APPENDIX V

AED LABORATORY OPERATING PROCEDURE FOR SEA URCHIN LARVAL DEVELOPMENT TEST

AED LABORATORY OPERATING PROCEDURE CONDUCTING THE SEA URCHIN LARVAL DEVELOPMENT TEST

POINT OF CONTACT:

Anne Kuhn-Hines
Diane Nacci
Atlantic Ecology Division
US Environmental Protection Agency
27 Tarzwell Dr.
Narragansett, RI 02882

1. OBJECTIVES

The purpose of the sea urchin larval development test is to determine the effects of effluents and water samples on survival, growth, and development of larvae of the sea urchin, Arbacia punctulata.

2. MATERIALS AND EQUIPMENT

- Facilities for holding and acclimating test organisms.
- Laboratory sea urchin culture unit -- See culturing LOP. To test effluent or receiving water toxicity, sufficient eggs and sperm must be available.
- Environmental chamber or equivalent facility with temperature control (20±1°C) for controlling temperature during exposure.
- Water purification system -- Millipore Super-Q, Deionized water (DI) or equivalent. - Balance -- Analytical, capable of accurately weighing to 0.000l g.
- Reference weights, Class S -- for checking performance of balance.
- Air pump -- for supplying air.
- Air lines, and air stones -- for aerating water containing adults.
- Vacuum suction device -- for washing eggs.
- pH and DO meters -- for routine physical and chemical measurements. Unless the test is being conducted to specifically measure the effect of one of the parameters, portable, field-grade instruments are acceptable.
- Transformer, 10-12 Volt, with steel electrodes -- for stimulating release of eggs and sperm.
- Centrifuge, bench-top, slant-head, variable speed -- for washing eggs.
- Fume hood -- to protect the analyst from formaldehyde fumes.
- Dissecting microscope -- for counting diluted egg stock.

- Compound microscope -- for examining and counting sperm cells and fertilized eggs.
- Compound microscope with CCD digital camera and low powered objectives (2-10x magnification) -- for use with image analyzer (quantification of growth endpoint).
- Cambridge Instruments Quantimet 520 image analyzer with IBM PC/AT (or equivalent) and video display -- for quantification of growth endpoint.
- Sedgwick-Rafter counting chamber -- for counting egg stock and final examination of larvae.
- Hemacytometer, Neubauer -- for counting sperm.
- Count register, 2-place -- for recording sperm and egg counts.
- Refractometer -- for determining salinity.
- Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.
- Thermometers, bulb-thermograph or electronic-chart type -- for continuously recording temperature.
- Ice bucket, covered -- for maintaining live sperm.
- Centrifuge tubes, conical, 15 mL -- for washing eggs.
- Cylindrical glass vessel, 8-cm diameter -- for maintaining dispersed egg suspension. - Beakers -- at least six Class A, borosilicate glass or non-toxic plasticware, 1000 mL for making test solutions.
- Glass dishes, flat bottomed, 20-cm diameter -- for holding adult urchins during gamete collection.
- Wash bottles -- for deionized water, for rinsing small glassware and instrument electrodes and probes.
- Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, l0-l000 mL for making test solutions.
- Syringes, 1-mL, and 10-mL, with 18 gauge, blunt-tipped needles (tips cut off) -- for collecting sperm and eggs.
- Pipets, volumetric -- Class A, 1-100 mL.
- Pipets, automatic -- adjustable, 1-100 mL.
- Pipets, serological -- 1-10 mL, graduated.
- Pipet bulbs and fillers -- PROPIPET®, or equivalent.
- Tape, colored -- for labelling tubes.
- Markers, water-proof -- for marking containers, etc.
- Sea Urchins (approximately 12 of each sex).
- Scintillation vials, 20 mL, disposable -- to prepare test concentrations.
- Parafilm -- to cover tubes and vessels containing test materials.
- Gloves, lab coat, disposable -- for personal protection from contamination.
- Safety glasses.
- Data sheets (one set per test) -- for data recording (Figure 1).
- Acetic acid, 10%, reagent grade, in sea water -- for preparing killed sperm dilutions.

- Formalin, 10% in seawater -- for preserving eggs.
- pH buffers 4, 7, and l0 (or as per instructions of instrument manufacturer) for standards and calibration check.
- Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms.
- Effluent, surface water, and dilution water.
- Saline test and dilution water -- The salinity of the test water must be 30‰. The salinity should vary by no more than ± 2‰ among the replicates.

3. PROCEDURE

A. Test Solutions

1. Samples are used directly as collected when sample salinity is between 28 and 32 parts per thousand. If samples do not require salinity adjustment natural seawater is used in all washing and diluting steps. Local uncontaminated water may be used as an additional control.

2. If salinity adjustment is required, prepare 3 L of control water at 30‰ using hypersaline brine (see Brine LOP). This water is used in all washing and diluting steps and as control water in the test. Natural sea water and uncontaminated local waters may be used as additional controls.

3. Effluent/receiving water samples are adjusted to salinity of 30 ^{o}/oo.

4. The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of 0.5 is used with this procedure, starting with a high concentration of 70% effluent (for freshwater effluents). If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used.

5. Three replicates are prepared for each test concentration, using 10 mL of solution in disposable liquid scintillation vials. A 50% (0.5) concentration series can be preparedby serially diluting test concentrations with control water.

6. All test samples are equilibrated at 20 ± 1°C before addition of sperm.

B. Collection and Preparation of Gametes for the Test

1. Select four females and place in shallow bowls, barely covering the shell with seawater. Stimulate the release of eggs by touching the test with electrodes from the transformer. Collect about 3 mL of eggs from each female using a syringe with a blunted needle. Remove the needle from the syringe before adding the eggs to a 15 mL conical centrifuge tube. Pool the eggs. The egg stock may be held at room temperature for several hours before use. Note: The egg suspension may be prepared during the l-h sperm exposure.

2. Select four males and place in shallow bowls, barely covering the animals with seawater. Stimulate the release of sperm by touching the shell with steel electrodes connected to a 12 V transformer (about 30 seconds each time). Collect the sperm (about 0.25 mL) from each male, using a l mL disposable syringe fitted with an 18-gauge, blunt-tipped needle. Maintain the syringe containing pooled sperm sample on ice. The sperm must be used in a toxicity test within 1 h of collection.

3. Using control water, dilute the pooled sperm sample to a concentration of about 5 X $l0^7$ sperm/mL (SPM). Estimate the sperm concentration as described below:

    a. Make a sperm dilutions of 1:50, 1:100, 1:200, and 1:400, using 30‰ seawater, as follows:

        1. Add 400 uL of collected sperm to 20 mL of sea water in Vial A. Mix by gentle pipetting using a 5-mL pipetter.

        2. Add 10 mL of sperm suspension from Vial A to 10 mL of seawater in Vial B. Mix by gentle pipetting using a 5-mL pipetter.

        3. Add 10 mL of sperm suspension from Vial B to 10 mL of seawater in Vial C. Mix by gentle pipetting using a 5-mL pipetter.

        4. Add 10 mL of sperm suspension from Vial C to 10 mL of seawater in Vial D. Mix by gentle pipetting using a 5-mL pipetter.

        5. Discard 10 mL from Vial D. (The volume of all suspensions is 10 mL).

b. Make a 1:2000 killed sperm suspension and determine the SPM.

1. Add 10 mL 10% acetic acid in seawater to Vial C. Cap Vial C and mix by inversion.

2. Add 1 mL of killed sperm from Vial C to 4 mL of seawater in Vial E. Mix by gentle pipetting with a 5-mL pipetter.

3. Add sperm from Vial E to both sides of the Neubauer hemacytometer. Let the sperm settle 15 min.

4. Count the number of sperm in the central 400 squares on both sides of the hemacytometer using a compound microscope (400X). Average the counts from the two sides.

5. SPM in Vial E = 10^4 x average count.

c. Calculate the SPM in all other suspensions using the SPM in Vial E above:

SPM in Vial A = 40 x SPM in Vial E
SPM in Vial B = 20 x SPM in Vial E
SPM in Vial D = 5 x SPM in Vial E
SPM in original sperm sample = 2000 x SPM in Vial E

d. Dilute the sperm suspension with a concentration greater than 5 x 10^7 SPM to 5 x 10^7 SPM.

Actual SPM/(5 x 10^7) = dilution factor (DF)

[(DF) x 10] - 10 = mL of seawater to add to vial.

4. Wash the pooled eggs three times using control water with gentle centrifugation (500xg or the lowest possible setting) for 3 min using a tabletop centrifuge). If the wash water becomes red, the eggs have lysed and must be discarded.

a. Dilute the egg stock, using control water, to about 2000 $\pm$ 200 eggs/mL.

1. Remove the final wash water from the eggs and transfer the washed eggs (by refilling the centrifuge tube with control water and repeatedly inverting to resuspend the eggs) to a beaker containing a small amount (about 50 mL) of control water. Add control water to bring the eggs to a volume of 200 mL ("egg stock").

2. Mix the egg stock using gentle aeration. Cut the point from a pipet tip and transfer l mL of eggs from the egg stock to a vial containing 9 mL of control water. (This vial contains an egg suspension diluted l:l0 from egg stock).

3. Mix the contents of the vial using gentle pipetting. Cut the point from a pipet tip and transfer l mL of eggs from the vial to a Sedgwick-Rafter counting chamber. Count all eggs in the chamber using a dissecting microscope at l0X ("egg count").

4. Calculate the concentration of eggs in the stock. Eggs/mL = l0x (egg count). Dilute the egg stock to 2000 eggs mL by the formula below.

b. If the egg count is equal to or greater than 200:

(egg count) - 200 = volume (mL) of control water to add to egg stock

c. If the egg count is less than 200, allow the eggs to settle and remove enough control water to concentrate the eggs to greater than 200, repeat the count, and dilute the egg stock as above. 100 mL of egg stock are required to perform this test.

d. Transfer 1 mL of the diluted egg stock to a vial containing 9 mL of control water. Mix well, then transfer 1 mL from the vial to a Sedgwick-Rafter counting chamber. Count all eggs using a dissecting microscope. Confirm that the final egg count = 200/mL $\pm$ 20.

5. Mix the egg stock well, subsample 100 mL, and place the subsample in a clean beaker. Add 10 mL of the proper sperm dilution to the beaker and mix well. This

will result in a egg:sperm ratio of 1:2500, which should allow acceptable egg fertilization 1 hr after sperm addition.

C. Start of the Test

1. Mix the diluted embryo suspension (2000 embryos/mL), using gentle aeration. Add 1 mL of diluted egg suspension to each test vial using a wide mouth pipet tip. Incubate covered for 48 hours 20 $\pm$ 1^{o}C.

D. Termination of the Test

1. Terminate the test and preserve the samples by adding 2 mL of l0% formalin in seawater to each vial.

2. Vials may be evaluated immediately or capped and stored for as long as one week before being evaluated.

3. Each vial is thoroughly mixed and a 1 mL aliquot added to a Sedgwick-Rafter counting chamber for microscopic observation and image analysis. The total number of larvae and of appropriately developed larvae (pluteii) are counted to determine survival and development for each treatment. Fifty larvae per replicate are also observed using the image analysis system and measured for maximum length, total area, and shape (a function relating observed shape to that of a circle).

4. QA/QC

A. STATISTICAL ANALYSIS AND DATA USAGE

1. Tabulate and summarize the data.

2. An estimate of the effluent concentration which would cause a 50% toxic effect (EC50) for each parameter is calculated using Trimmed Spearman-Karber analysis (Hamilton, Russo, and Thurston, 1977). One-way analysis of variance (ANOVA) followed by Dunnett's Procedure (Dunnett, 1955) is used to compare single

treatments to the control in order to estimate no effect and least effect concentrations (NOEC and LOEC values).

3. Data are used along with other toxicity tests in assessing the toxicity of an effluent or receiving water.

5. TROUBLE SHOOTING

1. Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment.

6. REFERENCES

Dunnett, C.W. 1955. A multiple comparisons procedure for comparing several treatments with a control. JASA 50:1096-1101.

Hamilton, M.A., R.C. Russo, and R.V. Thurston. 1977. Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. Environ. Sci. Technol. 11(7):714-719.

US EPA. 1988. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms. Weber, C.I., et al (eds). EPA Office of Research and Development EPA-600/4-87/028 (May 1988).