SEA URCHIN, *STRONGYLOCENTROTUS PURPURATUS*, DEVELOPMENT TEST

Copper Concentration (µg/L)	Replicate	No. Larvae Exposed	No. Larvae Normally Developed	Proportion Normal
Control	A	100	87	0.87
	B	100	89	0.89
	C	100	81	0.81
	D	101	89	0.88
	E	74	62	0.84
3.2	A	110	98	0.89
	B	100	82	0.82
	C	100	91	0.91
	D	100	83	0.83
	E	100	89	0.89
5.6	A	102	86	0.84
	B	100	89	0.89
	C	100	85	0.85
	D	107	90	0.84
	E	100	85	0.85
10.0	A	100	70	0.70
	B	100	71	0.71
	C	100	77	0.77
	D	100	74	0.74
	E	100	87	0.87
18.0	A	100	7	0.07
	B	100	12	0.12
	C	100	14	0.14
	D	100	16	0.16
	E	100	10	0.10
32.0	A	101	0	0.00
	B	102	0	0.00
	C	100	0	0.00
	D	100	0	0.00
	E	100	0	0.00

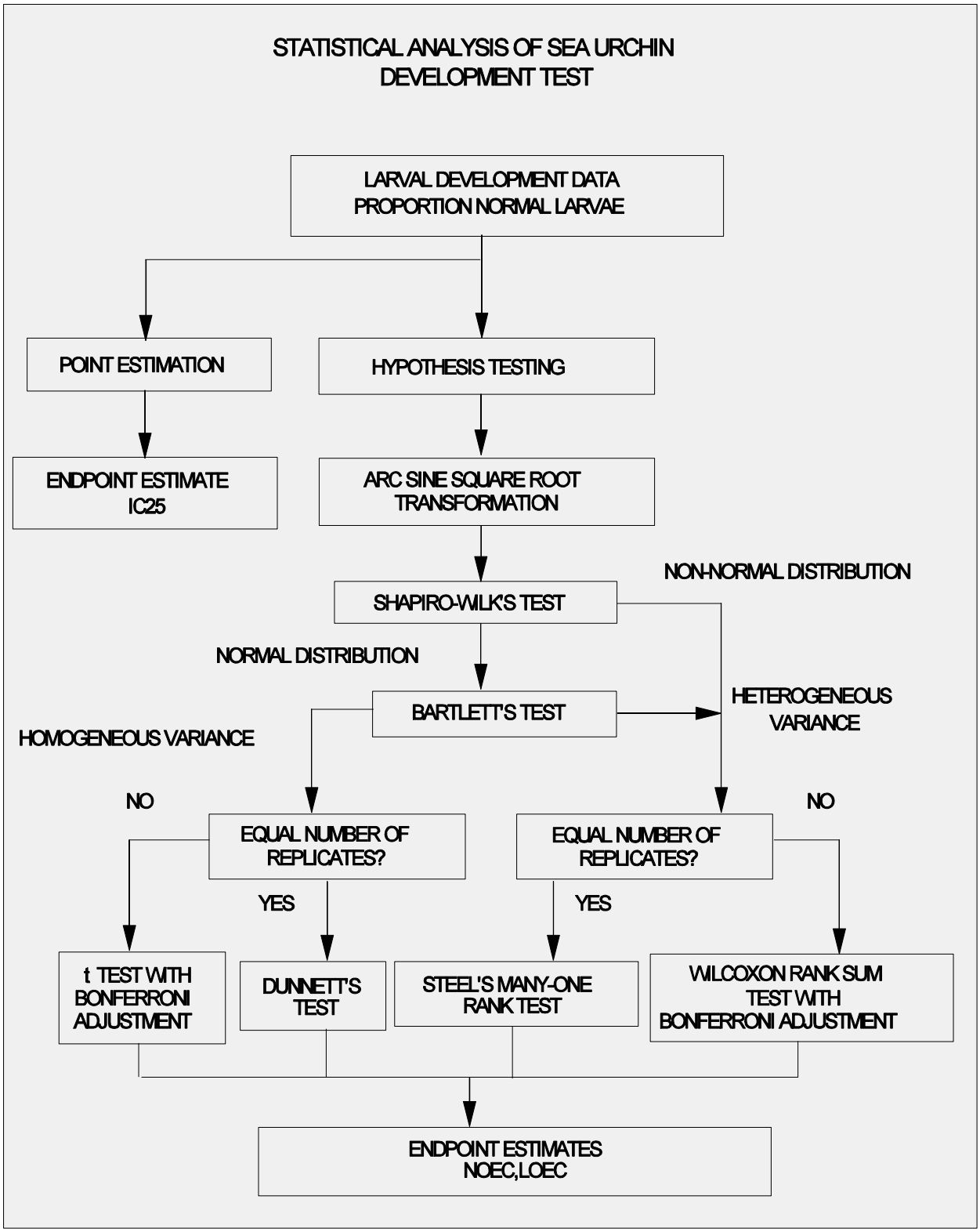


Figure 7. Flowchart for statistical analysis of sea urchin, *Strongylocentrotus purpuratus*, development test.

15.13.2.2 For the case of equal numbers of replicates across all concentrations and the control, the evaluation of the NOEC and LOEC endpoints is made via a parametric test, Dunnett's Procedure, or a nonparametric test, Steel's Many-one Rank Test, on the arc sine square root transformed data. Underlying assumptions of Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test, and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are estimated by the parametric procedure.

15.13.2.3 If unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a *t* test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative.

15.13.2.4 Example of Analysis of Development Data

15.13.2.4.1 This example uses toxicity data from a purple sea urchin, ***Strongylocentrotus purpuratus***, development test performed with copper. The response of interest is the proportion of normally developed larvae, thus each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix B. The raw and transformed data, means and variances of the transformed observations at each copper concentration and control are listed in Table 5. The data are plotted in Figure 8. Because there is zero normal development in all five replicates of the 32.0 µg/L copper concentration, it was not included in the statistical analysis and is considered a qualitative development effect.

15.13.2.5 Test for Normality

15.13.2.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 6.

15.13.2.5.2 Calculate the denominator, D, of the statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

& = the overall mean of the centered observations

n = the total number of centered observations

15.13.2.5.3 For this set of data, n = 25

$$\& = \frac{1}{25}(-0.001) = 0.000$$

D = 0.0680

TABLE 5. SEA URCHIN, *STRONGYLOCENTROTUS PURPURATUS*, DEVELOPMENT DATA

		Copper Concentration (µg/L)				
	Replicate	Control	3.2	5.6	10.0	18.0
RAW	A	0.87	0.89	0.84	0.70	0.07
	B	0.89	0.82	0.89	0.71	0.12
	C	0.81	0.91	0.85	0.77	0.14
	D	0.88	0.83	0.84	0.74	0.16
	E	0.84	0.89	0.85	0.87	0.10
ARC SINE SQUARE ROOT TRANSFORMED	A	1.202	1.234	1.159	0.991	0.268
	B	1.234	1.133	1.234	1.002	0.354
	C	1.120	1.266	1.173	1.071	0.383
	D	1.217	1.146	1.159	1.036	0.412
	E	1.159	1.234	1.173	1.202	0.322
Mean ($\&_i$)		1.186	1.203	1.180	1.060	0.348
S_i^2		0.00215	0.00351	0.00097	0.00725	0.00311
i		1	2	3	4	5

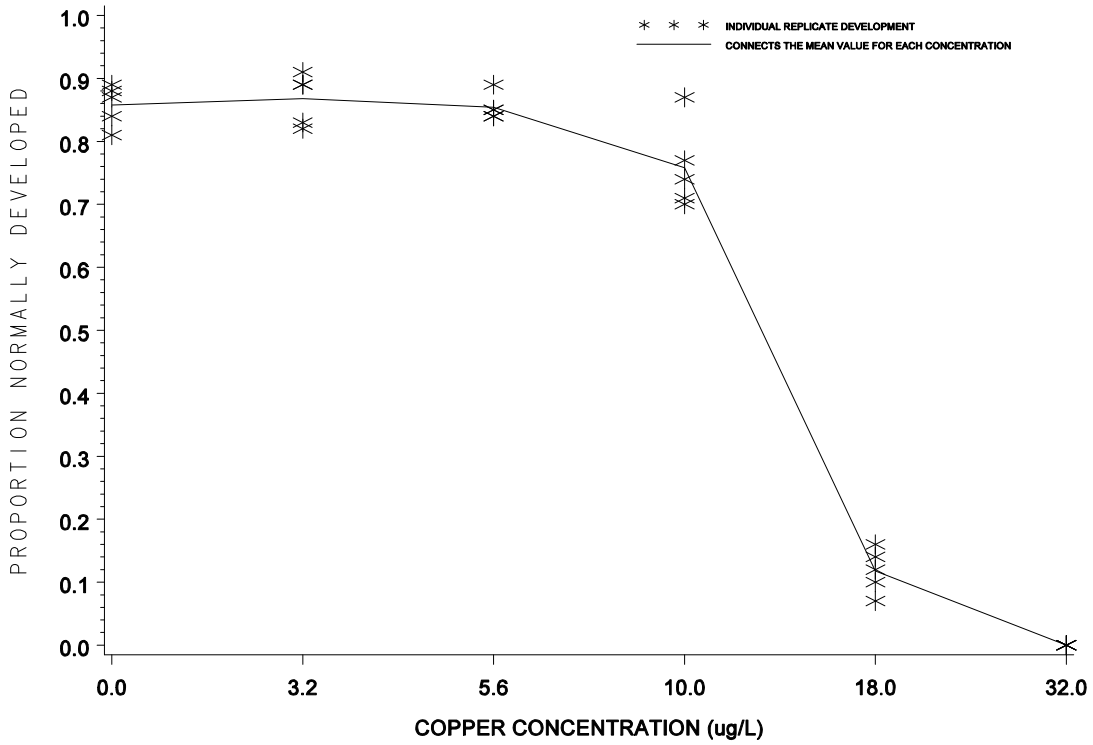


Figure 8. Plot of proportion of normally developed sea urchin, *Strongylocentrotus purpuratus*, larvae,

15.13.2.5.4 Order the centered observations from smallest to largest

$$X^{(1)} \# X^{(2)} \# \ldots \# X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 7.

TABLE 6. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

Replicate	Control	Copper Concentration (µg/L) 3.2	5.6	10.0	18.0
A	0.016	0.031	-0.021	-0.069	-0.080
B	0.048	-0.070	0.054	-0.058	0.006
C	-0.066	0.063	-0.007	0.011	0.035
D	0.031	-0.057	-0.021	-0.024	0.064
E	-0.027	0.031	-0.007	0.142	-0.026

15.13.2.5.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2, ... a_k where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 25 and k = 12. The a_i values are listed in Table 8.

15.13.2.5.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i\left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

The differences, $X^{(n-i+1)} - X^{(i)}$, are listed in Table 8. For the data in this example:

$$W = \frac{1}{0.0680}\ (0.2545)^2 = 0.953$$

TABLE 7. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.080	14	0.006
2	-0.070	15	0.011
3	-0.069	16	0.016
4	-0.066	17	0.031
5	-0.058	18	0.031
6	-0.057	19	0.031
7	-0.027	20	0.035
8	-0.026	21	0.048
9	-0.024	22	0.054
10	-0.021	23	0.063
11	-0.021	24	0.064
12	-0.007	25	0.142
13	-0.007		

TABLE 8. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4450	0.222	$X^{(25)} - X^{(1)}$
2	0.3069	0.134	$X^{(24)} - X^{(2)}$
3	0.2543	0.132	$X^{(23)} - X^{(3)}$
4	0.2148	0.120	$X^{(22)} - X^{(4)}$
5	0.1822	0.106	$X^{(21)} - X^{(5)}$
6	0.1539	0.092	$X^{(20)} - X^{(6)}$
7	0.1283	0.058	$X^{(19)} - X^{(7)}$
8	0.1046	0.057	$X^{(18)} - X^{(8)}$
9	0.0823	0.055	$X^{(17)} - X^{(9)}$
10	0.0610	0.037	$X^{(16)} - X^{(10)}$
11	0.0403	0.032	$X^{(15)} - X^{(11)}$
12	0.0200	0.013	$X^{(14)} - X^{(12)}$

15.13.2.5.7 The decision rule for this test is to compare W as calculated in Subsection 5.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For the data in this example, the critical value at a significance level of 0.01 and n = 25 observations is 0.888. Since W = 0.953

is greater than the critical value, conclude that the data are normally distributed.

15.13.2.6 Test for Homogeneity of Variance

15.13.2.6.1 The test used to examine whether the variation in the proportion of normally developed larvae is the same across all copper concentrations including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{[(\sum_{i=1}^{P} V_i) \ln \bar{S}^2 - \sum_{i=1}^{P} V_i \ln S_i^2]}{C}$$

Where: V_i = degrees of freedom for each concentration and control,

$V_i = (n_i - 1)$

p = number of concentration levels including the control

n_i = the number of replicates for concentration i.

ln = $\log_e$

i = 1,2, ..., p where p is the number of concentrations including the control

$$\bar{S}^2 = \frac{(\sum_{i=1}^{P} V_i S_i^2)}{\sum_{i=1}^{P} V_i}$$

$$C = 1 + [3(p-1)]^{-1}[\sum_{i=1}^{P} 1/V_i - (\sum_{i=1}^{P} V_i)^{-1}]$$

15.13.2.6.2 For the data in this example (see Table 5), all concentrations including the control have the same number of replicates (n_i = 5 for all i). Thus, V_i = 4 for all i.

15.13.2.6.3 Bartlett's statistic is, therefore:

$$B = [(20)\ln(0.00340) - 4\sum_{i=1}^{P} \ln(S_i^2)] / 1.100$$

$$= [20(-5.6840) - 4(-29.4325)]/1.100$$

$$= 4.050/1.100$$

$$= 3.6818$$

15.13.2.6.4 B is approximately distributed as chi-square with p-1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with 4 degrees of freedom, is 13.28. Since B = 3.6818 is less than the critical value of 13.28, conclude that the variances are not different.

15.13.2.7 Dunnett's Procedure

15.13.2.7.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 9.

Where: p = number of concentration levels including the control

TABLE 9. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_w^2 = SSW/(N-p)
Total	N - 1	SST	

N = total number of observations $n_1 + n_2 \ldots + n_p$

n_i = number of observations in concentration i

$SSB = \sum_{i=1}^{P} T_i^2/n_i - G^2/N$ Between Sum of Squares

$SST = \sum_{i=1}^{P} \sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$ Total Sum of Squares

$SSW = SST - SSB$ Within Sum of Squares

G = the grand total of all sample observations, $G = \sum_{i=1}^{P} T_i$

T_i = the total of the replicate measurements for concentration i

Y_{ij} = the jth observation for concentration i (represents the proportion of normal larvae for concentration i in test chamber j)

15.13.2.7.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = 5$

$N = 25$

$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} + Y_{15} = 5.932$
$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} + Y_{25} = 6.013$
$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} + Y_{35} = 5.898$
$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} + Y_{45} = 5.302$
$T_5 = Y_{51} + Y_{52} + Y_{53} + Y_{54} + Y_{55} = 1.739$

$G = T_1 + T_2 + T_3 + T_4 + T_5 = 24.884$

$SSB = \sum_{i=1}^{P} T_i^2/n_i - G^2/N$
$= (137.267)/5 - (24.884)^2/25 = 2.685$

$SST = \sum_{i=1}^{P} \sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$
$= 27.521 - (24.884)^2/25 = 2.752$

$SSW = SST - SSB = 2.752 - 2.685 = 0.067$

$S_B^2 = SSB/(p-1) = 2.685/(5-1) = 0.6713$

$S_W^2 = SSW/(N-p) = 0.067/(25-5) = 0.0034$

15.13.2.7.3 Summarize these calculations in the ANOVA table (Table 10).

TABLE 10. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	4	2.685	0.6713
Within	20	0.067	0.0034
Total	24	2.752	

15.13.2.7.4 To perform the individual comparisons, calculate the *t* statistic for each concentration, and control combination as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_w \sqrt{(1/n_1) + (1/n_i)}}$$

Where: $\bar{Y}_i$ = mean proportion normal larvae for concentration i

$\bar{Y}_1$ = mean proportion normal larvae for the control

S_w = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i.

Since we are looking for a decreased response from the control in the proportion of normally developed larvae, the concentration mean is subtracted from the control mean.

15.13.2.7.5 Table 11 includes the calculated t values for each concentration and control combination. In this example, comparing the 3.2 µg/L copper concentration with the control the calculation is as follows:

$$t_2 = \frac{(1.186 - 1.203)}{0.0583\sqrt{(1/5)+(1/5)}} = -0.461$$

TABLE 11. CALCULATED t VALUES

Copper Concentration (µg/L)	i	t_i
3.2	2	-0.461
5.6	3	0.163
10.0	4	3.417
18.0	5	22.727

15.13.2.7.6 Since the purpose of this test is to detect a significant decrease in the proportion of normally developed larvae, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix C. For an overall alpha level of 0.05, 20 degrees of freedom for error and four concentrations (excluding the control) the critical value is 2.30. The mean proportion of normally developed larvae for concentration i is considered significantly less than the mean proportion of normally developed larvae for the control if t_i is greater than the critical value. Therefore, the 10.0 µg/L and 18.0 µg/L concentrations have a significantly lower mean proportion of normally developed larvae than the control. Hence the NOEC is 5.6 µg/L copper and the LOEC is 10.0 µg/L copper.

15.13.2.7.7 To quantify the sensitivity of the test, the minimum significant difference (MSD) that can be statistically detected may be calculated:

$$MSD = d\,S_w\sqrt{(1/n_1)+(1/n)}$$

Where: d = the critical value for Dunnett's Procedure

S_w = the square root of the within mean square

n = the common number of replicates at each concentration (this assumes equal replication at each concentration)

n_1 = the number of replicates in the control.

15.13.2.7.8 In this example,

$$MSD \text{ ' } 2.30\,(0.0583)\sqrt{(1/5)\,\%\,(1/5)}$$

$$= 2.30\ (0.0583)(0.6325)$$

$$= 0.085$$

15.13.2.7.9 The MSD (0.085) is in transformed units. To determine the MSD in terms of proportion of normally developed larvae, carry out the following conversion.

1. Subtract the MSD from the transformed control mean.

$$1.186 - 0.085 = 1.101$$

2. Obtain the untransformed values for the control mean and the difference calculated in step 1 of 13.2.7.9.

$$[\ \text{Sine}\ (1.186)\ ]^2 = 0.859$$

$$[\ \text{Sine}\ (1.101)\ ]^2 = 0.795$$

3. The untransformed MSD (MSD_u) is determined by subtracting the untransformed values from step 2 in 14.2.7.9.

$$MSD_u = 0.859 - 0.795 = 0.064$$

15.13.2.7.10 Therefore, for this set of data, the minimum difference in mean proportion of normally developed larvae between the control and any copper concentration that can be detected as statistically significant is 0.064.

15.13.2.7.11 This represents a 7.5% decrease in the proportion of normally developed larvae from the control.

15.13.2.8 Calculation of the ICp

15.13.2.8.1 The development data in Table 4 are utilized in this example. As can be seen from Figure 9, the observed means are not monotonically non-increasing with respect to concentration.

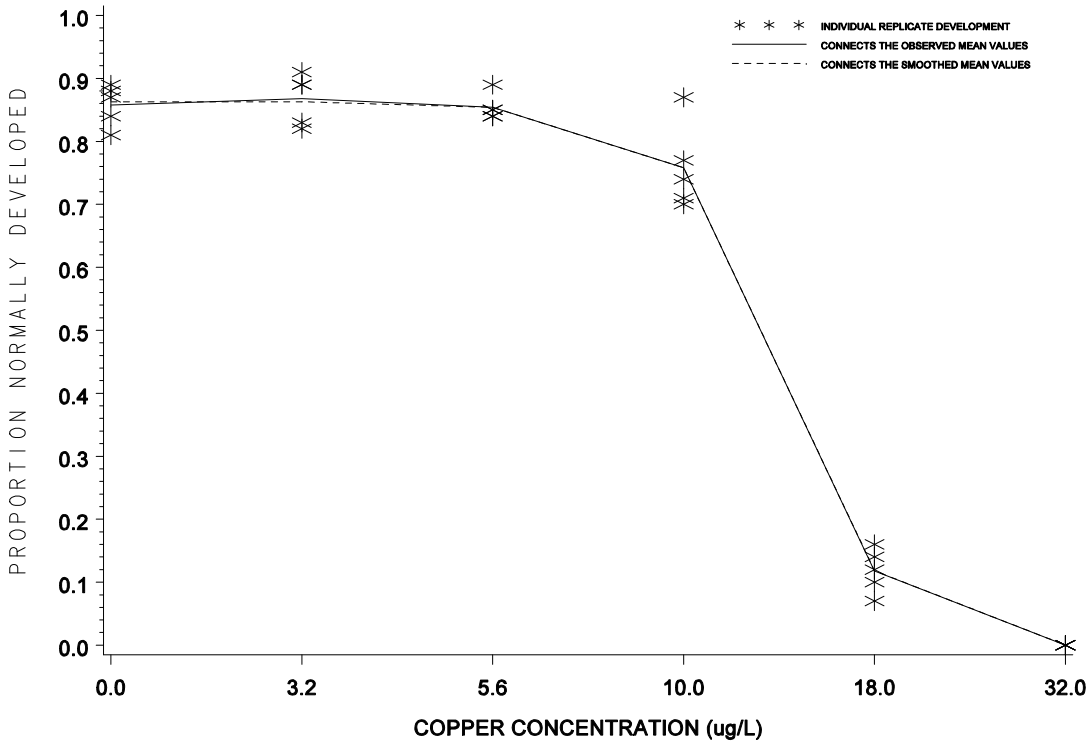


Figure 9. Plot of raw data, observed means, and smoothed means for the sea urchin, *Strogylocentrotus purpuratus*, larval development data from Tables 4 and 12.

Therefore, the means must be smoothed prior to calculating the IC.

15.13.2.8.2 Starting with the observed control mean, $\overline{Y}_1 = 0.858$, and the observed mean for the lowest copper concentration, $\overline{Y}_2 = 0.868$, we see that $\overline{Y}_1$ is less than $\overline{Y}_2$.

15.13.2.8.3 Calculate the smoothed means:

$$M_1 = M_2 = (\overline{Y}_1 + \overline{Y}_2)/2 = 0.863$$

15.13.2.8.4 Since $\overline{Y}_3 = 0.854 > \overline{Y}_4 = 0.758 > \overline{Y}_5 = 0.118 > \overline{Y}_6 = 0.0$, set $M_3 = 0.854$, $M_4 = 0.758$, $M_5 = 0.118$, and $M_6 = 0.0$. Table 12 contains the smoothed means and Figure 8 gives a plot of the smoothed means and the interpolated response curve.

15.13.2.8.5 An IC25 can be estimated using the Linear Interpolation Method. A 25% reduction in mean proportion of normally developed larvae, compared to the controls, would result in a mean proportion of 0.647, where $M_1(1\text{-}p/100) = 0.863(1\text{-}25/100)$. Examining the means and their associated concentrations (Table 12), the response, 0.647, is bracketed by $C_4 = 10.0$ µg/L copper and $C_5 = 18.0$ µg/L copper.

TABLE 12. SEA URCHIN, *STRONYLOCENTROTUS PURPURATUS*, MEAN PROPORTION OF NORMALLY DEVELOPED LARVAE

Copper Conc. (µg/L)	i	Response Means, $\overline{Y}_i$ (proportion)	Smoothed Means, M_i (proportion)
Control	1	0.858	0.863
0.05	2	0.868	0.863
0.10	3	0.854	0.854
0.15	4	0.758	0.758
0.20	5	0.118	0.118
0.40	6	0.000	0.000

15.13.2.8.6 Using the equation from Section 4.2 in Appendix L, the estimate of the IC25 is calculated as follows:

$$ICp = C_j + [M_1(1-p/100) - M_j]\frac{(C_{(j+1)} - C_j)}{(M_{(j+1)} - M_j)}$$

```
Conc. ID             1         2         3         4         5         6
-------------------------------------------------------------------------
Conc. Tested         0       3.2       5.6        10        18        32
-------------------------------------------------------------------------
Response  1        .87       .89       .84       .70       .07         0
Response  2        .89       .82       .89       .71       .12         0
Response  3        .81       .91       .85       .77       .14         0
Response  4        .88       .83       .84       .74       .16         0
Response  5        .84       .89       .85       .87       .10         0
-------------------------------------------------------------------------
*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Copper Chloride
Test Start Date:    Test Ending Date:
Test Species: Purple Sea Urchin, Strongylocentrotus purpuratus
Test Duration:        72 hours
DATA FILE: urch_dev.icp
OUTPUT FILE: urch_dev.i25
-------------------------------------------------------------------------
Conc.     Number     Concentration     Response      Std.       Pooled
 ID     Replicates            ug/L       Means       Dev.   Response Means
-------------------------------------------------------------------------
  1          5             0.000          0.858      0.033      0.863
  2          5             3.200          0.868      0.040      0.863
  3          5             5.600          0.854      0.021      0.854
  4          5            10.000          0.758      0.068      0.758
  5          5            18.000          0.118      0.035      0.118
  6          5            32.000          0.000      0.000      0.000
-------------------------------------------------------------------------
The Linear Interpolation Estimate:    11.3844   Entered P Value: 25
-------------------------------------------------------------------------
Number of Resamplings:   80
The Bootstrap Estimates Mean:  11.3702 Standard Deviation:     0.2898
Original Confidence Limits:   Lower:    10.7785 Upper:    11.9375
Expanded Confidence Limits:   Lower:    10.4756 Upper:    12.2141
Resampling time in Seconds:     0.16  Random_Seed: 83761380
```

Figure 10. ICPIN program output for the IC25.

$$IC25 = 10.0 + [0.863(1 - 25/100) - 0.758] (18.0 - 10.0)$$
$$S))))))))))))Q$$
$$(0.118 - 0.758)$$
$$= 11.38 \mu g/L.$$

15.13.2.8.7 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 11.3844 µg/L. The empirical 95.0% confidence interval for the true mean was 10.7785 µg/L to 11.9375 µg/L. The computer program output for the IC25 for this data set is shown in Figure 10.

15.14 **PRECISION AND ACCURACY**

15.14.1 PRECISION

15.14.1.1 Single Laboratory Precision

15.14.1.1.1 Data on the single-laboratory precision of the development test using copper as a reference toxicant is provided in Table 13. The NOEC varied by only one concentration interval indicating good precision. The coefficient of variation for the EC50 and EC25 were 22% and 21% indicating acceptable precision.

15.14.1.2 Multi-Laboratory Precision

15.14.1.2.1 Data on the multi-laboratory precision of the development test using copper as a reference toxicant is provided in Table 14. The NOEC for laboratory's A and B were identical. The difference in NOEC observed for lab C was probably due the wide range of concentrations used (See Footnote 4). The coefficient of variation for the EC50 was 39%, indicating acceptable interlaboratory precision.

15.14.2 ACCURACY

15.14.2.1 The accuracy of tox1icity tests cannot be determined.

Table 13. SINGLE-LABORATORY PRECISION OF THE PURPLE SEA URCHIN, *STRONGYLOCENTROTUS PURPURATUS*, DEVELOPMENT TEST WITH COPPER (CU µG/L) SULFATE AS A REFERENCE TOXICANT[1].

Test Number	NOEC (µg/L)	EC50 (µg/L)	EC25 (µg/L)
1	10.0	19.4	15.1
2	10.0	18.3	15.4
3	5.6	10.8	9.0
4	5.6	14.3	11.0
5	5.6	16.8	12.9
Mean CV(%)		15.9 22.0	12.7 21.0

[1] Tests performed by Marine Pollution Studies Laboratory, Granite Canyon, Monterey California.

TABLE 14. MULTI-LABORATORY PRECISION OF THE PURPLE SEA URCHIN, *STRONGYLOCENTROTUS PURPURATUS*, DEVELOPMENT TEST WITH COPPER (CU µG/L) SULFATE AS A REFERENCE TOXICANT.[1]

Lab	NOEC (µg/L)	EC50 (µg/L)
A[2]	10.0	22.5
B[3]	10.0	15.2
C[4]	1.8	10.1
Mean CV(%)		15.9 39.0

[1]Data from labs A and B are from an interlaboratory study using split reference toxicant samples and dilution water. Test performed in August, 1993. Test duration was 72 hr. Concentrations were 3.2, 5.6, 10, 18 and 32 Fg/L.

[2]Test performed by Southern California Coastal Water Research Project, Westminster, CA.

[3]Test performed by Marine Pollution Studies Laboratory, Granite Canyon, Monterey California.

[4] Test performed by MEC Analytical Systems, Inc., Tiburon, CA. Test performed in April, 1994. Test duration was 96 hr. Concentrations were 0.1, 0.32, 1.8, 18 and 56 Fg/L.

APPENDIX I. SEA URCHIN DEVELOPMENT: STEP-BY-STEP SUMMARY

PREPARATION OF TEST SOLUTIONS

A. Determine test concentrations and appropriate dilution water based on NPDES permit conditions and guidance from the appropriate regulatory agency.

B. Prepare effluent test solutions by diluting well mixed unfiltered effluent using volumetric flasks and pipettes. Use hypersaline brine where necessary to maintain all test solutions at 34 ± 2‰. Include brine controls in tests that use brine.

C. Prepare a copper reference toxicant stock solution.

D. Prepare a copper reference toxicant series. Add 10 mL of test solution each vial.

E. Sample effluent and reference toxicant solutions for physical/chemical analysis. Measure salinity, pH and dissolved oxygen of each test concentration.

F. Randomize numbers for test chambers and record the chamber numbers with their respective test concentrations on a randomization data sheet. Store the data sheet safely until after the test samples have been analyzed.

G. Place test chambers in a water bath or environmental chamber set to 15EC and allow temperature to equilibrate.

H. Measure the temperature daily in one random replicate (or separate chamber) of each test concentration. Monitor the temperature of the water bath or environmental chamber continuously.

I. At the end of the test, measure salinity, pH, and dissolved oxygen concentration from each test concentration.

PREPARTION AND ANALYSIS OF TEST ORGANISMS

A. Obtain test organisms and hold or condition as necessary for spawning.

B. Place six 100 mL beakers of dilution water in 15EC water bath or room. Select 6-8 sea urchins and place on tray covered with seawater moistened paper towels. Induce

spawning by injecting each sea urchin with 0.5 mL of 0.5 M KCl. Place animals back onto tray, oral side down.

C. When spawning begins, note time that each animal begins spawning. Leave males on tray for semen collection. Place spawning females oral side up on 100 mL beakers. Do not collect gametes more than 15 min after spawning begins.

D. Collect semen using either a Pasteur pipette or a 100 FL autopipette. Pipette semen from each male into a separate 5 mL conical test tube, stored in an ice water bath.

E. Check for the motility of sperm from each male.

F. Pool semen by pipetting equal amounts from each "good" male to another centrifuge tube. At least 0.025 mL should be taken from each male and a total of at least 0.05 mL should be available. Cover the tube and store in a refrigerator until ready for use.

G. Finish collecting eggs before diluting semen.

H. Mix pooled semen by agitating on a vortex mixer. Add about 0.025 mL of semen to a 100 mL beaker containing 50 mL of 15EC dilution water. Stir thoroughly with a Pasteur pipette. Test eggs from each female to determine if they can be fertilized.

I. Take 0.5 mL subsample of sperm dilution and add to 5 mL of 10% acetic acid in a 50 mL graduated cylinder. Bring to 50 mL with dilution water. Mix well by inversion and load a drop into each side of hemocytometer. Count the sperm in 80 small squares. Calculate the sperm density using Equation 2A.

J. Examine sample of eggs from each female. Do not use the eggs from any female whose eggs appear abnormal or that did not fertilize in Section G.

K. Decant water from eggs of each usable female and pour through Nitex® screen into a 1 liter beaker. Bring volume up to about 600 mL with dilution water. Allow to resettle, siphon about 400 mL of overlying water and bring back to 600 mL with dilution water.

L. Mix egg solution well and make an accurate 100x dilution using at least 10 mL of the egg solution. Mix the dilution well and count two different 1 mL subsamples in a counting

cell. Use the mean of the two counts in Equation 3 to determine the density of the egg stock.

M. Use Equation 4 to determine the egg dilution factor and make dilution of eggs with dilution water.

N. Use Equations 5 A-C to determine the volume of the sperm dilution that is necessary to fertilize the egg dilution. Add the appropriate volume of sperm and after 10 minutes, check fertilization success.

O. Gently mix the fertilized egg solution with a plunger and deliver 0.25 mL of egg solution to each vial. Make sure that the pipette tip is cut off to provide at least a 0.5 mm opening. Keep egg solution well mixed during addition period.

P. Incubate the embryos for 72 hours at 15 ± 1EC.

Q. Test termination and analysis

R. Perform water quality measurements as at the start.

S. After 72 hours, add 1.0 mL of 37% buffered formalin or 0.5 mL of 1.0% glutaraldehyde to each test chamber. Tightly cap and gently mix each vial.

T. Examine each sample with a microscope and determine the percentage of normally developed embryos.

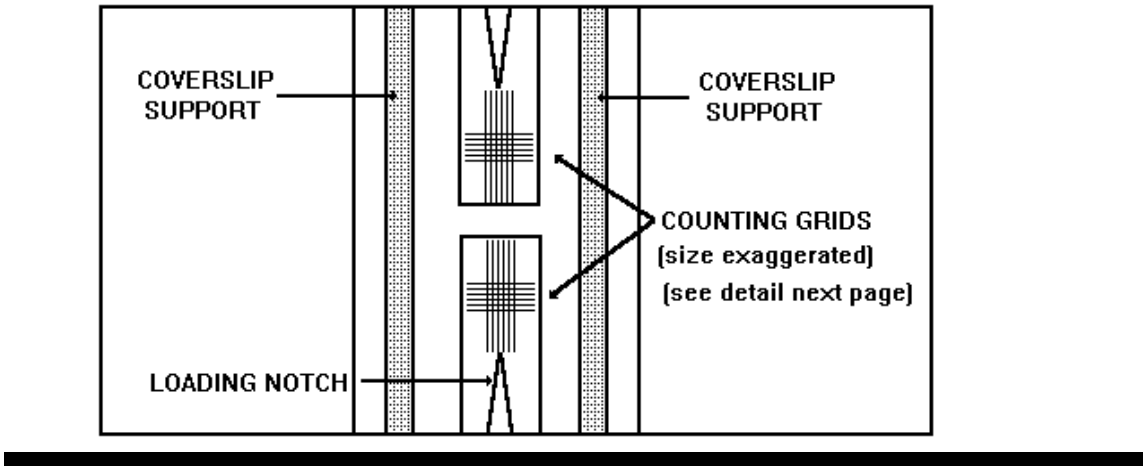
U. Analyze the data.

V. Include standard reference toxicant point estimate values in the standard quality control charts.

APPENDIX II. USING THE NEUBAUER HEMACYTOMETER TO ENUMERATE SEA URCHIN SPERM

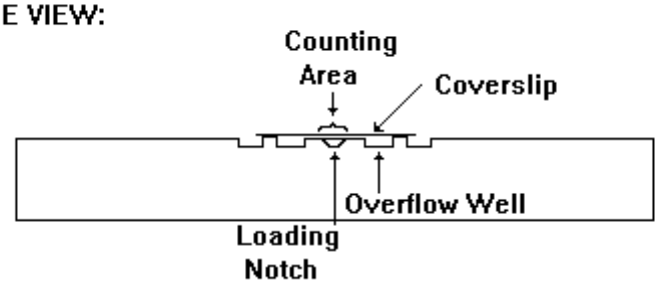
The Neubauer hemacytometer is a specialized microscope slide with two counting grids and a coverslip.

TOP VIEW:

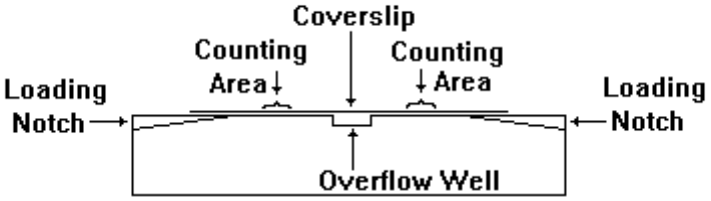


Together, the total area of each grid (1 mm^2) and the vertical distance between the grid and the coverslip (0.1 mm), provide space for a specific microvolume of aqueous sample (0.1 mm^3).

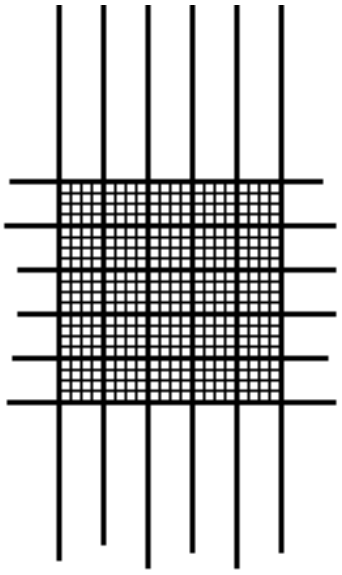
SIDE VIEW:



END VIEW THROUGH MID-CROSS SECTION:



This volume of liquid and the cells suspended therein (e.g., blood cells or sperm cells) represent 1/10,000th of the liquid volume and cell numbers of a full milliliter (cm^3) of the sampled material.



NEUBAUER
HEMACYTOMETER
GRID OF 400 SQUARES

If the full 400-squares of each grid are counted, this represents the number of sperm in 0.1 mm^3. Multiplying this value times 10 yields the sperm per mm^3 (and is the source of the hemacytometer factor of 4,000 squares/mm^3). If this product is multiplied by 1,000 mm^3/cm^3, the answer is the number of sperm in one milliliter of the sample. If the counted sample represents a dilution of a more concentrated original sample, the above answer is multiplied by the dilution factor to yield the cell density in the original sample. If the cells are sufficiently dense, it is not necessary to count the entire 400-square field, and the final calculation takes into account the number of squares actually counted:

$$\text{cells/mL} = \frac{\text{(dilution) (4,000 squares/mm}^3\text{) (1,000 mm}^3\text{/cm}^3\text{) (cell count)}}{\text{(number of squares counted)}}$$

Thus, with a dilution of 4000 (0.025 mL of semen in 100 mL of dilution water), 80 squares counted, and a count of 100, the calculation becomes:

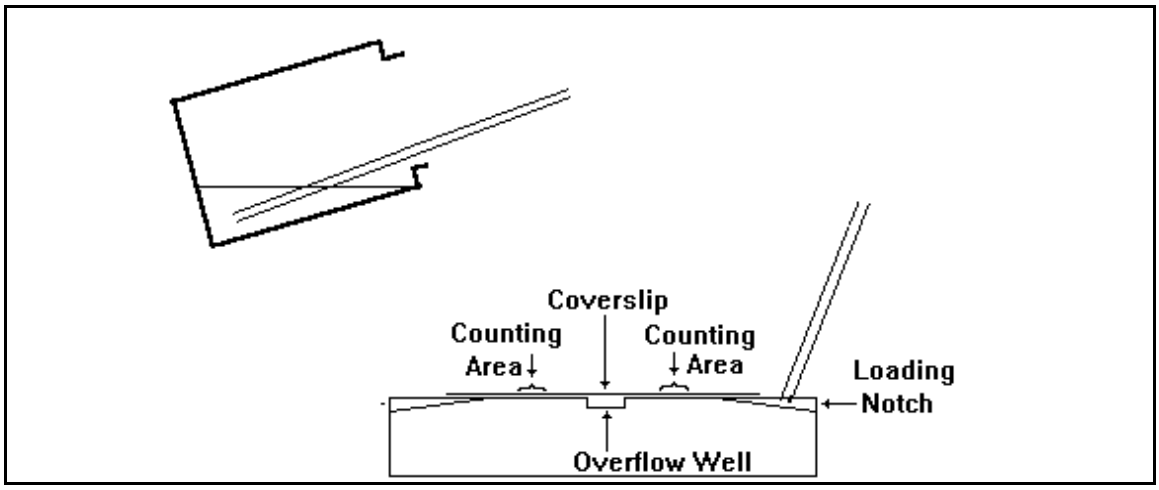
$$\text{cells/mL} = \frac{(4{,}000)\ (4{,}000)\ (1{,}000)\ (100)}{80}$$

$$= 20{,}000{,}000{,}000 \text{ cells/mL}$$

There are several procedures that are necessary for counts to be consistent within and between laboratories. These include mixing the sample, loading and emptying the hematocrit tube, cleaning the hemacytometer and cover slip, and actual counting procedures.

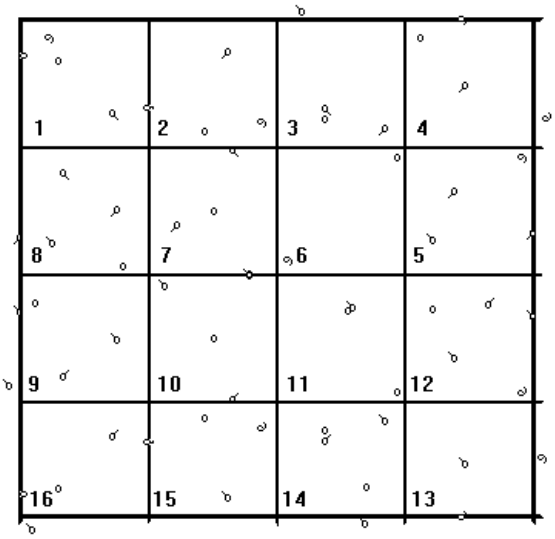
Obviously, if the sample is not homogeneous, subsamples can vary in sperm density. A few extra seconds in mixing can save a lot of wasted minutes in subsequent counting procedures. A full hematocrit tube empties more easily than one with just a little liquid, so withdraw a full sample. This can be expedited by tipping the sample vial.

Because the sperm are killed prior to sampling, they will slowly settle. For this reason, the sample in the hematocrit tube should be loaded onto the hemacytometer as rapidly as possible. Two replicate samples are withdrawn in fresh hematocrit tubes and loaded onto opposite sides of a hemacytometer.

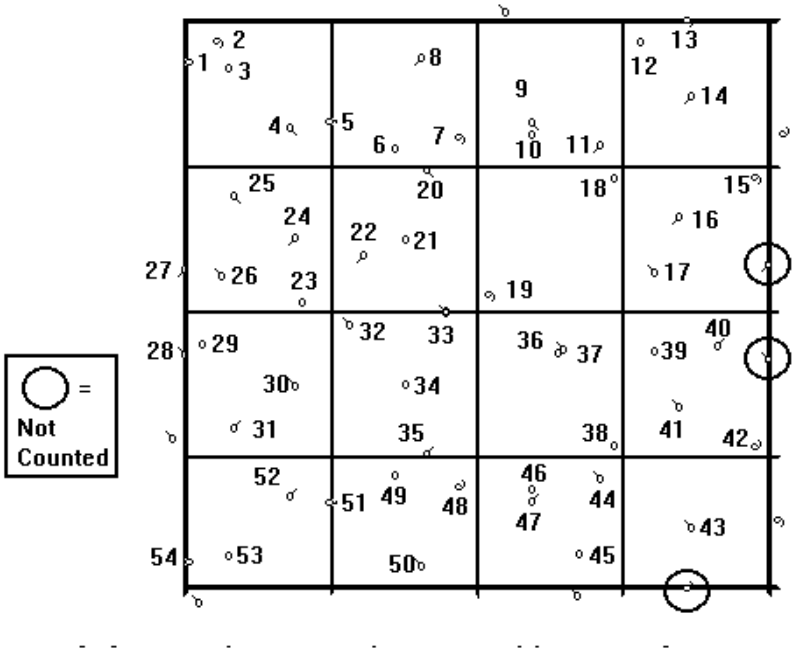


The loaded hemacytometer is left for 15 minutes to allow the sperm to settle onto the counting field. If the coverslip is moved after the samples are loaded, the hemacytometer should be rinsed and refilled with fresh sample. After 15 minutes, the hemacytometer is placed under a microscope and the counting grid located at 100x. Once the grid is properly positioned, the microscope is adjusted to 200x or 400x, and one of the corner squares is positioned for counting (any one of the four corners is appropriate). For consistency, use the same procedure each time (Many prefer to start in the upper left corner of the optical field, and this procedure will be used in the examples given below). Examine the first large square in the selected corner. If no sperm are visible, or if the sperm are so dense or clumped to preclude accurate counting, count a sample with a more appropriate dilution.

In making counts of sperm, it is necessary to adopt a consistent method of scanning the smaller squares and counting sperm that fall upon the lines separating the squares. Count the sperm in the small squares by beginning in the upper left hand corner (square 1) and proceding right to square 4, down to square 5, left to square 8, etc. until all 16 squares are counted.



Because sperm that appear <u>on</u> lines might be counted as being in either square, it is important to avoid double counting or non-counting. For this reason a convention is decided upon and used consistently: paraphrasing the instructions received with one (Hausser Scientific) counting chamber "to avoid counting (sperm) twice, the best practice is to count all touching the top and left, and none touching the lower and right, boundary lines." Whatever convention is chosen, it must be adhered to. The example below shows a sperm count based upon a selected convention of counting sperm that fall on the upper and left lines, but not on the lower or right lines:



In the above illustration, sperm falling on the lower and right lines are not counted. The count begins at the upper left as illustrated in the preceding figure. A typical count sequence is demonstrated by the numbers next to each sperm illustrated. Sperm identified as numbers 1, 5, 13, 20, 27, 28, 33, 51 and 54 touch lines and are counted as being in the square below them or to their right. The circled sperm are not counted as being in this field of 16 small squares (but they would be included in any counts of adjacent squares in which they would be on upper or left hand lines).

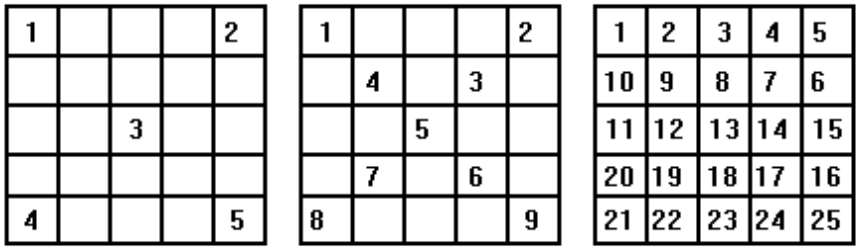
Once these counting conventions have been selected, it is advisable to follow another strict protocol outlining the number and sequence of large squares to be counted. Because the sperm may not be randomly distributed across the counting grid, it is recommended to count an array of squares covering the entire grid. The following procedure is recommended:

Count the number of sperm in the first large square.

1. If the number is less than 10, count all 25 squares using the same scanning pattern outlined above (left to right through squares 1 to 5, down to square 6, left through square 10, down to 11, etc.). See pattern no. 3.

2. If the number is between 10 and 19, count 9 large squares using pattern no. 2.

3. If the number is 20 or greater, count 5 large squares using pattern no. 1.



Pattern no. 1 Pattern no. 2 Pattern no. 3

The final consideration in achieving good replicate counts is keeping the hemacytometers and coverslips clean. They should be rinsed in distilled water soon after use. The coverslips should be stored in a good biocleanser such as hemasol. For an hour or so prior to use, the hemacytometer slides should also be soaked in the solution. Both slides and coverslips should then be rinsed off with reagent water, blotted dry with a lint-free tissue, and wiped with lens paper.