SECTION 16

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

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SECTION 16

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

16.1 SCOPE AND APPLICATION

16.1.1 This method estimates the chronic toxicity of effluents and receiving waters to the gametes of sea urchins, (*Strongylocentrotus purpuratus*), or sand dollars (*Dendraster excentricus*) during a static non-renewal 20 minute sperm exposure and a subsequent 20 minute exposure period following the addition of eggs for measuring the fertilizing capacity of the sperm. The effects include the synergistic, antagonistic, and additive effects of all chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms.

16.1.2 The purpose of the test is to determine the concentrations of a test substance that reduce egg fertilization by exposed sperm relative to that attained by sperm in control solutions. Concentrations of materials adversely affecting egg fertilization under the conditions of this test are usually acutely and chronically toxic to one or more of several common marine test species and, by extension, are presumably acutely and chronically toxic to other of the many untested marine species.

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

16.1.4 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.

16.1.5 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.

16.1.6 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.

16.2 **SUMMARY OF METHOD**

16.2.1 The method provides the step-by-step instructions for exposing sperm suspensions (appropriate sperm density may first be determined in a trial fertilization test) to effluents or receiving waters for 20 minutes. Eggs are then added to the sperm suspensions and, twenty minutes after the eggs are added, the test is terminated by the addition of a preservative. The percent fertilization is determined by microscopic examination of 100 eggs in an aliquot of eggs from each treatment. The test endpoint is normal egg fertilization.

16.3 **INTERFERENCES**

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

16.3.2 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).

16.4 **SAFETY**

16.4.1 See Section 3, Health and Safety

16.5 **APPARATUS AND EQUIPMENT**

16.5.1 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.

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

16.5.3 Constant temperature chambers or water baths -- for maintaining test solution temperature and keeping dilution water supply, gametes, and embryo stock suspensions at test temperature (12EC) prior to the test. (Incubators are usually unsatisfactory because test tubes must be removed for addition of sperm and eggs and the small test volumes can rapidly change temperature at normal room temperatures.)

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

16.5.5 Refractometer -- for determining salinity.

16.5.6 Hydrometer(s) -- for calibrating refractometer.

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

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

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

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

16.5.11 Winkler bottles -- for dissolved oxygen determinations.

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

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

16.5.14 Glass stirring rods -- for mixing test solutions.

16.5.15 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).

16.5.16 Volumetric flasks -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making test solutions.

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

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

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

16.5.20 Wash bottles -- for dilution water.

16.5.21 20-liter cubitainers or polycarbonate water cooler jugs -- for making hypersaline brine.

16.5.22 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. Strong solutions of NaOH and formaldehyde should not be held for several month periods in Cubitainers: interaction or leaching into solutions of 0.1 N or 1 N NaOH used for pH adjustment of dilution water has caused poor egg fertilization; formaldehyde similarly stored has induced abberant partial membrane elevation in eggs.

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

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

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

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

16.5.27 Compound microscope -- for examining gametes, counting sperm cells (200-400x) and eggs (100x), and examining fertilized eggs. Dissecting scopes are sometimes used to count eggs at a lower magnification.

16.5.28 Counter, two unit, 0-999 -- for recording sperm and egg counts.

16.5.29 Sedgwick-Rafter counting chamber -- for counting egg stock and examining eggs for fertilization at the end of the test.

16.5.30 Hemacytometers, Neubauer -- for counting sperm.

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

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

16.5.33 Perforated plunger -- for maintaining homogeneous distribution of eggs during sampling and distribution to test tubes.

16.5.34 60 µm NITEX® filter -- for filtering receiving water.

16.6 **REAGENTS AND SUPPLIES**

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

16.6.2 Data sheets (one set per test) -- for data recording (see Figures 1 and 2).

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

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

16.6.5 Parafilm -- to cover graduated cylinders and vessels containing gametes.

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

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

16.6.8 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.

16.6.9 Coverslips -- for microscope slides.

16.6.10 Lens paper -- for cleaning microscope optics.

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

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

16.6.13 pH buffers 4, 7, and 10 (or as per instructions of instrument manufacturer) -- for standards and calibration check (see USEPA Method l50.l, USEPA, l979).

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

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

16.6.16 Test chambers -- test tubes, borosilicate glass, 16 x 100 mm or 16 x 125 mm, with caps for conducting the test, four chambers per concentration.

Figure 1. Sample data sheet for spawning record.

Animal No.	Sex	Time Injected	Time Spawn	Comments
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				

Pooled eggs from female nos. _____.

Pooled (_____ mL) of sperm each from male nos. _____.

Figure 2. Sample data sheet for egg and sperm counts.

EGG COUNTS -

Sample	Dilution	Count	Eggs/mL

For 100 mL egg suspension at 2,240 eggs/mL use:

100 mL x 2,240 eggs/mL / (counted eggs/mL) = mL of egg stock

224,000 eggs / _____ eggs/mL = _____ mL

If required stock >100 mL, concentrate egg stock by settling the eggs and decanting off sufficient overlying water to retain:

(_____ eggs/mL / 2,240 eggs/mL) x 100 = _____ % volume

SPERM COUNTS -

Sample	Dilution	Count	Squares	Sperm/mL

$$\text{SPERM/mL} = \frac{(\text{DIL.FACT.})\ (\text{COUNT})\ (4000)\ (1000)}{(\text{NO. SQUARES COUNTED})}$$

16.6.17 Formaldehyde, 10%, in seawater -- for preserving eggs. 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.

16.6.18 Glutaraldehyde, 1% in seawater -- for preserving eggs.

16.6.13 pH buffers 4, 7, and l0 (or as per instructions of
16.6.19 Acetic acid, 10%, reagent grade, in filtered (10F) seawater -- for preparing killed sperm dilutions for sperm counts.

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

16.6.21 0.5 M KCl solution -- for inducing spawning.

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

16.6.23 Needles, 25 gauge -- for injecting KCl.

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

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

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

16.6.27 Reference toxicant solutions (see 16.10.2.4 and Section 4, Quality Assurance).

16.6.28 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 and Section 7, Dilution Water).

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

16.6.30 Dilution water and hypersaline brine -- see Section 7, Dilution Water and Section 16.6.24, Hypersaline Brines. The dilution water should be uncontaminated 1-µm-filtered natural seawater. Hypersaline brine should be prepared from dilution water.

16.6.31 HYPERSALINE BRINES

16.6.31.1 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.

16.6.31.2 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).

16.6.31.3 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.

16.6.31.4 Freeze Preparation of Brine

16.6.31.4.1 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).

16.6.31.4.2 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%.

16.6.31.4.3 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.

16.6.31.5 Heat Preparation of Brine

16.6.31.5.1 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 applied, use only oil-free air compressors to prevent contamination.

16.6.31.5.2 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.

16.6.31.5.3 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.

16.6.31.5.4 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.

16.6.31.6 Artificial Sea Salts

16.6.31.6.1 No data from sea urchin or sand dollar fertilization tests using sea salts are available for evaluation at this time, and their use should be considered provisional. The use of GP2 artificial seawater (Table 2) has been found to provide control fertilization equal to that of natural seawater.

16.6.31.6.2 The GP2 reagent grade chemicals (Table 2) should be mixed with deionized (DI) water or its equivalent in a single batch, never by test concentration or replicate. The reagent water used for hydration should be between 21-26EC. The artificial seawater must be conditioned (aerated) for 24 h before use as the testing medium. If the solution is to be autoclaved, sodium bicarbonate is added after the solution has cooled. A stock solution of sodium bicarbonate is made up by dissolving 33.6 g $NaHCO_3$ in 500 mL of reagent water. Add 2.5 mL of this stock solution for each liter of the GP2 artificial seawater.

16.6.31.7 Dilution Water Preparation from Brine

16.6.31.7.1 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.

16.6.31.7.2 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. Thus, the proportion is one 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.

16.6.31.8 Test Solution Salinity Adjustment

16.6.31.8.1 Table 3 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 3.

16.6.31.8.2 Check the pH of all brine mixtures and adjust to within 0.2 units of dilution water pH by adding, dropwise, dilute hydrochloric acid or sodium hydroxide.

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

16.6.31.8.3 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:

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

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

16.6.31.9 Preparing Test Solutions

TABLE 2. REAGENT GRADE CHEMICALS USED IN THE PREPARATION OF GP2 ARTIFICIAL SEAWATER FOR THE PURPLE URCHIN *STRONGYLOCENTROTUS PURPURATUS*, AND SAND DOLLAR *DENDRASTER EXCENTRICUS* TOXICITY TEST[1,2]

Compound	Concentration (g/L)	Amount (g) Required for 20 L
NaCl	23.90	478.0
Na_2SO_4	4.00	80.0
KCl	0.698	13.96
KBr	0.100	2.00
$Na_2B_4O_7 \cdot 10\ H_2O$	0.039	0.78
$MgCl_2 \cdot 6\ H_2O$	10.80	216.0
$CaCl_2 \cdot 2\ H_2O$	1.50	30.0
$SrCl_2 \cdot 6\ H_2O$	0.025	0.490
$NaHCO_3$	0.193	3.86

[1]Modified GP2 from Spotte et al. (1984)
[2]The constituent salts and concentrations were taken from USEPA (1990b). The salinity is 34.0 g/L.

16.6.31.9.1 Five 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.

16.6.31.9.2 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 3) 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.

16.6.31.10 Brine Controls

16.6.31.10.1 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, 16.6.33.8.3) 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.

16.6.32 TEST ORGANISMS, PURPLE URCHINS

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

16.6.32.2 Adult sea urchins (*Strongylocentrotus purpuratus*) can be obtained from commercial suppliers or collected from uncontaminated intertidal or subtidal 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.

TABLE 3. 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. Use either serially prepared dilutions of the highest test concentration or individual dilutions of 100% effluent.

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.

16.6.32.3 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.

16.6.32.4 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.

16.6.32.5 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.

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

16.6.32.7 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.

16.6.32.8 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.

16.6.33 TEST ORGANISMS, SAND DOLLARS

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

16.6.33.2 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.

16.6.33.3 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.

16.6.33.4 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.

16.6.33.5 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.

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

16.6.33.7 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.

16.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION AND STORAGE

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

16.8 CALIBRATION AND STANDARDIZATION

16.8.1 See Section 4, Quality Assurance.

16.9 QUALITY CONTROL

16.9.1 See Section 4, Quality Assurance.

16.10 TEST PROCEDURES

16.10.1 TEST DESIGN

16.10.1.1 The test consists of at least four effluent concentrations plus a dilution water control. Tests that use brine to adjust salinity must also contain four replicates of a brine control. In addition, four extra controls are prepared for egg controls.

16.10.1.2 Effluent concentrations are expressed as percent effluent.

16.10.2 TEST SOLUTIONS

16.10.2.1 Receiving waters

16.10.2.1.1 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 5 mL, and 400 mL for chemical analysis, would require approximately 420 mL or more of sample per test.

16.10.2.2 Effluents

16.10.2.2.1 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.

16.10.2.2.2 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%).

16.10.2.2.3 The volume in each test chamber is 5 mL.

16.10.2.2.4 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.

16.10.2.3 Dilution Water

16.10.2.3.1 Dilution water should be uncontaminated 1-µm-filtered natural seawater, or hypersaline brine prepared from uncontaminated natural seawater plus reagent water; or sea salts (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.

16.10.2.4 Reference Toxicant Test

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

16.10.2.4.2 The preferred reference toxicant for sea uchins and sand dollar 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.

16.10.2.4.3 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). Start with control solutions and progress to the highest concentration to minimize contamination.

16.10.2.4.4 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‰.

16.10.3 COLLECTION OF GAMETES FOR THE TEST

16.10.3.1 Spawning Induction

16.10.3.1.1 Pour seawater into 100 mL beakers and place in 12EC 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.

16.10.3.1.2 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.

16.10.3.1.3 Place each sand dollar, oral side up, on a 100 mL beaker filled with 12EC seawater or each sea urchin onto a clean tray covered with several layers of seawater moistened paper towels.

16.10.3.1.4 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.

16.10.3.1.5 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 3) 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 (sample data sheet, Figure 1).

16.10.3.1.6 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.

16.10.3.1.7 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.

16.10.3.1.8 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.

16.10.3.1.9 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.
16.10.3.1.10 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.

16.10.3.1.11 Collect the undiluted semen from each male sea urchin, using a 0.1 mL automatic pipet. Store the sperm from each male in a separate, labelled, conical, glass centrifuge tube, covered with a cap or parafilm, on ice. Air exposure of semen may alter its pH through gas exchange and reduce the viability of the sperm. Note: undiluted semen from *Strongylocentrotus purpuratus* typically contains about 4 x 10^{10} sperm/mL.

16.10.3.1.12 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.

16.10.3.2 Collection of Sperm

16.10.3.2.1 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.

16.10.3.3 Viability of Sperm

16.10.3.3.1 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. It is more important to use high quality sperm than it is to use a pooled population of sperm.

16.10.3.4 Pooling of Sperm

16.10.3.4.1 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).

16.10.3.5 Storage of Sperm

16.10.3.5.1 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 in a toxicity test within 4 h of collection.

16.10.4 PREPARATION OF EGG SUSPENSION FOR USE IN THE TEST

16.10.4.1 Acceptability of Eggs

16.10.4.1.1 Prior to pooling, a small sample of the eggs from each female should be examined for the presence of significant quantities of poor eggs (vacuolated, small, or irregularly shaped) and mixed with good sperm to determine extent of fertilization. If good quality eggs are available from one or more females, questionable eggs should not be used for the test. It is more important to use high quality eggs than it is to use a pooled population of eggs.

16.10.4.2 Pooling of Eggs

16.10.4.2.1 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. The sea urchin eggs are pooled into a 1 L beaker, and the volume brought to 600 mL with 12EC dilution water. The eggs are suspended by swirling and the eggs allowed

to settle for 15 minutes at 12EC. About 500 mL of the overlying water are siphoned off, the volume brought back to 600 mL with more 12EC dilution water, and the eggs resuspended and allowed to settle for a second 15 minute period. After again siphoning off the overlying 500 mL, the rinsed eggs are gently transferred to either a 100 or a 250 mL graduated cylinder and brought to volume with 12EC dilution water. Eggs are stored at 12EC throughout the pre-test period.

16.10.4.2.2 Pooled sand dollar eggs should be treated gently and no additional 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.

16.10.4.3 Density of Eggs

16.10.4.3.1 Subsamples of the egg stock are then taken for determining egg density. Place 9 mL of dilution water in each of two 22 mL liquid scintillation vials. Label A and B. Place 1 mL of well-mixed egg stock into vial A. Mix well. (The remaining egg stock is covered with parafilm and stored at 12EC.) Transfer 1 mL of egg suspension from vial A to vial B. Mix contents of vial B and transfer 1 mL of egg suspension B into a Sedgewick-Rafter counting chamber. Count eggs under a compound microscope. If count is <30, count a 1 mL sample from vial A (see sample data sheet, Figure 2).

16.10.4.3.2 Prepare 100 mL of egg stock in dilution water at the final target concentration of 2,240 eggs/mL (224,000 eggs in 100 mL). If the egg stock is >2,240 eggs/mL (A >224 or B >30 eggs/mL), dilute the egg stock by transferring:

$$224{,}000 \text{ eggs} / \frac{\quad D \quad}{} \text{ eggs/mL} = \underline{\quad\quad} \text{mL}$$

of well-mixed egg stock to a 100 mL graduated cylinder and bring the total volume to 100 mL with dilution water where:

$$D = (\text{Count A}) \times 10 \text{ or } (\text{Count B}) \times 100.$$

If the egg stock is <2,240 eggs/mL (A <224 eggs/mL), concentrate the eggs by allowing them to settle and then decant enough water to retain the following percent of the original volume:

(D eggs/mL / 2,240) x 100 = % volume.

16.10.4.3.3 Check the egg stock density. Place 9 mL of dilution water into a 22 mL scintillation vial; add 1 mL of the final egg stock. Mix well and transfer 1 mL into a Sedgewick-Rafter counting chamber. The egg count should be between 200 and 245. Adjust egg stock volume and recheck counts if necessary to obtain counts within this range. Because some eggs (especially sand dollar eggs) may be sensitive to handling, it is advisable to separately prepare egg stocks for the fertilization trial and the definitive test (but use the same pooled batch of eggs).

16.10.5 PREPARATION OF SPERM DILUTION FOR USE IN THE (OPTIONAL) TRIAL FOR ESTIMATING APPROPRIATE SPERM DENSITY FOR TEST

16.10.5.1 A trial fertilization is recommended to reduce the likelihood of a failed test due to inadequate control fertilization or exceeding the maximum acceptable sperm density. However, two other alternative approaches are acceptable:

1) Conduct the test at a low enough sperm density that oversperming does not create test insensitivity. This can be met by using a confirmed sperm stock density of #5.6x10^6/mL (this is equivalent to a sperm:egg ratio of #500:1 at 200 eggs/mL); or

2) Conduct the test, but include two extra sets of controls, one set receiving only 0.050 mL of the sperm stock and the other receiving 0.2 mL of the sperm stock. The control fertilization in the 0.050 mL sperm stock controls must be at least 5% lower than that in the 0.2 mL sperm stock controls or the test is unacceptable. Confirm that the sperm stock density did not exceed the maximum acceptable density of 3.36 x 10^7 sperm/mL.

16.10.5.2 Fertilization trial is conducted to determine the sperm density that will provide about 80-100 percent control egg fertilization while avoiding significant "oversperming" that can reduce test sensitivity. Although usually expressed as a sperm:egg ratio (e.g., 1,000:1), because egg density is held constant at 200/mL, the sperm:egg ratio is also a measure of sperm density.

16.10.5.3 It is unacceptable to conduct a definitive toxicity test if the sperm:egg ratio exceeds 3,000:1. This is a cut-off based on gradual loss of test sensitivity at higher sperm densities, even in cases where control fertilization is considerably below 100 percent.

16.10.5.4 It is unnecessary to conduct trials for definitive toxicity tests at sperm:egg ratios below 500:1, because this ratio should never cause significant "oversperming."

16.10.5.5 Sperm density of sea urchin semen or sand dollar sperm suspension is checked by hemocytometer counts and a replicated series of nominal S:E ratios set up (3,000, 1288, 550, 234, and 100:1) based upon appropriate dilution calculations.

16.10.5.6 For sea urchins and sand dollars, prepare a killed sperm preparation for determining the dilution required to obtain a sperm stock (3.36×10^7 sperm/mL) for the maximum sperm density (6×10^5 sperm/200 eggs/mL--3,000:1) needed for the trial. A sperm density of about 1×10^7 is convenient to count. If the approximate sperm density is known, the dilution procedures outlined in Table 4 can be followed without initial sperm counts; the actual trial sperm density must still be determined by subsequent counts. For example (Table 4), if expected sperm density is ca. 5×10^8 dilute 0.2 mL of sperm to 10 mL, if ca. 5×10^9 dilute 0.2 mL of sperm to 100 mL (or 0.025 mL of sperm to 10 mL), if ca. 5×10^{10} dilute 0.040 mL to 200 mL. Table 4 is provided for guidance as a quick reference for dilution volumes if sperm density of pooled semen is can be reasonably estimated, and as a check for mathematical accuracy of formula calculations for sperm dilution.

16.10.5.7 Mix the pooled sea urchin semen (16.10.3.8) by agitating the centrifuge tube for about 5 seconds using a vortex mixer. Very slowly withdraw a subsample of semen using an automatic pipet, wipe off the outside of the pipet tip with tissue, and empty the pipet contents into an Erlenmeyer flask containing the appropriate volume (Table 4) of a sperm killing solution of 1% glacial acetic acid in dilution water (e.g., 10 mL of 10% glacial acetic acid plus 90 mL of dilution water).

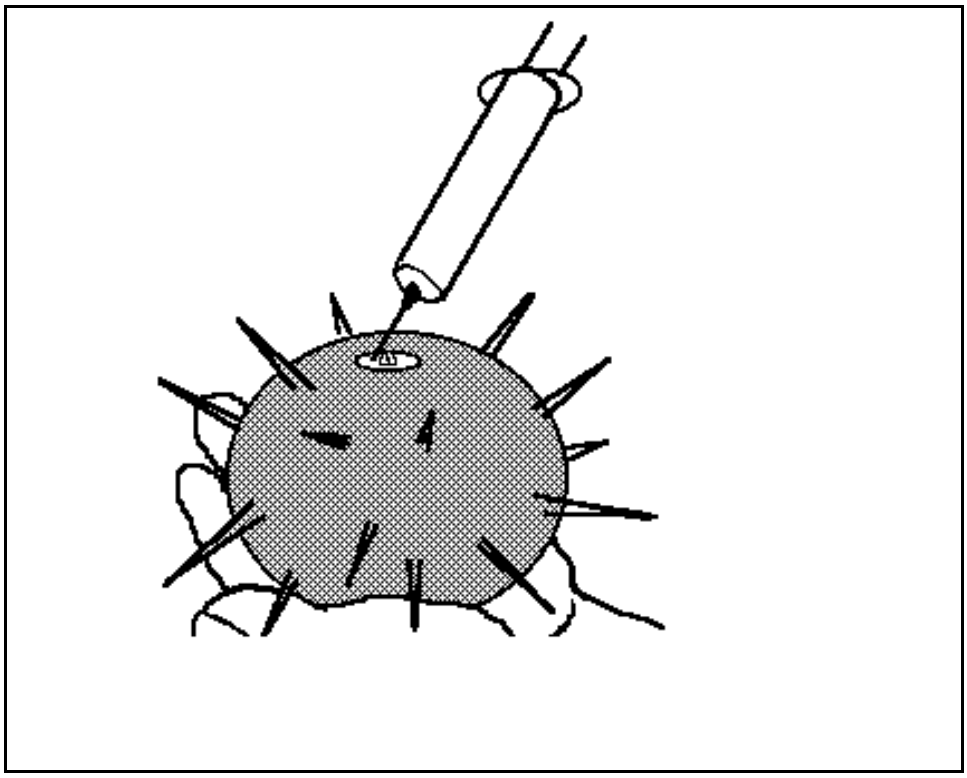


Figure 3. Showing the location and orientation used in the injection of KCl into sea urchins to stimulate spawning.

Repeatedly rinse the residual semen from the pipet tip by filling and emptying until no further cloudy solution is expelled from the pipet (this may require several dozen rinses). Cover the flask with parafilm and mix thoroughly by repeated inversion.

16.10.5.8 Mix the chilled suspension of pooled sand dollar sperm (16.10.5.6) using a stirring rod. Pipet the appropriate volume of sperm suspension (Table 4) into an Erlenmeyer flask containing the appropriate volume (Table 4) of a sperm killing solution of 1% glacial acetic acid in dilution water (e.g., 10 mL of 10% glacial acetic acid plus 90 mL of dilution water).

16.10.5.9 Transfer samples of well-mixed sperm suspension to both sides of two Neubauer hemacytometers. Let the sperm settle 15 min.

16.10.5.10 Count the sperm on one hemacytometer following procedures outlined in Appendix II. If the lower count is at

least 80% of the higher count use the mean count to estimate sperm density in semen and the required dilution volume for the test stock. If the two counts do not agree within 20%, count the two fields on the other hemacytometer. Calculate the sperm density in the semen or sperm suspension using the mean of all four counts unless one count can be eliminated as an obvious outlier.

16.10.5.11 Calculate the volume of dilution water necessary to dilute the sea urchin semen or the sand dollar sperm suspension to the sperm density (sperm/mL) required for the sperm stock for the trial. See Table 5 for recommended dilution procedures; it also provides a quick reference for dilution volumes once sperm density of pooled semen is known, or a check for mathematical accuracy of formula calculations for sperm dilution. Note: table values for sperm densities from $1x10^8$ to $9x10^9$ are for volume (mL) of sperm stock for total volume of 100 mL; table values for sperm densities $1x10^{10}$ are for dilution water volumes for 0.025 mL of semen. Table 5 is used as follows: given a sperm density in the semen stock (e.g., $4.7x10^9$) find the row containing the integer (characteristic) and the exponent ($4x10^9$) in the left hand column, then read across to the column coresponding to the mantissa (0.7). The value at the intersection of the row and column (0.71 mL) is the volume of semen per 100mL needed for sperm stock to achieve a 3000:1 sperm:egg ratio in the trial.

16.10.5.12 For the approximate sperm:egg ratios dilute the 3000:1 stock as follows:

1288:1	5 mL 3000:1 stock with 6.6 mL dilution water
550:1	2 mL 3000:1 stock with 9.9 mL dilution water
234:1	1 mL 3000:1 stock with 11.8 mL dilution water
100:1	0.5 mL 3000:1 stock with 16.5 mL dilution water

16.10.6 SPERM DENSITY TRIAL

16.10.6.1 The series of trial sperm:egg ratios should include 3,000:1 and several lower ratios. The ratios 100:1, 234:1, 550:1, 1288:1 and 3,000:1 are recommended because they evenly divide the log sperm:egg ratio. Fertilization appears to be a linear function of the log of sperm density (Figure 4). Recommended sperm dilution procedures are given in Table 5.

16.10.6.4 Quantitive evaluation of the sperm density trial should be obtained by counting 100 eggs from each tube until a suitable sperm density can be determined for the definitive test. Examples of sperm density selection are given in Table 6. Percent fertilization may be lower in the test than in the trial because the viability of the stored sperm may decrease during the period of the trial. If the sperm have very good viability (e.g., cases 1 and 2, Table 6), this loss of viability should be small. On the other hand, if viability is inherently poorer (cases 3, 4 and 5, Table 6), the loss of viability could be greater and probably should be taken into account in selecting the sperm density for the test. Case 6 (Table 6) represents a special case in which egg viability may affect the percent fertilization; in this case the asymptote of the fertilization curve is assumed to represent 100% fertilization for purposes of selection of sperm density for the test.

16.10.6.5 Prepare killed sperm preparations of the trial sperm stock suspensions to provide confirmation of the nominal sperm:egg ratios. It saves time if these can be prepared and loaded onto hemacytometers while the trial is being conducted. Alternatively, once the trial has been evaluated, the selected nominal sperm density can be confirmed by direct hemacytometer count.

16.10.6.6 Record all the counts made, select a target sperm:egg ratio for the test, and calculate the dilution of the stored sperm stock needed to provide the necessary sperm density for the definitive test.

16.10.6.7 Table 5 can be used for deriving the volumes needed for preparing the final sperm stock. For a pooled sperm suspension density of $4x10^9$ and a target sperm:egg ratio of 500:1, simply read the dilution for the 3000:1 sperm:egg ratio from Figure 5 (0.84 mL / 100 mL) and reduce the sperm volume by 3,000 / 500 = 6. In this case 0.84 / 6 = 0.14 mL; the dilution factor checks out (100 / 0.14 = 714).

TABLE 4. Dilution volume guide for initial count of sperm density to achieve recommended counting density of 1 x 10^7/mL.

Initial Sperm/mL	mL/10mL	mL/100mL	mL/200mL
1 x 10^8	1.000		
2 x 10^8	0.500		
3 x 10^8	0.333		
4 x 10^8	0.250		
5 x 10^8	0.200		
6 x 10^8	0.167		
7 x 10^8	0.143		
8 x 10^8	0.125		
9 x 10^8	0.111		
1 x 10^9	0.100	1.000	
2 x 10^9	0.050	0.500	1.000
3 x 10^9	0.033	0.333	0.667
4 x 10^9	0.025	0.250	0.500
5 x 10^9		0.200	0.400
6 x 10^9		0.167	0.333
7 x 10^9		0.143	0.286
8 x 10^9		0.125	0.250
9 x 10^9		0.111	0.222
1 x 10^{10}		0.100	0.200
2 x 10^{10}		0.050	0.100
3 x 10^{10}		0.033	0.067
4 x 10^{10}		0.025	0.050
5 x 10^{10}			0.040
6 x 10^{10}			0.033
7 x 10^{10}			0.029
8 x 10^{10}			0.025
9 x 10^{10}			0.022

Note: to obtain quantitatively repeatable samples of semen it is important that: (1) the pipet tip have an opening of at least 1 mm; (2) samples be withdrawn slowly to avoid cavitation and entrainment of air in the semen sample; (3) samples not include fragments of broken spines (which usually settle to the test tube bottom upon vortexing); and (4) wiping semen from the pipet tip with tissue be done with care to avoid wicking semen from within the pipet tip.

Bring the indicated volume of sperm stock to 100 mL

Density	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
1.00e+08	33.60	30.55	28.00	25.85	24.00	22.40	21.00	19.76	18.67	17.68
2.00e+08	16.80	16.00	15.27	14.61	14.00	13.44	12.92	12.44	12.00	11.59
3.00e+08	11.20	10.84	10.50	10.18	9.88	9.60	9.33	9.08	8.84	8.62
4.00e+08	8.40	8.20	8.00	7.81	7.64	7.47	7.30	7.15	7.00	6.86
5.00e+08	6.72	6.59	6.46	6.34	6.22	6.11	6.00	5.89	5.79	5.69
6.00e+08	5.60	5.51	5.42	5.33	5.25	5.17	5.09	5.01	4.94	4.87
7.00e+08	4.80	4.73	4.67	4.60	4.54	4.48	4.42	4.36	4.31	4.25
8.00e+08	4.20	4.15	4.10	4.05	4.00	3.95	3.91	3.86	3.82	3.78
9.00e+08	3.73	3.69	3.65	3.61	3.57	3.54	3.50	3.46	3.43	3.39
1.00e+09	3.36	3.05	2.80	2.58	2.40	2.24	2.10	1.98	1.87	1.77
2.00e+09	1.68	1.60	1.53	1.46	1.40	1.34	1.29	1.24	1.20	1.16
3.00e+09	1.12	1.08	1.05	1.02	0.99	0.96	0.93	0.91	0.88	0.86
4.00e+09	0.84	0.82	0.80	0.78	0.76	0.75	0.73	0.71	0.70	0.69
5.00e+09	0.67	0.66	0.65	0.63	0.62	0.61	0.60	0.59	0.58	0.57
6.00e+09	0.56	0.55	0.54	0.53	0.53	0.52	0.51	0.50	0.49	0.49
7.00e+09	0.48	0.47	0.47	0.46	0.45	0.45	0.44	0.44	0.43	0.43
8.00e+09	0.42	0.41	0.41	0.40	0.40	0.40	0.39	0.39	0.38	0.38
9.00e+09	0.37	0.37	0.37	0.36	0.36	0.35	0.35	0.35	0.34	0.34

To dilute dense semen: add 0.025 mL of semen into these volumes (mL) of dilution water

Density	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
1.00e+10	7.44	8.44	9.44	10.44	11.44	12.44	13.44	14.44	15.44	16.44
2.00e+10	14.88	15.88	16.88	17.88	18.88	19.88	20.88	21.88	22.88	23.88
3.00e+10	22.32	23.32	24.32	25.32	26.32	27.32	28.32	29.32	30.32	31.32
4.00e+10	29.76	30.76	31.76	32.76	33.76	34.76	35.76	36.76	37.76	38.76
5.00e+10	37.20	38.20	39.20	40.20	41.20	42.20	43.20	44.20	45.20	46.20
6.00e+10	44.64	45.64	46.64	47.64	48.64	49.64	50.64	51.64	52.64	53.64
7.00e+10	52.08	53.08	54.08	55.08	56.08	57.08	58.08	59.08	60.08	61.08
8.00e+10	59.52	60.52	61.52	62.52	63.52	64.52	65.52	66.52	67.52	68.52
9.00e+10	66.96	67.96	68.96	69.96	70.96	71.96	72.96	73.96	74.96	75.96

TABLE 5. DILUTION VOLUMES OF SPERM STOCK OF INDICATED DENSITY ($1.0X10^{8}$ TO $9.9X10^{10}$) TO ACHIEVE THE SPERM STOCK DENSITY ($3.36X10^{7}$) FOR A 3000:1 SPERM:EGG RATIO.

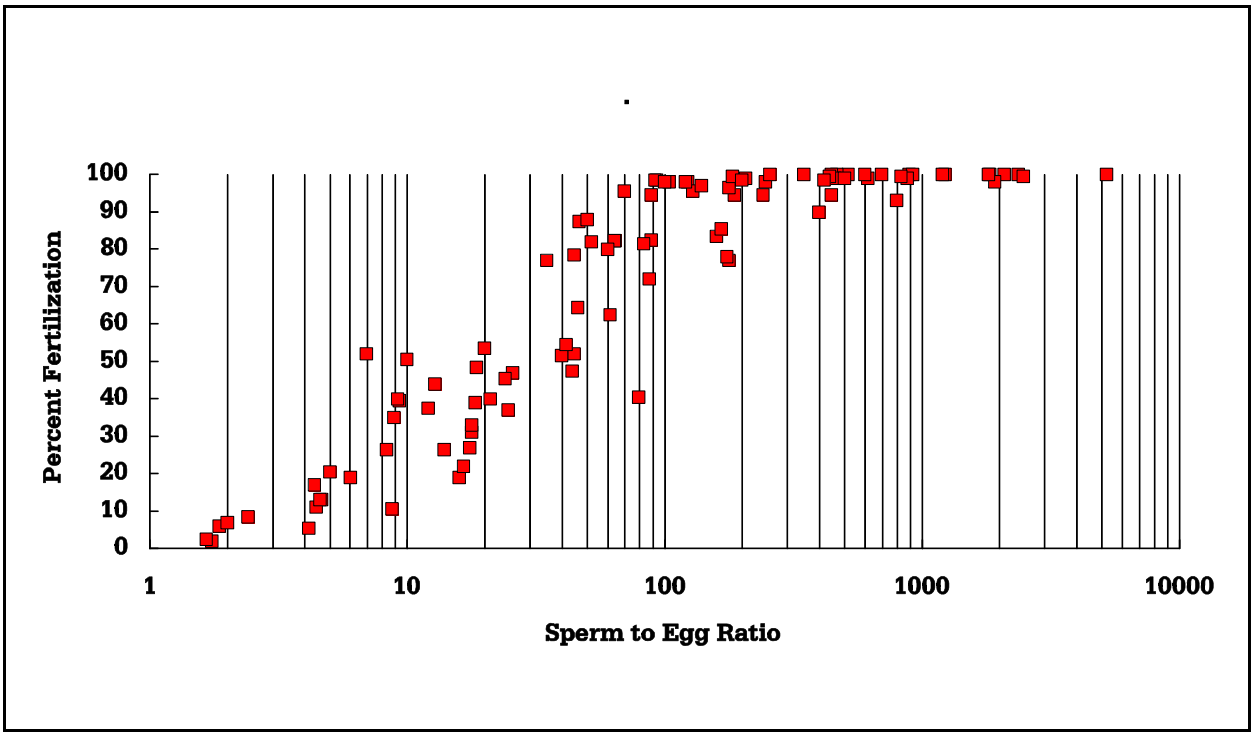


Figure 4. Relationship between sea urchin sperm:egg ration and percent control fertilization from 21 trials conducted by EPA (Feb-May 1991).

16.10.7 OBSERVATIONS DURING THE TEST

16.10.7.1 It is recommended that all observations be made on extra test solution remaining after the test tubes have been filled.

16.10.7.2 DO, pH, and salinity are measured at the beginning of the test. Due to the short duration of the test, no additional measurements of these parameters are required. Temperature is measured several times during the test as outlined in 16.10.7.

16.10.7.3 Record all measurements on the data sheet.

16.10.8 START OF THE DEFINITIVE TEST

16.10.8.1 Prior to Beginning the Test
produced good fertility, or if some produced good fertility.

TABLE 6. EXAMPLES OF RESULTS OF TRIAL FERTILIZATION TESTS WITH SPECIFIED SPERM DENSITIES AND TARGET SPERM DENSITY SELECTION (SPERM:EGG RATIO) FOR THE DEFINITIVE TEST.

sperm: egg	case 1	case 2	case 3	case 4	case 5	case 6
100:1	100*	95*	85	70	40	70
234:1	100	98	95*	80	64	85*
550:1	100	100	98	98*	82	89
1288:1	100	100	100	100	84	90
3000:1	100	100	100	100	88*	90

* recommended selection (interpolation to intermediate sperm:egg ratios may be used if found desirable)

1. If all trials exceed 90% fertilization, select 100:1 (case 1 and case 2).

2. If not all trials exceed 90% fertilization select the lowest sperm:egg ratio that does exceed 90% fertilization (case 3 and case 4).

3. If no trials exceed 90% fertilization, select the highest sperm:egg ratio (case 5) unless fertilization appears to become asymptotic below 100% (case 6).

4. If even the highest sperm:egg ratio fails to achieve 70% fertilization it is probable that an acceptable test cannot be conducted with these gametes.

   11,200 x target S:E ratio = target density; e.g., if target S:E = 500:1, target density = 11,200 x 500 = 5,600,000 sperm/mL. (11,200 = (1,120 eggs/tube)(0.1 mL of sperm stock/tube)).

   (stock sperm/mL)/(target sperm/mL) = dilution; e.g., if stock sperm has $4x10^9$ sperm/mL, then dilution = $4x10^9$ / $5.6x10^6$ = 714

16.10.8.1.1 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).

16.10.8.1.2 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 (12 ± 1EC) and maintained at that temperature during the addition of dilution water.

16.10.8.1.3 Increase the temperature of the water bath, room, or incubator to the required test temperature (12 ± 1EC).

16.10.8.1.4 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 and sand dollars have been examined at the end of the test.

16.10.8.1.5 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.

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

16.10.9.2 Sperm Exposure

16.10.9.2.1 Mix the iced sea urchin semen or sand dollar sperm suspension as described in 16.10.5.7 and 16.10.5.8 (do not kill the sperm). Combine the required volumes of sperm and dilution water and mix this sperm stock well by repeated inversion of the graduate cylinder or beaker. Begin test within 5 minutes. Table 5 (for 3000:1 sperm:egg ratio) can be used to aid in calculating appropriate volumes by reducing the sperm volume or increasing the dilution water volume by the factor:

f = 3000:1 / target sperm:egg ratio

16.10.9.2.2 The test tubes containing 5.0 mL of the various test solutions should have been equilibrated in a 12EC waterbath. Into each test tube, inject 0.100 mL of the sperm stock (except see 16.7.4 and 16.11.4) and note the time of first and last injection. It is important that the injection be performed with care that the entire volume goes directly into the test solution and not onto the side of the test tube. Similarly, the pipet tip should not touch the test solution or the side of the test tube, risking transfer of traces of test solution(s) into the sperm stock. Using repeated single 0.100 mL refill and injection, about 12 tubes per minute is a reasonable injection rate. More rapid rates of injection can be attained with repeating (single fill, multiple injection) pipets. Sperm injection rate (tubes/min) should not exceed that possible for egg injection.

16.10.9.2.3 Unless the test tubes are totally randomized, injection of sperm should be performed by replicate, i.e., the first set of replicates should receive sperm, then the second set, then the third set, etc. The sperm stock solution should be mixed frequently to maintain a homogeneous sperm stock.

16.10.9.2.4 Confirm the sperm density. Pipet 9 mL of sperm stock solution into a vial or test tube containing 1 mL of 10% acetic acid. Fill both sides of a hemacytometer with this dilution after mixing well. Let stand for 15 minutes. Count both sides of the hemacytometer using counting pattern no. 1 outlined in Appendix II and take the average count. For a sperm:egg ratio of 500:1 the stock sperm density will be 5,600,000 sperm/mL. (For counting pattern no. 1, this amounts to a total count of 102 sperm for the five large squares.) Calculate the sperm density in the sperm stock. If either: (1) the stock sperm density is greater than 33,600,000 sperm/mL (S:E

>3,000:1), or (2) the sperm density is more than 2x the target density, the test must be restarted with freshly diluted semen.

16.10.9.2.5 Check the temperature of the test solutions several times during the sperm exposure by including a temperature blank test tube containing 5 mL of dilution water with a thermometer.

16.10.9.3 Adding Eggs to the Test

16.10.9.3.1 Exactly 20 minutes after the sperm addition to the test was begun, begin to add the eggs, with every tube (including egg blanks - 11.7.4) receiving 0.5 mL of egg stock. Follow the same pattern of introduction for the eggs as used with the sperm so that each test tube has a sperm incubation period of 20 minutes. Note the time of start and finish of egg addition. This duration should be within one minute of that used for the sperm.

16.10.9.3.2 In order to maintain the same sperm:egg ratio in each test tube, the eggs must be maintained in a uniform distribution in the water column of the egg stock. Slow, gentle agitation of the egg stock in a beaker using a perforated plunger appears to be the best method of achieving a uniform distribution. Frequent inversion and mixing of egg stock in either a graduated cylinder or a multiple injection pipet may be acceptable.

16.10.9.3.3 The eggs should be injected using a pipet with an opening of at least 2 mm in order to avoid damaging the eggs and to provide sufficient flow to obtain a representative sample.

16.10.9.3.4 Two pair of egg blanks should be included in the test design, one at the beginning of the injection sequence (effluent blank) and one at the end of the injection sequence (egg blank). These tubes receive no sperm. The effluent blank contains the highest concentration of effluent and the egg blank contains dilution water. Examination of the effluent blank will indicate if the effluent induces a false fertilization membrane (a possible event, but probably rare) thus masking toxicity. Examination of the egg blank will indicate if accidentally fertilized eggs were used in the test (this is a minor factor unless a significant portion of the eggs were accidentally fertilized; it can indicate poor laboratory techniques). These

blanks are kept capped until the eggs are added in order to avoid contamination by sperm.

16.10.10 LIGHT, PHOTOPERIOD, SALINITY AND TEMPERATURE

16.10.10.1 The echinoderm fertilization test can be conducted in the dark or at ambient laboratory light levels. Due to its short duration, the fertilization test requires no photoperiod.

16.10.10.2 The water temperature in the test chambers should be maintained at 12 ± 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. A sensor placed in a temperature blank vial with standard volume of test solution can provide a direct measure of test solution temperature, one which may be more stable than the temperature in the air or water in the medium surrounding the test vials. Do not measure temperatures directly in a test vial, but prepare and handle the temperature blank(s) exactly as the normal control vials. Record the temperature several times between the beginning and the end of the test.

16.10.10.3 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.

16.10.10.4 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.

16.10.11 DISSOLVED OXYGEN (DO) CONCENTRATION

16.10.11.1 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.

16.10.12 OBSERVATIONS DURING THE TEST

16.10.12.1 Routine Chemical and Physical Observations

16.10.12.1.1 DO is measured at the beginning of the exposure period in one test chamber at each test concentration and in the control.

16.10.12.1.2 Temperature, pH, and salinity are measured at the beginning of the exposure period in one test chamber at each concentration and in the control. 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.

16.10.12.1.3 Record all the measurements on the data sheet.

16.10.13 TERMINATION OF THE TEST

16.10.13.1 Ending the Test

16.10.13.1.1 Record the time the test is terminated.

16.10.13.1.2 Because of the short test duration water quality measurements are not necessary at the end.

16.10.13.2 Sample Preservation

16.10.13.2.1 Exactly 20 minutes after the egg addition, the test should be stopped by the addition of a fixative to kill the sperm and eggs (both unfertilized and fertilized [zygotes]) and to preserve the eggs for examination. Again, the time allotted to fixative addition should be about the same as that for sperm and egg addition and the sequence of addition the same as for the introduction of the gametes.

16.10.13.2.2 The choice of formaldehyde or glutaraldehyde is up to the individual laboratory. There are at least two acceptable procedures: (1) the EPA *Arbacia* method of adding 10% formaldehyde in dilution water at the rate of 2 mL to each test tube; or (2) the addition of 1% glutaraldehyde (vol/vol) in clean seawater at the rate of 0.5 mL to each test tube. Glutaraldehyde should be made up fresh each day. Because concentrated glutaraldehyde is commonly only 25% strength, 1% glutaraldehyde is obtained by diluting the concentrate by 25x (e.g., 4 mL + 96 mL seawater).

16.10.13.2.3 It must be noted that formaldehyde has been identified as a carcinogen and that both glutaraldehyde and formaldehyde are irritating to skin and mucous membranes. Neither should be used at higher concentrations than needed to achieve morphological preservation of eggs for counting and only under conditions of maximal ventilation and minimal opportunity for volatilization into room air. Before using either compound in this method, the user should consult the latest material safety data available.

16.10.13.3 Counting

16.10.13.3.1 Immediately after termination of the test, the tubes are capped (or otherwise covered) and the contents mixed by inversion. They can be stored at room temperature until the eggs are examined for fertilization. Counts should be completed within 48 hours and, if counts extend over two days, should be made by replicate, i.e., count all replicate 1 tubes, then replicate 2, etc.

16.10.13.3.2 At least 100 eggs from each test tube are examined and scored for the presence or absence of an elevated fertilization membrane. Newly fertilized eggs will almost always have a completely elevated membrane around the egg (See Figures 5 and 6). Often a double membrane appears in sea urchin eggs, but following storage, even of only several hours, the inner (hyaline) membrane may disappear. Fertilized eggs may touch the outer membrane, or the membrane(s) may partially collapse. Because these phenomena only occur after preservation, eggs with any elevation of the fertilization membrane are counted as fertilized.

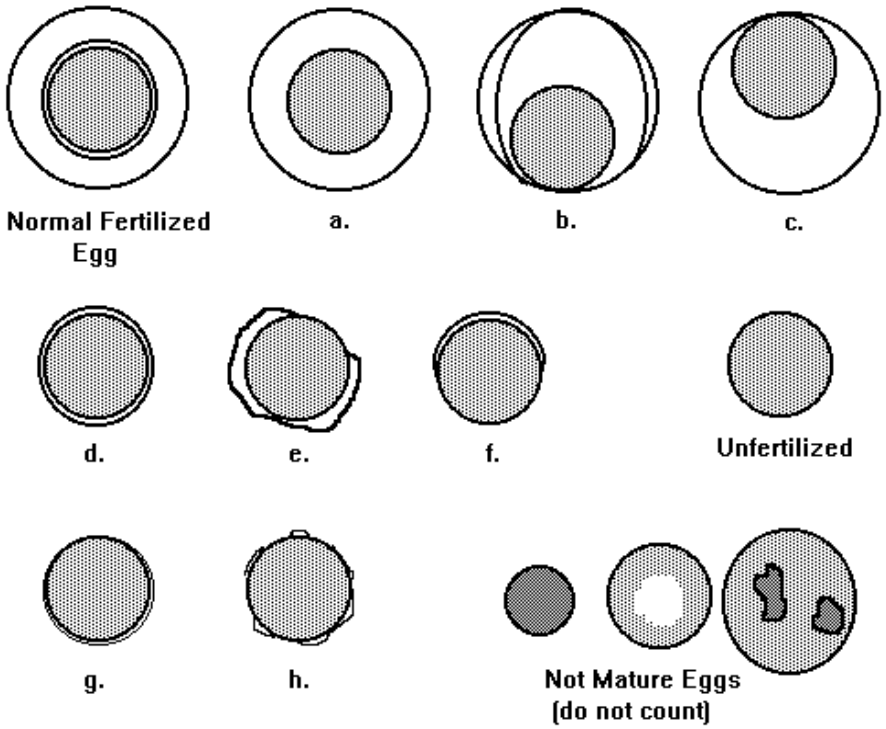


Figure 5. Examples of typical fertilized and unfertilized sea urchin eggs and a number of examples of atypical "fertilized" eggs (a through h). Normal fertilized eggs have an outer fertilization membrane and an inner hyaline membrane. After preservation, the hyaline membrane sometimes disappears (a); in other cases the egg is displaced from the center and contacts the perimeter either inside an enlarged hyaline envelope (b) or with no visible hyaline membrane (c). In some instances there appears to be only a slight elevation of the outer membrane or only the hyaline membrane appears, fully (d), partially (f), or only as a halo (g). In some batches of eggs the membrane(s) appear to be fragile and some collapse (e). In rare cases sperm appear to activate membrane elevation over only segments of the egg leading to a blistered appearance (h). When eggs appearing as those in examples f, g, and h are common in a test, the results should be examined closely to see if their occurrence appears to be dose-related (indicating an effect on fertilization), not dose-related (indicating a problem with egg quality or preservative), or is common in the effluent egg control (indicating an effluent- produced false fertilization). Eggs that are not mature are capable of being fertilized, but should never be counted. These include obviously smaller (often denser) eggs, normal sized eggs with a distinct, clear center, and very large eggs with often irregular color and density.

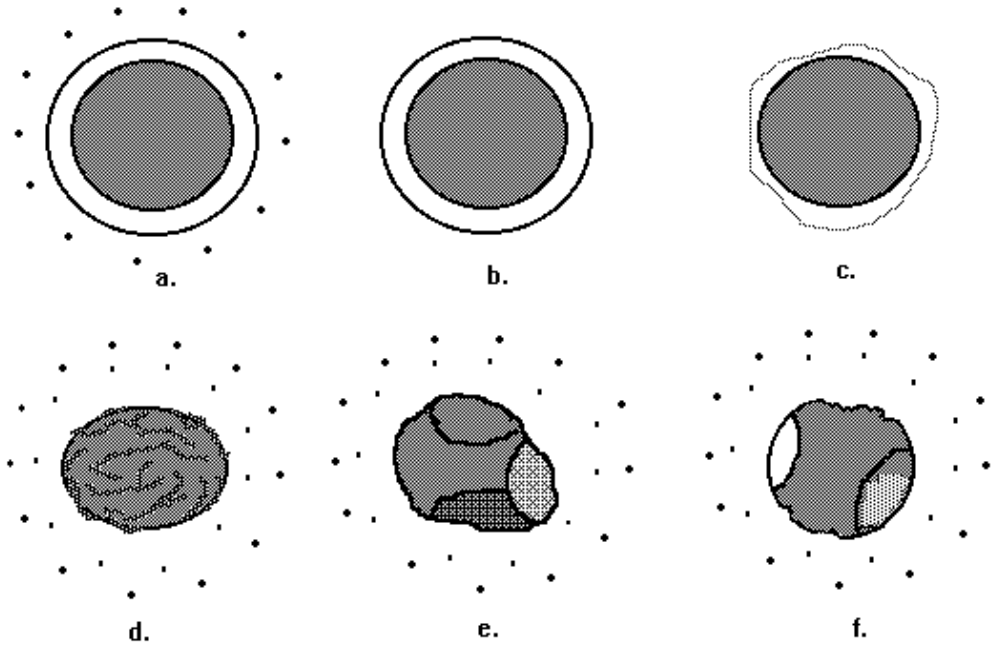


Figure 6. Examples of typical fertilized and unfertilized sand dollar eggs. Nearly all newly released eggs are characterized by a surrounding sphere of small purple chromatophores embedded within the transparent gelatinous coat surrounding the egg. The coat and the chromatophores may be lost or retained in the test and subsequent handling. Typical fertilized eggs are represented by (a) and (b). Some fertilized eggs (c) show only a wispy remnant of the fertilization membrane. Eggs when spawned usually appear as in (d) and (e) or somewhere in between. The more rounded "raisin" appearing egg in (d) is usually superior to the "asteroid" appearing egg in (e) although the latter can provide acceptable test results. However, the more irregularly shaped or vacuolated the eggs appear, the poorer the control fertilization is likely to be. The egg shown in (f), the "pitted olive," never shows a fertilization membrane and should not be counted.

16.10.13.3.3 It is convenient to concentrate the eggs prior to counting. If the eggs are allowed to completely settle (ca 30 minutes after termination and mixing), most of the overlying solution can be removed with a pipet, leaving the eggs concentrated in a much smaller volume. The eggs are then resuspended by filling and emptying a 1 mL pipet about 5 times from the remaining volume and finally transferring 1 mL of the egg suspension into a 1 mL Sedgewick-Rafter counting chamber (other volume counting chambers can be used).

16.10.13.3.4 Failure to completely resuspend the eggs can result in biasing the counts towards higher percent fertilization due to a tendency seen in rare batches of eggs in which unfertile eggs tend to be adhesive. This phenomenon may be further influenced by the choice of preservative, the strength of the preservative, and the period between preservation and counting. However, other sampling procedures may be used once demonstrated not to bias sampling and if no clumping of adhesive eggs is observed in a given test; for example, concentrated eggs may be picked up from the test tube and deposited in a small drop on a microscope slide, or eggs can be scored by examination with the test tubes laying on their sides and viewed at low power or with an inverted microscope.

16.10.13.4 Endpoint

16.10.13.4.1 In a count of at least 100 eggs, record the number of eggs with fertilization membranes and the number of eggs without fertilization membranes.

16.11 **SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA**

16.11.1 A summary of test conditions and test acceptability criteria is listed in Table 7.

TABLE 7. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR, *STRONGYLOCENTROTUS PURPURATUS* AND *DENDRASTER EXCENTRICUS*, FERTILIZATION TEST WITH EFFLUENTS AND RECEIVING WATERS

1. Test type:	Static non-renewal
2. Salinity:	34 ± 2‰
3. Temperature:	12 ± 1EC
4. Light quality:	Ambient laboratory light during test preparation

5.	Light intensity:	l0-20 uE/m^2/s (Ambient laboratory levels)
6.	Test chamber size:	16 x 100 or 16 x 125 mm
7.	Test solution volume:	5 mL
8.	Number of spawners:	Pooled sperm from up to four males and pooled eggs from up to four females are used per test
9.	No. egg and sperm cells per chamber:	About 1,120 eggs and not more than 3,360,000 sperm per test tube
10.	No. replicate chambers per concentration:	4
11.	Dilution water:	Uncontaminated 1-µm-filtered natural seawater or hypersaline brine prepared from natural seawater or artificial sea salts
12.	Test concentrations:	Effluents: Minimum of 5 and a control Receiving waters: 100% a control
13.	Dilution factor:	Effluents: $0.5 Receiving waters: None or $0.5
14.	Test duration:	40 min (20 min plus 20 min)
15.	Endpoint:	Fertilization of eggs
16.	Test acceptability criteria:	$70% egg fertilization in controls; %MSD of <25%; and appropriate sperm counts
17.	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, and Sample Preparation for Toxicity Tests)
18.	Sample volume required:	1 L

16.12 **ACCEPTABILITY OF TEST RESULTS**

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

(1) Egg fertilization at the NOEC must be greater than 80% of that in the controls.

(2) The minimum significant difference (%MSD) is <25% relative to the control.

(3) The sperm count for the final sperm stock must not exceed 33,600,000/mL.

(4) If the sperm count for the final sperm stock is between 5,600,000 and 33,600,000/mL it must not exceed 2x of the target density from the trial, or if no target density was specified for the test (see 11.5.1), the high sperm density controls (0.2 mL sperm stock) must have at least 5% higher fertilization than the low sperm density controls (0.05 mL sperm stock).

(5) Dilution water egg blanks and effluent egg blanks should contain essentially no eggs with fertilization membranes.

16.13 **DATA ANALYSIS**

16.13.1 GENERAL

16.13.1.1 Tabulate and summarize the data. Calculate the proportion of fertilized eggs for each replicate. A sample set of test data is listed in Table 8.

16.13.1.2 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.

16.13.1.3 The endpoints of toxicity tests using the sea urchin and the sand dollar are based on the reduction in proportion of eggs fertilized. The IC25 is calculated using the Linear Interpolation Method (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). LOEC and NOEC values for fecundity 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.

TABLE 8. DATA FROM SEA URCHIN, *STRONGYLOCENTROTUS PURPURATUS*, FERTILIZATION TEST

Effluent Concentration (%)	Replicate	No. of Eggs Counted	No. of Eggs Fertilized	Proportion Fertilized
Control	A	100	97	0.97
	B	100	90	0.90
	C	100	100	1.00
0.05	A	100	100	1.00
	B	100	100	1.00
	C	100	98	0.98
0.10	A	100	100	1.00
	B	100	97	0.97
	C	100	99	0.99
0.15	A	100	98	0.98
	B	100	96	0.96
	C	100	97	0.97
0.20	A	100	94	0.94
	B	100	88	0.88
	C	100	97	0.97
0.40	A	100	43	0.43
	B	100	63	0.63
	C	100	46	0.46
0.60	A	100	2	0.02
	B	100	1	0.01
	C	100	9	0.09
0.80	A	100	0	0.00
	B	100	0	0.00
	C	100	0	0.00

16.13.2 EXAMPLE OF ANALYSIS OF SEA URCHIN, *STRONGYLOCENTROTUS PURPURATUS*, AND SAND DOLLAR, *DENDRASTER EXCENTRICUS*, FERTILIZATION DATA

16.13.2.1 Formal statistical analysis of the fertilization data is outlined in Figure 7.

The response used in the analysis is the proportion of fertilized eggs 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 eggs fertilized in any of the test chambers are excluded from statistical analysis of the NOEC and LOEC, but included in the estimation of the IC25.

16.13.2.2 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.

16.13.2.3 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.

16.13.2.4 Example of Analysis of Fecundity Data

16.13.2.4.1 This example uses toxicity data from a sea urchin, *Strongylocentrotus purpuratus*, fertilization test performed with effluent. The response of interest is the proportion of fertilized eggs, 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 effluent concentration and control are listed in Table 9. The data are plotted in Figure 8. Because there is zero fertilization in all three replicates for the 0.80% effluent concentration, it was not included in the statistical analysis and is considered a qualitative fecundity effect.

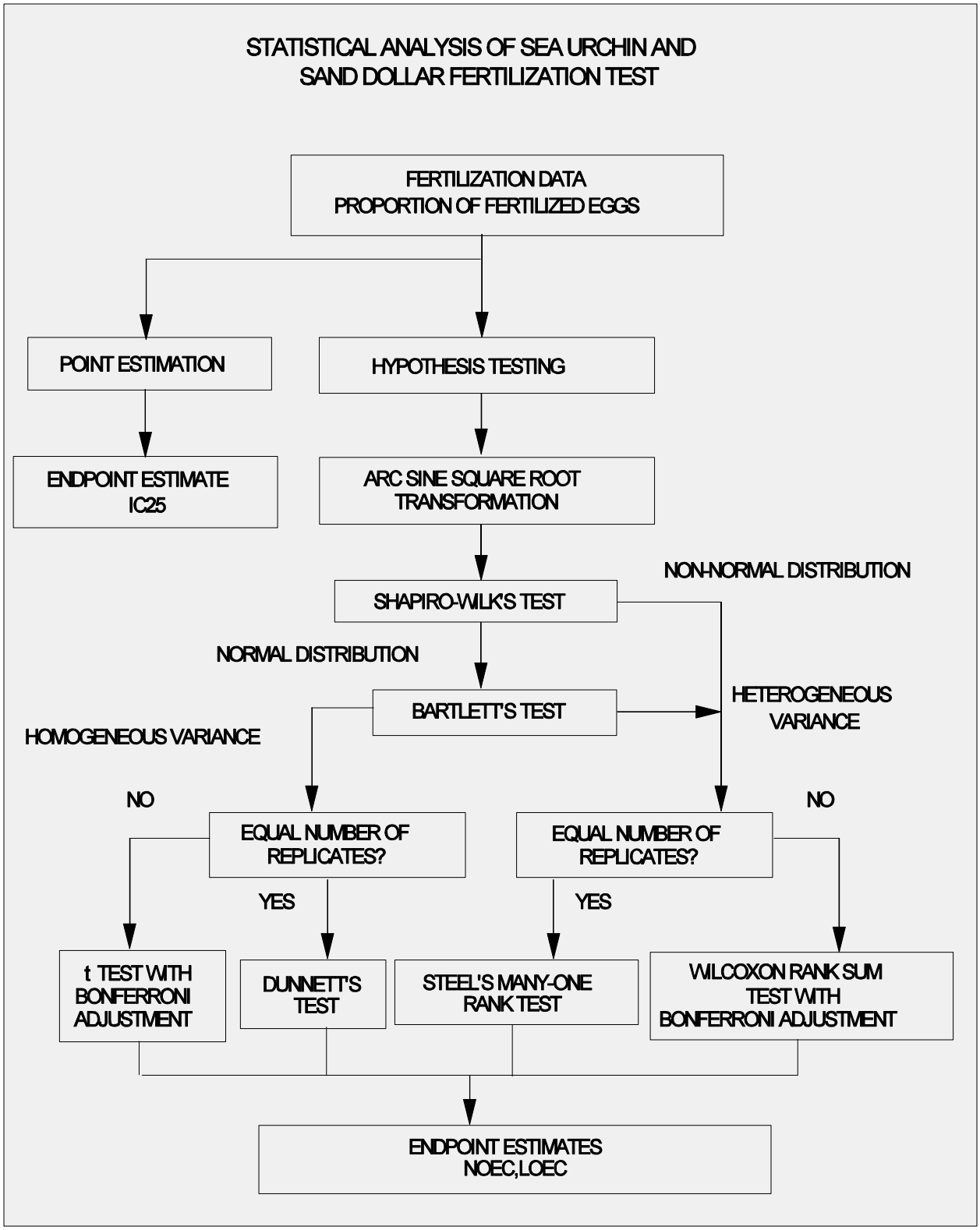


Figure 7. Flowchart for statistical analysis of sea urchin, *Strongylocentrotus purpuratus*, and sand dollar, *Dendraster excentricus*, test.

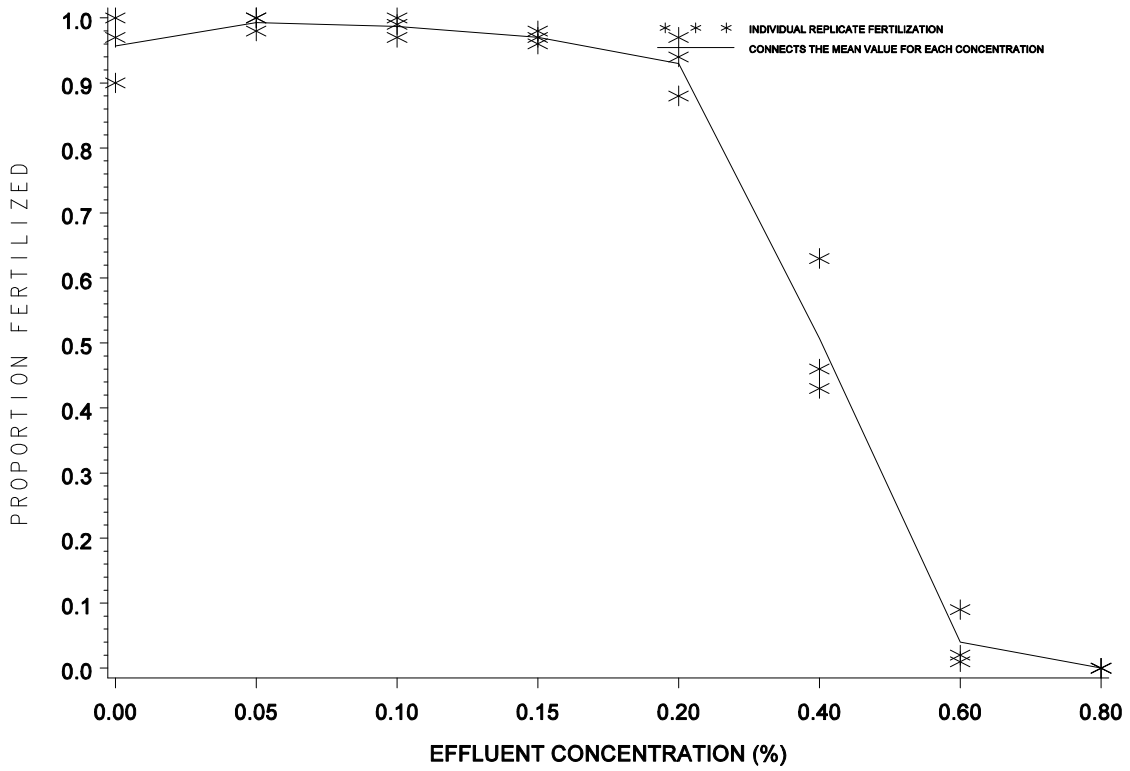


Figure 8. Plot of proportion of fertilized sea urchin, *Strongylocentrotus purpuratus*, eggs

16.13.2.5 Test for Normality

16.13.2.5.1 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 10.

TABLE 9. SEA URCHIN, *STRONGYLOCENTROTUS PURPURATUS*, FERTILIZATION DATA

		Effluent Concentration (%)						
	Rep.	Control	0.05	0.10	0.15	0.20	0.40	0.60
RAW	A	0.97	1.00	1.00	0.98	0.94	0.43	0.02
	B	0.90	1.00	0.97	0.96	0.88	0.63	0.01
	C	1.00	0.98	0.99	0.97	0.97	0.46	0.09
ARC SINE SQUARE ROOT TRANSFORMED	A	1.397	1.521	1.521	1.429	1.323	0.715	0.142
	B	1.249	1.521	1.397	1.369	1.217	0.917	0.100
	C	1.521	1.429	1.471	1.397	1.397	0.745	0.305
Mean ($\bar{x}_i$)		1.389	1.490	1.463	1.398	1.312	0.792	0.182
S_i^2		0.01854	0.00282	0.00389	0.00090	0.00819	0.01188	0.01173
i		1	2	3	4	5	6	7

TABLE 10. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

	Effluent Concentration (%)						
Replicate	Control	0.05	0.10	0.15	0.20	0.40	0.60
A	0.008	0.031	0.058	0.031	0.011	-0.077	-0.040
B	-0.140	0.031	-0.066	-0.029	-0.095	0.125	-0.082
C	0.132	-0.061	0.008	-0.001	0.085	-0.047	0.123

16.13.2.5.2 Calculate the denominator, D, of the statistic:

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

Where: X_i = the ith centered observation

$\bar{X}$ = the overall mean of the centered observations

n = the total number of centered observations

16.13.2.5.3 For this set of data, n = 21

$$\bar{X} = \frac{1}{21}(0.005) = 0.000$$

D = 0.1159

16.13.2.5.4 Order the centered observations from smallest to largest

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

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 11.

16.13.2.5.5 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 = 21 and k = 10. The a_i values are listed in Table 12.

16.13.2.5.6 Compute the test statistic, W, as follows:

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

The differences, $X^{(n-i+1)} - X^{(i)}$, are listed in Table 12. For the data in this example:

$$W = \frac{1}{0.1159}(0.3345)^2 = 0.9654$$

16.13.2.5.7 The decision rule for this test is to compare W as calculated in 2.6 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

TABLE 11. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.140	12	0.008
2	-0.095	13	0.011
3	-0.082	14	0.031
4	-0.077	15	0.031
5	-0.066	16	0.031
6	-0.061	17	0.058
7	-0.047	18	0.085
8	-0.040	19	0.123
9	-0.029	20	0.125
10	-0.001	21	0.132
11	0.008		

TABLE 12. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4643	0.272	$X^{(21)} - X^{(1)}$
2	0.3185	0.220	$X^{(20)} - X^{(2)}$
3	0.2578	0.205	$X^{(19)} - X^{(3)}$
4	0.2119	0.162	$X^{(18)} - X^{(4)}$
5	0.1736	0.124	$X^{(17)} - X^{(5)}$
6	0.1399	0.092	$X^{(16)} - X^{(6)}$
7	0.1092	0.078	$X^{(15)} - X^{(7)}$
8	0.0804	0.071	$X^{(14)} - X^{(8)}$
9	0.0530	0.040	$X^{(13)} - X^{(9)}$
10	0.0263	0.009	$X^{(12)} - X^{(10)}$

example, the critical value at a significance level of 0.01 and n = 21 observations is 0.873. Since W = 0.9654 is greater than the critical value, conclude that the data are normally distributed.

16.13.2.6 Test for Homogeneity of Variance

16.13.2.6.1 The test used to examine whether the variation in the proportion of fertilized eggs is the same across all effluent

concentrations including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

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

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

$$\overline{S}^2 = \frac{(\sum_{i=1}^{P} V_i S_i^2)}{\sum_{i=1}^{P} V_i} \qquad C = 1 + [3(p-1)]^{-1}[\sum_{i=1}^{P} 1/V_i - (\sum_{i=1}^{P} V_i)^{-1}]$$

16.13.2.6.2 For the data in this example (see Table 8), all effluent concentrations including the control have the same number of replicates (n_i = 3 for all i). Thus, V_i = 2 for all i.

16.13.2.6.3 Bartlett's statistic is, therefore:

$$B = [(14)\ln(0.008279) - 2\sum_{i=1}^{P} \ln(S_i^2)] / 1.1905$$

= [14(-4.7940) - 2(-36.1047)]/1.1905

= 5.0934/1.1905

= 4.2784

16.13.2.6.4 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 6 degrees of freedom, is 16.81. Since B = 4.2784 is less than the critical value of 16.81, conclude that the variances are not different.

16.13.2.7 Dunnett's Procedure

16.13.2.7.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 13.

TABLE 13. 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	

Where: p = number of concentration levels including the control

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 fertilized eggs for concentration i in test chamber j)

16.13.2.7.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = n_6 = n_7 = 3$

$N = 21$

$T_1 = Y_{11} + Y_{12} + Y_{13} = 4.167$
$T_2 = Y_{21} + Y_{22} + Y_{23} = 4.471$
$T_3 = Y_{31} + Y_{32} + Y_{33} = 4.389$
$T_4 = Y_{41} + Y_{42} + Y_{43} = 4.194$
$T_5 = Y_{51} + Y_{52} + Y_{53} = 3.937$
$T_6 = Y_{61} + Y_{62} + Y_{63} = 2.377$
$T_7 = Y_{71} + Y_{72} + Y_{73} = 0.547$

$G = T_1 + T_2 + T_3 + T_4 + T_5 + T_6 + T_7 = 24.082$

$$SSB = \sum_{i=1}^{P} T_i^2/n_i - G^2/N$$
$$= (95.656)/3 - (24.082)^2/21 = 4.269$$

$$SST = \sum_{ij} \sum^{n_i} Y_{ij}^2 - G^2/N$$
$$= 32.001 - (24.082)^2/21 = 4.385$$

$SSW = SST - SSB = 4.385 - 4.269 = 0.116$

$S_B^2 = SSB/(p-1) = 4.269/(7-1) = 0.7115$

$S_W^2 = SSW/(N-p) = 0.116/(21-7) = 0.0083$

16.13.2.7.3 Summarize these calculations in the ANOVA table (Table 14).

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

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

TABLE 14. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	6	4.269	0.7115
Within	14	0.116	0.0083
Total	20	4.385	

Where: $\bar{x}_i$ = mean proportion fertilized eggs for concentration i

$\bar{x}_1$ = mean proportion fertilized eggs 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 fertilized eggs, the concentration mean is subtracted from the control mean.

16.13.2.7.5 Table 15 includes the calculated *t* values for each concentration and control combination. In this example, comparing the 0.05% concentration with the control the calculation is as follows:

$$t_2 = \frac{(1.389 - 1.490)}{0.0911\sqrt{(1/3)+(1/3)}} = -1.358$$

16.13.2.7.6 Since the purpose of this test is to detect a significant decrease in the proportion of fertilized eggs, 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, 14 degrees of freedom for error and six concentrations (excluding the control) the critical value is 2.53. The mean proportion of fertilized eggs for concentration i is considered significantly less than the mean proportion of

fertilized eggs for the control if t_i is greater than the critical value. Therefore, the 0.40% and 0.60% concentrations have a significantly lower mean proportion of fertilized eggs than the control. Hence the NOEC is 0.20% effluent and the LOEC is 0.40% effluent.

TABLE 15. CALCULATED t VALUES

Effluent Concentration (%)	i	t_i
0.05	2	-1.358
0.10	3	-0.995
0.15	4	-0.121
0.20	5	1.035
0.40	6	8.026
0.60	7	16.227

16.13.2.7.7 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.

16.13.2.7.8 In this example,

$$MSD = 2.53\,(0.0911)\sqrt{(1/3) + (1/3)}$$

$$= 2.53\ (0.0911)(0.8165)$$

$$= 0.188$$

16.13.2.7.9 The MSD (0.188) is in transformed units. To determine the MSD in terms of proportion of fertilized eggs, carry out the following conversion.

1. Subtract the MSD from the transformed control mean.

$$1.389 - 0.188 = 1.201$$

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

$$[\ \text{Sine}\ (1.389)\ ]^2 = 0.967$$

$$[\ \text{Sine}\ (1.201)\ ]^2 = 0.869$$

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

$$MSD_u = 0.967 - 0.869 = 0.098$$

16.13.2.7.10 Therefore, for this set of data, the minimum difference in mean proportion of fertilized eggs between the control and any effluent concentration that can be detected as statistically significant is 0.098.

16.13.2.7.11 This represents a 10.2% decrease in the proportion of fertilized eggs from the control.

16.13.2.8 Calculation of the ICp

16.13.2.8.1 The fertilization data in Table 7 are utilized in this example. As can be seen from Figure 8, the observed means are not monotonically non-increasing with respect to concentration. Therefore, the means must be smoothed prior to calculating the IC.

16.13.3.8.2 Starting with the observed control mean, $\overline{Y}_1 = 0.957$, and the observed mean for the lowest effluent concentration, $\overline{Y}_2 = 0.993$, we see that $\overline{Y}_1$ is less than $\overline{Y}_2$.

16.13.3.8.3 Calculate the smoothed means:

$$M_1 = M_2 = (\overline{Y}_1 + \overline{Y}_2)/2 = 0.975$$

16.13.3.8.4 Since $\overline{Y}_3 = 0.987$ is larger than M_2, average $\overline{Y}_3$ with the previous concentrations:

$$M_1 = M_2 = M_3 = (M_1 + M_2 + \overline{Y}_3)/3 = 0.979.$$

16.13.3.8.5 Since $M_3 > \overline{Y}_4 = 0.970 > \overline{Y}_5 = 0.930 > \overline{Y}_6 = 0.507 > \overline{Y}_7 = 0.040 > \overline{Y}_8 = 0.0$, set $M_4 = 0.970$, $M_5 = 0.930$, $M_6 = 0.507$, $M_7 = 0.040$, and $M_8 = 0.0$. Table 16 contains the smoothed means and Figure 10 gives a plot of the smoothed means and the interpolated response curve.

16.13.2.8.6 An IC25 can be estimated using the Linear Interpolation Method. A 25% reduction in mean proportion of fertilized eggs, compared to the controls, would result in a mean

proportion of 0.734, where $M_1(1-p/100) = 0.979(1-25/100)$. Examining the means and their associated concentrations (Table 16), the response, 0.734, is bracketed by C_5 = 0.20% effluent and C_6 = 0.40% effluent.

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

$$ICp \text{ ' } C_j\%[M_1(1\&p/100)\&M_j]\frac{(C_{(j\%1)}\&C_j)}{(M_{(j\%1)}\&M_j)}$$

$$IC25 = 0.20 + [0.979(1 - 25/100) - 0.930] \frac{(0.40 - 0.20)}{(0.507 - 0.930)}$$

$$= 0.29\%.$$

TABLE 16. SEA URCHIN, *STRONYLOCENTROTUS PURPURATUS*, MEAN PROPORTION OF FERTILIZED EGGS

Effluent Conc. (%)	i	Response Means, $\bar{Y}_i$ (proportion)	Smoothed Means, M_i (proportion)
Control	1	0.957	0.979
0.05	2	0.993	0.979
0.10	3	0.987	0.979
0.15	4	0.970	0.970
0.20	5	0.930	0.930
0.40	6	0.507	0.507
0.60	7	0.040	0.040
0.80	8	0.000	0.000

16.13.2.8.8 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 0.2925%. The empirical 95.0% confidence interval for the true mean was 0.2739% to 0.3241%. The computer program output for the IC25 for this data set is shown in Figure 10.

16.14 **PRECISION AND ACCURACY**

16.14.1 PRECISION

16.14.1.1 Single-Laboratory Precision

16.14.1.1.1 Single-laboratory precision data for *Strongylocentrotus purpurtatus* with the reference toxicant copper, tested in natural seawater, are provided in Table 17. The coefficient of variation based on the EC25 is 29%, and on EC50 is 24%, showing acceptable precision. Single-laboratory precision data for *Dendraster excentricus* with the reference toxicant copper, tested in natural seawater, are provided in Tables 18 and 19. The coefficient of variation based on the EC25, is 18% to 29% and EC50, is 21% to 33%, showing acceptable precision.

16.14.1.2 Multi-laboratory Precision

16.14.1.2.1 Multi-laboratory precision data for *Strongylocentrotus purpuratus*, with the reference toxicant copper, tested in natural seawater, are provided in Table 20. The coefficient of variation for the EC25 was 52%, based on data from five laboratories.

16.14.2 ACCURACY

16.14.2.1 The accuracy of toxicity tests cannot be determined.

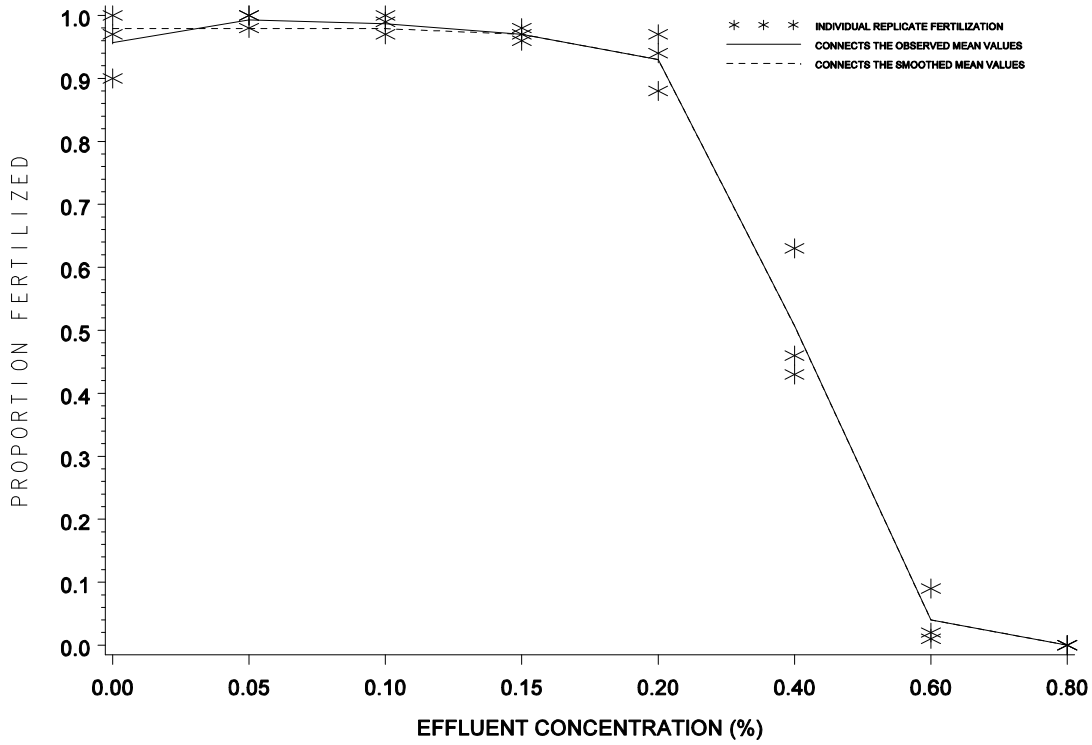


Figure 9. Plot of raw data, observed means, and smoothed means for the sea urchin, *Strongylocentrotus purpuratus*, eggs.

```
Conc. ID        1    2    3    4    5    6    7    8
-------------------------------------------------------
Conc. Tested    0  .05  .10  .15  .20  .40  .60  .80
-------------------------------------------------------
Response  1   .97 1.00 1.00  .98  .94  .43  .02    0
Response  2   .90 1.00  .97  .96  .88  .63  .01    0
Response  3  1.00  .98  .99  .97  .97  .46  .09    0
-------------------------------------------------------
*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Effluent
Test Start Date:    Test Ending Date:
Test Species: Sea Urchin, Strongylocentrotus purpuratus
Test Duration:      40 minutes
DATA FILE: urchin.icp
OUTPUT FILE: urchin.i25
-------------------------------------------------------------------------
Conc.     Number     Concentration     Response      Std.       Pooled
 ID     Replicates                %       Means       Dev.   Response Means
-------------------------------------------------------------------------
  1          3             0.000          0.957      0.051      0.979
  2          3             0.050          0.993      0.012      0.979
  3          3             0.100          0.987      0.015      0.979
  4          3             0.150          0.970      0.010      0.970
  5          3             0.200          0.930      0.046      0.930
  6          3             0.400          0.507      0.108      0.507
  7          3             0.600          0.040      0.044      0.040
  8          3             0.800          0.000      0.000      0.000
-------------------------------------------------------------------------
The Linear Interpolation Estimate:     0.2925   Entered P Value: 25
-------------------------------------------------------------------------
Number of Resamplings:   80
The Bootstrap Estimates Mean:   0.2917 Standard Deviation:     0.0141
Original Confidence Limits:   Lower:     0.2739 Upper:     0.3241
Expanded Confidence Limits:   Lower:     0.2533 Upper:     0.3589
Resampling time in Seconds:     0.22  Random_Seed: -25579058
```

Figure 10. ICPIN program output for the IC25.

TABLE 17. SINGLE LABORATORY PRECISION OF THE SEA URCHIN, *STRONGYLOCENTROTUS PURPURATUS* FERTILIZATION TEST PERFORMED IN SEAWATER USING GAMETES FROM ADULTS MAINTAINED IN SEAWATER AFTER BEING COLLECTED FROM NATURAL POPULATIONS WITH COPPER (CU µG/L) SULFATE AS THE REFERENCE TOXICANT

Test Number	NOEC (µg/L)	EC25 (µg/L)	EC50 (µg/L)
1	6.9	9.7	14.3
2	23.0	26.2	30.9
3	11.2	19.6	25.8
4	16.0	16.4	31.1
5	15.3	17.8	24.6
6	10.8	18.6	28.3
Mean CV(%)		18.1 29.0	25.8 24.0

Tests performed by Sally Noack, AScI, at EPA's Pacific Ecosystems Branch of ERL-Narragansett, Newport, OR.
Copper concentrations were measured and within 10% of nominal; nominal concentrations were 5, 8, 12, 17, 25, 35, and 50 µg/L.
These tests used only three replicates per concentration.

TABLE 18. SINGLE LABORATORY PRECISION OF THE SAND DOLLAR, *DENDRASTER EXCENTRICUS* FERTILIZATION TEST PERFORMED IN SEAWATER USING GAMETES FROM ADULTS MAINTAINED IN SEAWATER AFTER BEING COLLECTED FROM NATURAL POPULATIONS WITH COPPER (CU µG/L) SULFATE AS THE REFERENCE TOXICANT

Test Date	Test Number	NOEC (µg/L)	EC25 (µg/L)	EC50 (µg/L)
7/11/94	1* 2** 3***	5.0 5.0 -	9.4 14.6 16.0	12.6 17.5 18.6
7/14/94	1* 2** 3***	12.0 <5.0 17.0	16.7 19.6 23.0	20.9 25.8 30.5
7/17/94	1* 2** 3***	8.0 5.0 12.0	15.3 13.5 13.4	17.7 16.4 17.0
7/19/94	1* 2** 3***	12.0 17.0 12.0	12.8 18.6 13.3	15.6 22.1 16.0
Mean	1 2 3 overall		13.5 16.6 16.4	16.7 20.5 20.5
SD	1 2 3 overall		3.2 3.0 4.6	3.5 4.3 6.7
CV(%)	1 2 3 overall		24% 18% 28%	21% 21% 33%

Tests performed at National Council of the Paper Industry for Air and Stream Improvement, Inc. Anacortes, WA.
Copper concentrations were nominal; nominal concentrations were 5, 8, 12, 17, 25, 35, and 50 µg/L.

* Tests conducted with nominal S:E ratio of 147:1
** Tests conducted with nominal S:E ratio of 166:1
*** Tests conducted with nominal S:E ratio of 224:1

TABLE 19. SINGLE LABORATORY PRECISION OF THE SAND DOLLAR, *DENDRASTER EXCENTRICUS* FERTILIZATION TEST PERFORMED IN SEAWATER USING GAMETES FROM ADULTS MAINTAINED IN SEAWATER AFTER BEING COLLECTED FROM NATURAL POPULATIONS WITH COPPER (CU µG/L) SULFATE AS THE REFERENCE TOXICANT.

Test Number	NOEC (µg/L)	EC25 (µg/L)	EC50 (µg/L)
1	17.0	25.8	31.0
2	25.0	34.3	41.8
3	12.0	31.1	43.7
4	8.0	14.2	19.8
5	25.0	27.2	30.5
Mean CV(%)		26.5 29.0	33.4 29.0

Tests performed by Gary Chapman and Debra Denton at EPA's Pacific Ecosystems Branch of ERL-Narragansett, Newport, OR.

Copper concentrations were nominal; nominal concentrations were 5, 8, 12, 17, 25, 35, and 50 µg/L.

TABLE 20. MULTIPLE LABORATORY PRECISION OF THE SEA URCHIN, *STONGYLOCENTROTUS PURPURATUS*, FERTILIZATION TEST PERFORMED WITH COPPER (CU µG/L) SULFATE AS A REFERENCE TOXICANT

Lab	# of Tests	Statistic	EC25 (µg/L)
A	3	Mean SD CV(%)	7.8 3.0 38%
B	2	Mean SD CV(%)	4.0 - -
C	6	Mean SD CV(%)	18.0 5.4 30%
D	2	Mean NA CV(%)	14.9 - -
E	6	Mean SD CV(%)	19.3 10.5 54%

# of Lab Means	Statistic	EC25
5	Mean SD CV(%)	12.8 6.6 52%

Tests performed as part of a methods evaluation effort organized by the US EPA laboratory in Newport, Oregon; tests were conducted in 1991 by volunteer laboratories in California and Washington.

APPENDIX I. PURPLE URCHIN AND SAND DOLLAR TEST: 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 (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.

D. Prepare a control (0 Fg/L) plus 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).

E. 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.

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

G. Place test chambers in a water bath or environmental chamber set to 12EC and allow temperature to equilibrate.

H. Measure the temperature in several temperature blanks during the course of the test.

PREPARATION AND ANALYSIS OF TEST ORGANISMS

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

B. On day of test, spawn organisms, examine gametes, pool good eggs, pool good sperm.

C. Determine egg and sperm densities and adjust as necessary.

D. Run trial sperm:egg fertilization test (optional).

E. Adjust sperm density for definitive test.

F. Inject sperm into test solutions.

G. 20 minutes later inject eggs into test solutions.

H. 20 minutes after egg addition, stop the test by the addition of preservative.

I. Confirm sperm density in definitive test by hemacytometer counts.

J. Count at least 100 eggs in each test tube.

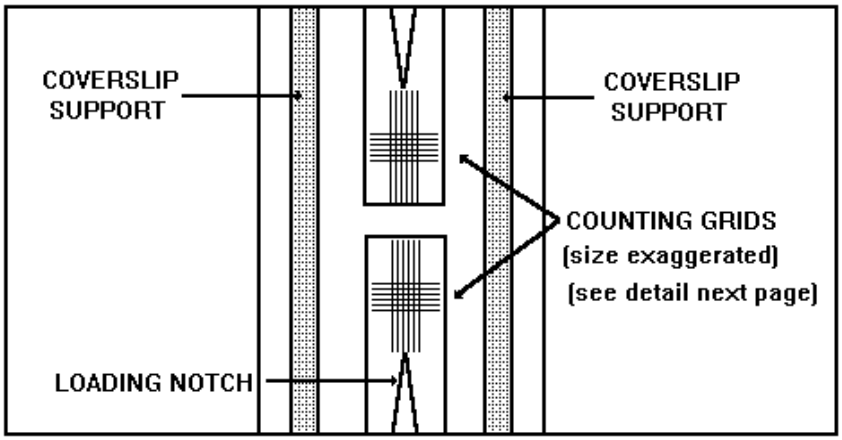
K. Analyze the data.

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

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

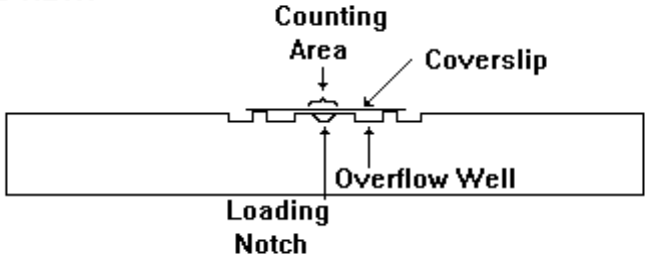
The Neubauer hemacytometer is a specialized microscope slide with two counting grids and a coverslip.

TOP VIEW:

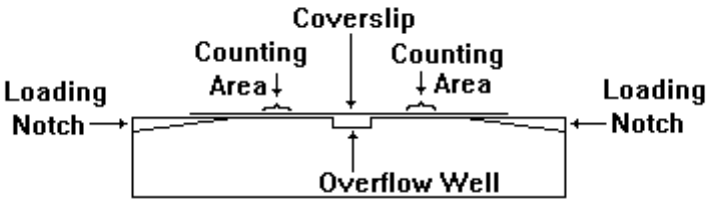


Together, the total area of each grid (1 mm^2) and the vertical distance between the grid and the coverslip (0.1 mm), provide space for a specific microvolume of aqueous sample (0.1 mm^3).

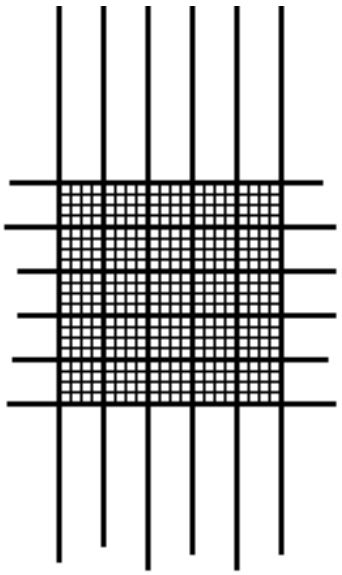
SIDE VIEW:



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:

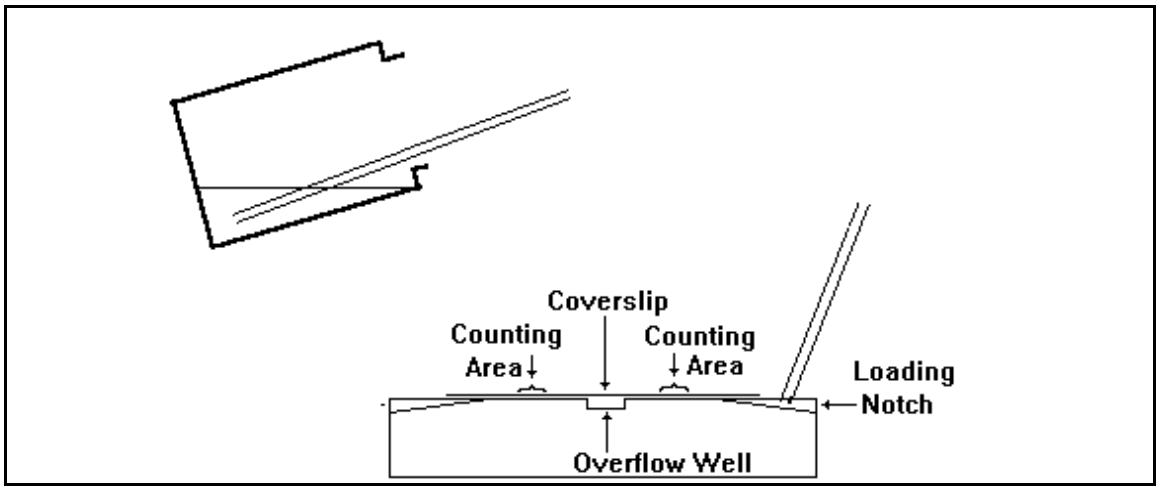
$$\text{cells/mL} = \frac{(4{,}000)\ (4{,}000)\ (1{,}000)\ (100)}{80}$$

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

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.

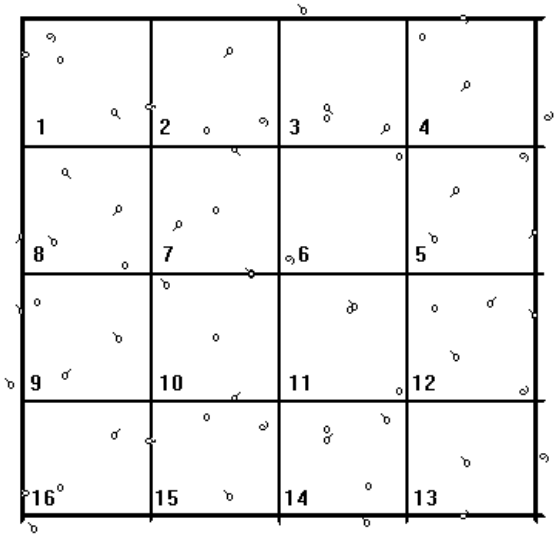
Because the sperm are killed prior to sampling, they will slowly settle. For this reason, the sample in the hematocrit tube should be loaded onto the hemacytometer as rapidly as possible. Two replicate samples are withdrawn in fresh hematocrit tubes and loaded onto opposite sides of a hemacytometer.



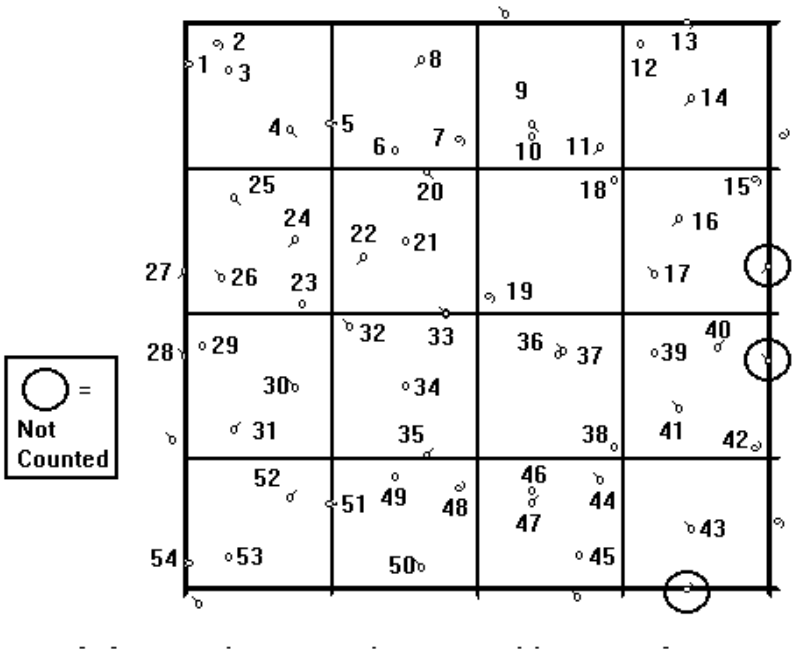
The loaded hemacytometer is left for 15 minutes to allow the sperm to settle onto the counting field. If the coverslip is moved after the samples are loaded, the hemacytometer should be rinsed and refilled with fresh sample. After 15 minutes, the hemacytometer is placed under a microscope and the counting 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).

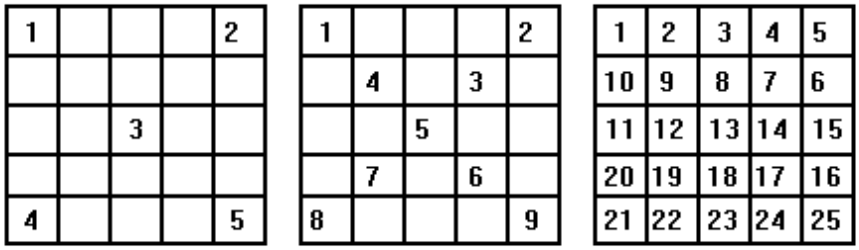
Once these counting conventions have been selected, it is advisable to follow another strict protocol outlining the number and sequence of large squares to be counted. Because the sperm may not be randomly distributed across the counting grid, it is recommended to count an array of squares covering the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