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TROPICAL COLLECTOR URCHIN, *Tripneustes gratilla,* FERTILIZATION TEST METHOD

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TROPICAL COLLECTOR URCHIN, *Tripneustes gratilla,* FERTILIZATION TEST METHOD

1.1 SCOPE AND APPLICATION

1.1.1 This fertilization method estimates the chronic toxicity of effluents and receiving waters to the gametes of the tropical sea urchin (*Tripneustes gratilla*). The test uses a static, non-renewal 60-minute sperm exposure and a subsequent 20-minute fertilization period, following the addition of eggs, for measuring the fertilizing capacity of the sperm. The purpose of the test is to determine the concentrations of a test substance that reduce fertilization of exposed gametes, relative to that of the control.

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

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

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

1.2 SUMMARY OF METHOD

1.2.1 The method provides the step-by-step instructions for exposing sperm suspensions to effluents or receiving waters for 60 minutes. Appropriate sperm density is first determined in a trial fertilization test. 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. The test results are reported as the concentration of the test substance that causes a statistically significant reduction in fertilization.

1.3 INTERFERENCES

1.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities, Equipment, and Supplies, USEPA, 1995).

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

Tests, USEPA, 1995).

1.4 **SAFETY**

1.4.1 See Section 3, Health and Safety (USEPA, 1995).

1.5 **APPARATUS AND EQUIPMENT**

1.5.1 Tanks, trays, or aquaria -- for holding and acclimating adult sea urchins, e.g., standard salt water aquarium (capable of maintaining seawater at 20-25°C), with appropriate filtration and aeration system.

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

1.5.3 Precisely-controlled temperature rooms, constant temperature chambers or water baths -- for maintaining test solution temperature and keeping dilution water supply and gametes stock suspensions at test temperature (23 $\pm$ 1°C) 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).

1.5.4 Water purification system -- Millipore Super-Q, deionized water (DI) or equivalent.

1.5.5 Refractometer -- for determining salinity.

1.5.6 Hydrometer(s) -- for calibrating refractometer.

1.5.7 Thermometers, bulb thermograph or electronic chart-type -- for continuously recording temperature.

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

1.5.9 Meters, pH and DO -- for routine physical and chemical measurements.

1.5.10 Standard or micro-Winkler apparatus -- for determining DO (optional).

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

1.5.12 Reference weights, Class S -- for checking performance of balance.

1.5.13 Fume hood -- to protect the analyst from effluent or glutaraldehyde fumes.

1.5.14 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).

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

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

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

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

1.5.19 Wash bottles -- for dilution water.

1.5.20 20-liter cubitainers, glass bottle, or polycarbonate water cooler jugs -- for making hypersaline brine.

1.5.21 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. Strong solutions of NaOH and glutaraldehyde should not be held for several month periods in cubitainers since they can cause poor egg fertilization. Seawater stored in plastic can also potentially cause poor egg fertilization.

1.5.22 Beakers, 100 mL borosilicate glass or flat bottomed, glass petri dishes, 20-cm diameter-- for spawning, to support sea urchins, and to collect sea urchin eggs. (Note: Not to be used interchangeably for gametes and test solutions).

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

1.5.24 Test tubes, borosilicate glass, 16 x 100 mm or 16 x 125 mm, with caps or 20 mL disposable scintillation vials with plastic-lined caps or similar vials-- for test chambers. Any test container and rinsing technique is acceptable as long as it meets test design and control performance criteria. Four chambers per concentration. (Note: All test containers should be leached by rinsing 3x or soaking them in seawater for at least 24 hours followed by 3 deionized water rinses. Just prior to using the test tubes, they should be rinsed 3x with filtered seawater. If test containers are not dried prior to use, follow deionized water rinses with a final seawater rinse.)

1.5.25 Vortex mixer -- to mix sea urchin sperm in tubes prior to sampling.

1.5.26 Compound microscope -- for examining gametes, counting sperm cells (200-400x) and

eggs (100x), and examining fertilized eggs (100x). A phase contrast microscope is highly recommended to observe the fertilization membranes clearly. An inverted microscope is recommended to examine eggs from below scintillation vials.

1.5.27 Counter, two unit, 0-999 -- for recording sperm and egg counts and counting fertilized and unfertilized eggs at the end of the test.

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

1.5.29 Hemacytometers, Neubauer -- for counting sperm.

1.5.30 Airline (e.g. Tygon®) tubing (3 mm i.d.) -- for removing wash water from settled eggs.

1.5.31 Centrifuge tubes, test tubes, or vials (conical, 3 mL) -- for holding sperm.

1.5.32 Perforated plunger -- for maintaining homogeneous distribution of eggs during sampling and distribution to test tubes. A perforated plunger is a perforated plastic disk, slightly smaller in diameter than the mixing beaker (that provides clearance between plunger and egg stock container), that has been attached to a plastic rod.

1.5.33 60-µm NITEX® filter -- for filtering receiving water.

1.5.34 1-µm filter--for filtering dilution seawater and hypersaline brine.

1.5.35 UV-VIS spectrophotometer -- capable of accommodating 1-5 cm cuvettes for sperm counts.

1.6 **REAGENTS AND SUPPLIES**

1.6.1 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests, USEPA, 1995).

1.6.2 Data sheets (one set per test) -- for data recording (see Figures 1, 2, 3, 4, 5, and 6).

1.6.3 Tape, colored -- for labeling test chambers and containers.

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

1.6.5 Parafilm -- for covering graduated cylinders and vessels containing gametes.

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

1.6.7 Safety glasses, lab coat -- for personal protection from contamination.

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

1.6.9 Pipet tips -- for automatic pipets. Note: Pipet tips for handling sperm 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 sperm, 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.

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

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

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

1.6.13 Membranes and filling solutions -- for dissolved oxygen probe (see USEPA Method 360.l, USEPA, l979).

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

1.6.15 Glutaraldehyde, 0.02% in seawater -- for preserving eggs (see Section 1.10.11.2.2).

1.6.16 Acetic acid, 1% and/or 0.1%, reagent grade, in filtered (10 μm) seawater -- for preparing killed sperm dilutions for sperm counts. See method for uses of 1% or 0.1% acetic acid.

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

1.6.18 0.5 M KCl solution -- for inducing spawning. To make a 100 mL solution, add 3.73 g KCl to a 100 mL volumetric flask and bring the solution to volume with deionized water.

1.6.19 Ice bucket or beaker -- for maintaining live sperm in ice.

1.6.20 Syringe, disposable, 3 or 5 mL and needles, 25 gauge -- for injecting KCl into sea urchins to induce spawning.

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

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

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

SPAWNING RECORD

Animal No.	Sex	Time Injected	Time Spawned	Appearance of gametes	Comments (hermaphrodites, sperm motility, maturity of eggs)

Pooled eggs from female nos. _____.

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

Figure 1. Sample data sheet for spawning record.

EGG DENSITY COUNTS

Egg Dilution

# eggs counted	_____	A (diluted 1:10–1 mL stock in 9 mL seawater)
	_____	B (diluted 1:100–1 mL A in 9 mL seawater)
Use (A x 10) or (B x 100)	_____	D = # eggs/mL in stock

If the egg stock is > 2,000 eggs/mL (A >200 or B > 20 eggs/mL), dilute the egg stock by transferring:

200,000 eggs/ _____ eggs/mL = _____ mL
(D)

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

If the egg stock is < 2,000 eggs/mL (A < 200 eggs/mL, B < 20 eggs/mL), concentrate the eggs by allowing them to settle and then decant enough water to retain the following percent of the original volume:

(_____ eggs/mL/ 2,000) x 100 = % volume
(D)

Final Egg Stock

Add 1 mL final egg stock to 9 mL dilution seawater. Count number of eggs in 1 mL sample.

eggs counted (C) _____

Final egg stock density (E) _____ * 10 = _____ eggs/mL
(C)

The egg count should be between 180 and 220 (=2,000 $\pm$ 200 eggs/mL in final stock). If not, adjust egg stock volume and recheck counts.

Figure 2. Sample data sheet for egg counts.

SPERM DENSITY COUNTS

Bioassay No. _____ Date _____

D*etermining Sperm Dilution using the Spectrophotometer*

1. $Y = [a + bx_i] \times 10^7$

Where: Y is the diluted sperm concentration (sperm/mL);
a is Y-intercept
b is the regression coefficient (slope)
x_i is the absorbance reading

2. Sperm/mL (SPM) in pooled stock = $Y * \frac{W1 \times W2}{S1 \times S2}$

Where: Y = sperm/mL in diluted sperm solution and

$\frac{W1 \times W2}{S1 \times S2}$ is derived from weights in Sections 1.10.5.5.3 & 1.10.5.5.4

The SPM should be greater than 5×10^7.

Sperm Count using Microscope

Add 0.05 mL sperm in 100 mL 0.1 % acetic acid (dilution = 2000). Load sperm onto each side of a Neubauer hemacytometer and average counts.

$$SPM = \frac{(\text{dilution})(4{,}000 \text{ squares/mm}^3)(1{,}000 \text{ mm}^3/\text{cm}^3)(\text{count})}{(\text{\# small squares counted})}$$

$$SPM = \frac{(\_\_\_\_\_)(4{,}000)(1{,}000)(\_\_\_\_\_)}{(\quad)}$$

The SPM should be greater than 5×10^7.

Figure 3. Sample data sheet for sperm counts.

SPERM DENSITY TRIAL WORKSHEET

S:E Ratio	% Fertilized	Sperm Count	Actual Sperm/mL	Predicted Sperm/mL
2500:1	_____	_____	_____	5×10^6
2000:1	_____	_____	_____	4×10^6
1500:1	_____	_____	_____	3×10^6
1000:1	_____	_____	_____	2×10^6
500:1	_____	_____	_____	1×10^6
250:1	_____	_____	_____	5×10^5
125:1	_____	_____	_____	2.5×10^5

**THE S:E RATIO CHOSEN FOR THE DEFINITIVE TEST IS CIRCLED

Add 500 μL of 1% acetic acid to each 5 mL solution of trial S:E ratio.

$$\text{sperm/mL (SPM)} = \frac{(\text{dilution})(\text{count})(\text{hemacytometer conversion})(\text{mm}^3/\text{mL})}{\#\text{ small squares counted}}$$

Where dilution = 1.1
hemacytometer conversion = 4000
$mm^3/mL = 1000$
small squares counted = see Appendix III

$$\text{SPM} = \frac{(1.1)(\quad)(4{,}000)(1{,}000)}{(\quad)} = \_\_\_\_\_ \text{sperm/mL}$$

Figure 4. Sample data sheet for sperm density trial.

DEFINITIVE SPERM COUNT

Add 0.1 mL final sperm stock to 9.9 mL 1 % acetic acid. Load sperm onto each side of a Neubauer hemacytometer and record counts below:

sperm counted _____

_____

_____

_____

Mean Count _____
(X)

sperm/mL (SPM) = $\frac{\text{(dilution)(4,000 squares/mm}^3\text{)(1,000 mm}^3\text{/cm}^3\text{)(mean count)}}{\text{(\# small squares counted)}}$

Final SPM (SPM) = $\frac{(100)(4000)(1000)(\quad)}{(\quad)}$ = _____
(SPM)

Final S:E Ratio

Sperm (S) = (0.1 mL sperm stock/test container) (Final SPM)

Eggs (E) = (Final egg stock density-Section 1.10.4.3.3)(1.0 mL)

Final S:E ratio = $\frac{S}{E}$ = _____ = _____

Figure 5. Sample data sheet for definitive sperm count using a microscope.

Test Concentration	Volume 100% Effluent (VE)	Volume HSB (VB)	Volume Dilution Water
Control	0.0 mL in 1L flask	_____	_____
Brine Control	0.0 mL in1L flask	_____ = highest volume in effluent with HSB	_____ (Use reagent water)
_____	_____	_____	_____
_____	_____	_____	_____
_____	_____	_____	_____
_____	_____	_____	_____
_____	_____	_____	_____

Salinity Adjustment Using Hypersaline Brine

Add hypersaline brine to those concentrations in which test solution salinity would otherwise fall below the minimum acceptable test salinity (32 ‰).

Calculate the volume of brine to be added, VB, for each concentration that requires salinity adjustment using the following equation.

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

Quantities known from dilutions required

VE = Volume of Effluent added for each concentration (mL)

Volume of Dilution Water = Volume of test container – VB – VE

Quantities to be measured

SB = Salinity of Brine (‰) = _____

SE = Salinity of Effluent (‰) = _____

Note: Always adjust the pH of the brine to equal that of the dilution water. Brine salinity should be 60 – 70 ‰.

Figure 6. Sample data sheet for brine adjustments.

1.6.24 Reference toxicant solutions -- see Section 1.10.2.4 (of this method) and Section 4, Quality Assurance (USEPA, 1995).

1.6.25 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, USEPA, 1995).

1.6.26 Effluent and receiving water -- see Section 8, Effluent and Surface Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests (USEPA, 1995).

1.6.27 Dilution water and hypersaline brine -- see Section 7, Dilution Water (USEPA, 1995) and Section 1.6.28, Hypersaline Brines (of this method). The dilution water should be uncontaminated 1-µm-filtered natural seawater. Hypersaline brine should be prepared from dilution water.

1.6.28 HYPERSALINE BRINES

1.6.28.1 Most industrial and sewage treatment effluents entering marine and estuarine systems have little measurable salinity. Exposure of organisms 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, Dilution Water, USEPA, 1995). 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.

1.6.28.2 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).

1.6.28.3 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.

1.6.28.4 Freeze Preparation of Brine

1.6.28.4.1 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

TABLE 1. MAXIMUM EFFLUENT CONCENTRATION (%) THAT CAN BE TESTED AT 34 °/₀₀ 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

freezing seawater at -10 to -20°C 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).

1.6.28.4.2 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 60-70 ‰ brine salinity, unless it is necessary to test effluent concentrations greater than 50%.

1.6.28.4.3 After the required salinity is attained, the HSB should be filtered through a 1 µm

filter and poured directly into portable containers (20 L glass bottles are suitable). The brine storage containers should be capped and labeled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4°C (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

1.6.28.5 Heat Preparation of Brine

1.6.28.5.1 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.

1.6.28.5.2 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.

1.6.28.5.3 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 40°C. 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 40°C. Additional seawater may be added to the brine to obtain the volume of brine required.

1.6.28.5.4 After the required salinity is attained, the HSB should be filtered through a 1 μm filter and poured directly into portable containers (20 L glass bottles are suitable). The brine storage containers should be capped and labeled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4°C (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

1.6.28.6 Artificial Sea Salts

1.6.28.6.1 No data from *T. gratilla* 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 with other species.

1.6.28.6.2 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 21-26°C. 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.

1.6.28.7 Dilution Water Preparation from Brine

1.6.28.7.1 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. A brine control must 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.

1.6.28.7.2 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, to make 1000 mL of brine at 34‰ starting with brine at 100‰, use the following formula:

$CxVx = CyVy$

Where Cx = Concentration of brine (‰)
Vx = Volume of brine (mL)
Cy = Desired salinity of the solution (‰)
Vy = Desired volume of the solution (mL)

$100\ ‰ Vx = 34\ ‰\ (1000\ mL)$

$$Vx = \frac{34\ ‰\ (1000\ mL)}{100\ ‰} = 340\ mL$$

Pour 340 mL of brine into a 1000 mL graduated cylinder and fill to 1000 mL with dilution water. Verify the salinity of the resulting mixture using a refractometer.

1.6.28.8 Test Solution Salinity Adjustment

1.6.28.8.1 Table 3 illustrates the preparation of test solutions (up to 50% effluent) at 34 ‰ by combining effluent (or ambient water), 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 equal to the effluent volume needed for each effluent concentration as in the example in Table 3.

1.6.28.8.2 Check the pH of all brine mixtures and adjust to within 0.2 units of dilution water pH by adding, dropwise, dilute (e.g., 1N) certified hydrochloric acid or sodium hydroxide.

TABLE 2. REAGENT GRADE CHEMICALS USED IN THE PREPARATION OF GP2 ARTIFICIAL SEAWATER FOR THE TROPICAL SEA URCHIN, *TRIPNEUSTES GRATILLA*, 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 (1990). The salinity is 34.0 g/L.

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 concentrated brine with reagent water or natural seawater to achieve a dilute brine of 68-x ‰ and a brine-based dilution water of 34 ‰. If the effluent is < 6 ‰ salinity and does not required hypersaline brine, add only natural seawater to the effluent.

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 (or ambient water) 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 (or ambient water) 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
Brine Control	None	400 mL	200 mL

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

1.6.28.8.3 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:

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

1.6.28.8.4 This calculation assumes that dilution water salinity is 34 $\pm$ 2 ‰. Figure 6 can be used to calculate brine adjustments.

1.6.28.9 Preparing Test Solutions

1.6.28.9.1 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 invert several times to mix. Pour into a (100-250 mL) graduated cylinder, cover, and invert several times. Distribute equal volumes into the replicate test chambers. The remaining test solution can be used for chemistry.

1.6.28.9.2 To prepare a test solution at higher effluent concentrations, hypersaline brine must usually be used. If lower effluent concentrations are within the salinity range specified in the method, it is acceptable to adjust only the higher effluent concentrations with hypersaline brine. For example, to prepare 40% effluent, add 400 mL of effluent to a 1 L volumetric flask. Then, assuming an effluent salinity of 2 ‰ and a brine salinity of 66 ‰, add 400 mL of brine (see equation in Section 1.6.28.8.3 above and Table 3) and top off the flask with dilution water. Stopper the flask and invert several times to mix. Pour into a (100-250 mL) graduated cylinder, cover, and invert several times. Distribute equal volumes into the replicate test chambers. The remaining test solution can be used for chemistry.

1.6.28.10 Brine Controls

1.6.28.10.1 Brine controls must be included 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 Section 1.6.28.8.3 above) setting SE = 0, and solving for VE.

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

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.

1.6.28.10.2. The dilution water control and brine control should be compared to determine whether a statistically significant difference exists before the analysis of test treatment data. The dual control comparison should be made using a t-test as described in Appendix G of USEPA (1995). More guidance on dual controls can be found in USEPA (2000).

1.6.28.11 Egg and Effluent Blanks

1.6.28.11.1 Two types of blanks with eggs only should be included in the test design: two replicates of an effluent blank at the beginning of the injection sequence and two replicates of an egg blank at the end of the injection sequence. 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 (which is a minor factor, unless a significant portion of the eggs were accidentally fertilized; it can indicate poor laboratory techniques or hermaphroditic egg use). Egg blanks will also indicated if any eggs have undergone cleavage, which indicates that eggs were accidentally fertilized early in the test. These blanks are kept capped until the eggs are added in order to avoid contamination by sperm.

1.6.29 TEST ORGANISMS, TROPICAL COLLECTOR SEA URCHINS

1.6.29.1 Tropical Collector Sea Urchins, *Tripneustes gratilla* (approximately 12 per test).

1.6.29.2 Adult sea urchins (*Tripneustes gratilla*), average adult size about 12.7 cm, can be obtained from commercial suppliers or collected from uncontaminated intertidal to shallow subtidal areas (e.g., contact the University of Hawaii, Pacific Biomedical Research Center for information on collection). *T. gratilla* spawn year-round depending upon local conditions (Dinnel, 1988). Since gamete availability can vary depending upon the locality, it may be necessary to collect urchins from different areas for each round of tests to obtain suitable gametes. Some laboratories spawn and collect gametes at the beach, maintain sperm on ice, and return urchins to the collection site. State collection permits are usually required for collection of sea urchins and collection is prohibited or restricted in some areas.

1.6.29.3 The animals are best transported "dry" (surrounded by either moist seaweed or paper towels moistened with seawater) in separate polyethylene containers to prevent cross contamination of gametes, should spawning occur during transit. Animals should be kept at approximately their collection or culture temperature to prevent thermal shock, which can prematurely induce spawning. Rough handling or abrupt pressure changes may also induce spawning.

1.6.29.4 The adult sea urchins are maintained in glass aquaria or fiberglass tanks. The tanks are

supplied continuously (approximately 5 L/min) with, or recirculated with, filtered natural seawater (≥ 32 ‰). The animals are checked daily and any obviously unhealthy animals are discarded.

1.6.29.5 Although ambient temperature seawater is usually acceptable, maintaining sea urchins in spawning condition usually requires holding at a relatively constant temperature in the dark. The culture unit should be capable of maintaining a constant temperature between 20 and 25°C with a water temperature control device. For more information about culturing *T. gratilla,* contact the Anuenue Fisheries Research Center.

1.6.29.6 *Tripneustes gratilla* will feed on seagrasses (i.e., *Thalassia*) and macroalgae (except *Sargassum*) (Klumpp et al., 1993).

1.7 EFFLUENTS AND RECEIVING WATER COLLECTION, PRESERVATION AND STORAGE

1.7.1 See Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sampling Preparation for Toxicity Tests (USEPA, 1995).

1.8 CALIBRATION AND STANDARDIZATION

1.8.1 See Section 4, Quality Assurance (USEPA, 1995).

1.9 QUALITY CONTROL

1.9.1 See Section 4, Quality Assurance (USEPA, 1995).

1.10 TEST PROCEDURES

1.10.1 TEST DESIGN

1.10.1.1 The test consists of four replicates of at least five 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, two replicates of egg blanks and effluent blanks are prepared (see Section 1.6.28.11).

1.10.1.2 Effluent concentrations are expressed as percent effluent.

1.10.2 TEST SOLUTIONS

1.10.2.1 Receiving waters

1.10.2.1.1 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.

1.10.2.2 Effluents

1.10.2.2.1 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 precision of ±100%, and testing of concentrations between 6.25% and 100% using only five effluent concentrations (6.25%, 12.5%, 25%, 50%, and 100%). Test precision shows little improvement as dilution factors are increased beyond 0.5 and declines rapidly if smaller dilution factors are used. **Therefore, the USEPA recommends the use of the ≥ 0.5 dilution factor.** If 100 ‰ HSB is used as a diluent, the maximum concentration of effluent that can be tested will be 66% at 34 ‰ salinity.

1.10.2.2.2 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%).

1.10.2.2.3 The volume in each test chamber is 5 mL.

1.10.2.2.4 Just prior to test initiation (approximately 1 h), the temperature of the sample should be adjusted to the test temperature (23 ± 1 ^{o}C) and maintained at that temperature during the addition of dilution water.

1.10.2.3 Dilution Water

1.10.2.3.1 Dilution water should be uncontaminated, 1 μm-filtered natural seawater with a salinity between 32-36 ‰, or hypersaline brine prepared from uncontaminated natural seawater plus reagent water (see Section 7, Dilution Water, USEPA, 1995). Natural seawater may be uncontaminated receiving water. This water is used in all dilution steps and as the control water.

1.10.2.4 Reference Toxicant Test

1.10.2.4.1 Reference toxicant tests should be conducted as described in Quality Assurance (see Section 4.7, USEPA, 1995). 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, USEPA, 1995).

1.10.2.4.2 The preferred reference toxicant for sea urchins is copper chloride ($CuCl_2 \cdot 2H_2O$). Prepare a 10,000 μg/L copper stock solution by adding 0.0268 g of copper chloride ($CuCl_2 \cdot 2H_2O$) to one liter of reagent water in a polyethylene volumetric flask. Alternatively, certified standard solutions can be ordered from commercial companies. Another toxicant may be specified by the appropriate regulatory agency.

1.10.2.4.3 Reference toxicant solutions should be at least four replicates each of 0 (control) and at least five consecutive copper reference toxicant solutions. For example, make the dilution series from 0 (control), 5, 10, 20, 40, and 80 µg/L total copper, by adding 0, 50 µL, 100 µL, 200 µL, 400 µL, and 800 µL of stock solution, respectively, to one hundred milliliter polyethylene volumetric flasks and fill with dilution water. Start with control solutions and progress to the highest concentration to minimize contamination. Dispense these into at least four replicates per concentration.

1.10.2.4.4 Alternatively, sodium dodecyl sulfate (SDS) can be used as an organic reference toxicant. Make a stock solution of 100 mg/L SDS. Mix stock solution gently with a stir bar during dilution preparations to make solutions as accurate as possible. Prepare a control (0 µg/L) and at least five consecutive SDS reference toxicant solutions. For example, make the dilution series from 0 (control), 0.38, 0.75, 1.5, 3.1, and 6.3 mg/L SDS by adding 0, 0.38, 0.75, 1.5, 3.1, and 6.3 mL of stock solution, respectively, to 100-mL volumetric flasks and filling with dilution water to 100 mL. Start with control solutions and progress to the highest concentration to minimize contamination. Mix solutions gently with a stir bar to avoid foaming as much as possible. Dispense 5 mL of each solution into at least four replicates per concentration.

1.10.2.4.5 Since 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‰.

1.10.3 COLLECTION OF GAMETES FOR THE TEST

1.10.3.1 Spawning Induction

1.10.3.1.1 Pour filtered seawater into 100 mL beakers or petri dishes and place in 23^{o}C water bath or room. Allow for temperature equilibration. Select approximately 12-24 sea urchins to ensure that three of each sex are likely to provide gametes of acceptable quantity and quality for the test.

1.10.3.1.2 Care should be exercised when removing sea urchins from holding tanks so that damage to tube feet is minimized.

1.10.3.1.3 Place each sea urchin onto a clean tray covered with several layers of seawater moistened paper towels.

1.10.3.1.4 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 (sperm) and females (eggs). Frequent washing of hands is a good practice.

1.10.3.1.5 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 7). Between each injection, rinse the

needle with hot tap water or alcohol. This will avoid the accidental injection of sperm from males into females. Note the time of injection (sample data sheet, Figure 1).

1.10.3.1.6 Spawning of sea urchins can also be induced by holding the sea urchin and vigorously shaking or swirling it in a circular, horizontal motion for several seconds. This may provide an enough physical stimulus to stimulate spawning, or may aid in distributing the KCl if the animals were injected.

1.10.3.1.7 Place the sea urchins oral side down onto flat bottomed petri dishes.

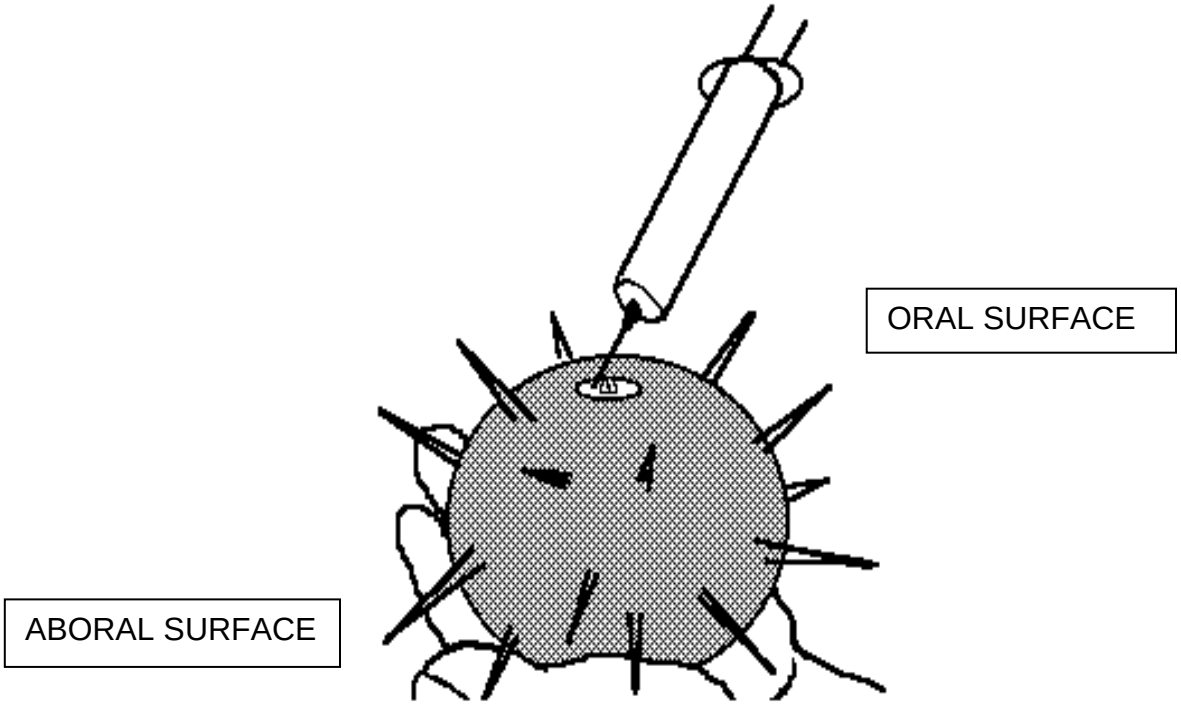


Figure 7. The location and orientation of the KCl injection into sea urchins to stimulate spawning.

1.10.3.1.8 Females will release clear or orange eggs, and males will release cream-colored sperm. As gametes begin to be shed, note the time on the data sheet and separate the sexes. Make sure spawning sea urchin males are turned oral side down for sperm collection. This ensures that sperm are only collected "dry" from the males. Female sea urchins are moved oral side up into the 100 mL beakers or flat bottomed petri dishes filled with seawater. The incidence of hermaphroditism in *Tripneustes gratilla* is 1 in 550 (Lawrence, 1987). To reduce risk of fertilizing eggs prior to the test, do not use any individuals that release both eggs and sperm.

1.10.3.1.9 If sufficient quantities of gametes are available, only collect gametes for the first 15 minutes 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.

1.10.3.1.10 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.

1.10.3.1.11 Sections 1.10.3.2 - 1.10.4 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.

1.10.3.2 Collection of Sperm

1.10.3.2.1 Sea urchin sperm should be collected dry (directly from the surface of the sea urchin), using either a Pasteur pipet or a 0.1-mL autopipet with the end of the tip cut off, so that the opening is at least 2 mm. Pipet sperm from each male into separate 1-15 mL conical test tubes covered with parafilm or microcentrifuge tubes, and store in an ice water bath. Note: Undiluted sperm from *Tripneustes gratilla* typically contains about 1×10^{10} sperm/mL.

1.10.3.3 Viability of Sperm

1.10.3.3.1 Early in the spawning process, place a very small amount of sperm from each male sea urchin into dilution water on a microscope slide (e.g., 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.

1.10.3.4 Pooling of Sperm

1.10.3.4.1 If only one male produces high quality and sufficient sperm for the test, there is no need to pool sperm for the test.

1.10.3.4.2 Pool equal quantities of sperm from each of the sea urchin males (up to 4) that has been deemed good. If possible, 0.25 mL should be pooled from each of those used, and a total of at least 1.0 mL of pooled sperm should be available. Vortex to mix all pooled sperm.

1.10.3.5 Storage of Sperm

1.10.3.5.1 Cover each test tube or beaker with a cap or parafilm, as air exposure of sperm may alter its pH through gas exchange and reduce the viability of the sperm. Keep sperm covered and on ice or refrigerated ($<5^{o}$C). The sperm should be used in a definitive toxicity test no later than 4 hours after collection from males.

1.10.4 PREPARATION OF EGG SUSPENSION FOR USE IN THE TEST

1.10.4.1 Acceptability of Eggs

1.10.4.1.1 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) or protozoa. Discard any egg samples that have protozoa or a fertilization membrane since contamination has occurred due to handling error or hermaphroditism.

1.10.4.1.2 The acceptable egg samples should be 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.

1.10.4.2 Pooling of Eggs

1.10.4.2.1 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 eggs from up to 4 urchins are pooled into a 1 L beaker, and the volume brought to 600 mL with 23°C dilution water. The eggs are suspended by swirling, and the eggs allowed to settle for 15 minutes at 23°C. About 500 mL of the overlying water are siphoned off (along with any feces or spines), and the rinsed eggs are gently transferred to either a 100 or a 250 mL graduated cylinder, and brought to volume with 23°C dilution water. Eggs are stored at 23°C throughout the pre-test period. NOTE: The egg suspension may be prepared and/or counted during the 1 h sperm exposure.

1.10.4.3 Density of Eggs

1.10.4.3.1 Subsamples of the egg stock are taken for determining egg density (see sample data sheet, Figure 2). Place 9 mL of seawater in each of two, 22 mL liquid scintillation vials labeled A and B. Mix egg stock well with a perforated egg plunger, without causing turbulent flow, and place 1 mL into vial A. This vial contains an egg suspension diluted 1:10 from egg stock. Mix vial A well and transfer 1 mL of egg suspension into vial B. This vial contains an egg suspension diluted 1:100 from egg stock. (The remaining egg stock is covered with parafilm and stored at 23°C) . 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 at 40x or 100x magnification. If count is <30, count a 1 mL sample from vial A.

1.10.4.3.2 Prepare 100 mL of egg stock in dilution water at the final target concentration of 2,000 eggs/mL (200,000 eggs in 100 mL). If the egg stock is >2,000 eggs/mL (A >200 or B >20 eggs/mL), dilute the egg stock by transferring:

200,000 eggs / __D__ eggs/mL = ___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 = (Count A) x 10 or (Count B) x 100 = # eggs/mL in stock solution.

If the egg stock is <2,000 eggs/mL (A < 200 eggs/mL, B < 20 eggs/mL), concentrate the eggs by allowing them to settle and then decant enough water to retain the following percent of the original volume:

$$\left(\frac{D}{\text{eggs/mL}} / 2{,}000\right) \times 100 = \%\ \text{volume}$$

1.10.4.3.3 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 200 $\pm$ 20 in the dilution (= 2000 $\pm$ 200 eggs/mL in the final stock). Adjust egg stock volume and recheck counts, if necessary, to obtain counts within this range.

1.10.5 PREPARATION OF SPERM DILUTION

1.10.5.1 A range-finder sperm density trial must be conducted with every test to ensure an optimum sperm control (see Section 1.10.6). The sperm density trial is conducted to determine the lowest sperm density that will provide about 80-90% control egg fertilization, to ensure that an optimum supply of sperm is used.

1.10.5.2 It is unacceptable to conduct a definitive toxicity test if the sperm:egg ratio exceeds 2,500:1. This threshold is based on gradual loss of test sensitivity at higher sperm densities, even in cases where control fertilization is considerably below 100 percent. The maximum acceptable sperm density is 5.0×10^7/mL with an egg density of 2,000 eggs/mL.

1.10.5.3 Figure 8 shows a flowchart for sperm preparation, counts, and dilutions.

1.10.5.4 Before a trial test is conducted, the sperm stock density must be determined, in order to dilute the sperm stock to a maximum concentration of 5.0×10^7 sperm/mL. A microscopic or more rapid spectrophotometric measurement can be used (Section 1.10.5.5 or Section 1.10.5.6). The final sperm concentration used in the toxicity test is always determined by a microscopic count (see Sections 1.10.7.2.3 – 1.10.7.2.5).

1.10.5.5 Spectrophotometric Measurement of Sperm

1.10.5.5.1 A rapid measurement using a spectrophotometer may be used to determine initial sperm density (Hall et al., 1993 and Vazquez, 2003). A regression equation is developed from a correlation between microscope counts and absorbance readings of sperm samples. A baseline collection of data must be developed over time pooling different batches of sperm, and the one regression of pooled batches must show a high degree of correlation ($R^2 \geq 0.95$). See Appendix III for more information.

1.10.5.5.2 Once a regression equation has been developed (Appendix III), the spectrophotometric measurement may be used to determine density of sperm prior to a test. The goal of the spectrophotometric method is to make a 2000x dilution of sperm followed by a measurement of this dilution using a spectrophotometer. The absorbance reading correlates to nominal concentration of sperm/mL.

18.10.5.5.3 Warm up the spectrophotometer for 30 minutes. Mix the pooled sea urchin sperm (1.10.3.4) by agitating the centrifuge tube for about 5 seconds using a vortex mixer. Withdraw a sample of sperm using an automatic pipet and empty approximately 0.1 g of the concentrated sperm into a tared scintillation vial. Note the initial sperm weight (S1). Dilute to about 20 g with dilution seawater and note the final weight (W1). Cap the vial and mix the contents.

1.10.5.5.4 Into a second tared scintillation vial, add between 2-4 g of the first dilution, and note the weight (S2). Dilute to about 20 g with dilution seawater and note the final weight (W2).

1.10.5.5.5 Read the absorbance of the diluted sperm (vial 2) in a 1-5 cm cuvette at 750 nm, using filtered (dilution) seawater as a blank.

1.10.5.5.6 Calculate the sperm cell density of the diluted sperm from the regression equation as follows:

$$Y = [a + bx_i]\ 10^7$$

Where: Y = the diluted sperm concentration (sperm/mL);
a = Y intercept
b = the regression coefficient (slope)
x_i = the absorbance reading

1.10.5.5.7 To determine the density of the pooled sea urchin sperm (see Section 1.10.3.4), multiply the diluted sperm density (Y) by the dilution factor, which is derived from weights calculated in Sections 1.10.5.5.3 & 1.10.5.5.4:

$$\text{Sperm/mL in pooled stock (SPM)} = Y \quad \frac{(W1)(W2)}{(S1)(S2)}$$

Where: Y = sperm/mL in diluted sperm solution

$\frac{(W1)(W2)}{(S1)(S2)}$ = dilution factor

1.10.5.5.7 The SPM should be greater than 5×10^7.

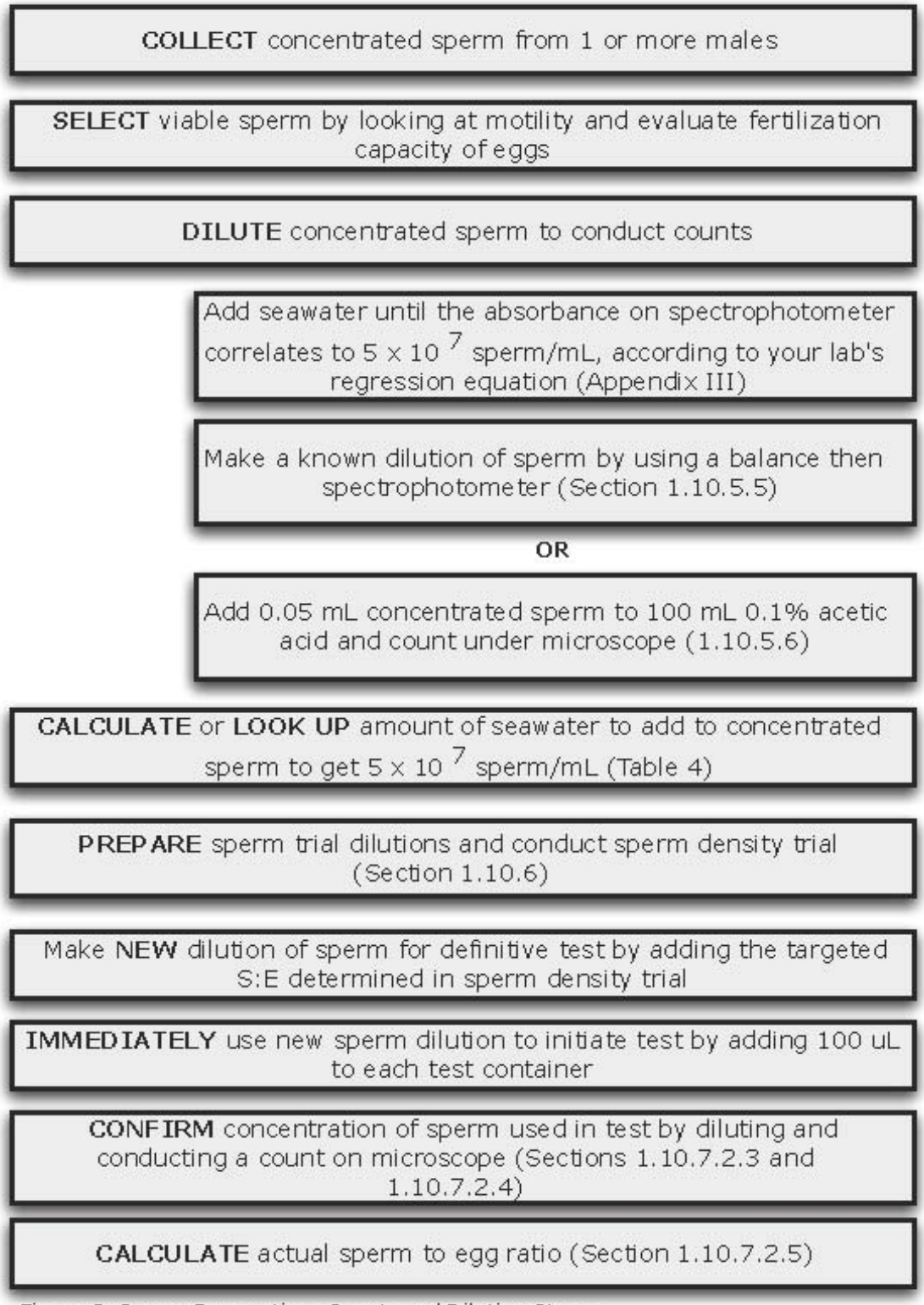


Figure 8. Sperm Preparation, Count, and Dilution Steps

1.10.5.6 Microscopic Measurement of Sperm

1.10.5.6.1 Microscopic measurement of sperm is more time consuming than the spectrophotometric measurement of sperm. If the sperm count and a sperm trial cannot be conducted within 4 hours of gamete collection, then the spectrophotometric method should be used to determine the initial sperm density. The final sperm concentration is always determined by a microscopic count (see Sections 1.10.7.2.3 – 1.10.7.2.5).

1.10.5.6.2 Mix the pooled sea urchin sperm (see Section 1.10.3.4) by agitating the centrifuge tube for about 5 seconds, using a vortex mixer. Very slowly withdraw a 0.05 mL subsample of sperm using an automatic pipet, wipe off the outside of the pipet tip with tissue, and empty the pipet contents into a vial containing a 100 mL solution of 0.1% acetic acid in filtered seawater (e.g., 1 mL of 10% glacial acetic acid plus 99 mL of dilution seawater). Repeatedly rinse the residual sperm from the pipet tip by filling and emptying until no further cloudy solution is expelled from the pipet. (Note: This may require several dozen rinses.) Cover the vial and mix thoroughly by repeated inversion. To obtain quantitatively repeatable samples of sperm, it is important that: (1) the pipet tips have an opening of at least 1 mm; (2) samples be withdrawn slowly to avoid cavitation and entrainment of air in the sperm sample; (3) samples not include fragments of broken spines (which usually settle to the test tube bottom upon vortexing); and (4) care be used when wiping sperm from the pipet tip with a tissue, to avoid wicking sperm from within the pipet tip.

1.10.5.6.3 Transfer a sample of the well-mixed sperm suspension to both sides of two Neubauer hemacytometers. Let the sperm settle 15 minutes.

1.10.5.6.4 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 sperm 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 sperm using the mean of all four counts unless one count can be eliminated as an obvious outlier. The formula for determining the density of sperm in the pooled stock is:

$$\text{SPM} = \frac{(\text{dilution})(4{,}000 \text{ squares/mm}^3)(1{,}000 \text{ mm}^3/\text{cm}^3)(\text{count})}{(\text{\# small squares counted})}$$

1.10.5.6.5 For example, if only 5 large squares are counted as in Pattern no. 1 Appendix II, the sperm concentration per mL is calculated as follows:

$$\text{SPM} = \frac{(\text{dilution factor})(\text{average count})(4000)(1000)}{80}$$

If 0.05 mL sperm is added to 100 mL, the dilution is 2000, so

$$\text{SPM} = \text{average count} \times 10^8$$

1.10.5.6.6 The SPM should be greater than 5×10^7.

1.10.5.7 Dilution of Sperm Stock

1.10.5.7.1 Calculate the volume of sperm stock or seawater necessary to achieve the sperm density required for the final test or trial.

1.10.5.7.2 Fixed Seawater Volume of 100 mL

1.10.5.7.2.1 To calculate the volume of sperm stock to add to 100 mL seawater, in order to achieve a sperm density of 5.0×10^7 sperm/mL, use the following formula:

$$\text{Volume of sperm stock} = \frac{A}{B}$$

Where: A = Target sperm/mL in 100 mL solution

B = Pooled sperm density (Section 1.10.5.5 or 1.10.5.6)

1.10.5.7.2.2 For example, if the target sperm:egg (S:E) ratio is 2500:1, the target density is $20{,}000 \times 2500 = 5.0 \times 10^7$ sperm/mL. {20,000 = (2,000 eggs/tube)/(0.1 mL of sperm stock/tube)}. Therefore,

$$A \text{ (sperm/100mL)} = 5.0 \times 10^7 * 100$$

$$= 5.0 \times 10^9$$

If the pooled sperm density (B) is 4.0×10^{10}, then

$$\text{Volume of sperm stock} = \frac{5.0 \times 10^9}{4.0 \times 10^{10}}$$

= 0.125 mL (brought to a volume of 100 mL with dilution water)

1.10.5.7.2.3 As an alternative, to calculate the proper dilution for any volume of sperm solution in order to achieve a 2500:1 sperm:egg ratio, use the following formula:

$$\text{Dilution} = \frac{\text{Stock Density (sperm/mL)}}{\text{Target Density (sperm/mL)}}$$

For example, if the target sperm:egg ratio is 2500:1,

Target Density = 20,000 x Target S:E Ratio

[20,000 = (2,000 eggs/tube)/(0.1 mL of sperm stock/tube)]

= 20,000 x 2500 = 50,000,000 sperm/mL

If the stock sperm density is 4×10^{10} sperm/mL, then

$$\text{Dilution} = \frac{4 \times 10^{10} \text{ sperm/mL}}{5 \times 10^{7} \text{ sperm/mL}} = 800\text{x}$$

1.10.5.7.3 Fixed Concentrated Sperm Volume of 0.025 mL

1.10.5.7.3.1 Use Table 4 to determine the volume of seawater that 0.025 mL concentrated sperm should be added to, in order to make 5.0×10^{7} sperm/mL.

1.10.5.7.3.2 Instead of using Table 4, you may also calculate the volume of seawater that 0.025 mL concentrated sperm should be added to in order to achieve a sperm stock of 5.0×10^{7} sperm/mL for a 2500:1 sperm:egg ratio, using the following formula:

$$\text{Volume of seawater (mL)} = \frac{(0.025 \text{ mL sperm})(\text{Density of sperm in concentrated sperm stock})}{5.0 \times 10^{7} \text{ sperm/mL}}$$

1.10.6 SPERM DENSITY TRIAL

1.10.6.1 In a sperm density trial, the density of sea urchin sperm is checked by hemacytometer counts, and a replicated series of nominal sperm:egg ratios is set up using 2500:1, 2000:1, 1500:1, 1000:1, 500:1, 250:1, and 125:1, based upon appropriate dilution calculations.

1.10.6.2 The series of trial sperm:egg ratios should include 2,500:1 and several lower ratios. Recommended sperm dilution procedures are given in Sections 1.10.5.7.2 and 1.10.5.7.3. Prepare 2 replicates (in a test tube or scintillation vial) for each sperm:egg ratio in the sperm density trial. For the S:E ratios in Table 5, add 10 mL of dilution water to 14 test tubes or scintillation vials. To prepare the trial sperm stock suspensions, add the volume of sperm stock (5×10^{7} sperm/mL) identified in Table 5 for the appropriate sperm:egg ratios. The laboratory may use different sperm:egg ratios based on experience.

1.10.6.3 One replicate from each S:E ratio is subsampled to determine the sperm density. 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 a direct hemacytometer count or spectrophotometric measurement. To prepare a killed sperm preparation, pipet 5 mL of the seawater/sperm solution from one replicate of each S:E ratio and add it to a second container labeled with the same sperm:egg ratio. Add 500 µL of 1% acetic acid to each of these replicates and load a sample onto a hemacytometer for counts. The formula that should be used for these counts is:

$$\text{sperm/mL} = \frac{(\text{dilution})(\text{count})(\text{hemacytometer conversion})(\text{mm}^3/\text{mL})}{\text{\# small squares counted}}$$

Where : dilution = 1.1

hemacytometer conversion = 4000

$mm^3/mL = 1000$

small squares counted = 80 or 400, see Appendix II

Record results in the worksheet (Figure 4).

1.10.6.4 If the first replicate is spilled or produces anomalous data, divide the second 10 mL replicate from each S:E ratio by pipetting 5 mL into a second test tube or scintillation vial. Count one or both of these extra replicates if necessary.

1.10.6.5 In the trial, an abbreviated sperm exposure of 45 minutes followed by a 20 minute fertilization period should be performed in order to conduct the definitive test within 4 hours of gamete collection. Except for less replicates and the abbreviated sperm exposure, the procedures, volume, etc. are as specified for the normal controls in the definitive test (see Section 1.10.7).

1.10.6.6 After the 45 minute sperm exposure, add 1 mL of egg stock, containing 2000 eggs/mL, to each of the three remaining replicates per S:E containing sperm (without acetic acid).

1.10.6.7 The trial is stopped by the addition of 0.5 mL of 0.02% glutaraldehyde in a fume hood after a 20 minute fertilization period.

TABLE 4. DILUTION WATER VOLUMES (mL) NECESSARY TO ACHIEVE A 5.0 x 10^7 SPERM/mL SOLUTION, FOR A 2500:1 SPERM:EGG RATIO, BY ADDING 0.025 ML CONCENTRATED SPERM[1].

Density	**0.0**	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**	**0.6**	**0.7**	**0.8**	**0.9**
1x 10^{10}	5.00	5.50	6.00	6.50	7.00	7.50	8.00	8.50	9.00	9.50
2 x 10^{10}	10.0	10.5	11.0	11.5	12.0	12.5	13.0	13.5	14.0	14.5
3 x 10^{10}	15.00	15.50	16.00	16.50	17.00	17.50	18.00	18.50	19.00	19.50
4 x 10^{10}	20.00	20.50	21.00	21.50	22.00	22.50	23.00	23.50	24.00	24.50
5 x 10^{10}	25.00	25.50	26.00	26.50	27.00	27.50	28.00	28.50	29.00	29.50
6 x 10^{10}	30.00	30.50	31.00	31.50	32.00	32.50	33.00	33.50	34.00	34.50
7 x 10^{10}	35.00	35.50	36.00	36.50	37.00	37.50	38.00	38.50	39.00	39.50
8 x 10^{10}	40.00	40.50	41.00	41.50	42.00	42.50	43.00	43.50	44.00	44.50
9 x 10^{10}	45.00	45.50	46.00	46.50	47.00	47.50	48.00	48.50	49.00	49.50

[1]For example, if the density of the concentrated sperm stock is 3.2 x 10^{10}, go to the intersection of row thirteen (3 x 10^{10}) and column four (0.2) to find that the volume of dilution seawater that 0.025 mL concentrated sperm should be added to is 16 mL.

TABLE 5. EXAMPLE OF SPERM STOCK (5 X 10^7 SPERM/mL) NEEDED TO ACHIEVE TRIAL SPERM:EGG RATIOS WHEN ADDED TO 10 mLSEAWATER

Volume Sperm Stock, µL (Density=5 x 10^7 sperm/mL)	Sperm:Egg Ratio (S:E)	Nominal Sperm Concentration, sperm/mL
5	125	2.5 x 10^5
10	250	5 x 10^5
20	500	1 x 10^6
40	1000	2 x 10^6
60	1500	3 x 10^6
80	2000	4 x 10^6
100	2500	5 x 10^6

1.10.6.8 After the addition of preservative, quantitative evaluation of the sperm density trial should be obtained by counting 100 eggs from one test tube or vial for each S:E, until a suitable sperm density can be determined for the definitive test. Record all counts made using the example data sheet in Figure 4.

1.10.6.9 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.

1.10.6.10 After selecting a target sperm:egg ratio for the test, use the examples in Sections 1.10.5.7.2 or 1.10.5.7.3 to calculate the dilution of the pooled sperm stock needed to provide the necessary sperm density for the definitive test (see sample data sheet, Figure 5).

1.10.6.11 Table 4 can be used for deriving the dilution water volumes needed for preparing the final sperm stock for the definitive test. For a pooled sperm suspension density of $4x10^{10}$ and a target sperm:egg ratio of 500:1, simply read the dilution for the 2500:1 sperm:egg ratio from Table 4 (20 mL dilution water/0.025 mL sperm stock) and reduce the sperm volume by 2500/500 = 5. In this case, 20 mL/5 = 4 mL dilution water/0.025 mL sperm stock.

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
125:1	100*	95*	85	70	40	70
250:1	100	98	95*	80	64	85*
500:1	100	100	98	98*	82	89
1000:1	100	100	100	100	84	90
1500: 1	100	100	100	100	85	90
2500:1	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 125:1 (case 1 and case 2).

2. If all trials do not 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.

1.10.7 START OF THE DEFINITIVE TEST

1.10.7.1 Prior to Beginning the Test

1.10.7.1.1 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, USEPA, 1995).

1.10.7.1.2 The definitive test should immediately follow the sperm density trial and should begin by four hours after sperm collection.

1.10.7.1.3 Just prior to test initiation (approximately 1 h), the temperature of the sample should be adjusted to the test temperature ($23 \pm 1^{o}C$) and maintained at that temperature during the addition of dilution water.

1.10.7.1.4 Increase the temperature of the water bath, room, or incubator to the required test temperature ($23 \pm 1^{o}C$).

1.10.7.1.5 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, USEPA, 1995, 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 urchin eggs have been examined at the end of the test.

1.10.7.1.6 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.

1.10.7.1.7 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.

1.10.7.1.8 Measure temperature of a temperature blanks to ensure that the test chambers have been equilibrated at $23 \pm 1^{o}C$.

1.10.7.2 Sperm Exposure

1.10.7.2.1 A new dilution of sperm must be prepared for the definitive test. Mix the iced sea

urchin sperm suspension by agitating the centrifuge tube for about 5 seconds, using a vortex mixer. Very slowly withdraw the required volume of sperm with an autopipet. Combine sperm with the required volume of dilution water in a graduated cylinder or beaker, rinsing the autopipet several times to expel all sperm. (Note: Sperm and dilution water volumes determined in Section 1.10.5.7.2, 1.10.5.7.3 or 1.10.6.10.) Cover the graduated cylinder or beaker and mix the sperm stock well, by repeated inversion. To obtain quantitatively repeatable samples of sperm, it is important that: (1) the pipet tips have an opening of at least 1 mm; (2) samples be withdrawn slowly to avoid cavitation and entrainment of air in the sperm sample; (3) samples not include fragments of broken spines (which usually settle to the test tube bottom upon vortexing); and (4) care be used when wiping sperm from the pipet tip with a tissue, to avoid wicking sperm from within the pipet tip. Begin test within 5 minutes of this final sperm dilution preparation and within 4 hours of initial sperm collection from the male(s).

1.10.7.2.2 Into each test chamber (except egg blanks, see Section 1.6.28.11), inject 0.100 mL of the sperm stock, and note the time of first and last injection. It is important that the injection be performed with care so 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. The sperm stock solution should be mixed frequently to maintain a homogeneous sperm stock.

1.1.10.7.2.3 Confirm the sperm density by sampling from the sperm test stock (see sample data sheet, Figure 5). Add 0.1 mL of test stock to 9.9 mL of 1% acetic acid in sea water (0.1 mL 10% acetic acid in 9.9 mL seawater). After mixing well, fill both sides of a hemacytometer with this dilution. 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.

1.10.7.2.4 Calculate the sperm density in the sperm stock using the following formula, where the dilution is 100:

$$\#\text{ sperm per mL (SPM)} = \frac{(\text{dilution})(4{,}000\text{ squares/mm}^3)(1{,}000\text{ mm}^3/\text{cm}^3)(\text{mean count})}{(\#\text{ small squares counted on hemacytometer})}$$

For a sperm:egg ratio of 2500:1, the stock sperm density will be 5×10^7 sperm/mL. For counting pattern no. 3 in Appendix II (all 25 squares), this amounts to an total count average of 50 sperm. For counting pattern no. 1 (5 squares), this amounts to a total count average of 10.

1.10.7.2.5 Calculate the actual sperm to egg ratio used in the test using the data sheet in Fig. 5:

$$\text{Final S:E ratio} = \frac{(0.1\text{ mL sperm stock/test container})\ (\text{Final sperm/mL from Section 1.10.7.2.4})}{(\text{Final egg stock density from Section 1.10.4.3.3})(1.0\text{ mL per test tube})}$$

1.10.7.2.6 Check the temperature of the test solutions either continuously during the sperm exposure or at the beginning and end of exposure by including two temperature blank test tubes

containing 5 mL of dilution water and a thermometer.

1.10.7.3 Adding Eggs to the Test

1.10.7.3.1 Exactly 60 minutes after the sperm addition to the test was begun, begin to add the eggs, with every test chamber (including egg and effluent blanks – see Section 1.6.28.11) receiving 1.0 mL of egg stock. Follow the same pattern of introduction for the eggs as was used with the sperm, so that each test tube has a sperm incubation period of 60 minutes. Gently swirl test tube rack or scintillation vial holder to ensure mixing of eggs and sperm. Note the time of start and finish of egg addition. This duration should be within one minute of that used for the sperm.

1.10.7.3.2 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 with a perforated plunger is the recommended method of achieving a uniform distribution. Frequent inversion of egg stock in a graduated cylinder may be acceptable.

1.10.7.3.3 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.

1.10.8 LIGHT, PHOTOPERIOD, SALINITY AND TEMPERATURE

1.10.8.1 The sea urchin 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.

1.10.8.2 The water temperature in the test chambers should be maintained at $23 \pm 1^{o}C$. If a water bath is used to maintain the test temperature, the water depth surrounding the test tubes or vials should be as deep as possible, without floating the containers. A sensor placed in two temperature blank vials with a 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 in the water surrounding the test vials. Do not measure temperatures directly in test vials, but prepare and handle the temperature blank(s) exactly as the normal control vials. Record the temperature either continuously or at the beginning and the end of the test.

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

1.10.8.4 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.

1.10.9 DISSOLVED OXYGEN (DO) CONCENTRATION

1.10.9.1 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. 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, USEPA, 1995). 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.

1.10.10 OBSERVATIONS DURING THE TEST

1.10.10.1 Routine Chemical and Physical Observations

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

1.10.10.1.2 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.

1.10.10.1.3 Temperature should be monitored continuously or measured in at least two test chambers at the beginning and the end of the test to determine temperature variation in the environmental chamber as outlined in Section 1.10.8.2.

1.10.10.1.4 Record all the measurements on the water quality data sheet.

1.10.11 TERMINATION OF THE TEST

1.10.11.1 Ending the Test

1.10.11.1.1 Record the time the test is terminated.

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

1.10.11.2 Sample Preservation

1.10.11.2.1 Exactly 20 minutes after the egg addition, the test should be stopped by the addition of a fixative to kill the sperm and eggs, 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.

1.10.11.2.2 Sample preservation is achieved by adding 0.5 mL of 0.02% glutaraldehyde (vol/vol) in clean seawater to each test tube, to give a final glutaraldehyde concentration of 0.002% in each test tube. Glutaraldehyde should be made up fresh each day. Because concentrated glutaraldehyde is commonly only 25% strength, 0.02% glutaraldehyde is obtained by diluting the concentrate by 1250x (e.g., 200 µL of 25% glutaraldehyde + 248.98 mL seawater). Formaldehyde or higher concentrations of glutaraldehyde should not be substituted since they can cause difficulty seeing an elevated fertilization membrane.

1.10.11.2.3 Note: Glutaraldehyde is irritating to skin and mucous membranes. It should not 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 (a fume hood is recommended). Before using this compound, the user should consult the latest material safety data available.

1.10.11.3 Counting

1.10.11.3.1 Immediately after termination of the test, the tubes are capped (or otherwise covered) and the contents mixed by inversion. Eggs can be stored at room temperature until they are examined for fertilization. Counts should be completed as soon as possible to avoid difficulty reading the fertilization membrane. Counts should be completed within 48 hours and, if counts extend over a period of two days, they should be made by replicate, i.e., count all replicate 1 tubes, then replicate 2, etc.

1.10.11.3.2 At least 100 eggs from each test tube are examined under a compound microscope (100 x) 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 Figure 9). A phase contrast microscope is highly recommended for examining fertilization membranes. If a membrane is difficult to detect, a hypersaline salt solution or India ink may be added to the sample.

1.10.11.3.3 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. When eggs with a partial fertilization membrane 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 common in the effluent egg blank (indicating an effluent-produced false fertilization).

1.10.11.3.4 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.

18.10.11.3.5 It is convenient to concentrate the eggs prior to counting. If the eggs are allowed to completely settle (for approximately 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 with the remaining volume and finally, transferring 1 mL of the egg suspension into a 1 mL Sedgewick-Rafter counting chamber (other volume counting chambers or slides can be used).

1.10.11.3.6 Eggs can also be transferred to scintillation vials and read from below the vial with an inverted microscope.

1.10.11.3.7 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 unfertilized 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.

1.10.11.4 Endpoint

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

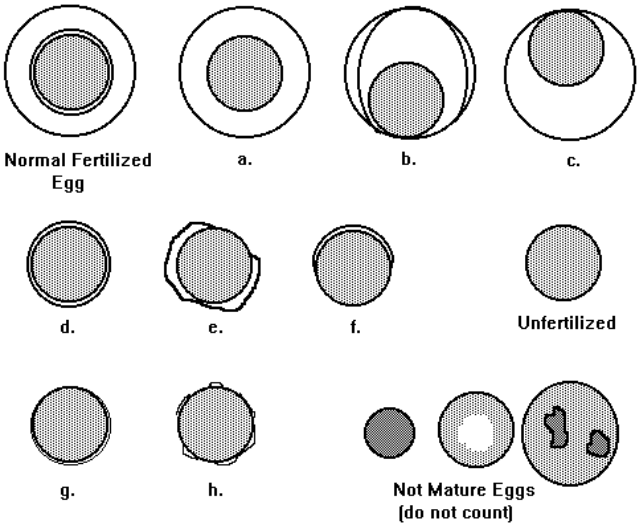


Figure 9. Examples of typical fertilized and unfertilized sea urchin eggs and a number of atypical "fertilized" eggs (a through h). Normal fertilized eggs have an outer fertilization membrane and an inner hyaline membrane. After preservation, the hyaline membrane sometimes disappears (a); in other cases, 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 only appears to be a slight elevation of the outer membrane or the hyaline membrane only appears, fully (d), partially (f), or 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 segments of the egg only, 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 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.

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

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

1.12 **ACCEPTABILITY OF TEST RESULTS**

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

(1) Mean control fertilization must be $\geq$ 70%.

(2) The sperm count for the final sperm stock must not exceed 50,000,000 sperm/mL.

(3) Dilution seawater egg blanks and effluent egg blanks should contain essentially no eggs with fertilization membranes or cleavage.

TABLE 7. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR TROPICAL SEA URCHIN, *TRIPNEUSTES GRATILLA,* FERTILIZATION TEST WITH EFFLUENTS AND RECEIVING WATERS[1]

1.	Test type:	Static non-renewal (required)
2.	Salinity:	34 $\pm$ 2‰ (recommended)
3.	Temperature:	23 $\pm$ 1°C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required)
4.	Light quality:	Ambient laboratory light during test preparation (recommended)
5.	Light intensity:	l0-20 μE/m^2/s (Ambient laboratory levels) (recommended)
6.	Test chamber size:	16 x 100 mm or 16 x 125 mm test tubes or 22 mL scintillation vials (recommended)
7.	Test solution volume:	5 mL (recommended)
8.	Number of spawners:	Pooled sperm from up to four males and pooled eggs from up to four females are used per test (recommended)
9.	No. egg and sperm cells per chamber:	About 2000 eggs and not more than 5,000,000 sperm per chamber (recommended)

10. No. replicate chambers per concentration:	4 (required minimum)
11. Dilution water:	Uncontaminated 1-µm-filtered natural seawater or hypersaline brine prepared from natural seawater or artificial sea salts (available options)
12. Test concentrations:	Effluents: 5 and a control (recommended) Receiving waters: 100% receiving water (or minimum of five) and a control (recommended)
13. Dilution factor:	Effluents: >0.5 (recommended) Receiving waters: None or >0.5 (recommended)
14. Test duration:	80 min (60 min plus 20 min) (required)
15. Endpoint:	Fertilization of sea urchin eggs (required)
16. Test acceptability criteria:	1) > 70 mean control fertilization in reference toxicant and effluent tests 2) The sperm count for the final sperm stock is < 50,000,000 sperm/mL
17. Sampling requirements:	For on-site tests, one sample collected at test initiation, and used within 24 h of the time it is removed from the sampling device. For off-site tests, holding time must not exceed 36 h before first use (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests, Subsection 8.5.4, USEPA, 1995) (required)
1. Sample volume required:	1 L per test (recommended)

[1]For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 10.2, USEPA, 1995, for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

1.13 **DATA ANALYSIS**

1.13.1 GENERAL

1.13.1.1 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.

1.13.1.2 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.

1.13.1.3 The endpoints of toxicity tests using the sea urchin 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, USEPA, 1995). 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, USEPA, 1995). 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 TROPICAL SEA URCHIN, *TRIPNEUSTES GRATILLA*, FERTILIZATION TEST USING SODIUM DODECYL SULFATE REFERENCE TOXICANT

SDS Concentration (mg/L)	Replicate	No. of Eggs Counted	No. of Eggs Fertilized	Proportion Fertilized
Control	A	100	98	0.98
	B	100	96	0.96
	C	100	93	0.93
	D	100	91	0.91
0.25	A	100	89	0.89
	B	100	92	0.92
	C	100	94	0.94
	D	100	97	0.97
0.5	A	100	90	0.90
	B	100	88	0.88
	C	100	92	0.92
	D	100	94	0.94
1.0	A	100	91	0.91
	B	100	88	0.88
	C	100	90	0.90
	D	100	89	0.89
2.0	A	100	56	0.56
	B	100	65	0.65
	C	100	60	0.60
	D	100	72	0.72
4.0	A	100	10	0.10
	B	100	4	0.04
	C	100	8	0.08
	D	100	12	0.12

1.13.2 EXAMPLE OF ANALYSIS OF TROPICAL SEA URCHIN, *TRIPNEUSTES GRATILLA*, FERTILIZATION DATA

1.13.2.1 Formal statistical analysis of the fertilization data is outlined in Figure 10. 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.

1.13.2.2 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.

18.13.2.3 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, USEPA, 1995). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative.

1.13.2.4 Example of Analysis of Fertilization Data

1.13.2.4.1 This example uses toxicity data from a tropical sea urchin, *Tripneustes gratilla*, fertilization test performed with sodium dodecyl sulfate. 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 (USEPA, 1995). The raw and transformed data, and the means and variances of the transformed observations, at each toxicant concentration and control are listed in Table 9. The data are plotted in Figure 11.

1.13.2.5 Test for Normality

1.13.2.5.1 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.

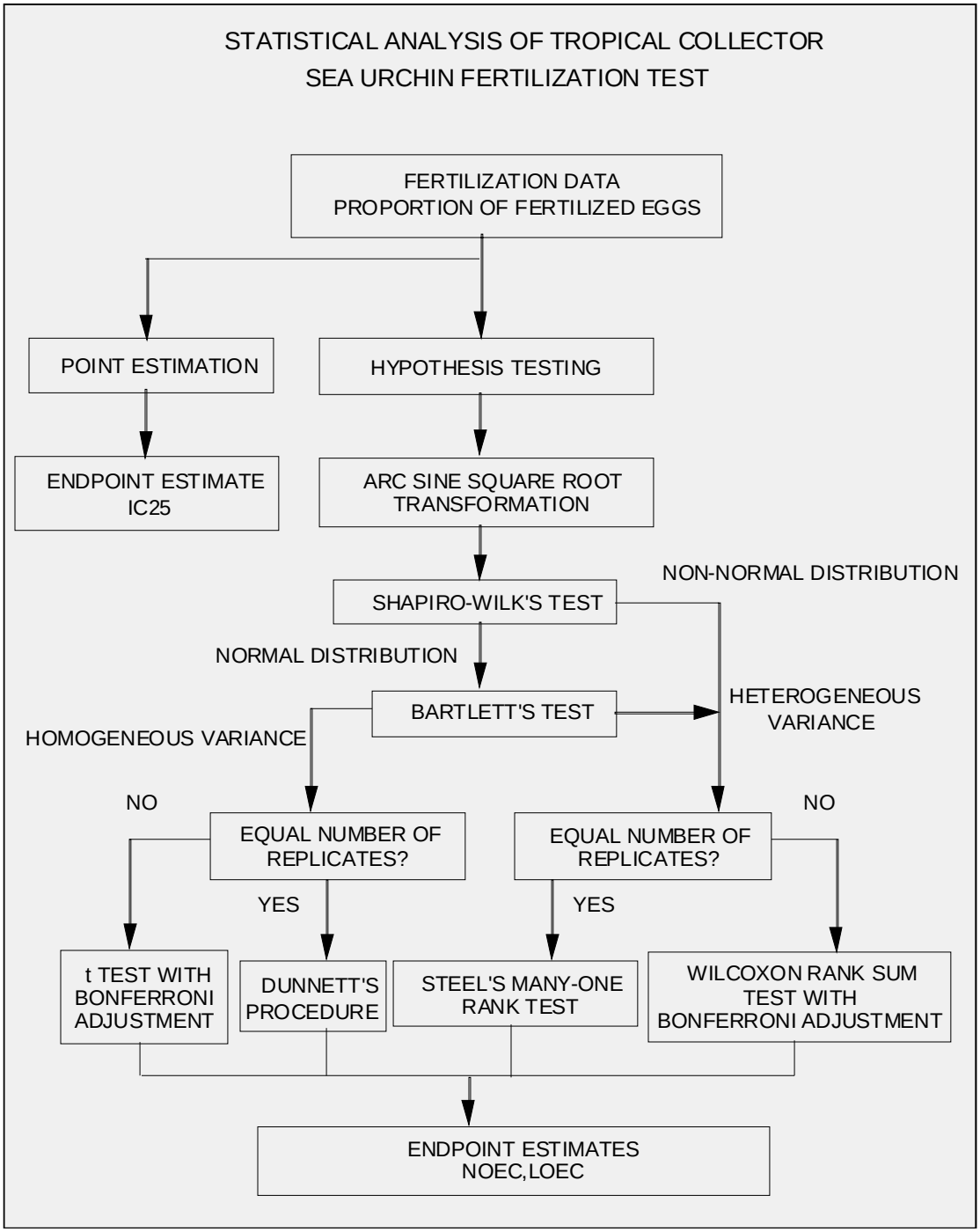


Figure 10. Flowchart for statistical analysis of tropical sea urchin, *Tripneustes gratilla*, fertilization test.

TABLE 9. TROPICAL SEA URCHIN, *TRIPNEUSTES GRATILLA*, FERTILIZATION DATA.

		SDS Concentration (mg/L)					
	Rep.	Control	0.25	0.5	1.0	2.0	4.0
	A	0.98	0.89	0.90	0.91	0.56	0.10
RAW	B	0.96	0.92	0.88	0.88	0.65	0.04
	C	0.93	0.94	0.92	0.90	0.60	0.08
	D	0.91	0.97	0.94	0.89	0.72	0.12
ARC SINE	A	1.429	1.233	1.249	1.266	0.846	0.322
SQUARE ROOT	B	1.369	1.284	1.217	1.217	0.938	0.201
TRANSFORMED	C	1.303	1.323	1.284	1.249	0.886	0.287
	D	1.266	1.397	1.323	1.233	1.013	0.354
Mean ($\overline{Y}_i$)		1.342	1.309	1.268	1.241	0.921	0.291
s_i^2		0.00520	0.00478	0.00208	0.00044	0.00520	0.00435
i		1	2	3	4	5	6

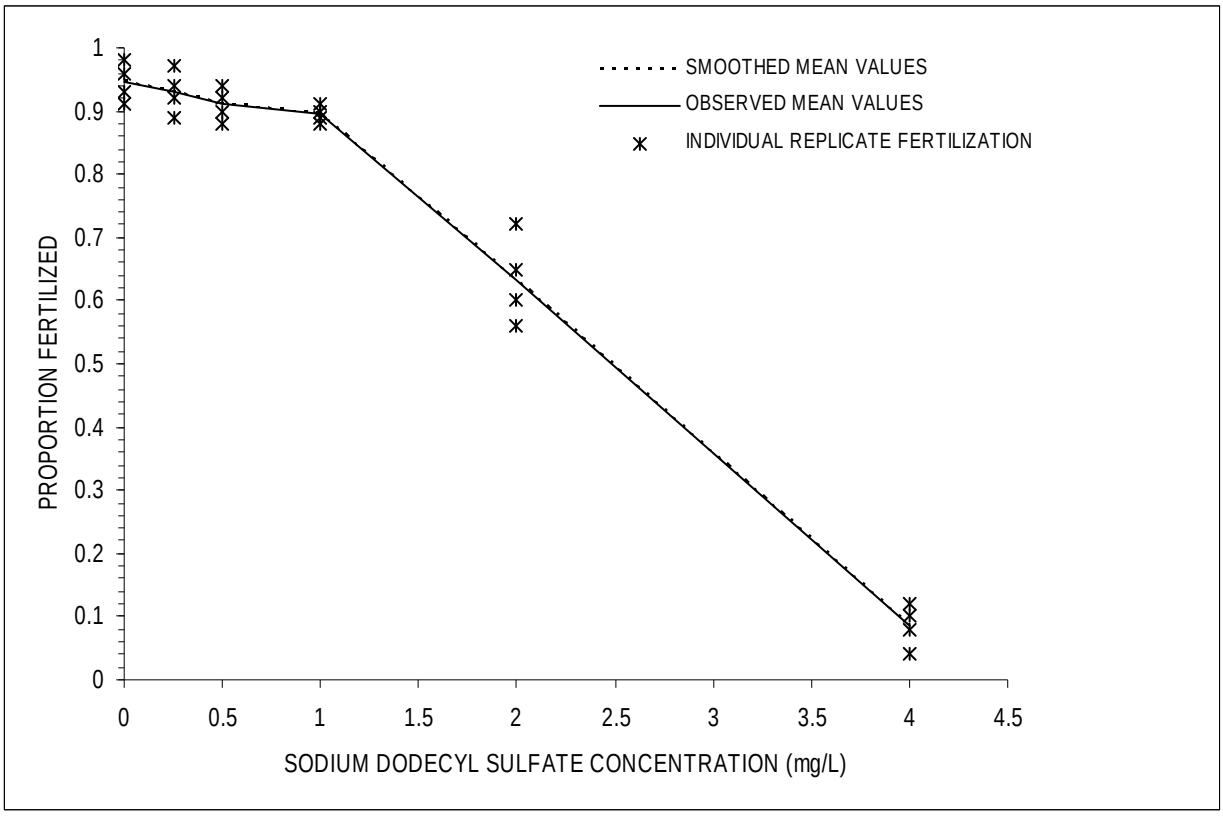


Figure 11. Plot of raw data, observed means, and smoothed means for the sea urchin, *Tripneustes gratilla*, eggs fertilized.

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

	SDS Concentration (mg/L)					
Replicate	Control	0.25	0.5	1.0	2.0	4.0
A	0.087	-0.076	-0.019	0.025	-0.075	0.031
B	0.027	-0.025	-0.051	-0.024	0.017	-0.090
C	-0.039	0.014	0.016	0.008	-0.035	-0.004
D	-0.076	0.088	0.055	-0.008	0.092	0.063

1.13.2.5.2 Calculate the denominator, D, of the statistic:

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

Where: X_i = the ith centered observation

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

n = the total number of centered observations

1.13.2.5.3 For this set of data, n = 24

$$\overline{X} = \frac{1}{24}(0.001) = 0.000$$

$$D = 0.0666$$

1.13.2.5.4 Order the centered observations from smallest to largest

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

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

1.13.2.5.5 From Table 4, Appendix B (USEPA, 1995), 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 = 24 and k = 12. The a_i values are listed in Table 12.

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

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.090	13	0.008
2	-0.076	14	0.014
3	-0.076	15	0.016
4	-0.075	16	0.017
5	-0.051	17	0.025
6	-0.039	18	0.027
7	-0.035	19	0.031
8	-0.025	20	0.055
9	-0.024	21	0.063
10	-0.019	22	0.087
11	-0.008	23	0.088
12	-0.004	24	0.092

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

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4493	0.182	$X^{(24)} - X^{(1)}$
2	0.3098	0.164	$X^{(23)} - X^{(2)}$
3	0.2554	0.163	$X^{(22)} - X^{(3}$
4	0.2145	0.138	$X^{(21)} - X^{(4)}$
5	0.1807	0.106	$X^{(20)} - X^{(5)}$
6	0.1512	0.070	$X^{(19)} - X^{(6)}$
7	0.1245	0.062	$X^{(18)} - X^{(7)}$
8	0.0997	0.050	$X^{(17)} - X^{(8)}$
9	0.0764	0.041	$X^{(16)} - X^{(9)}$
10	0.0539	0.035	$X^{(15)} - X^{(10)}$
11	0.0321	0.022	$X^{(14)} - X^{(11)}$
12	0.0107	0.012	$X^{(13)} - X^{(12)}$

1.13.2.5.6 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, $X^{(n-i+1)} - X^{(i)}$, and Shapiro-Wilk's coefficients (a_i) are listed in Table 12. For the data in this example:

$$W = \frac{1}{0.0666} (0.2521)^2 = 0.9543$$

1.13.2.5.7 The decision rule for this test is to compare W as calculated in Section 1.13.2.5.6 (of this method) to a critical value found in Table 6, Appendix B (USEPA, 1995). If the computed W is less than the critical value, conclude that the data are not normally distributed. For the data in this example, the critical value at a significance level of 0.01 and n = 24 observations is 0.884. Since W = 0.9543 is greater than the critical value, conclude that the data are normally distributed.

1.13.2.6 Test for Homogeneity of Variance

1.13.2.6.1 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.

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

ln = $\log_e$

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

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

1.13.2.6.2 For the data in this example (see Table 8), all effluent concentrations including the control have the same number of replicates ($n_i = 4$ for all i). Thus, $V_i = 3$ for all i.

1.13.2.6.3 Bartlett's statistic is, therefore:

$$B = [(18)\ln(0.003675) - 3\sum_{i=1}^{p}\ln(S_i^2)] \ / \ 1.1296$$

$$= [18(-5.6062) - 3(-35.2032)]/1.1296$$

$$= 4.6980/1.1296$$

$$= 4.1590$$

1.13.2.6.4 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 5 degrees of freedom, is 15.09. Since B = 4.1590 is less than the critical value of 15.09, conclude that the variances are not different.

1.13.2.7 Dunnett's Procedure

1.13.2.7.1 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 $n_1 + n_2 ... + n_p$

n_i = number of observations in concentration i

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

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

$$SSW = SST - SSB \qquad \text{Within Sum of Squares}$$

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)

1.13.2.7.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = n_6 = 4$

$N = 24$

$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} = 5.367$
$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} = 5.237$
$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} = 5.073$
$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} = 4.965$
$T_5 = Y_{51} + Y_{52} + Y_{53} + Y_{54} = 3.683$
$T_6 = Y_{61} + Y_{62} + Y_{63} + Y_{64} = 1.164$

$G = T_1 + T_2 + T_3 + T_4 + T_5 + T_6 = 25.489$

$$SSB = \sum_{i=1}^{p} T_i^2 / n_i - G^2 / N$$

$= (121.537)/4 - (25.489)^2/24 = 3.314$

$$SST = \sum_{i=1}^{p} \sum_{j=1}^{n_i} Y_{ij}^2 - G^2 / N$$

$= 30.450 - (25.489)^2/24 = 3.380$

$SSW = SST - SSB = 3.380 - 3.314 = 0.066$

$S_B^2 = SSB/(p-1) = 3.314/(6-1) = 0.663$

$S_W^2 = SSW/(N-p) = 0.066/(24-6) = 0.0037$

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

TABLE 14. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE.

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	5	3.314	0.663
Within	18	0.066	0.0037
Total	23	3.380	

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

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

Where: $\overline{Y}_i$ = mean proportion fertilized eggs for concentration i

$\overline{Y}_1$ = 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.

1.13.2.7.5 Table 15 includes the calculated *t* values for each concentration and control combination. In this example, comparing the 0.25 mg/L concentration with the control the calculation is as follows:

$$t_2 = \frac{(1.342 - 1.309)}{0.0608\ \sqrt{(1/4) + (1/4)}} = 0.768$$

TABLE 15. CALCULATED *t* VALUES.

SDS Concentration (mg/L)	i	t_i
0.25	2	0.768
0.5	3	1.721
1.0	4	2.349
2.0	5	9.792
4.0	6	24.446

1.13.2.7.6 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 (USEPA, 1995). For an overall alpha level of 0.05, 18 degrees of freedom for error and five concentrations (excluding the control) the critical value is 2.41. 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 2.0 mg/L and 4.0 mg/L concentrations have a significantly lower mean proportion of fertilized eggs than the control. Hence the NOEC is 1.0 mg/L SDS and the LOEC is 2.0 mg/L SDS.

1.13.2.7.7 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.

1.13.2.7.8 In this example,

$$MSD = 2.41\ (0.0608)\ \sqrt{(1/4)\ +\ (1/4)}$$

$$= 2.41\ (0.0608)(0.7071)$$

$$= 0.104$$

1.13.2.7.9 The MSD (0.104) 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.

$$1.342 - 0.104 = 1.238$$

2. Obtain the untransformed values for the control mean and the difference calculated in step 1, above.

$$[\ \text{Sine}\ (1.342)\ ]^2 = 0.9486$$

$$[\ \text{Sine}\ (1.238)\ ]^2 = 0.8936$$

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

$$MSD_u = 0.9486 - 0.8936 = 0.0553$$

1.13.2.7.10 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 0.0553.

1.13.2.7.11 This represents a 4.12% decrease in the proportion of fertilized eggs from the control.

1.13.2.8 Calculation of the ICp

1.13.2.8.1 The fertilization data in Table 9 are utilized in this example. As can be seen from Table 9 and Figure 11, the observed means are monotonically non-increasing with respect to concentration (mean response for each higher concentration is less than or equal to the mean response for the previous concentration, and the responses between concentrations follow a linear trend). Therefore, the means do not require smoothing prior to calculating the IC. In the following discussion, the observed means are represented by $\overline{Y}_i$ and the smoothed means by M_i.

1.13.2.8.2 Since $\bar{Y}_6 = 0.085 < \bar{Y}_5 = 0.633 < \bar{Y}_4 = 0.895 < \bar{Y}_3 = 0.910 < \bar{Y}_2 = 0.930 < \bar{Y}_1 = 0.945$, set $M_1 = 0.945$, $M_2 = 0.930$, $M_3 = 0.910$, $M_4 = 0.895$, $M_5 = 0.633$, and $M_6 = 0.085$. Table 16 contains the response means and smoothed means and Figure 11 gives a plot of the smoothed means.

1.13.2.8.3 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 0.709 where $M_1(1\text{-}p/100) = 0.945(1\text{-}25/100)$. Examining the means and their associated concentrations (Table 16), the response, 0.709, is bracketed by $C_4 = 1.0$ mg/L SDS and $C_5 = 2.0$ mg/L SDS.

TABLE 16. TROPICAL SEA URCHIN, *TRIPNEUSTES GRATILLA*, MEAN PROPORTION OF FERTILIZED EGGS.

SDS Conc. (mg/L)	i	Response Means, $_i$ (proportion)	Smoothed Means, M_i (proportion)
Control	1	0.945	0.945
0.25	2	0.930	0.930
0.5	3	0.910	0.910
1.0	4	0.895	0.895
2.0	5	0.633	0.633
4.0	6	0.085	0.085

1.13.2.8.4 Using the equation from Section 4.2 in Appendix L (USEPA, 1995), the estimate of the IC25 is calculated as follows:

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

Where: C_j = tested concentration whose observed mean response is greater than $M_1(1 - p/100)$

C_{j+1} = tested concentration whose observed mean response is less than $M_1(1 - p/100)$

M_1 = smoothed mean response for the control

M_j = smoothed mean response for concentration j

M_{j+1} = smoothed mean response for concentration j + 1

p = percent reduction in response relative to the control response

ICp = estimated concentration at which there is a percent reduction from the smoothed mean control response

$$IC25 = 1.0 + [0.945(1 - 25/100) - 0.895]\ \frac{(2.0 - 1.0)}{(0.633 - 0.895)} = 1.71 \text{ mg/L}$$

1.13.2.8.5 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 1.7095 mg/L. The empirical 95.0% confidence interval for the true mean was 1.5750 mg/L to 1.8631mg/L. The computer program output for the IC25 for this data set is shown in Figure 12.

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	0.25	0.5	1.0	2.0	4.0
Response 1	0.98	0.89	0.9	0.91	0.56	0.1
Response 2	0.96	0.92	0.88	0.88	0.65	0.04
Response 3	0.93	0.94	0.92	0.90	0.6	0.08
Response 4	0.91	0.97	0.94	0.89	0.72	0.12

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: SDS
Test Start Date: Test Ending Date:
Test Species: Tripneustes gratilla
Test Duration:
DATA FILE: icpout.icp
OUTPUT FILE: ICPout.i25

Conc. ID	Number Replicates	Concentration	Response Means	Std. Dev.	Pooled Response Means
1	4	0.000	0.945	0.031	0.945
2	4	0.250	0.930	0.034	0.930
3	4	0.500	0.910	0.026	0.910
4	4	1.000	0.895	0.013	0.895
5	4	2.000	0.633	0.069	0.633
6	4	4.000	0.085	0.034	0.085

The Linear Interpolation Estimate: 1.7095 Entered P Value: 25
Number of Resamplings: 80
The Bootstrap Estimates Mean: 1.7142 Standard Deviation: 0.0852
Original Confidence Limits: Lower: 1.5750 Upper: 1.8631
Expanded Confidence Limits: Lower: 1.4943 Upper: 1.9552
Resampling time in Seconds: 0.00 Random_Seed: -363164480

Figure 12. ICPIN program output for the IC_{25}.

1.14 **PRECISION AND ACCURACY**

1.14.1 PRECISION

1.14.1.1 Single-Laboratory Precision

1.14.1.1.1 Single-laboratory precision data (IC_{25} and IC_{50}) for *Tripneustes gratilla*, with the reference toxicant sodium dodecyl sulfate (SDS), tested in natural seawater is provided in Table 17. The coefficient of variation, based on the IC_{25}, is 8.2% , and IC_{50} is 11.6%, showing acceptable precision.

TABLE 17. SINGLE LABORATORY PRECISION OF THE TROPICAL SEA URCHIN, *TRIPNEUSTES GRATILLA* FERTILIZATION TEST WITH SODIUM DODECYL SULFATE (mg/L) AS THE REFERENCE TOXICANT AND 2500:1 SPERM:EGG RATIO. DATA FROM VAZQUEZ (2003).

Test Number	IC_{25} (mg/L)	IC_{50} (mg/L)
1	2.57	3.32
2	2.51	3.09
3	2.42	2.97
4	2.35	2.95
5	2.34	2.90
6	2.89	3.87
Mean CV (%)	2.5 8.2	3.2 11.6

1.14.1.2 Multi-laboratory Precision

1.14.1.2.1 In 2002-2003, EPA conducted an interlaboratory variability study of the Tropical Collector Urchin, *Tripneustes gratilla*, Fertilization Test Method. Participation in this study was limited to the number of labs that conducted this test in Hawaii at the time. In this study, each of the four participating labs tested four blind test samples that included some combination of effluent, inorganic reference toxicant, organic reference toxicant, and receiving water sample types. The effluent sample was a Hawaiian primary treated wastewater sample, spiked with $CuS0_4$; the inorganic reference toxicant consisted of seawater spiked with $CuS0_4$; the organic

reference toxicant consisted of seawater spiked with sodium dodecyl sulfate; and the receiving water sample was natural seawater from Hawaii, filtered to 0.2 μm. Of the 16 Tropical Collector Urchin, *Tripneustes gratilla*, fertilization tests conducted in this study, 75% were successfully completed and met the required test acceptability criteria and test conditions. Of the tests that were successfully completed on blank samples (Blind Sample 4), none showed false positive results for the fertilization endpoint. Results from the effluent, inorganic reference toxicant, and organic reference toxicant were used to calculate precision of the method. Table 18 shows the precision of the EC_{50} for each of these sample types. Averaged across sample types, the interlaboratory variability (expressed as %CV), was 25.5% for EC_{50} results. The range and mean CVs in this interlaboratory study fall in the range of "excellent" according to the criteria developed for West coast marine chronic toxicity tests, including a fertilization sea urchin test (BSAB, 1994).

1.14.2 ACCURACY

1.14.2.1 The accuracy of toxicity tests cannot be determined.

TABLE 18. MULTIPLE LABORATORY PRECISION OF THE TROPICAL SEA URCHIN, *TRIPNEUSTES GRATILLA*, FERTILIZATION TEST PERFORMED WITH VARIOUS SAMPLE TYPES. [1,2]

Test Date	Interlab Comparison	Laboratory	S:E Ratio	NOEC	EC50[3] (95% CI)	Coefficient of Variation
8/8/2002	Blind Sample 1 Effluent spiked w/copper	A B C D E (Referee)	2550:1 2332:1 905:1 2325:1	1.5% 0.38% F[5] <0.19% (S)[6] 0.38%	>6% (N/C)[4] **2.0%** F 0.61% (S) **1.8%**	7.4%
10/2/2002	Blind Sample 2 Copper	A B C D	3301:1 2428:1 2300:1 2645:1	>10ug/L (S) 0.625ug/L 2.5 ug/L 0.625 ug/L	>10 ug/L (S) **3.3 ug/L** **5.8 ug/L** **1.3 ug/L**	38.9%
2/4/2003	Blind Sample 3 Sodium Dodecyl Sulfate	A B C D	2524:1 2316:1 1137:1	6.25 mg/L 6.25mg/L F <6.25mg/L (S)	**10.85 mg/L** **16.72 mg/L** F 2.4 mg/L (S)	30.11%
5/1/2003	Blind Sample 4 Filtered Seawater	A B C D	 2572:1 504:1	F >100% F >100%	F N/C F N/C	N/C

[1] Mean interlaboratory CV = 25.5%. Interlaboratory variability based on first 3 samples for which CVs could be calculated (CVs could not be calculated for a receiving water sample since toxicity was not expected)

[2] Sperm to egg ratio of 2500:1 and test temperature of 23 $\pm$ 1 ^{o}C.

[3] Grey highlighted values are those that were used to calculate a % coefficient of variation (%CV)

[4] N/C-Indicates that the CV could not be calculated, because the laboratory did not observe a 50% effect

[5] F - Laboratory failed to run the test

[6] S- Laboratory did not use standard S:E as per study requirement (2500:1 S:E)

APPENDIX I. TROPICAL SEA URCHIN FERTILIZATION 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 pipets. Use hypersaline brine where necessary to maintain all test solutions at 34 $\pm$ 2 ‰. Include brine controls in tests that use brine.

C. Prepare a copper reference toxicant stock solution (10,000 µg/L) by adding 0.0268 g of copper chloride ($CuCl_2 \bullet 2H_2O$) to one liter of reagent water, in a polyethylene volumetric flask.

D. Prepare a control (0 µg/L) and at least five consecutive copper reference toxicant solutions. For example, make the dilution series from 0 (control), 5, 10, 20, 40, and 80 µg/L total copper by adding 0, 50 µL, 100 µL, 200 µL, 400 µL, and 800 µL of stock solution, respectively, to one hundred milliliter polyethylene volumetric flasks and fill with dilution water.

E. Alternatively, prepare a stock solution of 50 mg/L sodium dodecyl sulfate. Prepare a control (0 µg/L) and at least five consecutive SDS reference toxicant solutions. For example, make the dilution series from 0 (control), 0.38, 0.75, 1.5, 3.1, and 6.3 mg/L SDS by adding 0, 0.75, 1.5, 3.1, 6.3, and 12.5 mL of stock solution, respectively, to 100-mL volumetric flasks and fill with dilution water to 100 mL.

F. Dispense 5 mL of test solutions into at least 4 replicates per concentration. Randomize numbers for test chambers and record the chamber numbers, with their respective test concentrations, on a randomization data sheet. Store the data sheet safely until after the test samples have been analyzed.

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

H. Place test chambers in a water bath or environmental chamber set to 23^{o}C and allow temperature to equilibrate.

I. Measure the temperature in two or more 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, and pool good eggs and sperm.

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

D. Run trial sperm:egg fertilization test.

E. Adjust sperm density for definitive test.

F. Inject 0.1 mL sperm into test solutions.

G. 60 minutes later, inject 1.0 mL eggs into test solutions.

H. 20 minutes after egg addition, stop the test by the addition of 0.5 mL 0.02% glutaraldehyde preservative.

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

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

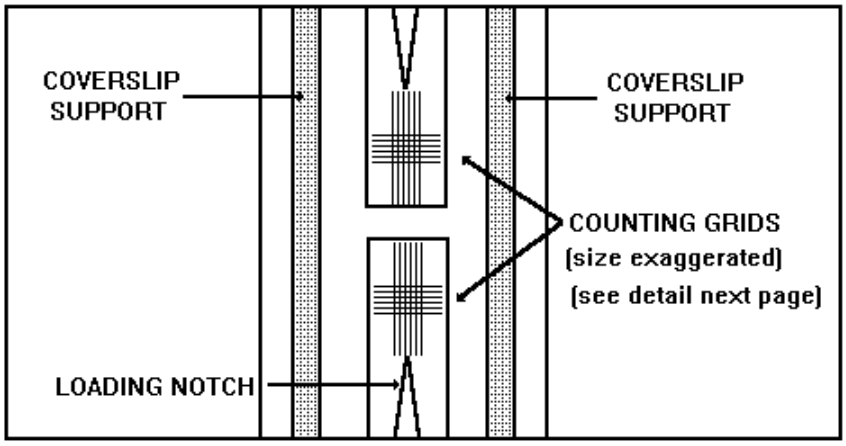
K. Analyze the data.

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

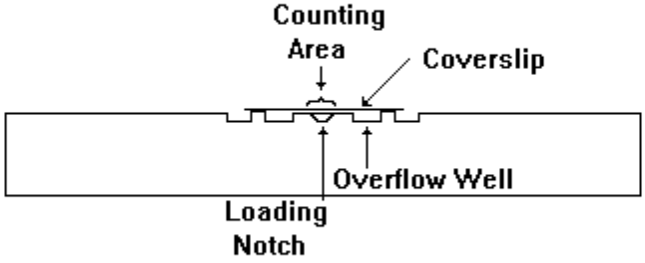
The Neubauer hemacytometer is a specialized microscope slide with two counting grids and a coverslip.

TOP VIEW:

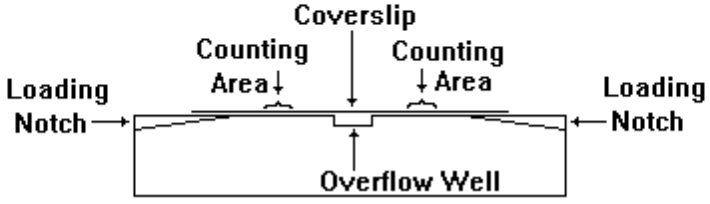


Together, the total area of each grid (1 mm^2) and the vertical distance between the grid and the coverslip (0.1 mm), provide space for a specific microvolume of aqueous sample (0.1 mm^3).

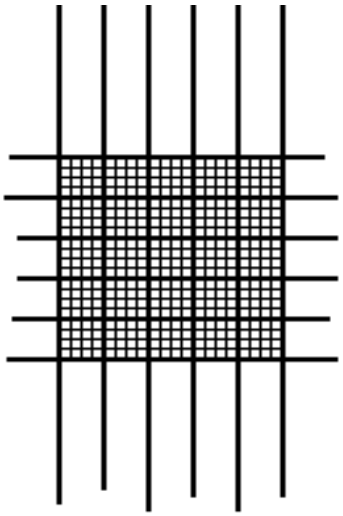
SIDE VIEW:



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.



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 sperm in 100 mL of dilution water), 80 squares counted, and a count of 100, the calculation becomes:

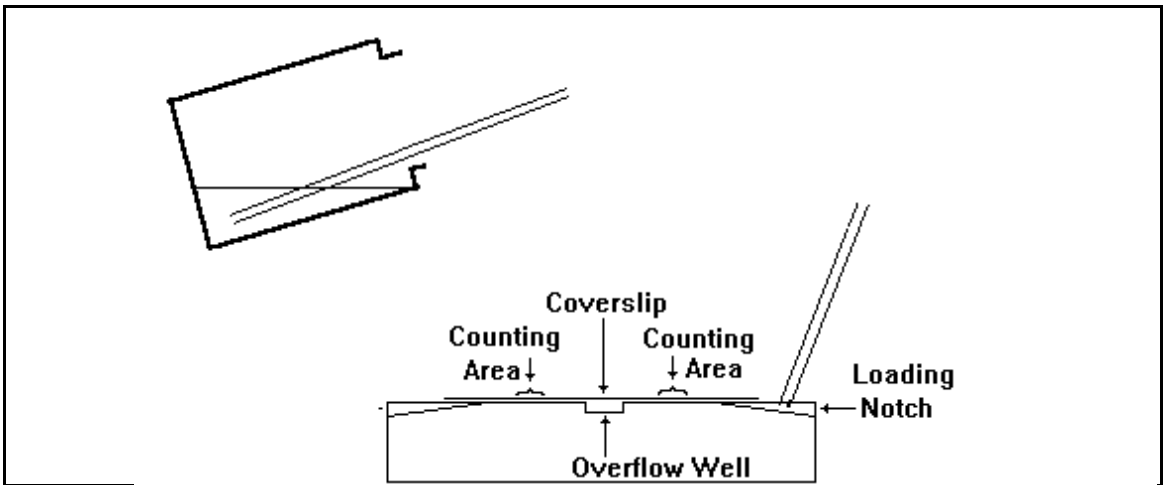
$$\text{cells/mL} = \frac{(4{,}000)\,(4{,}000)\,(1{,}000)\,(100)}{80}$$

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

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 or pipet, 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 or pipet 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 or pipet should be loaded onto the hemacytometer as rapidly as possible. Two replicate samples are withdrawn in fresh hematocrit tubes or pipets and loaded onto opposite sides of a hemacytometer.

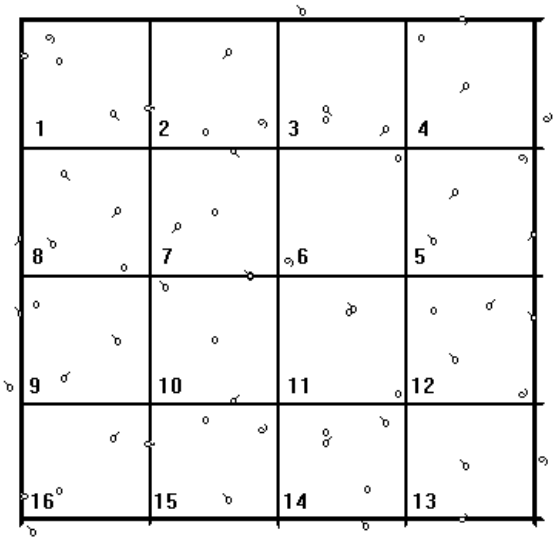


The loaded hemacytometer is left for 15 minutes to allow the sperm to settle onto the counting field. If the coverslip is moved after the samples are loaded, the hemacytometer should be rinsed and refilled with fresh sample. After 15 minutes, the hemacytometer is placed under a microscope and the counting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

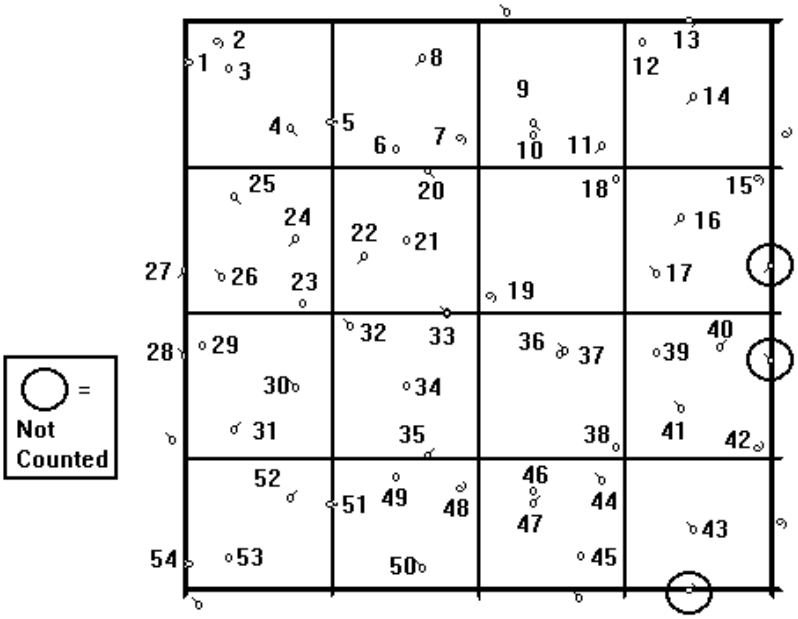
Examine the first large square in the selected corner. If no sperm are visible, or if the sperm are so dense or clumped to preclude accurate counting, count a sample with a more appropriate dilution.

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 proceeding 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).

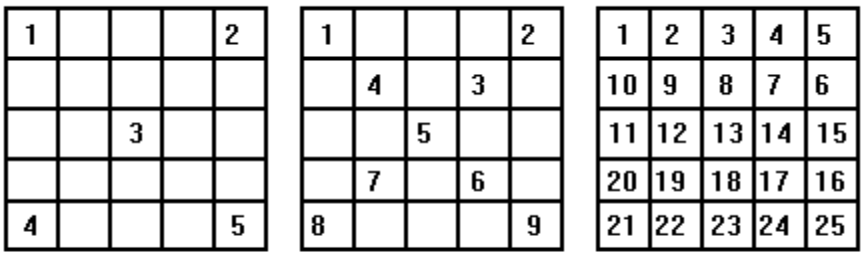
Once these counting conventions have been selected, it is advisable to follow another strict protocol outlining the number and sequence of large squares to be counted. Because the sperm may not be randomly distributed across the counting grid, it is recommended to count an array of squares covering the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

APPENDIX III. DEVELOPMENT OF STANDARD REGRESSION CURVE BETWEEN MICROSCOPIC SPERM COUNTS AND ABSORBANCE ON A SPECTROPHOTOMETER (Supplemental information to Section 1.10.5.5)

1. A rapid measurement using a spectrophotometer may be used to determine initial sperm density (Hall et al., 1993 and Vazquez, 2003). A least squares regression curve equation is developed by plotting microscope counts and 750 nm absorbance readings of sperm samples. Develop the standard curve specific to your laboratory, on a day when toxicity testing is not being performed.

2. A baseline collection of data must be developed over time from different batches of sperm, in an approximate range of 0.1-10 x 10^7 sperm/mL. It is acceptable to estimate dilutions (since there is no correct dilution), as long as the sperm are in the range of 0.1-10 x 10^7 sperm/mL. A 2000-fold dilution with seawater should be sufficient to dilute the sperm in the 10^7 sperm/mL range. The main objective is to get a range of dilutions so a regression plot can be developed.

3. First measure the absorbance of all dilutions on the spectrophotometer. Warm up the spectrophotometer for 30 minutes. Set the spectrophotometer at 750 nm absorbance and the same sensitivity every time (e.g. low, medium, high). It is preferable to use a low or medium setting, especially if a large zero adjustment is necessary. Zero the spectrophotometer using a cuvette filled with filtered seawater. Cover the sperm dilution with parafilm, invert the solution several times, and add the sperm solution to a cuvette. Wipe the cuvette with a Kimwipe, before putting the cuvette in the spectrophotometer (Note: fingerprints may alter the reading). Take an absorbance reading before the sperm settle.

4. Conduct a sperm count of all sperm dilutions on the microscope. To read dilutions on the microscope, you must kill the sperm with acetic acid. Do not add acetic acid until after spectrophotometer readings (acetic acid kills sperm and causes them to sink). Add 1 mL of well-mixed sperm dilution to a 9 mL solution of 1% acetic acid in seawater. If the acetic acid causes sperm clumping, try a more dilute concentration of acetic acid. Follow procedures in Appendix II and Section 1.10.5.6. Determine the concentration of sperm by using the following formula:

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

The dilution factor in this example is 10
hemacytometer conversion = 4000
mm3/mL = 1000
small squares counted = 80 or 400, see Appendix II

5. Prepare a regression curve by plotting sperm x 10^7/mL on the y-axis and absorbance (at 750 nm) on the x-axis. Determine whether there is a good correlation between spectrophotometric absorbance values and microscopic counts by looking at the R^2 value (R^2 must be ≥ 0.95). Generate a regression equation and use the equation for future testing to determine what sperm concentration the absorbance correlates to.

6. DO NOT use a curve generated by another lab, because spectrophotometers differ.

Worksheet for generating correlation between spectrophotometer readings and microscopic sperm count

Vial #	Microscopic Sperm Count	Absorbance Reading Spectrophotometer (750 nm)	Low, Medium, or High setting	Comments

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