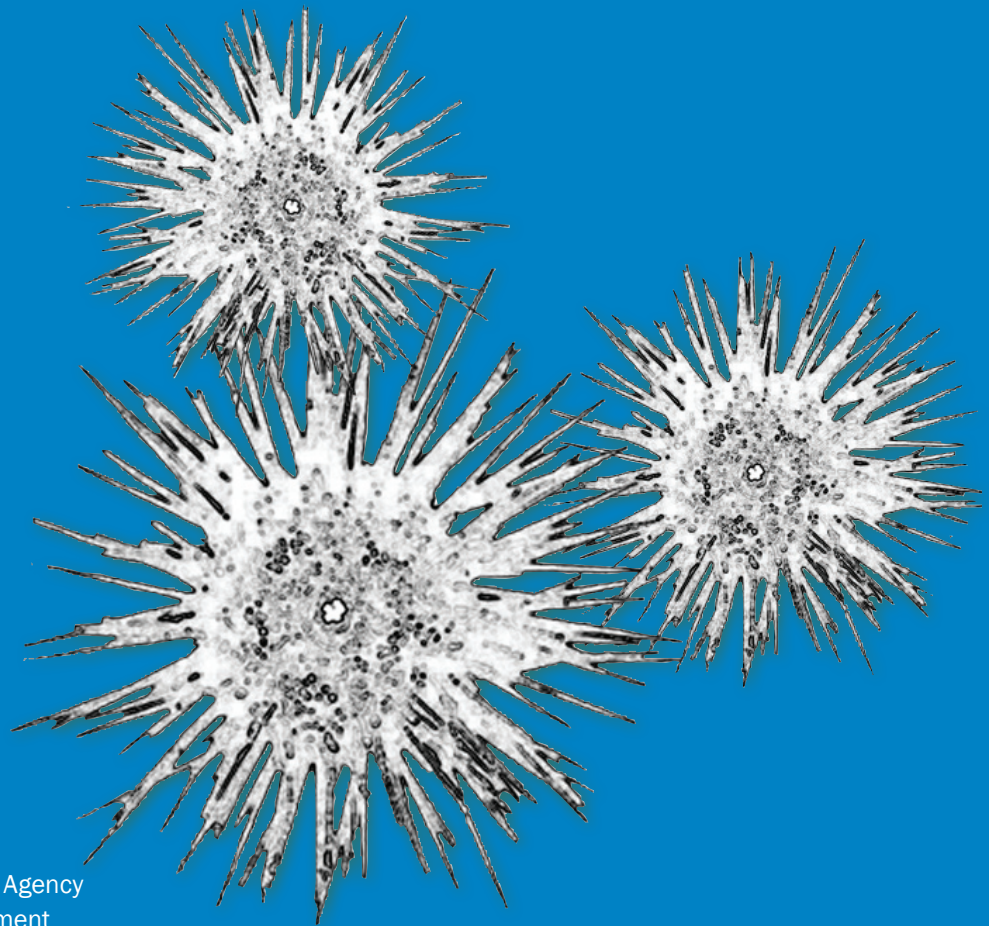




# Sperm Cell Toxicity Tests Using the Sea Urchin (*Arbacia punctulata*)

## Supplement to Training Video



U.S. Environmental Protection Agency  
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## NOTICE

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## Foreword

This guide serves as a supplement to the video “Sperm Cell Toxicity Tests Using the Sea Urchin, *Arbacia punctulata*” (EPA, 2009). The methods illustrated in the video and described in this supplemental guide support the methods published in the U.S. Environmental Protection Agency’s (EPA’s) *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms, Third Edition* (EPA, 2002a), referred to as the Saltwater Chronic Methods Manual. The video and this guide provide details on preparing for and conducting the test based on the expertise of personnel at the following EPA Office of Research and Development (ORD) laboratories:

National Health and Environmental Effects Research Laboratory (NHEERL) – Atlantic Ecology Division in Narragansett, Rhode Island

NHEERL – Gulf Ecology Division in Gulf Breeze, Florida

National Exposure Research Lab (NERL) – Ecological Exposure Research Division (EERD) in Cincinnati, Ohio

This guide and its accompanying video are part of a series of training videos produced by EPA’s Office of Wastewater Management. This Saltwater Series includes the following videos and guides:

“Mysid (*Americamysis bahia*) Survival, Growth, and Fecundity Toxicity Tests”

“Culturing *Americamysis bahia*”

“Sperm Cell Toxicity Tests Using the Sea Urchin, *Arbacia punctulata*”

“Red Algal (*Champia parvula*) Sexual Reproduction Toxicity Tests”

“Sheepshead Minnow (*Cyprinodon variegatus*) and Inland Silverside (*Menidia beryllina*) Larval Survival and Growth Toxicity Tests”

The Freshwater Series, released in 2006, includes the following videos and guides:

“*Ceriodaphnia* Survival and Reproduction Toxicity Tests”

“Culturing of Fathead Minnows (*Pimephales promelas*)”

“Fathead Minnow (*Pimephales promelas*) Larval Survival and Growth Toxicity Tests”

All of these videos are available through the National Service Center for Environmental Publications (NSCEP) at 800 490-9198 or nscep@bps-lmit.com.



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## Introduction

This supplemental guide accompanies the Environmental Protection Agency's (EPA's) video training for conducting sea urchin (*Arbacia punctulata*) fertilization toxicity tests (EPA, 2009). The test method is found in Section 15 of EPA's *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms, Third Edition* (EPA, 2002a). The test was developed at EPA's Office of Research and Development's (ORD's) National Health and Environmental Effects Research Laboratory-Atlantic Ecology Division (NHEERL-AED) in Narragansett, Rhode Island, and is based on the freshwater tests developed at the EPA Mid-Continent Ecology Division (MED) in Duluth, Minnesota. The material presented in both the video and this guide summarizes the methods but does not replace a thorough review and understanding of the methods by laboratory personnel before conducting the test.

## Background

Under the National Pollutant Discharge Elimination System (NPDES) program (Section 402 of the Clean Water Act), EPA uses toxicity tests to monitor and evaluate effluents for their toxicity to biota and their impact on receiving waters. By determining acceptable or safe concentrations for toxicants discharged into receiving waters, EPA can establish NPDES permit limitations for toxicity. These WET (Whole effluent toxicity) permit limitations regulate pollutant discharges on a whole effluent effect basis rather than by a chemical-specific approach only.

Whole effluent toxicity methods measure the synergistic, antagonistic, and additive effects of all the chemical, physical, and additive components of an effluent that adversely affect the physiological and biochemical functions of the test organisms. Therefore, healthy organisms and correct laboratory procedures are essential for valid test results. Laboratory personnel should be very familiar with the test methods and with sea urchin handling techniques before conducting a test.

This supplemental guide covers the procedures for conducting the test according to EPA's promulgated methods (*40 CFR Part 136*; EPA, 2002c) and also provides some helpful information that is not presented in the *Saltwater Chronic Methods Manual* (EPA, 2002a).

This test method examines the effect of effluent or receiving waters on the reproduction of sea urchin gametes after exposure in a static system for 1 hour and 20 minutes. Sperm cells are exposed to a series of effluent concentrations for 1 hour. The eggs are then introduced to the test chambers which contain the sperm cells. After 20 minutes, the test is ended and the effects on exposed gametes are compared to controls to determine if the effluent concentrations had any effect on fertilization.

This guide and the accompanying video describe how the test is set up, initiated, terminated, and reviewed, including suggestions on maintaining healthy cultures of test animals.

## Water and Light

### OBTAINING AND MAINTAINING SEA URCHINS

Before conducting tests, healthy sea urchin cultures should be established. Adult sea urchins can be ordered from commercial biological supply houses, or collected along the Atlantic coast. Keep male and female animals in separate tanks. To determine the sex of each animal, briefly stimulate each with a 12-volt transformer. This causes the immediate release of masses of gametes from genital pores on the top of the animal. The eggs are red and the sperm are white. Separate the animals into 20 L aerated fiberglass tanks; each can hold about 20 adults.

## CULTURE WATER

The quality of water used for maintaining sea urchins is very important. Culture water and all water used for washing and dilution steps and for control water in the tests should be maintained at a salinity of  $30‰ \pm 2‰$  using natural seawater, hypersaline brine (HSB), or artificial sea salts. Instructions for making dilution water and HSB are provided in Appendix A of this document and Section 7 of the Saltwater Chronic Methods Manual (EPA, 2002a).

## PHOTOPERIOD

The sea urchin conditions should include a photoperiod of 16 hours light and 8 hours darkness. The light quality and intensity should be at ambient laboratory levels, which is approximately  $10 - 20 \text{ E}/\mu\text{m}^2/\text{s}$  or 50 to 100 foot candles (ft-c) (EPA, 2002a).

## CULTURE VESSELS

Adult sea urchins are kept in natural or artificial seawater in a flow-through or recirculating aerated 40-L glass aquarium.

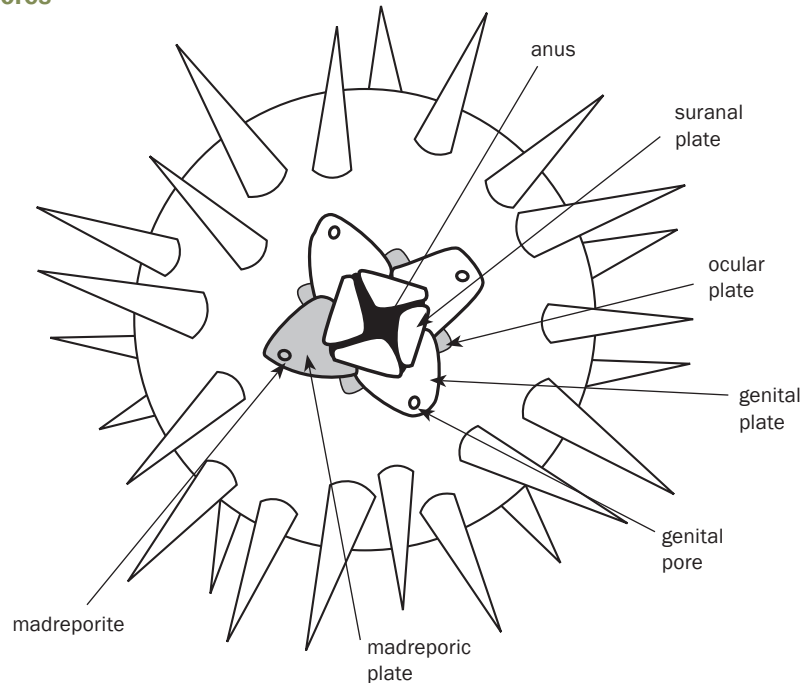
Allow filtered seawater to flow into the tanks at a rate of 5 L per minute and maintain the temperature at  $15^\circ\text{C} \pm 3^\circ\text{C}$ .

## WATER DELIVERY SYSTEMS

Equip the adult sea urchin aquarium with an under-gravel or outside biological filter, or cartridge filter. A stock of at least 12 males and 12 females are needed for routine testing. If the animals will be used for an on-site test, transport them separated by sex in separate or partitioned coolers packed with wet kelp and paper towels. Once on site, the sea urchins should be transferred into separate 10-gallon aquarium tanks with gravel-bed filtration. Even with filtration, the water should be changed periodically to maintain good water quality.

Collect eggs first to avoid any possible pre-fertilization.

**Figure 1. Schematic of the aboral surface of *Arbacia punctulata*, with spines partly removed to show structure, especially the genital pores**



## FOOD PREPARATION

Sea urchins are fed kelp of the species *Laminaria* obtained from uncontaminated coastal waters or ordered from commercial supply houses, or romaine lettuce. Supply the urchins with ample food, renewing the kelp each week and removing decaying kelp as necessary. Healthy sea urchins will attach to kelp or aquarium walls within hours — any unhealthy animals should be removed and should not be used for testing. Every 1 to 2 weeks, empty and clean the tanks.





## Test Method

### OBTAINING GAMETES

To prepare for the test, all vials, pipets, and pipet tips should be soaked in clean, 30‰ seawater overnight. Collect eggs and sperm from healthy animals by transferring the animals into a shallow bowl filled with enough control seawater to just cover their shells. Eggs are obtained from female sea urchins using electrical stimulation by touching the shells close to the genital pores with electrodes from a 10 – 12-volt transformer for about 30 seconds.

*At AED, staff use the data sheet included in Appendix E for calculating and recording dilutions.*

The red eggs pool on the sea urchin shell above the genital pores. These are collected from the shell using a 10 mL disposable syringe with an 18-gauge, blunt-tipped needle with the tip cut off so that it will rest on the shell without puncturing it. After collection, the needle

is removed and the eggs emptied into conical centrifuge tubes. Pool the eggs and keep them at room temperature until use, but not longer than a few hours. Four females should yield enough eggs to test five test dilutions plus one control, with four replicates.

Obtain sperm from four male sea urchins. Again, place the animals in a shallow bowl with their shells barely covered with control seawater. Like the females, the males are induced to spawn by placing electrodes from a 10 – 12-volt transformer against their shells for 30 seconds. The sperm appear white. Collect the concentrated sperm that pools on top of the shell using a syringe fitted with an 18-gauge, blunt-tipped needle. Pool the sperm, keep the sample on ice, and record the collection time. The sperm must be used in a toxicity test within 1 hour of collection.

### MAKING STOCK SOLUTIONS OF SPERM AND EGGS

To ensure reproducibility in the test results, the sperm and eggs must be concentrated to known dilutions using the 30‰ seawater. During the exposure period, 2,500 sperm should be present for every one egg. Figure E-1, presented in Appendix E, provides a sample data sheet used to calculate the sperm and egg deliveries.

After collection, the sperm should be in a volume of about 0.5 to 1 mL of control water in the collecting syringe. This is called the “sperm stock” solution. Perform a 50 percent serial dilution for counting the sperm cell density using the following dilution method (see Table 1).

Add sperm from Vial E to both sides of a Neubauer hemacytometer. Let the sperm settle 15 minutes. Count the number of sperm in the central 400 squares on both sides of the hemacytometer under a compound microscope (100X).

The average of the two sperm cell counts (sperm/mL or SPM) from Vial E = # x 10<sup>4</sup>.

Calculate the SPM in all the other suspensions based on this count:

*The egg solution can be prepared during the first hour of the test after the sperm exposure has started.*

Vial A = 40 x SPM of Vial E

Vial B = 20 x SPM of Vial E

Vial D = 5 x SPM of Vial E

SPM of original sample = 2000 x SPM of Vial E

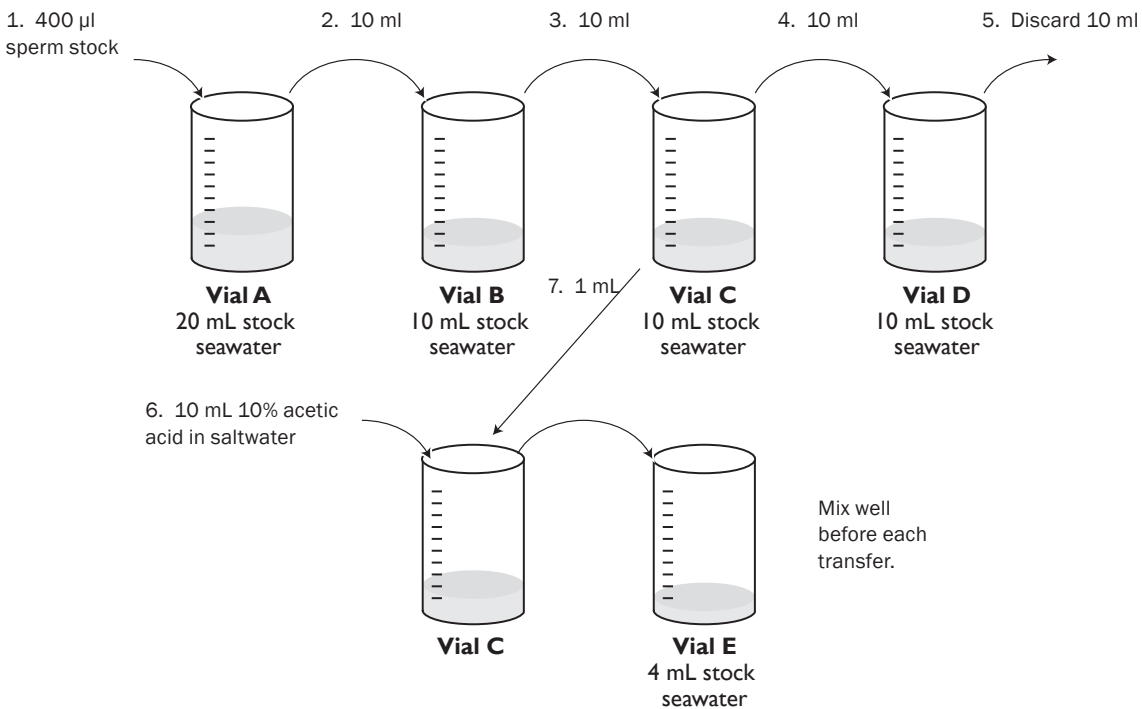
To prepare the sperm suspension for the test, select the vial containing an SPM greater than 5 x 10<sup>7</sup> SPM. To determine the dilution needed for the test:

$\frac{\text{The calculated SPM}}{(5 \times 10^7)}$  = Dilution Factor (DF)

$[(DF) \times 10] - 10$  = mL of seawater to add to selected vial

**Table 1. Fifty Percent Serial Dilution Method for Counting Sperm Cell Density.**

1.	Add 400 $\mu$ L of sperm stock to 20 mL of seawater to create Vial A. Mix by gently pipetting using a 5-mL pipettor, or by inversion.
2.	Add 10 mL from Vial A to 10 mL of seawater to create Vial B. Mix by gently pipetting using a 5-mL pipettor, or by inversion.
3.	Add 10 mL from Vial B to 10 mL of seawater to create Vial C. Mix by gently pipetting using a 5-mL pipettor, or by inversion.
4.	Add 10 mL from Vial C to 10 mL of seawater to create Vial D. Mix by gently pipetting using a 5-mL pipettor, or by inversion.
5.	Discard 10 mL from Vial D so that all vials now contain 10 mL.
6.	Vial C is used to create a final dilution that is killed and counted. Add 10 mL 10% acetic acid in seawater to Vial C; cap the vial and mix by inversion.
7.	Add 1 mL of the killed sperm in Vial C to 4 mL of seawater in Vial E. Mix by gently pipetting using a 4-mL pipettor.



The sperm cell count in the test stock should be confirmed. Add 0.1 mL of test stock to 9.9 mL of 10 percent acetic acid in seawater and count the sperm cells using a hemacytometer. This count should average  $50 \pm 5$  cells. Only about 2.5 mL of sperm test stock solution is needed for testing 5 test solutions and a control, with 4 or more replicates. Hold the test stock on ice until the test begins, but no longer than 1 hour.

The eggs must be washed before preparing the standard egg dilution needed for the test (2,000 eggs/mL). To wash the eggs, first remove the supernatant water from the settled eggs. Add seawater and mix carefully by inversion. Spin the vial in a tabletop centrifuge at the lowest possible setting (e.g., 500xg) for 3 minutes to form a lightly packed pellet. Wash and spin the eggs twice more. If at any time the wash water appears red the eggs are lysing (the membranes have been disturbed) and the eggs are unsuitable for testing; discard these eggs and start again.

After washing, transfer the washed eggs to a beaker containing 200 mL of control seawater. This is called the "egg test stock." Mix the stock solution using gentle aeration until the egg solution is homogenous. The aeration device used in Narragansett is a 3-pronged diffuser attached by flexible tubing to an air pump.



Make a 1:10 dilution of the test stock for the purpose of counting the eggs. Cut the point from a wide-mouth pipet tip to make sure the eggs will not be damaged and transfer 1 mL of egg solution to a vial containing 9 mL of control water. Mix by inversion.

Transfer 1 mL of the egg solution to a Sedgewick-Rafter counting chamber. Count the number of eggs under a dissecting microscope at 25X magnification. Ten times the number of eggs in that milliliter equals the number of eggs/mL in the egg stock. The target concentration for test initiation is 2,000 eggs/mL.

If the egg count is greater than or equal to 200 eggs, add the proper volume of water:

$$(\# \text{ of eggs counted}) - 200 = \text{volume (mL) of control water to add}$$

If less than 200 eggs were counted, allow the eggs to settle in the beaker, remove the supernatant water to concentrate the eggs to greater than 200, repeat the count, and dilute the egg test stock as described above.

Verify the concentration by counting 1 mL of a 1:10 dilution of the adjusted stock solution. The count for the final dilution should equal  $100 \pm 20$  eggs/mL. The test requires 24 mL of egg test stock for a control and five exposure concentrations.

## EFFLUENT PREPARATION

Effluent sampling should be conducted according to Section 8 of the Saltwater Chronic Methods Manual (EPA, 2002a) and any specific requirements of a NPDES permit. The effluent or receiving waters should be held at  $0^{\circ}\text{C} - 6^{\circ}\text{C}$  until used for testing. Under the NPDES program, lapsed time from sample collection to first use in the test must not exceed 36 hours. Under special conditions or variances, samples may be held longer but should never be used for testing if held for more than 72 hours.

Maintain the salinity of the test samples to  $30\text{‰} \pm 2\text{‰}$ . To do this, effluent samples may need to be adjusted using hypersaline brine (HSB). A recipe for HSB is provided in Appendix A of this manual.

Approximately 1 hour before the test is to begin, adjust approximately 1 L of effluent to the test temperature of  $20^{\circ}\text{C} \pm 1^{\circ}\text{C}$  and maintain that temperature while preparing the test concentrations. To test a series of decreasing concentrations of effluent, use a dilution factor of  $\geq 0.5$ . When starting with effluent that has  $0\text{‰}$  salinity and using HSB, the maximum effluent concentration that can be prepared at  $30\text{‰}$  is 70 percent effluent. Table A-1 presents the volumes needed for the test concentrations using HSB.

### Dilution Water

The type of dilution water used to make the test concentrations is dependent on the objectives of the test. Any specific requirements included in NPDES permits should be followed. The Saltwater Chronic Methods Manual (Section 7) provides the following guidelines:

- If the test is conducted to estimate the **absolute chronic toxicity of the effluent**, synthetic dilution water should be used. If the cultures were maintained in different water than used for dilution water, a second set of control replicates should be conducted using the culture water.
- If the test is conducted to estimate the **chronic toxicity of the effluent in uncontaminated receiving waters**, the test can be conducted using a grab sample of the receiving waters collected outside the influence of the outfall, other uncontaminated waters, or standard dilution water with the same salinity as the receiving waters. If the cultures were maintained in different water than used for dilution water, a second set of control replicates should be conducted using the culture water.
- If the test is conducted to estimate the **additive or mitigating effects of the effluent on already contaminated receiving waters**, the test must be conducted using receiving waters collected outside the influence of the outfall. Controls should be conducted using both receiving water and culture water.



## BEGINNING THE TEST

In Narragansett, disposable glass vials are used as test chambers. They are labeled with concentration and replicate numbers and arranged in the partitioned cardboard box in which they are shipped. Prepare the effluent dilutions for four replicates of each concentration and the control solution to reduce variability among replicates. Each concentration should be prepared in one beaker and 5 mL distributed to each of the test chambers. Be sure the effluent temperature has been brought up to 20°C before beginning the test.

Within 1 hour of collecting and preparing the sperm test stock, add 100 µL of the well-mixed sperm test stock to each test and control vial. Cover the chambers, record the time, and maintain the chambers at 20°C ± 1°C for 1 hour.

At the end of the hour, mix the egg test stock using gentle aeration and add 1 mL of the egg solution to each exposure vial using a wide-mouth pipet. When all of the vials contain eggs, lift the storage box and gently move it in circles to “swirl” the egg-sperm suspension. Cover the chambers, record the time, and incubate the eggs and sperm at 20°C ± 1°C for 20 minutes.

## ROUTINE CHEMISTRIES

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

## TERMINATING THE TEST

After 20 minutes, end the test and preserve the samples by adding 2 mL of 1% formalin in seawater to each vial. Cap the vials and record the time. The test should be evaluated immediately but can be evaluated up to 48 hours later.

## Test Acceptability and Data Review

This test demonstrates the effluent or receiving water’s effect on sea urchin fertilization. To evaluate this, exposed and control eggs are examined under a microscope and the number of unfertilized eggs in each test chamber is recorded.

For each replicate, transfer about 80 – 120 µL of the preserved eggs to a multiple-chamber counting slide. If a Sedgewick-Rafter counting chamber is used, transfer about 1 mL. Using a compound microscope at 100X magnification, observe 100 – 200 eggs per sample. This should be done with adequate ventilation, preferably under a hood, to reduce exposure to the formalin fumes.

For each test chamber, record the total number of eggs counted, and the number that were not fertilized. Fertilized eggs are surrounded by a fertilization membrane, while unfertilized eggs lack this membrane. Abnormal eggs are not counted. Figure E-2 in Appendix E provides a sample data collection sheet.

Test data are reviewed to verify that test acceptability criteria (TAC) requirements for a valid test have been met. For the test to be acceptable, the control chambers are required to have between 70% and 90% fertilization of the eggs. The concentration-response relationship generated for each multi-concentration test must be reviewed to ensure that calculated test results are interpreted appropriately. In conjunction with this requirement, EPA has provided recommended guidance for concentration-response relationship review (EPA, 2000b).

EPA’s promulgated toxicity testing method manuals (EPA, 2002a, b) recommend the use of point estimation technique approaches for calculating endpoints for effluent toxicity tests under the NPDES program. The promulgated methods also require a data review of toxicity data and concentration-response data, and require calculating the percent minimum significant difference (PMSD) when point estimation (e.g., LC<sub>50</sub>, IC<sub>25</sub>) analyses are not used. EPA specifies the PMSD must be calculated when NPDES permits require sub-lethal hypothesis testing. EPA also requires that variability criteria be applied as a test review step when



NPDES permits require sub-lethal hypothesis testing endpoints (i.e., no observed effect concentration [NOEC] or lowest observed effect concentration [LOEC]) and the effluent has been determined to have no toxicity at the permitted receiving water concentration (EPA, 2002b). This reduces the within-test variability and increases statistical sensitivity when test endpoints are expressed using hypothesis testing rather than the preferred point estimation techniques.

The sea urchin sperm cell test is currently used to assess the potential toxic effects of complex chemical mixtures on marine and estuarine organisms. Used in conjunction with chemical-specific methods, this test can provide a comprehensive and effective approach to assessing the impact of complex effluents discharged to the marine and estuarine environments.

## Citations and Recommended References

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EPA references are available online at [www.epa.gov/npdes](http://www.epa.gov/npdes).

If you need additional copies of this document, you can download it at:  
[www.epa.gov/npdes/wqbasedpermitting](http://www.epa.gov/npdes/wqbasedpermitting).



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## Glossary

**Acute toxicity.** An adverse effect measured on a group of test organisms during a short-term exposure in a short period of time (96 hours or less in toxicity tests). The effect can be measured in lethality or any variety of effects.

***Arbacia punctulata.*** A species of *Arbacia* genus of purple-spined sea urchins. Its natural habitat is in the Western Atlantic Ocean. *Arbacia punctulata* can be found in shallow water from Massachusetts to Cuba and the Yucatan Peninsula, from Texas to Florida in the Gulf of Mexico, the coast from Panama to French Guiana and in the Lesser Antilles, usually on rocky, sandy, or shelly bottoms.

**Chronic toxicity.** An adverse effect that occurs over a long exposure period. The effect can be lethality, impaired growth, reduced reproduction, etc.

**Diluent water.** Dilution water used to prepare the effluent concentrations.

**Effluent concentrations.** Concentrations or dilutions of an effluent sample to which test organisms are exposed to determine the biological effects of the sample on the test organism.

**Effluent sample.** A representative collection of the discharge that is to be tested.

**Flow-through water delivery system.** An open water flow system that delivers fresh water or seawater to culture tanks and is disposed of after it leaves those tanks.

**Hypothesis testing.** Technique (e.g., Dunnett's test) that determines what concentration is statistically different from the control. Endpoints determined from hypothesis testing are NOEC and LOEC.

**IC<sub>25</sub> (Inhibition Concentration, 25%).** The point estimate of the toxicant concentration that would cause a 25% reduction in a non-quantal biological measurement (e.g., reproduction or growth) calculated from a continuous model.

***Laminaria.*** The scientific name for a species of kelp given as food to laboratory sea urchins.

**LC<sub>50</sub> (Lethal Concentration, 50%).** The concentration of toxicant or effluent that would cause death to 50% of the test organisms at a specific time of observations (e.g., 96-hour LC<sub>50</sub>).

**Lowest Observed Effect Concentration (LOEC).** The LOEC is the lowest concentration of toxicant to which organisms are exposed in a test, which causes statistically significant adverse effects on the test organisms (i.e., where the values for the observed endpoints are statistically significantly different from the control). The definitions of NOEC and LOEC assume a strict dose-response relationship between toxicant concentration and organism response.

**Minimum Significant Difference (MSD).** The MSD is the magnitude of difference from the control where the null hypothesis is rejected in a statistical test comparing a treatment with a control. MSD is based on the number of replicates, control performance and power of the test. MSD is often measured as a percent and referred to as PMSD.

**No Observed Effect Concentration (NOEC).** The NOEC is the highest tested concentration of toxicant to which organisms are exposed in a full life-cycle or partial life-cycle (short-term) test, that causes no observable adverse effect on the test organism (i.e., the highest concentration of toxicant at which the values for the observed responses are not statistically significantly different from the controls). NOECs calculated by hypothesis testing are dependent upon the concentrations selected.



**NPDES (National Pollutant Discharge Elimination System) Program.** The national program for issuing, modifying, revoking, and reissuing, terminating, monitoring and enforcing permits, and imposing and enforcing pretreatment requirements, under Sections 307, 318, 402, and 405 of the Clean Water Act.

**Point Estimation Techniques.** This technique is used to determine the effluent concentration at which adverse effects (e.g., fertilization, growth or survival) occurred, such as Probit, Interpolation Method, Spearman-Kärber. For example, a concentration at which a 25% reduction in reproduction and survival occurred.

**Receiving Water Concentration (RWC).** The RWC is the concentration of a toxicant or the parameter toxicity in the receiving water (i.e., riverine, lake, reservoir, estuary or ocean) after mixing.

**Recirculating water delivery system.** A water flow system that treats water after it passes through the culture tanks (usually with sand and biofilters) and delivers the same treated water back to the tanks.

**Toxicity test.** A procedure to measure the toxicity of a chemical or effluent using living organisms. The test measures the degree of response of an exposed organism to a specific chemical or effluent.

**WET (Whole effluent toxicity).** The total toxic effect of an effluent measured directly with a toxicity test.





## Appendix A: Preparing Hypersaline Brine (HSB)

Salinity adjustments are a vital part of using marine and estuarine species for toxicity testing. Because the majority of industrial and sewage treatment effluents entering marine and estuarine waters contain little or no measurable salts, the salinity of these effluents must be adjusted before exposing estuarine or marine plants and animals to the test solutions. It also is important to maintain constant salinity across all treatments throughout the test for quality control. Finally, matching the test solution's salinity to the expected receiving water's salinity may require salinity adjustments. NHEERL-AED uses HSB, prepared from filtered natural seawater, to adjust exposure solution salinities.

HSB has several advantages over artificial sea salts that make it more suitable for use in toxicity testing. Concentrated 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 test organisms. HSB can be held for prolonged periods without any apparent degradation, added directly to the effluent to increase the salinity, or used as control water by diluting to the desired salinity with deionized water. The brine can be made from any high quality, filtered seawater supply through simple heating and aerating.

### GENERATING THE BRINE

The ideal container for making brine from natural seawater has a high surface-to-volume ratio, is made of a non-corrosive material, and is easily cleaned. Shallow fiberglass tanks are ideal.

Thoroughly clean the tank, aeration supply tube, heater, and any other materials that will be in direct contact with the brine before adding seawater to the tank. Use a good quality biodegradable detergent, followed by several thorough deionized-water rinses.

Collect high-quality (and preferably high-salinity) seawater on an incoming tide to minimize the possibility of contamination. Special care should be used to prevent any toxic materials from coming in contact with the seawater. The water should be filtered to at least 10  $\mu\text{m}$  before placing into the brine tank. Fill the tank with seawater, and slowly increase the temperature to 40°C. If a heater is immersed directly into the seawater, make sure that the heater components will not corrode or leach any substances that could contaminate the brine. A thermostatically controlled heat exchanger made from fiberglass is suggested.

Aeration prevents temperature stratification and increases the rate of evaporation. Use an oil-free air compressor to prevent contamination. Evaporate the water for several days, checking daily (or more or less often, depending on the volume being generated) to ensure that the salinity does not exceed 100‰ and the temperature does not exceed 40°C. If these changes are exceeded, irreversible changes in the brine's properties may occur. One such change noted in original studies at NHEERL-AED was a reduction in the alkalinity of seawater made from brine with salinity greater than 100‰, and a resulting reduction in the animals' general health. Additional seawater may be added to the brine to produce the volume of brine desired.

When the desired volume and salinity of brine is prepared, filter the brine through a 1-mm filter and pump or pour it directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are most suitable). Cap the containers, and record the measured salinity and the date generated. Store the brine in the dark at room temperature.



## SALINITY ADJUSTMENTS USING HYPERSALINE BRINE

To calculate the volume of brine ( $V_b$ ) to add to a 0‰ sample to produce a solution at a desired salinity ( $S_f$ ), use this equation:

$$V_b * S_b = S_f * V_f$$

Where:  $V_b$  = volume of brine, mL  
 $S_b$  = salinity of brine, ‰  
 $S_f$  = final salinity, ‰  
 $V_f$  = final volume needed, mL

Table A-1 presents volumes needed to make 30‰ test solutions from effluent (0‰), deionized water, and 100‰ HSB. At 30‰ salinity, the highest achievable concentration is 70% effluent.

**Table A-1. Preparation of Test Solutions at a Salinity of 30‰ Using HSB for a Final Test Concentration Volume of 1000 mL.**

Exposure Concentration (%)	Effluent (0 ‰) (mL)	Deionized Water (mL)	Hypersaline Brine (100 ‰) (mL)
70	700	—	300
25	250	450	300
7	70	630	300
2.5	25	675	300
0.7	7	693	300
Control	—	1,000	—

Table A-2 gives examples of attainable exposure concentrations and dilution volumes needed when an effluent salinity is raised to 30‰ using artificial sea salts and using 0.5 serial dilution.

**Table A-2. Preparation of Test Solutions at a Salinity of 30‰ Using Natural Seawater or Artificial Sea Salts.<sup>1</sup>**

Effluent Solution	Effluent Concentration (%)	Solutions To Be Combined	
		Volume of Effluent Solution (mL)	Volume of Diluent Seawater (30‰) (mL)
1	100	840	—
2	50	420	Solution 1 + 420
3	25	420	Solution 2 + 420
4	12.5	420	Solution 3 + 420
5	6.25	420	Solution 4 + 420
Control	0.0		420
<b>Total</b>			<b>2,080</b>

<sup>1</sup>This illustration assumes: 1) the use of 5 mL of test solution in each of four replicates (total of 20 mL) for the control and five concentrations of effluent, 2) an effluent dilution factor of 0.5, 3) the effluent lacks appreciable salinity, and 4) 400 mL of each test concentration is used for chemical analysis. A sufficient initial volume (840 mL) of effluent is prepared by adjusting the salinity to 30‰. In this example, the salinity is adjusted by adding artificial sea salts to the 100% effluent, and preparing a serial dilution using 30‰ seawater (natural seawater, HSB, or artificial seawater). Stir solutions 1 hour to ensure that the salts dissolve. The salinity of the initial 840 mL of 100% effluent is adjusted to 30‰ by adding 25.2 g of dry artificial sea salts (FORTY FATHOMS®). Test concentrations are then made by mixing appropriate volumes of salinity adjusted effluent and 30‰ salinity dilution water to provide 840 mL of solution for each concentration. If HSB alone (100%) is used to adjust the salinity of the effluent, the highest concentration of effluent that could be tested would be 70% at 30‰ salinity.

Source: EPA, 2002a.



## Appendix B: Apparatus and Equipment

**Air lines, and air stones.** For aerating water containing adults, or for supplying air to test solutions with low DO.

**Air pump.** For oil-free air supply.

**Balance.** Analytical, capable of accurately weighing to 0.00001 g.

**Beakers or flasks.** Six, borosilicate glass or non-toxic plasticware, 1000 mL for making test solutions.

**Centrifuge.** Bench-top, slant-head, variable speed for washing eggs.

**Centrifuge tubes.** Conical for washing eggs.

**Compound microscope.** For examining and counting sperm cells and fertilized eggs (25X and 100X).

**Count register.** 2-place for recording sperm and egg counts.

**Cylindrical glass vessel.** 8-cm diameter for maintaining dispersed egg suspension.

**Dissecting microscope.** For counting diluted egg stock (100X).

**Environmental chamber or equivalent facility with temperature control** ( $20^{\circ}\text{C} \pm 1^{\circ}\text{C}$ ).

**Fume hood.** To protect from formaldehyde fumes.

**Glass dishes.** Flat bottomed, 20-cm diameter for holding sea urchins during gamete collection.

**Hemocytometer, Neubauer.** For counting sperm.

**Ice bucket.** Covered for maintaining live sperm after collection until test initiation.

**Laboratory sea urchins, *Arbacia punctulata*, culture unit.** To test effluent or receiving water toxicity, sufficient eggs and sperm must be available from healthy adult animals.

**Meters: pH and DO, and specific conductivity.** For routine physical and chemical measurements.

**Pipets, automatic.** Adjustable 1 – 100 mL.

**Pipets, serological.** 1 – 10 mL, graduated.

**Pipets, volumetric.** Class A, 1 – 100 mL.

**Pipet bulbs and filters.** Propipet<sup>®</sup>, or equivalent.

**Reference weights, Class S.** For checking performance of balance. Weights should bracket the expected weights of materials to be weighed.

**Refractometer or other method.** For determining salinity.

**Samplers.** Automatic sampler, preferably with sample cooling capability, that can collect a 24-hour composite sample of 5 L.



**Sedgwick-Rafter counting chamber.** For counting egg stock and examining fertilized eggs.

**Syringes.** 1 mL, and 10 mL, with 18 gauge, blunt-tipped needles (tips cut off) for collecting sperm and eggs.

**Thermometers.** National Bureau of Standards Certified (see EPA 2002a). Used to calibrate laboratory thermometers.

**Thermometers, glass or electronic, laboratory grade.** For measuring water temperatures.

**Transformer, 10–12 Volt.** With steel electrodes for stimulating release of eggs and sperm.

**Vacuum suction device.** For washing eggs.

**Volumetric flasks and graduated cylinders.** Class A, Borosilicate glass or non-toxic plastic labware, 10 – 1000 mL for making test solutions.

**Wash bottles.** For deionized water, for washing organisms from containers and for rinsing small glassware and instrument electrodes and probes.

**Water purification system.** Millipore® Milli-Q® deionized water or equivalent.



## Appendix C: Reagents and Consumable Materials

**Acetic acid.** 10%, reagent grade, in seawater for preparing killed sperm dilutions.

**Buffers pH 4, pH 7, and pH 10.** (Or as per instructions of instrument manufacturer) for standards and calibration check.

**Data sheets (one set per test).** For data recording (see Appendix E).

**Effluent, receiving water, and dilution water.** Test waters, including effluent, receiving, and dilution water should be analyzed to ensure its quality prior to using in tests. Dilution water containing organisms that might prey upon or otherwise interfere with the test organisms should be filtered through a fine mesh (with 150  $\mu\text{m}$  or smaller openings).

**Food.** Kelp, *Laminaria* sp., or romaine lettuce for the sea urchin, *Arbacia punctulata*.

**Formalin.** 1%, in 2 mL of seawater for preserving eggs at end of test.

**Gloves, disposable; lab coat and protective eyewear.** For personal protection from contamination.

**Laboratory quality assurance samples and standards.** For calibration of the above methods.

**Markers, waterproof.** For marking containers, etc.

**Parafilm.** To cover tubes and vessels containing test materials.

**Reagent water** Distilled or deionized water that does not contain substances which are toxic to the test organisms.

**Reference toxicant solutions.** Reference toxicants such as sodium chloride (NaCl), potassium chloride (KCl), cadmium chloride ( $\text{CdCl}_2$ ), copper sulfate ( $\text{CuSO}_4$ ), sodium dodecyl sulfate (SDS), and potassium dichromate ( $\text{K}_2\text{Cr}_2\text{O}_7$ ), are suitable for use in the NPDES Program and other Agency programs requiring aquatic toxicity tests.

**Saline test and dilution water.** The salinity of the test water must be in the range of 20‰ – 30‰. 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.

It is important to maintain a constant salinity across all treatments during a test. It is desirable to match the test salinity with that of the receiving water. Two methods are available to adjust salinities — a hypersaline brine (HSB) derived from natural seawater or artificial sea salts. Both are described in EPA, 2002.

**Sample containers.** For sample shipment and storage.

**Sea Urchins.** *Arbacia punctulata*, minimum of 12 of each sex.

**Scintillation vials.** 20 mL, disposable; to prepare test concentrations.

**Standard salt water aquarium or Instant Ocean Aquarium.** Capable of maintaining seawater at 15°C, with appropriate filtration and aeration system.

**Tape, colored.** For labeling tubes.



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## Appendix D: Summary of Test Conditions and Test Acceptability Criteria

Summary of Test Conditions and Test Acceptability Criteria for Sea Urchin, *Arbacia punctulata*, Fertilization Test with Effluent and Receiving Waters (Test Method 1008.0)<sup>1</sup>

Test type	Static, non-renewal ( <i>required</i> )
Salinity	30‰ ± 2‰ of the selected test salinity ( <i>recommended</i> )
Temperature (C°)	20°C ± 1°C ( <i>recommended</i> ) Test temperatures must not deviate by more than 3°C during the test (i.e., max. temp – min. temp ≤ 3°C) ( <i>required</i> )
Light quality	Ambient laboratory light during test preparation ( <i>recommended</i> )
Light intensity	10 – 20 µE/m <sup>2</sup> /s, or 50 – 100 ft-c (Ambient laboratory levels) ( <i>recommended</i> )
Test chamber size	Disposable (glass) liquid scintillation vials (20 mL capacity), pre-soaked in control water ( <i>recommended</i> )
Test solution volume	5 mL ( <i>recommended</i> )
Number of sea urchins	Pooled eggs from 4 females and pooled sperm from 4 males per test ( <i>recommended</i> )
Number of eggs and sperm cells per chamber	About 2,000 eggs and 5,000,000 sperm cells per vial ( <i>recommended</i> )
Number of replicate chambers per concentration	4 ( <i>required minimum</i> )
Dilution water	Uncontaminated source of natural seawater; deionized water mixed with HSB or artificial sea salts ( <i>available options</i> )
Test concentrations	Effluents: 5 and a control ( <i>required minimum</i> ) Receiving waters: 100% receiving water ( <i>or minimum of 5</i> ) and a control ( <i>recommended</i> )
Dilution factor	Effluents: ≥ 0.5 ( <i>recommended</i> ) Receiving Waters: None or ≥ 0.5 ( <i>recommended</i> )
Test duration	1 hour and 20 minutes ( <i>required</i> )
Endpoint	Fertilization of sea urchin eggs ( <i>required</i> )
Test acceptability criteria	70% – 90% egg fertilization in controls ( <i>required</i> )
Sampling requirements	For on-site tests, one sample collected at test initiation, and used within 24 hr of the time it is removed from the sampling device. For off-site tests, holding time must not exceed 36 hr before first use for NPDES compliance testing. ( <i>required</i> )
Sample volume required	1 L per test ( <i>recommended</i> )

<sup>1</sup>Source: EPA, 2002a. For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended. Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.



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# Appendix E: Data Sheets

Figure E-1. Sperm Cell Toxicity Test, Sample Data Sheet #1

Test ID: \_\_\_\_\_

Performed By: \_\_\_\_\_

**Sperm Dilutions:**

Hemacytometer Count, E: \_\_\_\_\_ X 10<sup>4</sup> = SPM "E"

Sperm Concentrations \_\_\_\_\_ "E" X 40 = A = \_\_\_\_\_ SPM

\_\_\_\_\_ "E" X 20 = B = \_\_\_\_\_ SPM

\_\_\_\_\_ "E" X 5 = D = \_\_\_\_\_ SPM

Solution Selected for Test (> 5 X 10<sup>7</sup> SPM): \_\_\_\_\_

Dilution: SPM/(5 X 10<sup>7</sup>) = \_\_\_\_\_ DF

\_\_\_\_\_ ((DF) X 10) - 10 = \_\_\_\_\_ + SW, mL

Final Sperm Counts = \_\_\_\_\_

**Egg Dilutions:**

Initial Egg Count: = \_\_\_\_\_

Egg Stock Concentration = Egg Count (1 mL of 1:10 dilution) X 10: = \_\_\_\_\_

(Allow eggs to resettle and recount until count ≤ 200)

Volume of SW to Add to Dilute Egg Stock to 2000/mL: Egg Count - 200: = \_\_\_\_\_

Verify Final Egg Count (in 1 mL of 1:10 dilution): = \_\_\_\_\_

(Count should = 100 ± 20 eggs/mL)

**Test Stocks:**

Sperm Stock: \_\_\_\_\_ (5 X 10<sup>7</sup> SPM)

Volume Added/Test Vial: \_\_\_\_\_ (100 µL)

Egg Stock: \_\_\_\_\_ (2000/mL)

Volume Added/Test Vial: \_\_\_\_\_ (1 mL)

**Test Times:**

Sperm Collection: \_\_\_\_\_

Egg Collection: \_\_\_\_\_

Sperm Added: \_\_\_\_\_

Eggs Added: \_\_\_\_\_

Fixative Added: \_\_\_\_\_

Samples Read: \_\_\_\_\_

**Salinities:**



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