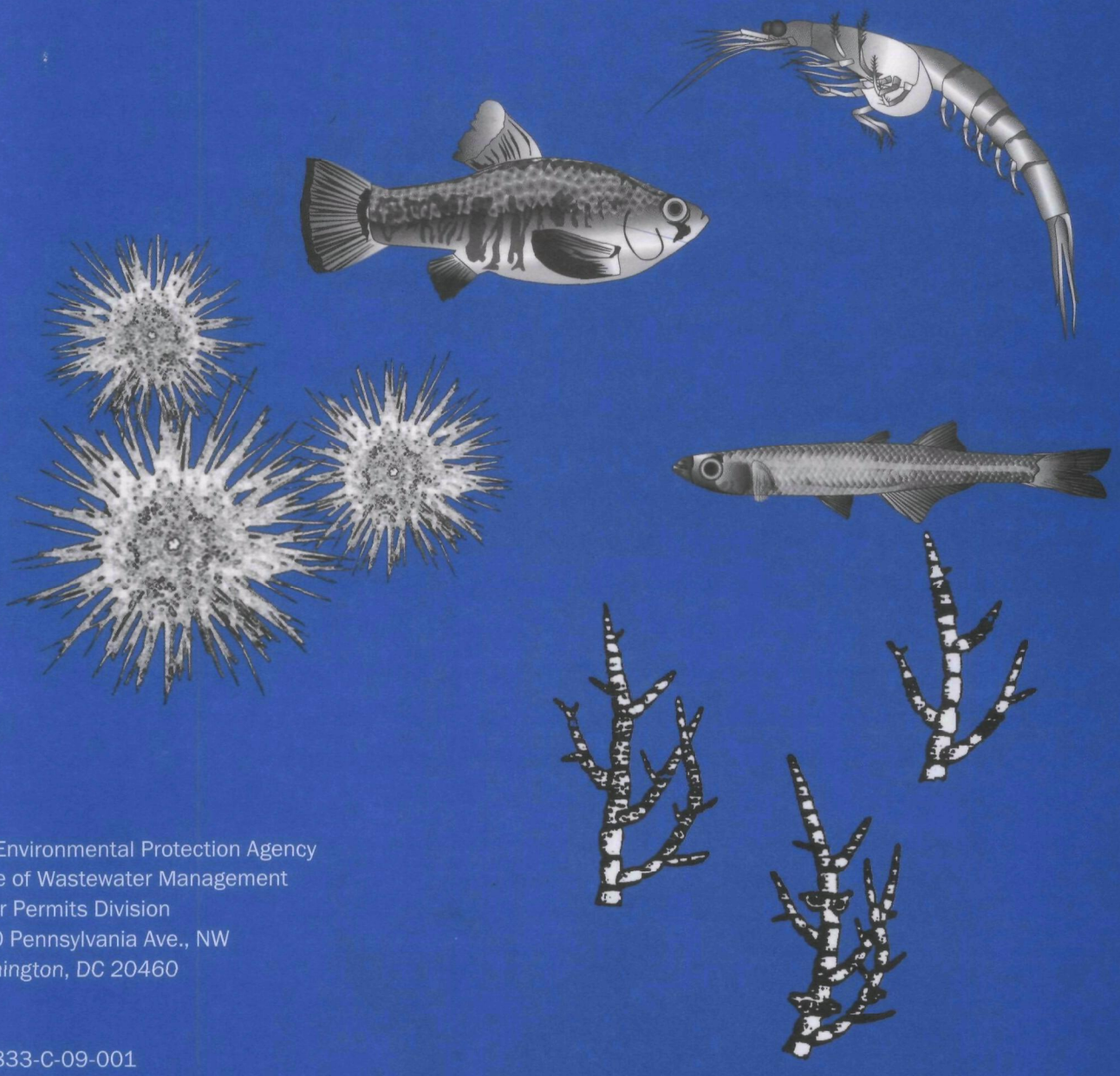
Whole Effluent Toxicity Training Video Series

Saltwater Series



U.S. Environmental Protection Agency
Office of Wastewater Management
Water Permits Division
1200 Pennsylvania Ave., NW
Washington, DC 20460

EPA 833-C-09-001
March 2009

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

Supplement to Training Video

U.S. Environmental Protection Agency
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NOTICE

The revision of this guide has been funded wholly or in part by the Environmental Protection Agency under Contract EP-C-05-063. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

Foreword

This guide serves as a supplement to the video "Red Algal (*Champia parvula*) Sexual Reproduction Toxicity Tests" (EPA, 2009). The methods illustrated in the video and described in this 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 guide accompanies the Environmental Protection Agency's (EPA's) video training for conducting red algal (*Champia parvula*) sexual reproduction toxicity tests (EPA, 2009). The test method is found in Section 16 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 by 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. 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 Whole effluent toxicity (WET) 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 red algae 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 guide summarizes methods developed at NHEERL-AED for estimating the chronic toxicity of marine or estuarine effluents and receiving waters on the sexual reproduction of the marine macroalga, *Champia parvula*. Males and females are exposed to effluents or receiving waters for 2 days, followed by a 5- to 7-day recovery period for the female plants in a control medium. Cystocarp production by the female, which indicates sexual reproduction, is used as the endpoint. The test results determine the effluent concentration causing a statistically significant reduction in the number of cystocarps formed.

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

Culturing *Champia parvula*

There are three macroscopic stages in the life history of *Champia*. The adult plant body (thallus) is hollow, septate, and highly branched. Only the mature male and female plants are used in toxicity testing. Mature plants are illustrated in Figure 1.

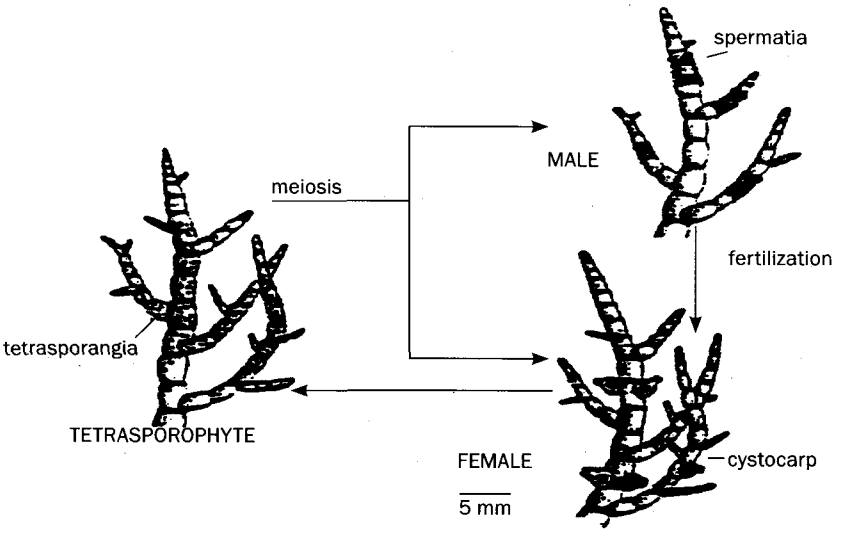
To keep a constant supply of plant material available, maintain several unialgal stock cultures of males and females simultaneously. Also, new cultures should be started weekly from excised branches so that cultures are available in different stages of development.

Culture Water

Natural seawater, or a 50-50 mixture of natural and artificial seawater, makes optimal culturing media. Seawater for cultures is filtered at least to 0.45 µm to remove most particulates and autoclaved for 30 minutes at 15 psi (120°C). Carbon stripping the seawater may be necessary before autoclaving to enhance

its water quality (EPA, 1990). Instructions for carbon stripping are provided in the Saltwater Chronic Methods Manual (EPA, 2002a). Nutrients should be added to the water to ensure healthy cultures. Recipes for the culturing medium and nutrient solutions are provided in Appendix A. The water temperature should be maintained at 23°C ± 1°C and the salinity at 30‰ ± 2‰

Figure 1. Life History of the Red Macroalga, *Champia parvula*. Left: Size and Degree of Branching in Female Branch Tips Used For Toxicity Tests



Source: EPA 1987.

Gently aerate the cultures. Change alternate cultures' medium every week so that if a stock solution should become contaminated, the entire batch will not be lost. While replenishing the medium, divide the growing algae in half with sharp forceps or discard half of the biomass to prevent overcrowding. New cultures also can be started at this time using 1 cm branch tips. Add nutrients using a pipet; NHEERL-AED has found a squeeze bottle is quick and easy to use. At the end of approximately three weeks, there should be enough plant material to conduct the test.

PHOTOPERIOD

The culture conditions should include a photoperiod 16 hours of light and 8 hours of darkness. The light level should not exceed 500 ft-candles (75 $\mu E/m^2/s$) and may have to be adjusted to that level, depending on the reflecting characteristics of the incubators.

CULTURE VESSELS

Maintain stock cultures of males and females in separate, aerated, 1 L Erlenmeyer flasks containing 800 mL of the culture medium. All glass must be acid-stripped in 15 percent HCl and rinsed in deionized water before use because some detergent residues can be toxic to the *Champia*. At least every 6 months, the glass should be cleaned to remove organic materials that can build up on the surface. Always use sterile techniques when culturing the algae (i.e., autoclave all stock solutions and flame all tools before cutting or transferring plants) to guard against microalgal contamination.

PREPARING ALGAE FOR TESTING

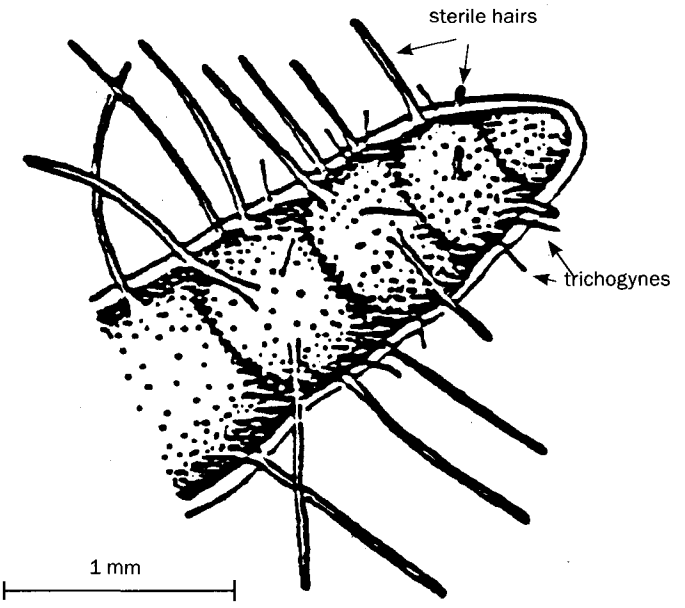
Examine the stock cultures to determine their readiness for testing. Place a few female branch tips in seawater in a petri dish, and examine them under a compound microscope to determine if trichogynes are present. An inverted scope works best with the petri dishes, although standard slides and microscopes also can be used. Trichogynes are the short, fine reproductive hairs to which the spermatia attach (see Figure 2). They should be seen easily near the apex of the branch tip. Although both sterile hairs and trichogynes occur on the apex, sterile hairs occur over the entire plant thallus. Sterile hairs are wider and generally much longer than trichogynes, and appear hollow, except at their tip, where they seem to be plugged.

Males should be visibly producing spermatia. Sometimes, the presence of spermatia sori can be determined by placing some male tissue in a petri dish and holding it against a dark background. Mature sori can be easily identified under a microscope along the edge of the thallus. The sorus areas are generally thicker and lighter in color than the rest of the plant body. At higher magnification, the spermatia themselves can be seen (see Figures 3 and 4).

The readiness of the male stock culture can also be assessed by placing a portion of a female plant into a portion of the solution from the male culture for a few seconds. Under a microscope, numerous spermatia should be seen attached to the sterile hairs and trichogynes of the female plant (see Figure 5).

Once readiness is established for both males and females, the test can begin.

Figure 2. Apex of Branch of Female Plant, Showing Sterile Hairs and Reproductive Hairs (Trichogynes)



Sterile hairs are wider and generally much longer than trichogynes, and appear hollow except at the tip. Both types of hairs occur on the entire circumference of the thallus, but are seen easiest at the "edges." Receptive trichogynes occur only near the branch tips.

Source: EPA 1987.

Conducting the Test

Collecting the Algae

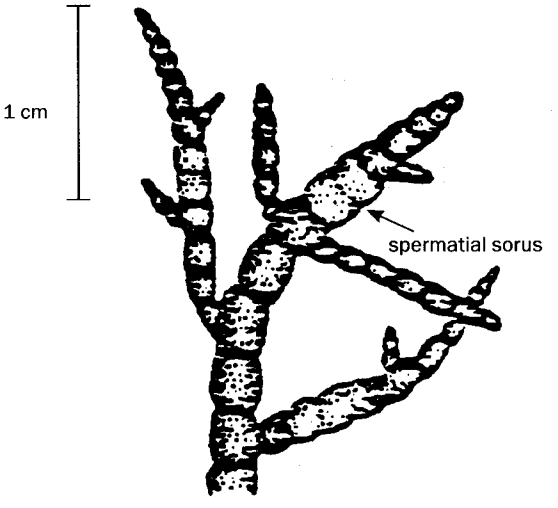
Prepare cuttings from the most healthy-looking plants. Prepare the female cuttings first to minimize the chances of contaminating them with water containing spermatia from the male stock cultures. Place each plant in a petri dish containing a small amount of seawater. Using a fine-point forceps or scalpel, prepare five cuttings from the female plants for each treatment replicate, severing the plant 7 – 10 mm from the ends of the branch. Try to be consistent in the degree of branching in the cuttings, since cystocarps form at the branch tips.

For male plants, use one cutting for each treatment replicate, severing the plant about 2 – 3 cm from the end of the branch. If there are few branches, or the spermatial sori appear sparse, larger male cuttings may be needed. The cuttings can be kept at room temperature for up to an hour.

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°C – 6°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.

Figure 3. A Portion of the Male Thallus Showing Spermatial Sori. The Sorus Areas Are Generally Slightly Thicker and Somewhat Lighter in Color



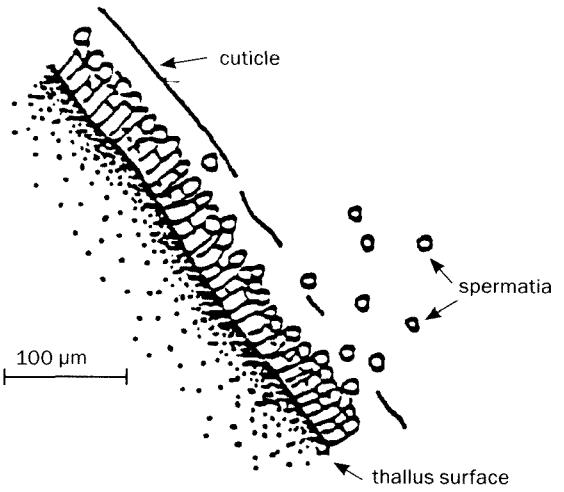
Source: EPA 1987.

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:

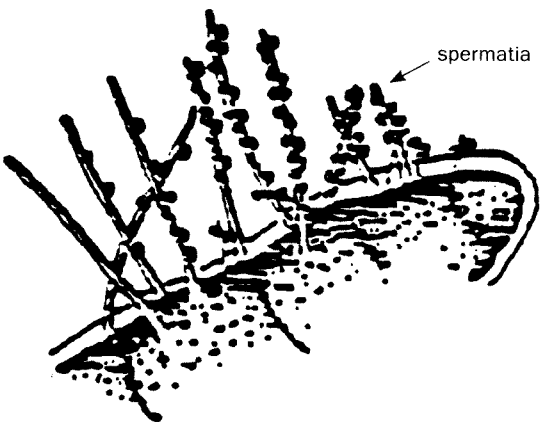
- *If the test is conducted to estimate the* ***absolute chronic toxicity of the effluent****, synthetic dilution water should be used. If the cultures were maintained in different water than used for dilution water, a second set of control replicates should be conducted using the culture water.*
- *If 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.*

Figure 4. A Magnified Portion of a Spermatial Sorus. Note the Rows of Cells that Protrude from the Thallus Surface



Source: EPA 1987.

Figure 5. Apex of a Branch on a Mature Female Plant That Was Exposed To Spermatia from a Male Plant



The sterile hairs and trichogynes are covered with spermatia. Note that few or no spermatia are attached to the older hairs (those more than 1 mm from the apex).

Source: EPA 1987.

Maintain the salinity of the test samples to 30‰ ± 2‰. 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 23°C ± 1°C and maintain that temperature while preparing the test concentrations. To test a series of decreasing concentrations of effluent, use a dilution factor of ≥ 0.5. When starting with effluent that has 0‰ salinity, the maximum effluent concentration that can be prepared at 30‰ is 70 percent effluent. A table for preparing the samples is provided in Appendix A.

The Exposure Period

A 125 mL Erlenmeyer flask is used for each test chamber, but any clean container can be used. The test chambers should be labeled using colored tape and marking pens to identify each treatment and replicate. These should be placed in randomized positions for the duration of the test.

Under a hood, prepare five dilutions using a ≥ 0.5 dilution factor in 300 or 400 mL replicates. Approximately 1800 mL of effluent is required for a test conducted using a 0.5 dilution factor. This allows for enough of each prepared effluent concentration to provide four replicates at 100 mL and 400 mL for chemical analyses and water quality data. Record the water quality data on a form such as the one provided in Figure 6.

Figure 6. Receiving Water Data Form for the Red Macroalga, *Champia parvula*, Sexual Reproduction Test.

Site: _____

Collection Date: _____

Test Date: _____

Locations	***Initial Salinity***	***Final Salinity***	***Source of Salts for Salinity Adjustment[1]***

[1]*Natural seawater, GP2 brine, GP2 salts, etc. (include some indication of amount.)*

Source: EPA, 2002a.

The 2-day exposure period starts when the algae are added to the test chambers. Add five female branches and one male branch to each prepared chamber. Pick up the branch at the base or cut end to avoid injuring the tips. The effluent must be in the test chamber before the algae are added.

Cover the chambers with aluminum foil or a foam stopper, exposing the cultures to 16 hours of cool white light and 8 hours of darkness each day for the 2-day exposure, as well as the 5- to 7-day recovery periods. Maintain the temperature at 23°C ± 1°C, and the salinity between 28‰ and 32‰ with the variance between chambers on any day maintained at ≤ 2‰.

Check on the chambers twice a day, and gently hand-swirl the chambers, or shake continuously at 100 rpm on a rotary shaker. Spermatia are not motile, so some motion is critical during the exposure period for reproduction to occur. If desired, the media can be changed after 24 hours. Record the temperature daily from a thermometer placed in a flask of water among the chambers.

pH should be measured in the effluent sample before any new test solutions are made to determine changes in the effluent sample.

Routine chemical and physical observations should be made during the test. Dissolved oxygen (DO) is measured at the beginning and end of each 24-hour exposure period in one test chamber at each concentration and in the control. Temperature, pH, and salinity are measured at the end of each 24-hour exposure period, also in one test chamber at each concentration and in the control. Temperature also should be monitored continuously, observed and recorded daily for at least two locations in the environmental control system or the samples. The locations for determining temperature should be sufficient to indicate any temperature variations in the environmental chamber.

The Recovery Period

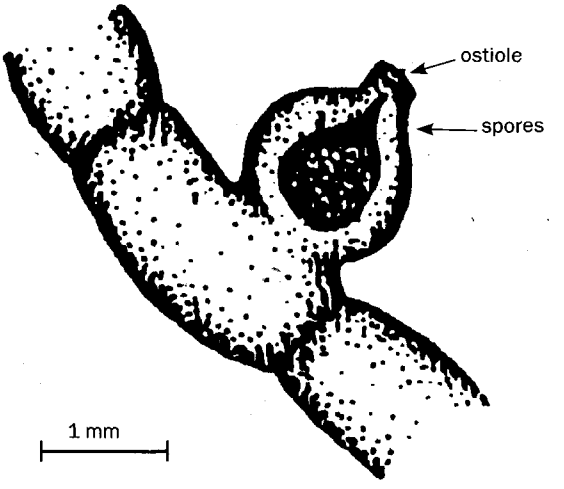
Prepare recovery bottles by labeling clean 100 – 200 mL vessels with the effluent concentrations tested, and fill them with 150 mL of natural seawater and nutrients. Smaller volumes can be used but may require changes of the medium to allow for adequate growth.

After the 48-hour exposure period, use forceps to gently remove all of the females from each test chamber, and place them into recovery bottles. When all the replicates have been transferred, place the vessels under cool white light and aerate or shake for the 5- to 7-day recovery period. Aeration will enhance the growth rate of plants in the recovery bottles, although adequate growth will occur using a shaker. Aerate using plastic tubes held in place by foam stoppers.

Terminating the Test

At the end of the recovery period, drain the chambers and remove the females with forceps, starting with the control plants and ending with those in the highest concentration. Place the female plants between the inverted halves of a petri dish containing a small amount of seawater, and count the cystocarps under a stereomicroscope. Cystocarps are distinguished from young branches by the darkly pigmented spores enclosed in the nodule, and the apical opening for spore release (ostiole). Figures 7 through 9 provide illustrations to help identify cystocarps.

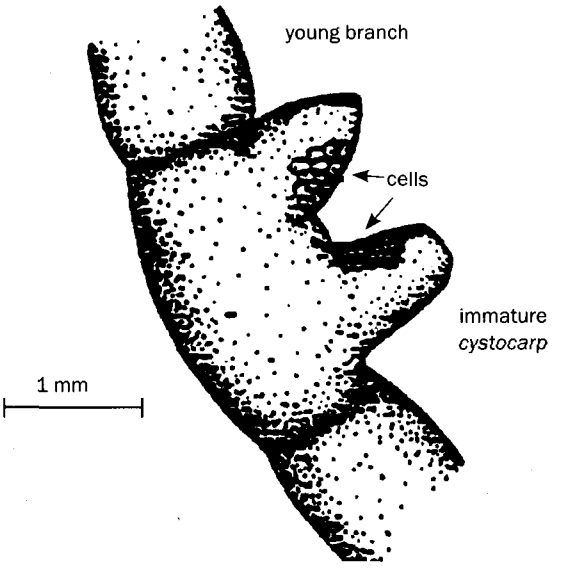
Figure 7. A Mature Cystocarp



In the controls and lower effluent concentrations, cystocarps often occur in clusters of 10 or 12.

Source: EPA 1987.

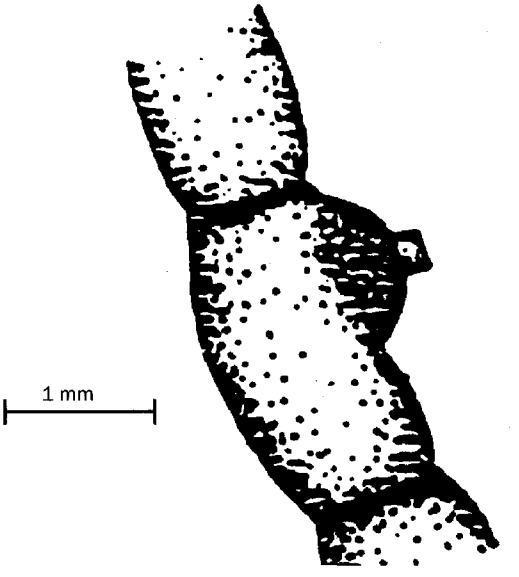
Figure 8. Comparison of a Very Young Branch and an Immature Cystocarp



Both the young branch and immature cystocarp can have sterile hairs. Trichogynes might or might not be present on a young branch, but are never present on an immature cystocarp. Young branches are more pointed at the apex and are made up of larger cells than immature cystocarps, and never have ostioles.

Source: EPA 1987.

Figure 9. An Aborted Cystocarp.



A new branch will eventually develop at the apex.

Source: EPA 1987.

If there is doubt about the identification of an immature cystocarp, aerate the plants a little longer in the recovery bottles. Within 24 to 48 hours, the suspected cystocarp will look more like a mature cystocarp or a young branch, or will have changed very little, if at all, indicating an aborted cystocarp. Occasionally cystocarps will abort, and these should not be included in the counts. Aborted cystocarps are easily identified by their dark pigmentation and/or by the formation of a new branch at the apex. Dead plants lose their pigmentation and appear white.

Record all counts for the test on a form such as the one provided in Figure 10.

Figure 10. Cystocarp Data Sheet for the Red Macroalga, *Champia parvula*, Sexual Reproduction Test

Collection Date: _____ Recovery Began (date): _____

Exposure Began (date): _____ Counted (date): _____

Effluent or Toxicant: _____

Treatment (% Effluent, mg/L, or receiving water sites)

Replicates	***Control***						
A 1							
2							
3							
4							
Mean							
B 1							
2							
3							
4							
Mean							
C 1							
2							
3							
4							
Mean							
D 1							
2							
3							
4							
Mean							
Overall Mean							

Temperature: _____

Salinity: _____

Light: _____

Source of Dilution Water: _____

Source: EPA, 1987.

Test Acceptability and Data Review

Test data are reviewed to verify that EPA's WET test methods' test acceptability criteria (TAC) requirements for a valid test have been met. The algal sexual reproduction test requires that several criteria be met before the test results are considered acceptable.

- Control plants should average 10 or more cystocarps per plant and survival in the control must be 80 percent or greater.
- Control and lowest-concentration exposed algae should be in good physical condition—for example, the branches should not be fragmented. Broken or fragmented branches could indicate that the plants were unhealthy or stressed from the beginning of the test.
- The results from the replicate control chambers should be similar.
- All replicates from the affected concentration chambers should show effect.

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_{50}, IC_{25}) 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.

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EPA references are available online at **www.epa.gov/npdes**.

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www.epa.gov/npdes/wqbasedpermitting.

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.

Champia parvula. The scientific name for red algae. *Champia parvula* have soft, gelatinous, pinkish red, much-branched fronds that are densely matted, with blunt apices, to 100 mm high. Their axes are segmented, with nodal diaphragms. The segments are about as broad as long, filled with a watery mucilage. Red algae are found epiphytic on smaller algae in lower intertidal pools. They are found widely distributed in the Atlantic and Pacific marine environments.

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.

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_{25} (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.

LC_{50} (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_{50}).

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-Karber. 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.

Toxicity test. A test 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: Nutrients and Media

The following instructions for nutrients are provided in the Saltwater Chronic Methods Manual (EPA, 2002a). Table A-1 lists the additional nutrients to be added to natural or artificial seawater for stock cultures and test media. The concentrated stock solution is autoclaved at standard temperature and pressure for 15 minutes before the vitamins are added. Adjust the solution to about pH 2 before autoclaving to minimize the possibility of precipitation.

Table A-1. Nutrient Stock Solution

Nutrient Stock Solution[a]	*Amount/L Concentrated Nutrient Stock Solution*	
	Stock Solution for Culture Medium	*Stock Solution for Test Medium*
$NaNO_3$	6.35 g	1.58 g
$NaH_2PO_4 \cdot H_2O$	0.64 g	0.16 g
$Na_2EDTA \cdot 2\ H_2O$	133 mg	—
$Na_3C_6H_5O_7 \cdot 2\ H_2O$	51 mg	12.8 mg
Iron[b]	9.75 mL	2.4 mL
Vitamins[c]	10 mL	2.5 mL

[a] *Add 10 mL of appropriate nutrient stock solution per liter of culture or test medium.*

[b] *A stock solution of iron is made that contains 1 mg iron/mL. Ferrous or ferric chloride can be used.*

[c] *A vitamin stock solution is made by dissolving 4.88 g thiamine HCl, 2.5 mg biotin, and 2.5 mg B_{12} in 500 mL deionized water. Adjust vitamin stock to approximately pH 4, divide into 10 mL subsamples, and autoclave for 2 minutes before it is added to the nutrient stock solution.*

Preparing Hypersaline Brine (HSB)

BACKGROUND

Champia parvula cannot be cultured in 100% artificial seawater. However, 100% artificial seawater can be used during the 2-day exposure period. This allows 100% effluent to be tested.

Salinity adjustments are a vital part of using marine and estuarine species for toxicity testing. The majority of industrial and sewage treatment effluents entering marine and estuarine waters contain little or no measurable salts. Therefore, the salinity of these effluents must be adjusted before exposing estuarine or marine plants and animals to the solutions. The salinity of the effluent can be adjusted by adding HSB prepared from natural seawater (100‰), concentrated (triple strength) salt solution (GP2 described in table below), or dry GP2 salts (also below). Adjust the salinity of the effluent to 30‰. Control solutions should be prepared with the same percentage of natural seawater and at the same salinity as the effluent solutions.

Constant salinity should be maintained across all treatments throughout the test for quality control. Matching the test solutions' salinity to the expected receiving water's salinity may require salinity adjustments. EPA 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. It may be held for prolonged periods without any apparent degradation. Brine may be added directly to the effluent to increase the salinity, or may be 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.

Table A-2. GP2 Artificial Seawater for Use in Conjunction with Natural Seawater for the Red Macroalga, *Champia parvula*, Sexual Reproduction Toxicity Test

Compound	*Concentration (g/L)*	*Amount (g) Required for 20-L*
NaCl	21.03	420.6
Na_2SO_4	3.52	70.4
KCl	0.61	12.2
KBr	0.088	1.76
$Na_2B_4O_7 \cdot 10\ H_2O$	0.034	0.68
$MgCl_2 \cdot 6\ H_2O$	9.50	190.0
$CaCl_2 \cdot 2\ H_2O$	1.32	26.4
$SrCl_2 \cdot 6\ H_2O$	0.02	0.400
$NaHCO_3$[a]	0.17	3.40

The original formulation calls for autoclaving anhydrous and hydrated salts separately to avoid precipitation. However, if the sodium bicarbonate is autoclaved separately (dry), all of the other salts can be autoclaved together. Since no nutrients are added until needed, autoclaving is not critical for effluent testing. To minimize microalgal contamination, the artificial seawater should be autoclaved when used for stock cultures. Autoclaving (120°C) should be for at least 10 minutes for 1-L volumes, and 20 minutes for 10- to 20-L volumes.

Artificial seawater should be prepared in 10- to 20-L batches. Effluent salinity adjustment to 30‰ can be made by adding the appropriate amount of dry salts from this formulation, by using a triple-strength brine prepared from this formulation, or by using a 100‰ salinity brine prepared from natural seawater.

Nutrients listed in Table A-1 should be added to the artificial seawater in the same concentration described for natural seawater.

[a] *A stock solution of 68 mg/mL sodium bicarbonate is prepared by autoclaving it as a dry powder, and then dissolving it in sterile deionized water. For each liter of GP2, use 2.5 mL of this stock solution.*

Source: EPA, 2002a. Modified from Spotte et al., 1984. Constituents salts and concentrations were taken from EPA 1990.

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.

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 µm before placing into the brine tank. 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. 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 would contaminate the brine. A thermostatically controlled heat exchanger made from fiberglass works well.

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 ERL-N 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 10-µm filter and pump or pour it directly into portable containers (5-gallon cubitainers or polycarbonate water cooler jugs are most suitable). Cap the containers, and record the measured salinity and the date the brine was generated. Store the brine in the dark at room temperature until used.

SALINITY ADJUSTMENTS USING HSB

To calculate the volume of brine (V_b) to add to 0‰ sample to produce a solution at certain 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, mL (brine brought to this volume with 0 ‰ sample).

Table A-3 gives 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-3. 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	—

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Appendix B: Apparatus and Equipment

Air lines, and air stones. For aerating cultures, brood chambers, and holding tanks, and 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, Class A. Borosilicate glass or non-toxic plasticware, 1000 mL for making test solutions.

Bottles. Borosilicate glass or disposable polystyrene cups (200 – 400 mL) for use as recovery vessels.

Compound microscope. For examining the condition of plants.

Count register. 2-place for recording cystocarp counts.

Dissecting (stereomicroscope) microscope. For counting cystocarps.

Drying oven. To dry glassware.

Erlenmeyer flasks, 250 mL, or 200 mL disposable polystyrene cups, with covers. For use as exposure chambers.

Environmental chamber or equivalent facility with temperature control (23 ± 1°C).

Facilities for holding and acclimating test organisms.

Filtering apparatus. For use with membrane filters (47 mm).

Forceps, fine-point, stainless steel. For cutting and handling branch tips.

Laboratory red macroalga, *Champia parvula*, culture unit. To test effluent or receiving water toxicity, sufficient number of sexually mature male and female plants must be available.

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

Micropipettors, digital, 200 and 1000 µL. To make dilutions.

Pipet bulbs and filters. Propipet®, or equivalent.

Pipets, automatic. Adjustable 1 – 100 mL.

Pipets, serological. 1 – 10 mL, graduated.

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

Reference weights, Class S. For checking performance of balance.

Refractometer or other method. For determining salinity.

Rotary shaker. For incubating exposure chambers (hand-swirling twice a day can be substituted).

Samplers. Automatic samplers, preferably with sample cooling capability, that can collect a 24-hour composite sample of 1 L.

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

Thermometers. Bulb-thermograph or electronic-chart type for continuously recording temperature.

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

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

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

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

Appendix C: Reagents and Consumable Materials

Aluminum foil, foam stoppers, or other closures. To cover cultures and test flasks.

Artificial seawater. A slightly modified version of the GP2 medium (Spotte, et al., 1984) has been used successfully to perform the red macroalga sexual reproduction test. The preparation of artificial seawater (GP2) is described in Table A-2.

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 Figures 6 and 10).

Disposable tips for micropipettors.

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 µm or smaller openings).

Laboratory quality assurance samples and standards. For the above methods.

Markers, waterproof. For marking containers, etc.

Mature red macroalga, *Champia parvula*, plants.

Petri dishes, polystyrene. To hold plants for cystocarp counts and to cut branch tips. Other suitable containers may be used.

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

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 ($CdCl_2$), copper sulfate ($CuSO_4$), sodium dodecyl sulfate (SDS), and potassium dichromate ($K_2Cr_2O_7$), are suitable for use in the NPDES Program and other Agency programs requiring aquatic toxicity tests.

Saline test and dilution factor. The use of natural seawater is recommended for this test. A recipe for the nutrients that must be added to the natural seawater is given in Table A-1. The salinity of the test water must be 30‰ and vary no more than ± 2‰ among the replicates. If effluent and receiving water tests are conducted concurrently, the salinity of these tests should be similar.

The overwhelming majority of industrial and sewage treatment effluents entering marine and estuarine systems contain little or no measurable salts. Therefore, exposure of the red macroalga, *Champia parvula*, to effluents will usually require adjustments in the salinity of the test solutions. Although the red macroalga, *Champia parvula*, cannot be cultured in 100% artificial seawater, 100% artificial seawater can be used during the 2-day exposure period. This allows 100% effluent to be tested. It is important to maintain a constant salinity across all treatments. The salinity of the effluent can be adjusted by adding HSB prepared from natural seawater (100‰), concentrated (triple strength) salt solution (GP2 described in Table A-2), or dry GP2 salts (Table

A-2), to the effluent to provide a salinity of 30‰. Control solutions should be prepared with the same percentage of natural seawater and at the same salinity (using deionized water adjusted with dry salts, or brine) as used for the effluent dilutions.

Sample containers. For sample shipment and storage.

Tape, colored. For labeling test chambers.

Appendix D:
Summary of Test Conditions and Test Acceptability Criteria for the Red Macroalga, *Champia parvula*, Sexual Reproduction Test With Effluents and Receiving Waters

(Note: this test is not listed at 40 CFR Part 136 for nationwide use)

Test type	Static, non-renewal *(required)*
Salinity	30‰ ± 2 ‰ of the selected test salinity *(recommended)*
Temperature (C°)	23°C ± 1°C *(recommended)*
Light source	Cool-white fluorescent lights *(recommended)*
Light intensity	About 75 μE/m²/s (500 ft-c) *(recommended)*
Photoperiod	16 hr light, 8 hr dark *(recommended)*
Test chamber size	200 mL polystyrene cups (with covers) or 250 mL Erlenmeyer flasks *(recommended)*
Test solution volume	100 mL *(minimum required)*
Number of organisms per test chamber	5 female branch tips and one male plant *(recommended)*
Number of replicates per concentration	4 *(3 required minimum)*
Number of organisms per concentration	24 *(18 required minimum)*
Aeration	None; chambers are either shaken at 100 rpm on a rotary shaker or hand-swirled twice a day
Dilution water	30‰ salinity natural seawater, or a combination of 50% of 30‰ salinity natural seawater and 50% of 30‰ salinity GP2 artificial seawater
Test concentrations	Effluents: 5 and a control *(required minimum)*
Receiving waters	100% receiving water *(or minimum of 5)* and a control *(recommended)*
Dilution factor	Effluents: ≥ 0.5 *(recommended)* Receiving Waters: None or ≥ 0.5 *(recommended)*
Test duration	2-day exposure to effluent followed by 7-day recovery period in control medium for cystocarp development *(required)*
Endpoints	Reduction in cystocarp production compared to controls *(required)*
Test acceptability criteria	80% or greater survival, and an average of 10 cystocarps per plant in controls *(required)*
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 test, holding time must not exceed 36 hr before test use. *(required)*
Sample volume required	2 L per test *(recommended)*

Source: EPA, 2002a. Saltwater Chronic Methods Manual.

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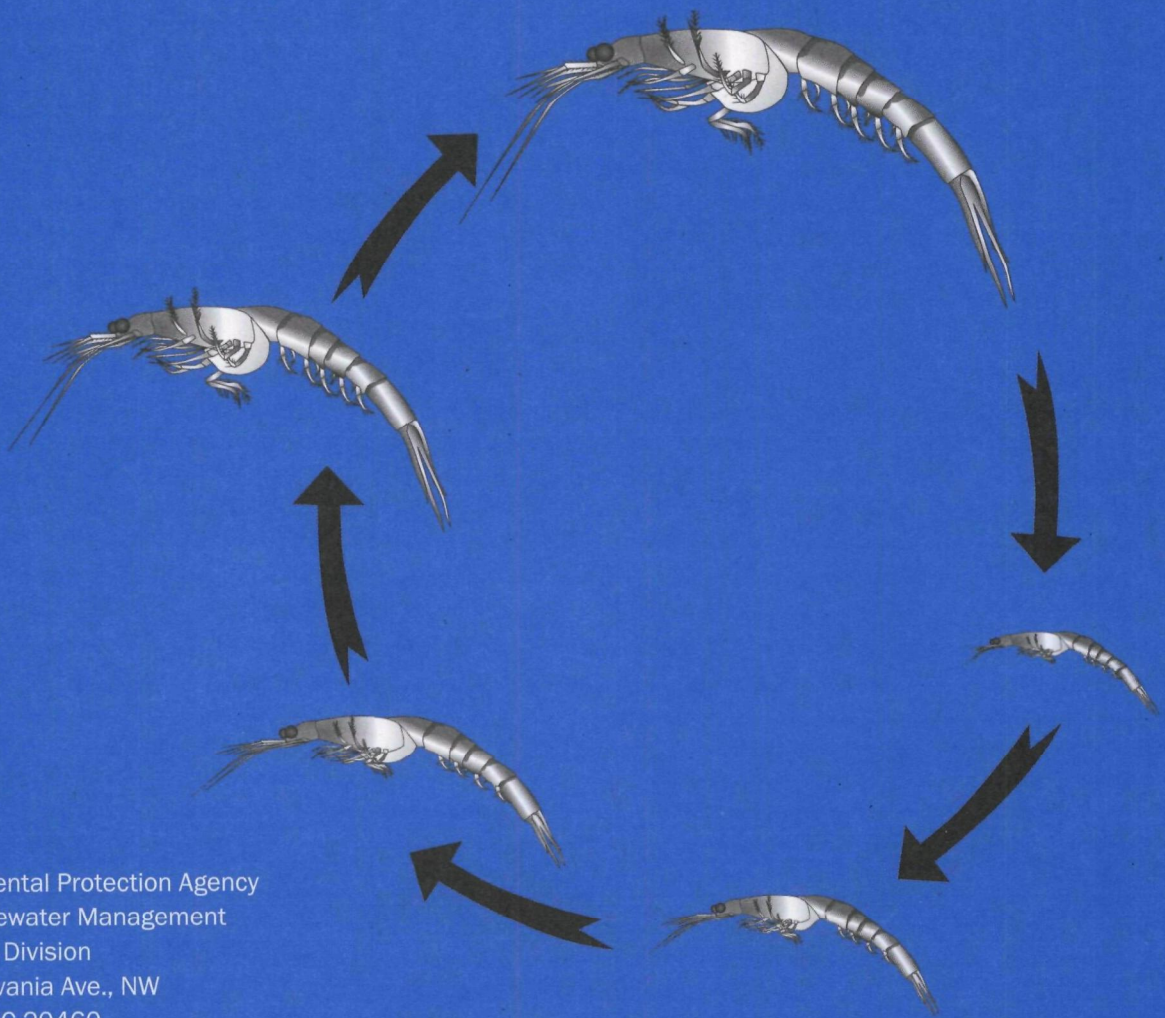
www.epa.gov/npdes/wqbasedpermitting

Culturing *Americamysis bahia*

Supplement to Training Video



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NOTICE

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Foreword

This guide serves as a supplement to the video "Culturing *Americamysis bahia*" (EPA, 2009a). 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) *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, Fifth Edition* (2002a), referred to as the Acute Methods Manual. The video and this guide provide details on culturing of mysids for the use in conducting tests 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. The video entitled "Mysid (*Americamysis bahia*) Survival, Growth, and Fecundity Toxicity Tests" (EPA 2009b) complements the material in this video by explaining the 7-day short-term chronic toxicity test method using mysids. 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 supplemental 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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Contents

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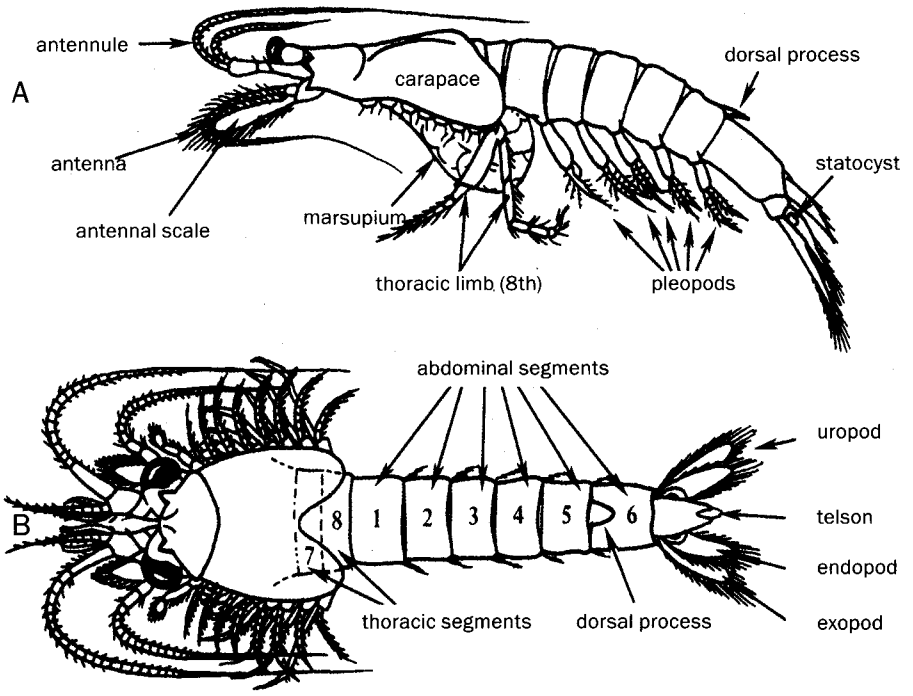
Introduction

Americamysis bahia, A. almyra, A. bigelowi, Metamysidopsis ilongata, and *Neomysis americana*, called mysids or opossum shrimp, have all been used in toxicity tests. This guide focuses on *Americamysis bahia*, the EPA-recommended species used in the mysid survival, growth, and fecundity toxicity test (Method 1007 in EPA, 2002b). *Americamysis bahia* are found in the coastal waters of the Gulf of Mexico and along the Atlantic coast as far north as Rhode Island.

As shown in Figure 1, mysids usually appear transparent with a yellow, brown, or black tint and range from 4.4 mm to 9.4 mm in length (Molenock, 1969). *Americamysis bahia* differ from the other *Americamysis* species by the armature of the telson and the spine-setaes on the thoracic and uropodal endopods (Molenock, 1969; Price et al., 1994).

The culturing procedures presented in this supplemental guide and illustrated in the video were developed to meet the specific needs of the mysid in each of its life stages. This guide and the video "Culturing *Americamysis bahia*" (EPA, 2009a) were produced by EPA to clarify and expand on methods explained in the EPA manual *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, Fifth Edition* (EPA, 2002a). Laboratory personnel who are familiar with the culturing and handling procedures of the test species and the use of healthy test organisms are critical for valid and successful toxicity test results.

Figure 1. The General Morphology of Mysids: (A) Lateral View; (B) Dorsal View.



Source: Heard and Price, 2006 as modified from Stuck et al., 1979a.

The first section of this guide covers the selection and preparation of the water for culturing and presents options for water delivery systems. The second section explains how to set up and maintain mysid cultures specifically for providing healthy test organisms. The third section provides instructions for collecting young of the same age for testing. The fourth section provides details on the food preparation methods used at NHEERL-AED in Narragansett, Rhode Island. This guide also includes a glossary and additional references. Appendix A provides a list of the apparatus and equipment needed to culture mysids.

Water and Light

Culture Water

Culture water is a primary consideration when starting mysid cultures. EPA recommends using natural seawater. However, hypersaline brine may be used to make up culture water if natural seawater is not available. If natural seawater is used, it must be contaminant-free and filtered through a 0.45 µm screen before use to remove particulates and possible predators. The source of the culture water should be uncontami-

nated, consistent, reliable, and periodically checked to ensure the water supports adequate performance of the test organisms with respect to survival, growth, and reproduction. More specific instructions for the preparation of artificial seawater are listed in EPA's Acute Methods Manual (EPA, 2002a) or can be obtained from commercial suppliers. Optimum culture conditions, including water quality, are provided in Table 1.

Table 1. Recommended Culture Conditions for *Americamysis bahia*

Parameter	*Culture Conditions*
Salinity	25 g/l (20‰ – 30‰)
Temperature	26°C ± 1°C
pH	7.8-8.2
Dissolved oxygen	7.1 mg/L
Ammonia	0.1-0.3 mg/L
Nitrite	<0.05 mg/L
Nitrate	<20 mg/L
Alkalinity	150 mg/L
Photoperiod	12-hr light:12-hr dark to 16-hr light:8-hr dark
Filtration	20 µm
Tank Size	10-55 gal
Substrate	Dolomite, oyster shells, coral
Biological filter / algal mat	*Spirulina subsalsa*

Source: Lussier et al., 1988.

Reference toxicant tests should be conducted at least once each month to analyze both the culture water being used and to check the mysid mass culture's sensitivity. Recommended reference toxicants are copper sulfate, cadmium chloride, or sodium dodecyl sulphate.

PHOTOPERIOD

For optimum growth and fecundity, the photoperiod for mysid cultures should be 16 hours light and 8 hours dark with a light intensity of about 50 – 100 foot-candles. EPA recommends using a system that turns the lights on and off gradually so as not to startle the mysids, which can cause them to jump out of the culture vessels. Alternatively, the light cycle can be provided using overhead room lights (cool-white fluorescent bulbs, approximately 50 ft-c), supplemented with individual grow lights placed over each tank (approximately 65 ft-c). This arrangement allows the overhead lights to turn on one hour before the aquaria lights turn on and to turn off one hour after they are extinguished.

CULTURE VESSELS

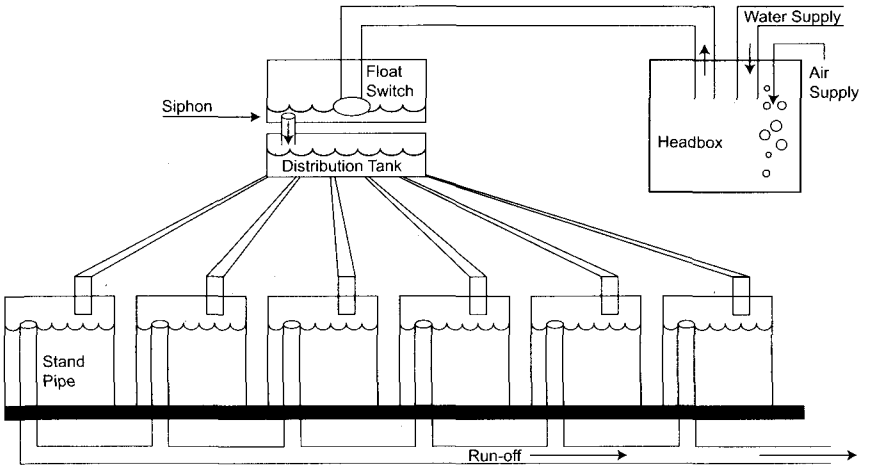
Mysids can be cultured in tanks of various sizes. The most commonly used are 20 and 29 gallon aquaria. Wider tanks are more suitable for culturing than taller ones because a large surface area to volume ratio provides both good oxygen exchange and a larger surface area for these epibenthic organisms that prefer to hover over the bottom of the tank. Tanks, as with all culturing equipment, should be cured in the culture water for approximately 3 – 5 days before being used for organisms.

WATER DELIVERY SYSTEMS

Mysids can be cultured in flow-through, recirculating, or static systems. The preferred system is the flow-through arrangement where water is delivered to the tanks at a measured rate and the runoff is discharged out of the system (see Figure 2). The flow rate through the culture tanks should be no less than 4 – 5 liters per hour or two complete turn-overs per day. Non-toxic materials such as glass, fiberglass, Teflon®, and polyvinylchloride (PVC) pipe are recommended for the water delivery system. Materials such as rubber, cop-

per, brass, or plastic should not be used because they could become a source of toxicity.

Figure 2. Intermittent Flow-Through Water Delivery System



Source: EPA 2002b.

Recirculating systems also can be used to culture mysids and should be designed to provide the same flow rate as the flow-through system. However, recirculating systems must also provide a biofiltering system that can be constructed out of any non-toxic, high-surface-area material such as crushed coral, pea gravel, or dolomite. This biological filtration system serves to oxidize the ammonia and nitrites that can build up in a closed system. A sand filter also may be added to the system.

Static systems are made of a series of tanks that are independently filtered and supplied with water. The advantage of this type of system is that problems such as disease are confined to one tank and complete culture "crashes" (sudden death of a culture) are less common. Each tank in a static system should be supplied with an under gravel filter and water changes should be made by replacing one-half of the tank's volume of water with fresh culture water every other day. Static systems are harder to maintain than flow-through or recirculating systems due to evaporation. Tanks should be covered and care must be taken to avoid the concentration of salts as the water evaporates.

Culture Start Up and Maintenance

Starting Cultures

Once the culture system and water source are designed, obtained, and seasoned, mysids can be purchased from a number of sources. A reliable supplier will certify that the correct species has been shipped. Records of the verification should be retained with a few preserved organisms. If test animals are not needed immediately, cultures should be started with juveniles to allow laboratory personnel to become familiar with mysid handling and maintenance requirements before learning to collect the young.

Mysids should be shipped in Nalgene® containers packed inside coolers or polyfoam boxes within cardboard shipping cartons. The shipping density should be <100 mysids per liter and the container should have 2 - 4 cm of airspace to ensure a supply of oxygen throughout the shipping period. No food should be added to the containers. A reliable overnight delivery service should be used for shipment so that the mysids are not in transit without food for more than 24 hours.

After the shipment is received, the mysids must be acclimated to the receiving laboratory's culture water and conditions. The temperature and salinity of the water used for shipping must be measured. Slow adjustment of the water temperature can be accomplished by placing the container in a water bath. The salinity can be adjusted by adding new culture water to the water used for shipment. Increases or decreases in temperature and salinity should not exceed 2°C or 2‰ - 3‰, respectively, per day.

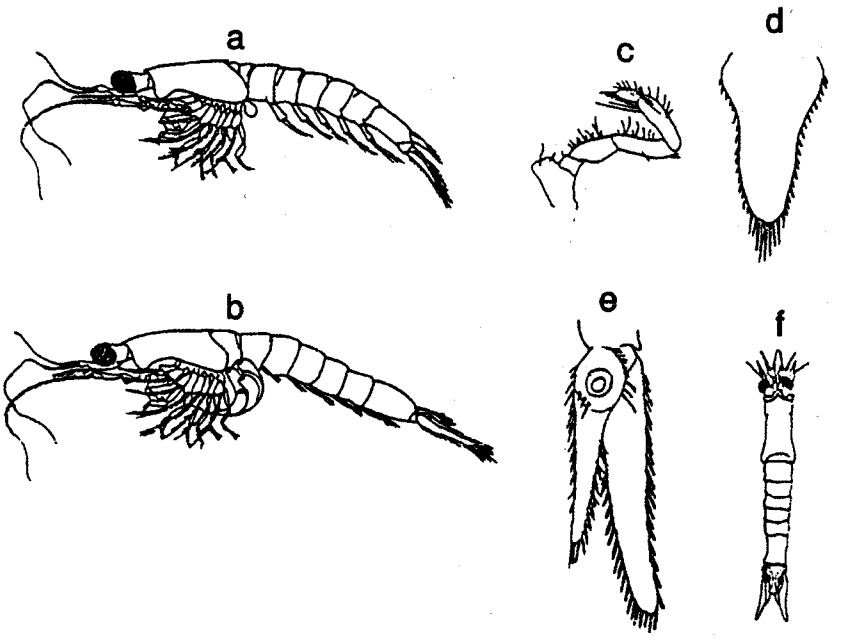
For optimum growth and reproduction, the stocking density for adult mysids should be approximately 20 mysids per liter. Juveniles can be stocked at higher densities than adults. A healthy, unstressed culture should have at least 70% of the females carrying eggs in their brood pouch.

TAXONOMY

Mysids usually appear transparent with a yellow, brown, or black tint and range from 4.4 mm to 9.4 mm in length (Molenock, 1969). The morphological characteristics used to distinguish *A. bahia* from other mysids are presented in Figure 3.

Figure 4 shows the life cycle of a mysid. Mysids produce live young called early juveniles. These juveniles are planktonic for the first 24 hours post-release and then settle to the bottom where they orient to the current in the tank and begin to feed. Depending on water temperature and diet, females reach sexual maturity in about 20 days. Brood pouches appear at the age of 12 – 16 days and young are released at approximately 20 days. A gravid female is identified by an enlarged and darkened brood pouch containing the developing embryos. The female is ready to release the young when the eyespots can be identified in the brood pouch. Females average 5 – 7 young per brood, but can produce as many as 20 in one brood. Broods are produced for several months at a rate of one every 4 – 6 days.

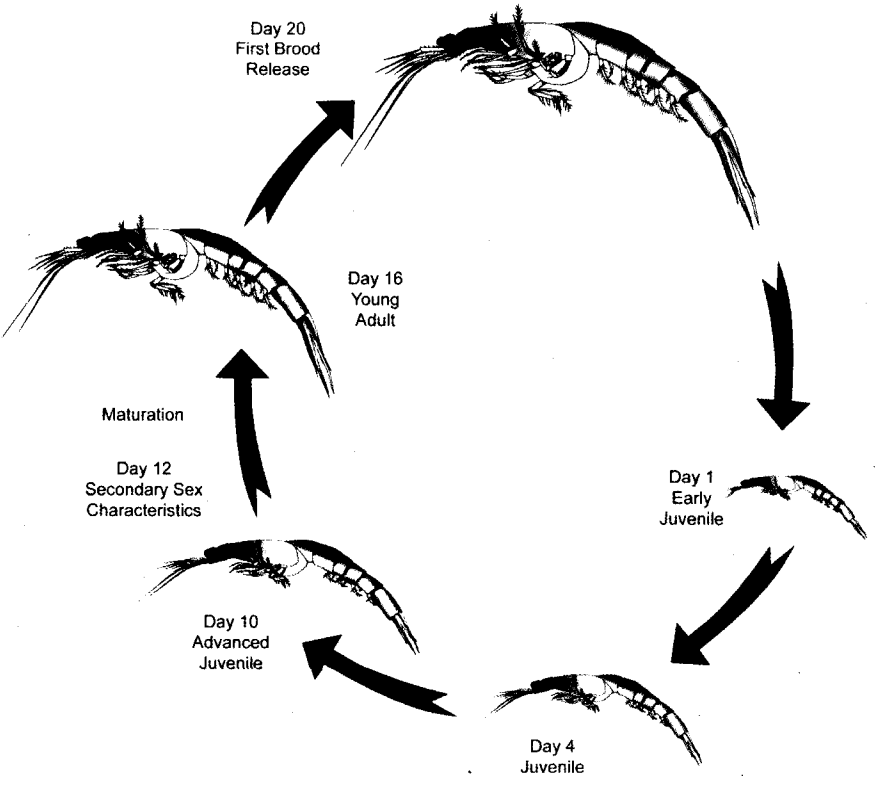
Figure 3. Morphological Characteristics Used in Mysid Identification



Morphological features most useful in identifying Americamysis bahia. *a. male; b. female; c. thoracic leg 2; d. telson; e. right uropod, dorsal; f. male, dorsal (redrawn from Molenock, 1969; Heard et al, 1987). Note testes in area where marsupium is located on female and length of male pleopods as compared to female. Also note the three spines on the endopod of the uropod (e).*

Source: Molenock, 1969; Price et al., 1994

Figure 4. Life Cycle of a Mysid

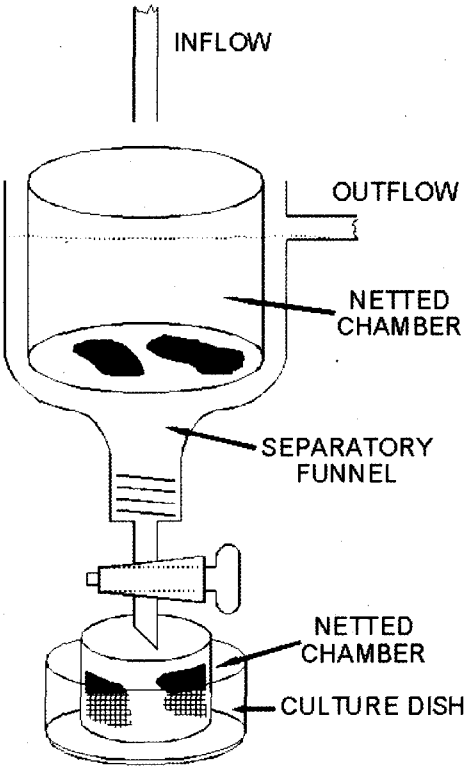


Collecting Test Organisms

To conduct toxicity tests using mysids, organisms of the same age must be collected and pooled. To accomplish this, gravid females are collected from the culture tanks and their young are collected and held until the proper age for starting tests. For testing

needs, assume a reproduction rate of two juveniles per female per day because not all females will release their young on the same day. Collect the gravid females from a minimum of three culture tanks. While identifying and selecting gravid females for the brood chamber, the sex ratio and density of each tank should be determined and adjusted, if needed, to maintain a ratio of 2 females:1 male.

Figure 5. Illustration of Mysid Brood Chamber



Source: Lussier, et al., 1987.

Brood chambers such as the one illustrated in Figure 5 are used to collect test animals. Gravid females are collected from a minimum of three culture tanks and placed in a 4 L Nalgene® beaker that is placed inside a separatory funnel containing culture water. The solid plastic bottom of the Nalgene® beaker is replaced by 1 mm mesh screen. The screen allows the newly released young to pass through while preventing the adults from leaving the beaker.

Once the females are placed in the brood chamber, provide food and gentle aeration by either placing an airstone in the neck of the separatory funnel or providing water inflow and outflow to the funnel. The females should be left overnight and the young collected the next day.

To harvest the young, remove the airstone or stop the flow of water and slowly drain the separatory chamber into a 300 µm mesh cup placed in a culture dish. To prevent injury to the young mysids, partially submerge the mesh cup in culture water within the culture dish before draining the brood chamber. While the water is draining, gently lift and dunk the beaker containing the females to wash any remaining young out through the screen. As the water drains from the funnel, gently rinse the sides 2 – 3 times with clean seawater to wash out any mysids that may stick to the sides. The females should be placed back into the culture tanks. The young can be used immediately for testing or grown out in a separate tank to the desired age. The harvested young should be maintained at conditions similar to the regular cultures.

An alternative system for collecting young is a siphon entrapment system, or a "mysid generator" (see Figure 6). The siphon inlet is covered by a 750 µm screen that excludes adults and allows juveniles to pass through to a collection vessel. In the collection vessel juveniles are deposited into a 350 – 370 µm Nitex® screen cup. The juveniles in the screen cup are collected daily for test use. When using mysid generators, care must be taken to siphon all of the juveniles out of the tank each day. Otherwise, the collected juveniles' ages may not be within 24 hours of each other as test methods require.

Tank Cleaning

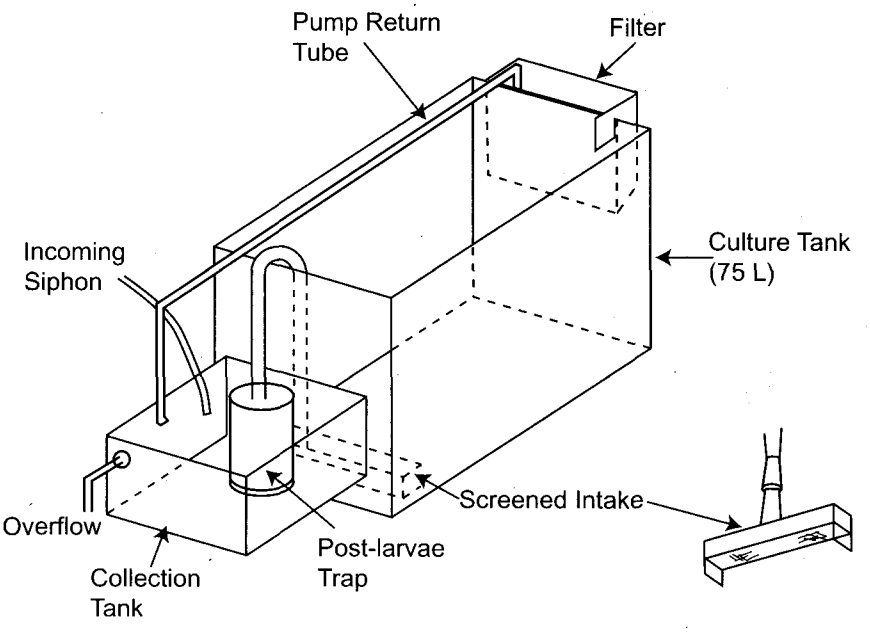
Culture tanks should be cleaned at least once each month. The sides of each tank should be scraped to remove any algal growth and the gravel should be stirred to dislodge the accumulated debris, which will clear the dolomite filter.

Approximately twice each year, the tanks should be completely emptied and scrubbed. At this time the gravel should also be replaced. It is important to cure any new materials as described in the previous section "*Water and Light, Culture Vessels*," before using them in culture tanks.

Record Keeping

Culture tanks should be monitored and all conditions recorded on data forms that are kept in a permanent file. These forms are used to assess any problems that may occur with the cultures and assist in eliminating possible causes. The forms also serve as a record for testing laboratories to verify that their test organisms were raised using proper culture techniques.

Figure 6. Illustration of Mysid Generator



Source: Lussier et al., 1988.

Figure 7 is a data sheet adapted from the one used by AED-Narragansett for mysid cultures. Each of the conditions is checked daily and initialed by the technician taking the reading, checking the condition, or performing the task. Daily tasks performed are measurement of temperature, pH, salinity, and dissolved oxygen; seawater and air flow checks; and feeding (twice daily).

Figure 7. Data Form for Mysid Cultures

Date	*Temp °C*	*pH SU*	*Salinity ‰*	*DO mg/L*	*SW Flow*	*Air Flow*	*Mysids Fed*	*Comments*

Food Preparation

Mysid cultures are fed *Artemia* nauplii (newly-hatched brine shrimp) twice each day at a rate that ensures live *Artemia* are always available in the tanks (approximately 150 *Artemia* nauplii per mysid per day). The *Artemia* should be cultured in the laboratory in order to provide 24 - 48 hour old nauplii on a daily basis. *Artemia* cysts are available from commercial suppliers. Each shipment of *Artemia* received should be analyzed for priority pollutants and should be tested on a small batch of mysids to ensure that good mysid growth and reproduction occur before the *Artemia* are fed to entire mysid cultures. Food supplements are commercially available and are used more often when using artificial seawater for culturing.

Culture the *Artemia* by adding dry cysts to clean seawater at a rate of approximately 10 mL cysts to 1 L seawater (ASTM, 1998). A separatory funnel works well for culturing *Artemia*. Inverted two-liter plastic bottles also have been used by cutting out their bottoms and inserting a rubber stopper with a flexible tube and pinch clamp.

After placing the water and cysts into the culture chamber, aerate vigorously to keep the cysts (and eventually the newly-hatched nauplii) in suspension. Deliver the filtered air through a 1 mL pipet by resting the tip of the pipet at the bottom of the neck of the chamber. This keeps the nauplii from settling and depleting the oxygen supply.

IMPORTANT NOTE:
The nauplii must be aerated if they remain unused for more than a few minutes. Without aeration the nauplii will begin to die.

The cysts will hatch in approximately 24 hours. Newly-hatched *Artemia* nauplii are more nutritious than older ones and are the appropriate size for feeding early juvenile mysids. To harvest the nauplii for feeding, remove the air supply and allow the cysts and nauplii to separate for five minutes. The empty cysts will float and the nauplii will descend to the neck of the chamber. The nauplii are attracted to light, so a light source placed at the bottom of the chamber and/or a dark cover or hood placed on the top will hasten the separation process.

Drain the nauplii through the stop clamp or siphon them from the bottom of the chambers. If the nauplii are drained through the stop cock, the first plug of unhatched cysts that collect at the neck of the chamber should be discarded and not mixed with the nauplii. Drain only the hatched nauplii (the bright orange suspension), leaving behind the empty cysts. The nauplii should be drained through a 150 µm screen and rinsed with clean seawater to remove any chemicals released during hatching.

To determine the correct amount of *Artemia* for feeding, an aliquot of the hatched *Artemia* should be counted under a microscope to determine the density of the culture. This density will serve as a reference to ensure that future cultures are hatching at the same rate and that mysids are being fed a consistent amount of food.

Once the *Artemia* are rinsed, the volume of clean seawater that is added determines the volume of food provided to each tank. From the calculated and adjusted density of the diluted food supply, determine the volume of food needed for each tank by estimating a feeding rate of 150 *Artemia* per mysid per day, or 75 *Artemia* per mysid per feeding. Feeding the mysids in two feedings, 8 - 12 hours apart ensures there are always live *Artemia* available for the mysids.

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EPA references are available online at **www.epa.gov/npdes**.

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Glossary

Artemia. The marine invertebrate (referred to as brine shrimp) used as the recommended food source for mysid cultures and test organisms; Brazilian or Colombian strains are preferred because the supplies are found to have low concentrations of chemical residues and nauplii are of suitably small size.

Crash. Sudden (overnight) death of cultured organisms in a tank.

Cyst. The life stage of unhatched *Artemia*.

Epibenthic. Pertaining to the area just above the sediment.

Fecundity. Productivity or fertility as measured in the mysid test as the percentage of females with eggs in the oviduct and/or brood pouch.

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

Mysid (*Americamysis bahia*). An estuarine crustacean, formerly known as *Mysidopsis bahia*, ranging 4.4 mm to 9.4 mm in length found from the Gulf of Mexico and along the Atlantic coast as far north as Rhode Island; used in test procedures as an indicator species for aquatic toxicity.

Nauplii. Free-swimming microscopic larvae stage characteristic of copepods, ostracods, barnacles, etc. typically with only three pairs of appendages.

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.

Static water system. An enclosed system contained within one culture tank. The water is filtered through an underground or charcoal filter and is delivered back to the same tank.

WET (Whole effulent toxicty). The total toxic effect of an effluent measured directly with a toxicty text.

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Appendix A
Apparatus and Equipment List

Air line and air stones. For aerating cultures, brood chambers, and holding tanks, and 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, 2 – 3 L for making test solutions.

Brine shrimp (*Artemia*) culture unit. See *"Food Preparation"* section.

Depression glass slides or depression spot plates. Two for observing organisms.

Dissecting microscope (240 – 400X magnification). For examining organisms to determine their sex and to check for the presence of eggs in the oviducts of the females.

Droppers, and glass tubing with fire polished edges. 4 mm inner diameter (ID), for transferring organisms.

Environmental chamber or equivalent facility with temperature control (26 ± 1°C).

Facilities for holding and acclimating test organisms.

Light box. For illuminating organisms during examination.

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

Mysid (*Americamysis bahia*) culture unit. See *"Culture Start Up and Maintenance"* section. The test requires a minimum of 240 7-day old (juvenile) mysids.

NITEX® or stainless steel mesh sieves. 150 µm and 100 µm for concentrating organisms; 1 mm mesh and 300 µm mesh for collection of juveniles.

Pipet bulbs and fillers. Propipet®, or equivalent.

Pipets, automatic. Adjustable, 1 – 100 mL.

Pipets, serological. 1 – 10 mL, graduated.

Pipets, volumetric, Class A. 100 mL.

Reference weights, Class S. For checking performance of balance.

Refractometer or other method. For determining salinity.

Separatory funnels, 2-liters. Two to four for culturing *Artemia*.

Standard or micro-Winkler apparatus. For determining DO and checking DO meters.

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

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

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

Volumetric flasks and graduated cylinders. Class A, borosilicate glass or non-toxic plastic labware, 50 - 2000 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.

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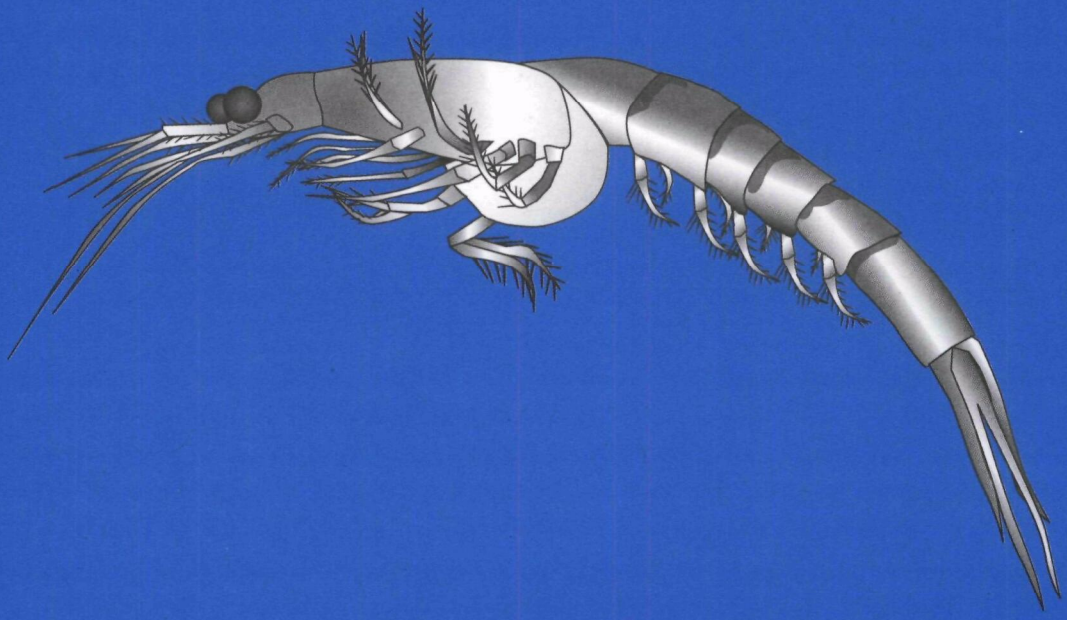
Mysid (*Americamysis bahia*) Survival, Growth, and Fecundity Toxicity Tests

Supplement to Training Video



U.S. Environmental Protection Agency
Office of Wastewater Management
Water Permits Division
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NOTICE

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Foreword

This guide serves as a supplement to the video "Mysid (*Americamysis bahia*) Survival, Growth, and Fecundity Toxicity Tests" (EPA, 2009a). 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 supplemental 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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Contents

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Introduction

This supplemental guide accompanies the Environmental Protection Agency's (EPA's) video to provide instructions for conducting the standard 7-day survival, growth, and fecundity toxicity test using the mysid, *Americamysis bahia* (EPA, 2009a; EPA, 2009b). The test method is found in *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms, Third Edition* (EPA, 2002a). The methods presented in this guide and the video are based on the experience and standardized practices 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. 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 whole effluent toxicity (WET) permit limitations regulate pollutant discharges on a whole effluent effect basis rather than solely by a chemical-specific approach.

The mysid survival, growth, and fecundity toxicity test (Test Method 1007.0 in EPA, 2002a) is used by EPA for determining the toxicity of marine or estuarine discharges by measuring specified endpoints after a 7-day exposure period. 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 mysid 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).

Maintaining and Feeding Cultures

Culture Maintenance

Americamysis bahia (mysids, or opossum shrimp) are estuarine invertebrates generally found in the coastal waters of the Gulf of Mexico and along the Atlantic coast as far north as Rhode Island (see Figure 1). They usually appear transparent with a yellow, brown, or black tint and range from 4.4 mm to 9.4 mm in length (Molenock, 1969). Adult mysids can be collected from the field, however, they must be verified taxonomically as the correct species before being placed in cultures for test use (Price et al., 1994). Alternatively, commercial suppliers provide adults for cultures and juveniles for cultures or testing. The supplier should verify that the correct species is sent.

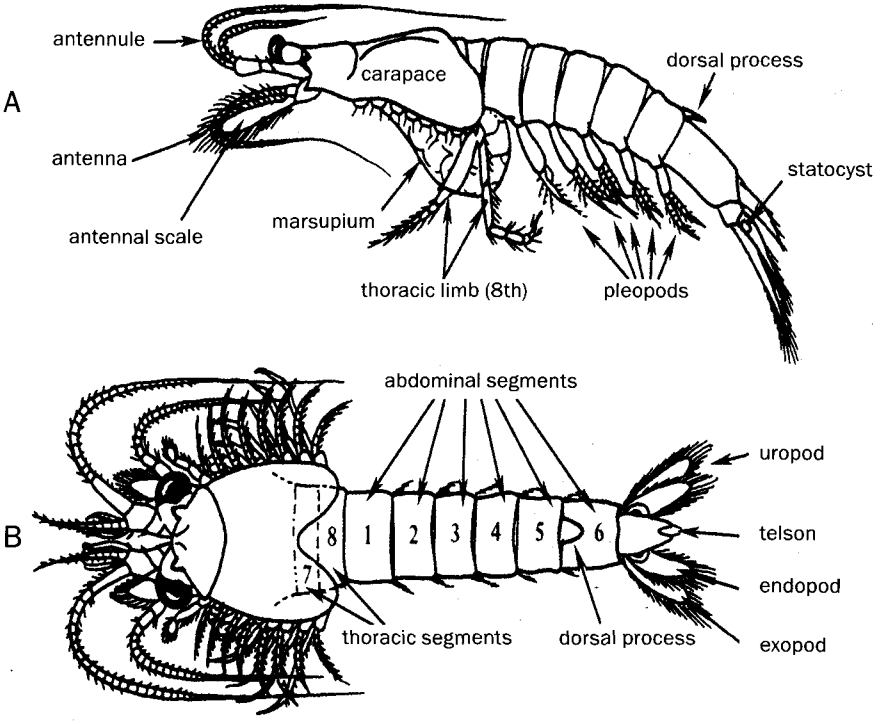
Cultures should be maintained in glass aquaria supplied with flow-through or recirculating seawater (Lussier et al., 1988). The water temperature should be 26°C and salinity between 20‰ to 30‰ and should not fluctuate more than 2°C or 2‰ per day, respectively. The light regime recommended for culturing is 16 hours light and 8 hours dark. The light should be phased on and off gradually so as not to startle the mysids.

FEEDING

Mysids are fed <24-hr old *Artemia* nauplii (newly hatched brine shrimp) twice daily. Feeding amounts should be adequate to provide live food at all times for the mysids to feed upon. Approximately 150 *Artemia* per mysid per day is recommended. *Artemia* supplies should be checked periodically for contamination and hatch rates.

Detailed instructions on culturing *Artemia* are presented in the video "Culturing *Americamysis bahia*," and its accompanying supplemental guide, and in the EPA manual *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, Fifth Edition* (EPA, 2009b; EPA, 2002b).

Figure 1. The General Morphology of Mysids. (A) Lateral View; (B) Dorsal View



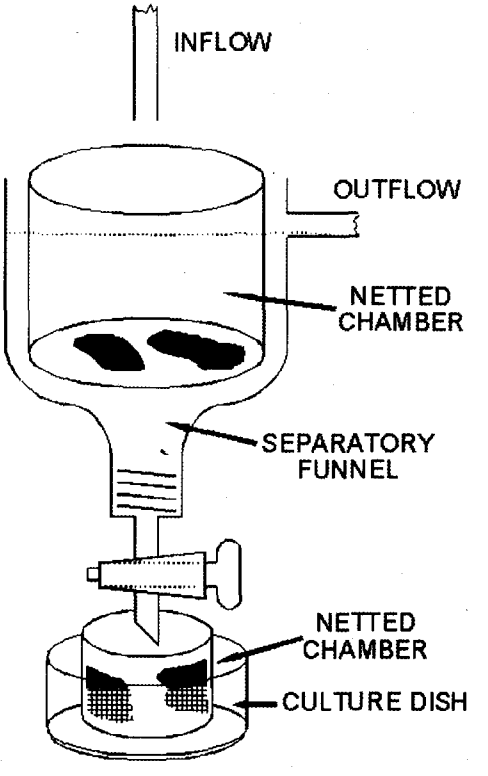
Source: Heard and Price, 2006 as modified from Stuck et al., 1979.

Collecting Juveniles for Test Use

The 7-day survival, growth, and fecundity toxicity test must be started with 7-day old mysids that are all within 24 hours age of each other. Seven-day old juveniles are needed in sufficient number to randomly select five juveniles for each replicate. For a test with five effluent concentrations and one control, with 8 replicates at each concentration, it is recommended to have approximately 240 – 300, 7-day old mysids available to choose from. Avoid using any mysids that appear injured.

To collect juveniles and to be assured of their age range (within 24-hours age), a brood chamber is used (see Figure 2). The brood chamber is set up eight days before the start of the test.

Figure 2. Apparatus for Collection of Juvenile Mysids from Gravid Females



Source: Lussier et al., 1987.

Gravid females selected from a minimum of three culture tanks are placed in a netted chamber inside a funnel. Gravid females are those ready to release their young and are identified by dark spots in their brood pouches. Because not all of the females will release young on the same day, an estimate of two juveniles per female per day should be used to determine the number of gravid females needed. Therefore, to have sufficient mysids for test initiation, approximately 125 - 150 gravid females should be placed in the brood chamber.

Twenty-four hours after placing the females in the brood chamber, or seven days before the test start date, remove the netted chamber containing the gravid females from the brood chamber allowing the juveniles to escape through the screened bottom. Return the females to the culture tanks and drain the juveniles from the funnel into a mesh cup placed in a dish containing culture water. To prevent injury to the test animals, gently rinse the sides of the funnel as it drains. These juveniles, all born within the last 24 hours should be counted and transferred into a separate tank where they will be held for the next seven days. Because stocking density is very important to the rate of juvenile development, no more than 300 juveniles should be held in a 10-gallon tank. If the holding tank used is a static system, half of the water must be replaced every other day with new culture water.

Nutrition and temperature are important factors in mysid development (Lussier et al., 1999). During the 7-day holding period maintain the holding tanks at 26°C - 27°C with a salinity similar to the culture/test water. If necessary, the salinity should be gradually adjusted (≤2‰/day) to the desired test salinity (20‰ - 30‰) during this holding period. Feed the juveniles <24-hour old *Artemia* nauplii twice daily.

Conducting the Test

Under the NPDES program, lapsed time from sample collection to first use of that sample in a toxicity test (i.e., test initiation) must not exceed 36 hours. If stored correctly, the sample may be used for test renewals at 24 hours, 48 hours, and/or 72 hours after test initiation.

Effluent Sampling

Effluent sampling should be conducted according to the EPA Saltwater Chronic Methods Manual (EPA, 2002a) and any conditions specified in a regulatory permit. In static renewal tests, each grab or composite sample may be used to prepare test solutions for renewal at 24, 48, and/or 72 hours after first use if stored between 0°C - 6°C, with minimum head space. According to the EPA 2002 promulgated methods, for WET samples with a specified storage temperature of 4°C, storage at a temperature above the freezing point of water to 6°C shall be acceptable (0°C - 6°C). EPA has further clarified that hand-delivered samples used on the day of collection do not need to be cooled to 0°C - 6°C prior to test initiation (EPA, 2002c).

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 (cont.)*

Dilution Preparation

To start a test, warm the effluent to 26°C ± 1°C slowly to avoid exceeding the desired temperature. This is accomplished using a water bath and monitoring the temperature closely. A temperature of 26°C ± 1°C should be maintained throughout the 7-day test period and the instantaneous temperature must not deviate by more than 3°C during the test.

Once the effluent and the dilution water reach the desired temperature, the dilutions are prepared.

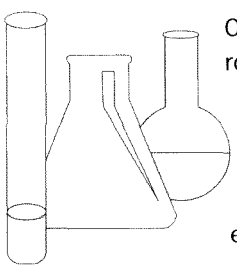
Dilution Water (cont.)
can be conducted using a grab sample of the receiving waters collected outside the influence of the outfall, other uncontaminated waters, or standard dilution water with the same salinity as the receiving waters. If the cultures were maintained in different water than used for 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.*

Because the marine/estuarine species used for testing are salinity sensitive, the effluent must be adjusted to the proper salinity before preparing the test concentrations. Hypersaline brine is recommended for adjusting the effluent salinity. Appendix D provides instructions for preparing the brine solution (EPA, 2002a). To prepare test concentrations at the desired salinity, adjust the diluent (deionized water) with the hypersaline brine before adding it to the effluent. Using hypersaline brine instead of seawater allows the test to be run at higher effluent concentrations because less dilution is needed to adjust to the proper salinity.

Use a minimum of five exposure concentrations and a control with a minimum of eight replicates per concentration. The Saltwater Chronic Methods Manual recommends the use of a 0.5 dilution factor, which provides precision of ± 100%. Test precision shows little improvement as the dilution factor is increased beyond 0.5, and declines rapidly if a smaller dilution factor is used. Approximately 3 L of test solution are needed each day for a test conducted with 8 replicates of 5 concentrations and a control.

Routine Chemistries



Once the various concentrations are prepared, set aside one aliquot of each for conducting routine chemistries. By setting these aside, the chemistries can be performed without contaminating the actual test solutions with the probe. For test initiation and renewals, measure and record the dissolved oxygen (DO) at the beginning and end of each 24-hour renewal in at least one test chamber of each test concentration and in the control. If the DO falls below 4.0 mg/L in any replicate, aerate all concentrations and the control. Take care not to cause excess turbulence that can cause physical stress to the organisms.

Dissolved oxygen, temperature, pH, and salinity must be measured on each new sample. Dissolved oxygen is measured at the beginning and end of each 24-hour renewal in at least one test chamber of each test concentration and in the control. Measuring salinity at the beginning and end of each 24-hour renewal is preferred but not required. The salinity, temperature, and pH of the effluent sample must be measured at the end of each 24-hour exposure period in one test chamber at each concentration and in the control. See Table 1.

Table 1. Monitoring Schedule

	Monitoring Frequency		
		24-hr Exposure Period	
Parameter	***Each New Sample***	***Beginning***	***End***
Dissolved Oxygen[1,2]	X	X	X
Temperature[1,3]	X		X
pH[1,3]	X		X
Salinity[1,2]	X	X	X

1 Measured in each new sample (100% effluent or receiving water) and in control.
2 Beginning and end measurement on one replicate in each concentration and the control.
3 End measurement on one replicate in each concentration and the control.

These parameters should fall within the recommended ranges for conducting the test and they should be recorded on the test data sheet. The recommended test conditions are presented in Appendix A and a sample water quality data sheet is provided in Figure 3.

Figure 3. Data Form for the Mysid Survival and Fecundity Toxicity Test – Water Quality Data

Test: _____

Start Date: _____ Salinity: _____

	TRTMT	TEMP	Salinity	DO	pH	TRTMT	TEMP	Salinity	DO	pH
Day 1	REP									
	REP									
Day 2	REP									
	REP									
Day 3	REP									
	REP									
Day 4	REP									
	REP									
Day 5	REP									
	REP									
Day 6	REP									
	REP									
Day 7	REP									
	REP									
	TRTMT	TEMP	Salinity	DO	pH	TRTMT	TEMP	Salinity	DO	pH
Day 1	REP									
	REP									
Day 2	REP									
	REP									
Day 3	REP									
	REP									
Day 4	REP									
	REP									
Day 5	REP									
	REP									
Day 6	REP									
	REP									
Day 7	REP									
	REP									

Source: EPA, 2002a.

TEST CHAMBERS

The test chambers should be readied before the effluent concentrations are prepared. EPA recommends using 8 oz disposable plastic drinking cups or 400 mL glass beakers to conduct this test. The test chambers are presoaked in clean seawater and labeled with colored tape. Each concentration is indicated by a different color tape with the replicate number (1 – 8) written on it. The use of different colored tape makes renewals easier because all of the replicates of one concentration can be identified quickly.

Once the cups are prepared and the effluent solutions have been adjusted to within the proper parameter ranges, each test solution is distributed to eight replicate cups. Each replicate should contain approximately 150 mL. The cups are placed in holding trays that are randomly placed in a temperature-controlled water bath. The holding trays should be labeled with the same colored tape and replicate numbers as the cups which allows for easier collection and replacement of the randomized cups during renewals. The cups will stay in the same randomized positions for the duration of the test. Specific directions for test randomization are provided in Appendix A of the Saltwater Chronic Methods Manual (EPA, 2002a).

TEST ORGANISMS

Juvenile mysids should be collected from gravid females obtained from at least three separate culturing tanks. To begin a test with five effluent concentrations and a control, each with eight replicates, a minimum of 240 juveniles are needed. Having more than 240 juveniles allows for extra juveniles from which to choose. Select juveniles at random, but avoid using any that appear injured.

Juvenile mysids are assigned to the test chambers at a density of five mysids per chamber. The juveniles are randomly selected from the 7-day old juvenile pool and pipetted using a large bore (4 mm inner diameter [ID]) pipet into small presoaked ampules, two to three at a time. The open covers of the ampules serve as handles. This random selection and assignment is continued until all of the ampules contain five mysids. As the mysids are placed in these ampules, a minimum amount of water should be transferred with them so that the effluent concentrations are not diluted.

To transfer the mysids to the test chambers, the ampules should be dipped below the water level in each cup and gently rinsed to deposit the mysids. Pouring the mysids from above the water surface may cause injury. The test chambers should remain in the water bath while this transfer is made.

FEEDING

Once the test has been set-up, the mysids are fed. The initial feeding rate is 0.5 mL of a food solution made from 4.0 mL concentrated *Artemia* nauplii in 80 mL of uncontaminated, filtered seawater. This concentration of nauplii should yield a level of approximately 150 24-hr old nauplii per mysid per day. This amount of food solution should provide the test organisms with a sufficient number of live *Artemia* for the next 24 hours until test renewal. Immediately after renewal each day, feed the mysids 0.25 mL of food solution. Another 0.25 mL should be fed 8 – 12 hours later. The food should be dispensed using an automatic pipet and the food solution should be swirled before pipetting to ensure an even distribution of the *Artemia*. After feeding the mysids, cover the test chambers to prevent evaporation or contamination.

RENEWALS

To conduct the daily renewals, collect the test cups from the water bath starting with the control and working toward the higher concentrations. Measure and record the temperature, salinity, DO, and pH in a composite aliquot of a minimum of two randomly selected replicates from each concentration (see Figure 3). If the DO concentration falls below 4 mg/L in any one of the exposure chambers, all chambers must be gently aerated at a rate of approximately 100 bubbles/minute. During renewals the mysids in each chamber should be counted and the survival recorded on the test data sheets. Any dead animals should be discarded. A sample survival and fecundity data sheet is presented as Figure 4.

To renew the effluent, pour or siphon off the old effluent solution into a white tray or a large beaker placed on a light table. Either of these receptacles will clearly show any mysids that are accidentally removed. Slowly pouring the effluent from the cups works well because mysids tend to swim against the current and will swim towards the back of the cups. If a mysid is poured out with the old effluent it should be pipetted back into the exposure chamber and recorded as "returned during renewal" on the test data sheet. When removing the old effluent, a pipet should be used to clean any uneaten *Artemia* from the bottom of the chamber.

To add the new effluent solution to the chamber, gently pour approximately 150 mL of the appropriate solution down the side of the chamber avoiding as much turbulence as possible. This renewal procedure must

be repeated on days two through six of the exposure period. All data should be carefully recorded on the data sheets each day.

Immediately after renewal each day, feed the mysids 0.25 mL of food solution. Another 0.25 mL should be fed 8 – 12 hours later. If the survival rate in any replicate drops below 50%, the food provided to that replicate should be reduced by half. Detailed instructions for culturing *Artemia* are provided in the video "Culturing *Americamysis bahia*" and in its supplemental guide (EPA, 2009b).

Figure 4. Data Form for the Mysid Survival and Fecundity Toxicity Test – Survival and Fecundity Data

Test: _____

Start Date: _____ Salinity: _____

Treatment/ Replicate		Day 1 Alive	Day 2 Alive	Day 3 Alive	Day 4 Alive	Day 5 Alive	Day 6 Alive	Day 7 Alive	Females w/eggs	Females No eggs	Males	Immatures
Control	1											
	2											
	3											
	4											
	5											
	6											
	7											
	8											
1	1											
	2											
	3											
	4											
	5											
	6											
	7											
	8											
2	1											
	2											
	3											
	4											
	5											
	6											
	7											
	8											
5	2											
	3											
	4											
	5											
	6											
	7											
	8											

Source: EPA, 2002a.

Terminating the Test

On the last day of the 7-day exposure, the replicates are checked for survival and fecundity and the animals are prepared for growth measurements. The mysids are not fed on the last day of the test so that total weights do not reflect the added weight of any undigested *Artemia*.

In preparation for the test termination, prepare small pieces (1 cm^2) of clean, light-weight aluminum foil by labeling them with sequential numbers. Gloves should be worn or forceps should be used to handle the aluminum because oils from skin could affect weight differences. After they are numbered, these pieces of foil should all be dried, tared, and their weights recorded on the growth-data sheet. The sample growth-data sheet is presented as Figure 5.

Figure 5. Data Form for the Mysid Survival and Fecundity Toxicity Test – Dry Weight Measures

Test: _____

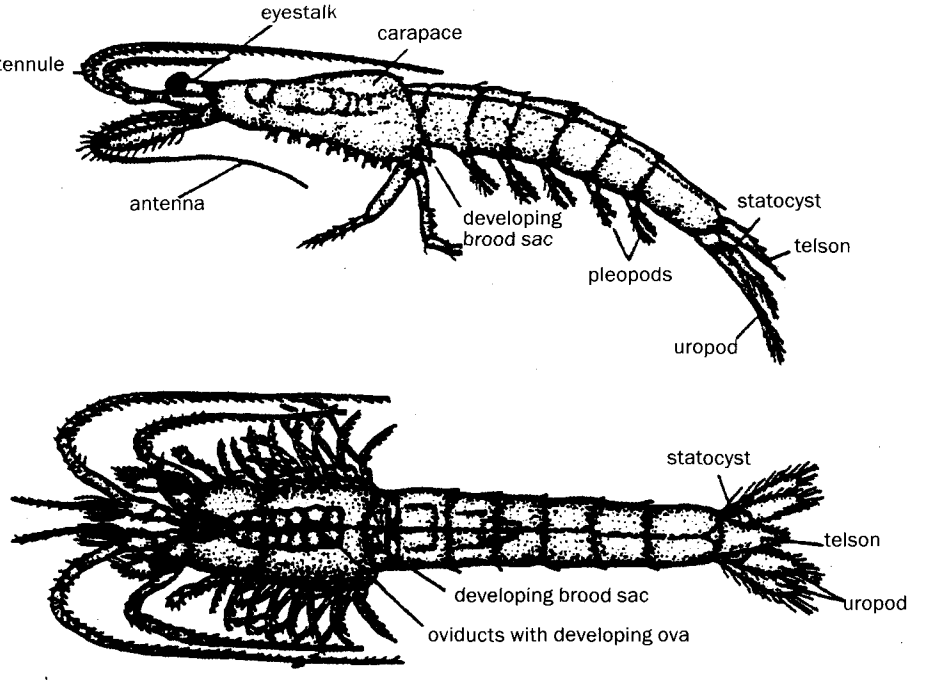
Start Date: _____ Salinity: _____

Treatment/ Replicate		*Pan #*	*Tare Wt.*	*Total Wt.*	*Organism Wt.*	*# of Organisms*	*Wt./Organism*
Control	1						
	2						
	3						
	4						
	5						
	6						
	7						
	8						
1	1						
	2						
	3						
	4						
	5						
	6						
	7						
	8						
2	1						
	2						
	3						
	4						
	5						
	6						
	7						
	8						
5	6						
	7						
	8						

Source: EPA, 2002a.

After the aluminum is prepared, pick up the test chambers in the same manner as for conducting a renewal. That is, collect all of the replicates of one concentration at one time, starting with the control. Final water quality measurements, including DO, temperature, salinity, and pH should be measured on aliquots taken from several test chambers in each concentration and the control and recorded (see Figure 3).

Figure 6. Mature Female *A. bahia* with Eggs in Oviducts. Lateral view (top) Dorsal view (bottom)



Source: Lussier, Kuhn, and Sewall, 1987.

First, remove dead mysids from the test chambers and record the final survival count for each replicate on the test data sheet (see Figure 4). The minimum requirement for an acceptable test is 80% survival in the controls.

Second, determine the sexual development and fecundity of each mysid in each replicate. The effluent should be poured off in the same manner as during renewals. For each replicate remove the mysids and place each one in a separate well of a multi-well slide. Any excess water transferred with the mysid can be removed from the well to make viewing under a microscope easier.

Using a stereomicroscope at 240X, determine the sexual development of each mysid and record it on the test data sheet (see Figure 4). This must be conducted while the mysids are alive because they turn opaque upon dying. Figures 6 through 9 illustrate the sexual characteristics used to determine the maturity and fecundity of the mysids.

Figure 6 is a mature female with eggs in the oviducts. This is most easily determined when viewed from above and is determined by large, dark, oval-shaped bodies in the mid-section of the thorax.

Figure 7 shows a mature female with eggs in the brood pouch, and is characterized by the presence of dark pigmented spots on the lateral sides of the body. These can be seen both from above and from the side. Females that have no eggs or embryos have an empty brood pouch and empty oviducts. These females can be identified by a single dark spot on each half of the brood pouch. These spots can be seen from both above and from the side, although from the top is easiest. The video provides examples of females with, and without, eggs and embryos.

Figure 8 presents a mature male mysid. Males are determined by the presence of testes that appear either as clear circles, when viewing them from above, or as appendages at the junction of the thorax and abdomen when viewing them from the side.

Figure 9 presents a diagram of an immature mysid. Immature mysids are those that do not have characteristics that determine their classification as either mature males or females. Care must be taken, however, not to mistake a barren female for an immature mysid. As the sex of each mysid is determined it should be recorded on the survival and fecundity data sheet (see Figure 4).

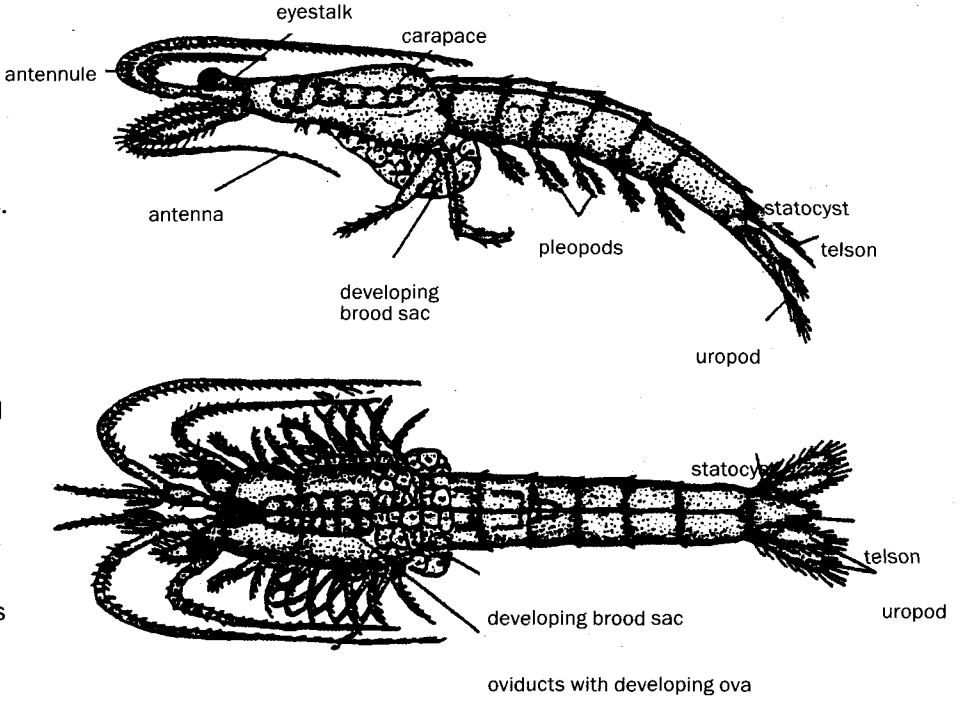
After the sex, maturity, and fecundity of each mysid from one replicate is determined, all of the mysids from that replicate should be placed on a Nitex® screen that rests on top of a beaker. Rinse the mysids with deionized water to remove any salts that may interfere with the dry weights. After the animals are rinsed they are placed on the designated pre-tared piece of aluminum foil for that replicate. Note that all of the mysids from one replicate are placed on the same piece of foil.

Once this process has been repeated for all of the replicates the mysids are dried in an oven at 60°C for 24 hours or 105°C for at least six hours. The mysids must be completely dried before they are weighed but they should not be overdried.

The mysids should be transported and stored in a desiccator when weighing them. This prevents moisture from reabsorbing into the mysids. The mysids are weighed, one replicate at a time, to the nearest milligram (0.001 g.). Because small differences in weight or appearance can easily change the test results, it is critical to record observations and measurements clearly and accurately. See Figure 5 for a sample data sheet for recording weights. The minimum requirement for an acceptable test is an average weight of at least 0.20 mg/mysid in the controls.

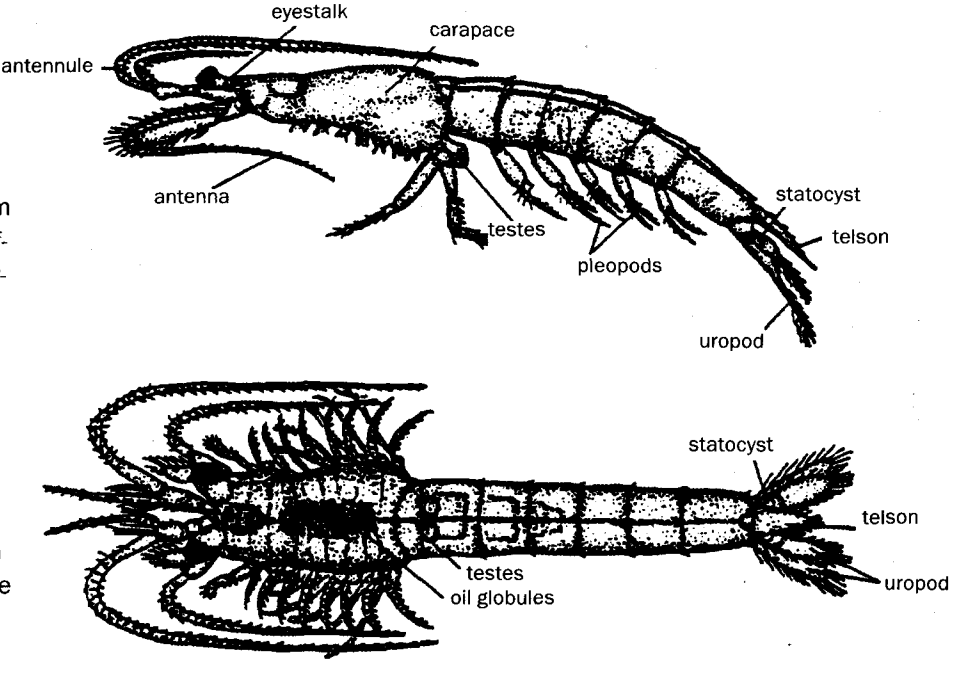
The analysis of this test compares the maturity, fecundity, growth, and survival of the

Figure 7. Mature Female *A. bahia* with Eggs in Oviducts and Developing Embryos in Brood Sac. Lateral view (top) Dorsal view (bottom)



Source: Lussier, Kuhn, and Sewall, 1987.

Figure 8. Mature Male *A. bahia*. Lateral view (top) Dorsal View (bottom)



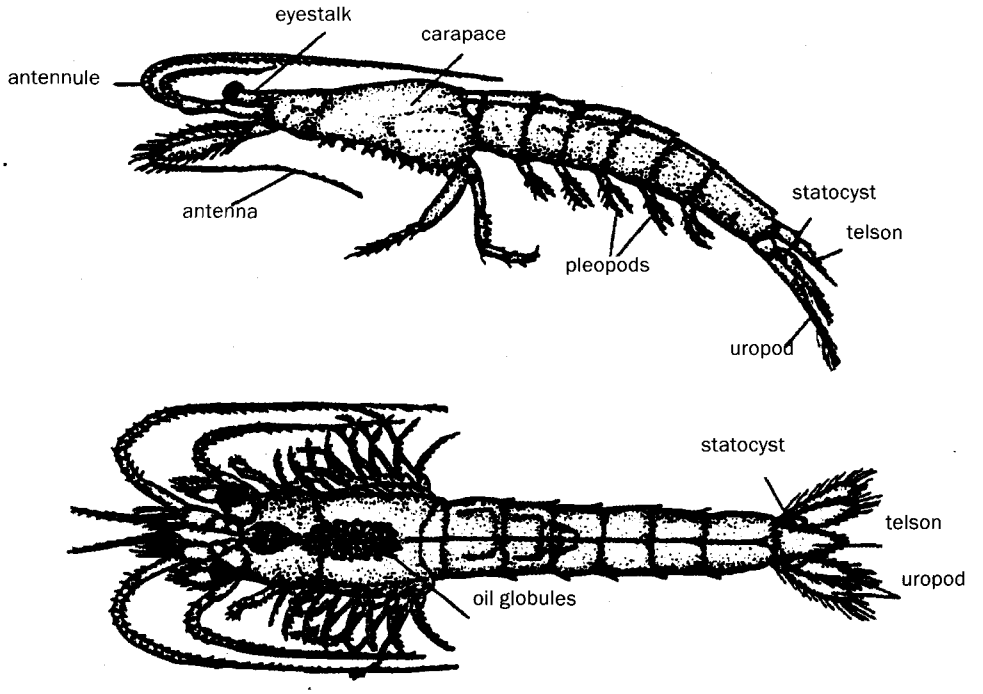
Source: Lussier, Kuhn, and Sewall, 1987.

exposed mysids to the control mysids. The Saltwater Chronic Methods Manual (EPA, 2002a) provides instructions for statistical analysis of the survival, growth, and fecundity data.

Test Acceptability and Data Review

Test data are reviewed to verify that EPA's WET test methods' acceptability criteria (TAC) requirements for a valid test have been met. For instance, the TAC requires 80% or greater survival in controls with an average weight of at least 0.20 mg/mysid and 50% or more of the females in the controls must have eggs.

Figure 9. Immature *A. bahia*. Lateral view (top) Dorsal view (bottom)



Source: Lussier, Kuhn, and Sewall, 1987)

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, 2000a).

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_{50}, IC_{25}) 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.

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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.

Artemia. The marine invertebrate (referred to as brine shrimp) used as the recommended food source for mysid cultures and test organisms; Brazilian or Colombian strains are preferred because the supplies are found to have low concentrations of chemical residues and nauplii are of suitably small size.

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

Cyst. The life stage of unhatched *Artemia*.

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.

Fecundity. Productivity or fertility as measured in this test as the percentage of females with eggs in the oviduct and/or brood pouch.

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_{25} (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.

LC_{50} (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_{50}).

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.

Mysid (*Americamysis bahia*). An estuarine crustacean, formerly known as *Mysidopsis bahia*, ranging 4.4 mm to 9.4 mm in length found from the Gulf of Mexico and along the Atlantic coast as far north as Rhode Island, used in test procedures as an indicator species for marine or estuarine aquatic toxicity.

Nauplii. Free-swimming microscopic larvae stage characteristic of copepods, ostracods, barnacles, etc. typically only with three pairs of appendages.

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-Karber. 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.

Static renewal. The exposure medium is replaced each day by moving the test animal to a new test cup prepared with the proper effluent concentration.

Static water system. An enclosed system contained within one culture tank. The water is filtered through an underground or charcoal filter and is delivered back to the same tank.

Toxicity test. A test 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
Summary of Test Conditions and Test Acceptability Criteria

Table A-1. Summary of Test Conditions and Test Acceptability Criteria for *Americamysis bahia* 7-day Survival, Growth, and Fecundity Toxicity Test

Test type	Static renewal *(required)*
Salinity	20‰ – 30‰ ± 2‰ *(recommended)*
Temperature (C°)	26 ±1°C *(recommended)*[1]
Photoperiod	16 hours light; 8 hours dark, with phase on/off period *(recommended)*
Light intensity (quality)	10 – 20 μE/m²/s (50 – 100 ft-c) (ambient lab levels) *(recommended)*
Test chamber size	8 oz plastic disposable cups, or 400 mL glass beakers *(recommended)*
Test solution volume	150 mL per replicate cup *(recommended minimum)*
Renewal of test solutions	Daily *(required)*
Age of test organisms	7 days at start of test *(required)*
Number of concentrations per study	Minimum of 5 concentrations and a control *(required minimum)*
Number of organisms per test chamber	5 (40 per concentration) *(required minimum)*
Number of replicate chambers per concentration	8 *(required minimum)*
Source of food	Newly hatched *Artemia* nauplii *(<24-hr old; required)*
Feeding regime	Feed 150 24-hr old nauplii per mysid daily, half after test solution renewal and half after 8 – 12 hr *(recommended)*
Aeration	None unless DO falls below 4.0 mg/L, then gently aerate all cups *(recommended)*
Dilution water	Natural seawater, or hypersaline brine diluted with deionized water, or artificial seasalts *(available options)*
Effects measured	Survival and growth *(required)*; egg development *(recommended)*
Cleaning	Pipet excess food from cups daily immediately before test solution renewal and feeding *(recommended)*
Sample volume needed	3 L per day *(recommended)*
Test concentrations	Effluents: 5 and a control *(required)* Receiving waters: 100% receiving water (or minimum of 5) and a control *(recommended)*
Dilution factor	Effluents: ≥ 0.5 series *(required)* Receiving waters: None, or ≥ 0.5 *(recommended)*
Test duration	7 days *(required)*
Endpoints	Survival and growth *(required)*; and egg development *(recommended)*
Test acceptability criteria	80% or greater survival, average dry weight 0.20 mg or greater in controls *(required)*; fecundity may be used if 50% or more of females in controls produce eggs *(required if fecundity endpoint used)*
Sampling requirements	For on-site tests, samples collected daily and used within 24 hr of the time they are removed from the sampling device. For off-site tests, a minimum of three samples (e.g., collected on days one, three, and five) with a maximum holding time of 36 hr before first use (see Saltwater Chronic Methods Manual, Section 8, Effluent and Receiving Water Sampling, Sample Handling and Sample Preparation for Toxicity Test, Subsection 8.5.4) *(required)*

Source: Adapted from EPA, 2002a.

[1]Lussier at al, 1999 found that test conducted at 26°C – 27°C exhibited higher probability of meeting test acceptability criteria for fecundity than tests conducted at 26 ±1°C.

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Appendix B
Apparatus and Equipment List

Air line, and air stones. For aerating cultures, brood chambers, and holding tanks, and 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, 2 – 3 L for making test solutions.

Brine shrimp (*Artemia*) culture unit. See section on "Maintaining and Feeding Cultures."

Depression glass slides or depression spot plates. Two for observing organisms.

Desiccator. For holding dried organisms.

Dissecting microscope (240 – 400X magnification). For examining organisms in the test vessels to determine their sex and to check for the presence of eggs in the oviducts of the females.

Droppers, and glass tubing with fire polished edges. 4 mm inner diameter for transferring organisms.

Drying oven. 50 – 105°C, for drying organisms.

Environmental chamber or equivalent facility with temperature control (26 ± 1°C).

Facilities for holding and acclimating test organisms.

Forceps (fine tips such as jewelers forceps). For transferring organisms to weighing boats.

Light box. For illuminating organisms during examination.

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

Mysid (*Americamysis bahia*) culture unit. See section on "Maintaining and Feeding Cultures". The test requires a minimum of 240 7-day old (juvenile) mysids.

NITEX® or stainless steel mesh sieves. 150 µm and 100 µm for concentrating organisms; 1 mm mesh and 300 µm mesh for collection of juveniles.

Pipet bulbs and fillers. Propipet®, or equivalent.

Reference weights, Class S. For checking performance of balance.

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.

Separatory funnels, 2-liters. Two to four funnels for culturing *Artemia*.

Standard or micro-Winkler apparatus. For determining DO and checking DO meters.

Test vessels. 200 mL borosilicate glass beakers or 8 oz disposable plastic cups or other similar containers. Cups must be rinsed thoroughly in distilled or deionized water and then pre-soaked (conditioned) overnight in dilution water before use. Forty-eight (48) test vessels are required for each test (eight replicates at each of five effluent concentrations and a control). To avoid potential contamination from the air and excessive evaporation of test solutions during the test, the chambers should be covered with safety glass plates or sheet plastic (6 mm thick).

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

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

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

Trays. For test vessels: one large enough to transport eight vessels at one time; one to hold 56 test vessels (approximately 90 x 48 cm).

Volumetric flasks and graduate cylinders. Class A. Borosilicate glass or non-toxic plastic labware, 50 – 2000 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

Data sheets. One set per test for recording data

Effluent, receiving water, and dilution water. Dilution water containing organisms that might prey upon or otherwise interfere with the test organisms should be filtered through a fine mesh (with 150 µm or smaller openings).

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 ±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, 2002a.

Food source. Feed the mysids *Artemia* nauplii that are less than 24-hour-old.

Laboratory quality assurance samples and standards

Markers, waterproof. For marking containers, etc.

Membranes and filling solutions for DO probe. Or reagents, for modified Winkler analysis (See EPA, 2002a).

pH buffers 4, 7, and 10 – (Or as per instructions of instrument manufacturer) for standards and calibration check (see EPA 2002a).

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 ($CdCl_2$), copper sulfate ($CuSO_4$), sodium dodecyl sulfate (SDS), and potassium dichromate ($K_2Cr_2O_7$), are suitable for use in the NPDES Program and other Agency programs requiring aquatic toxicity tests.

Sample containers. For sample shipment and storage.

Tape, colored. For labeling test containers.

Test organisms. The test is begun with 7-day-old juvenile *Americamysis bahia* (mysids).

Weighing pans, aluminum. To determine the dry weight of the organisms

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Appendix D: 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 µm 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 D-1 gives volumes needed to make 20‰ test solutions from effluent (0‰), deionized water, and 100‰ HSB. The highest effluent exposure concentrations achievable are 80% effluent at 20‰ salinity and 70% effluent at 30‰ salinity. Test solutions presented in Table D-1 are not meant as recommendations, rather as examples.

Table D-1. Preparation of Test Solutions at a Salinity of 20‰ Using HSB for a Final Test Concentration Volume of 2000 mL.

Exposure Concentration (% effluent)	*Effluent (assumes 0‰ salinity) (mL)*	*Deionized Water (mL)*	*HSB (100‰ salinity) (mL)*
80	1,600	0	400
40	800	800	400
20	400	1,200	400
10	200	1,400	400
5	100	1,500	400
Control	—	2,000	400

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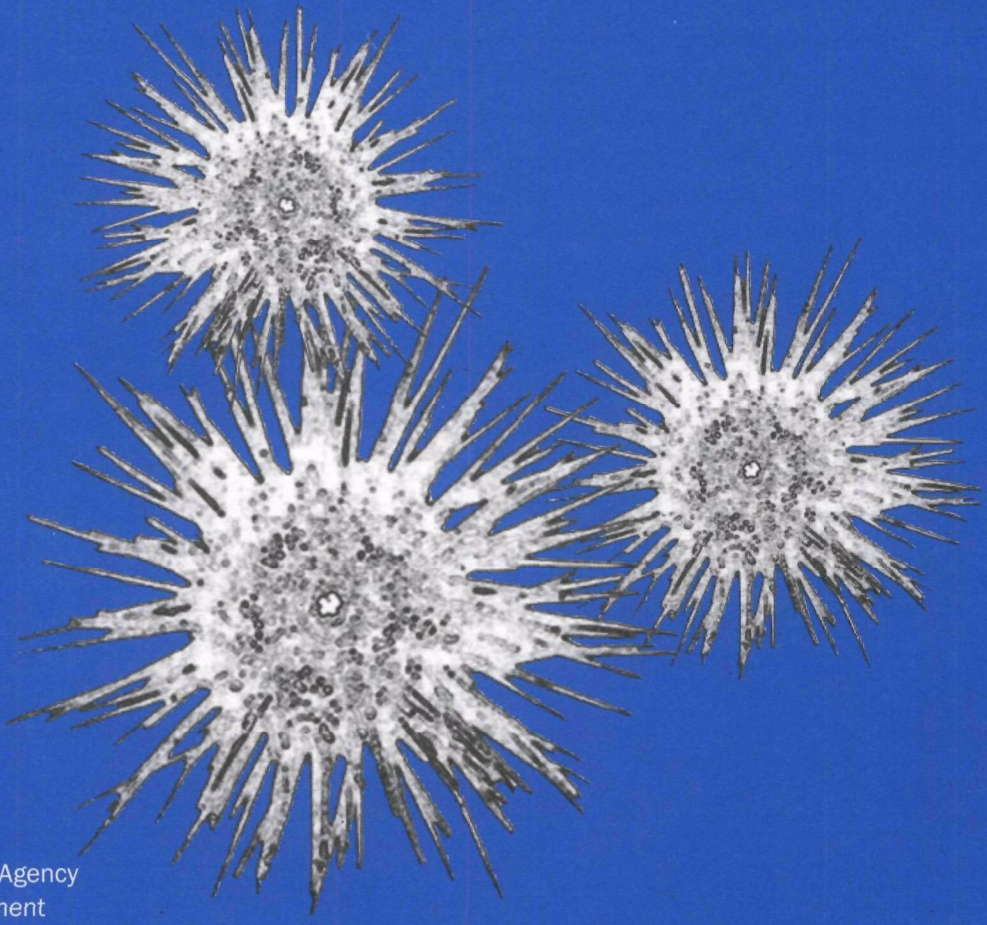
Sperm Cell Toxicity Tests Using the Sea Urchin (*Arbacia punctulata*)

Supplement to Training Video



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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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Contents

FIGURES

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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

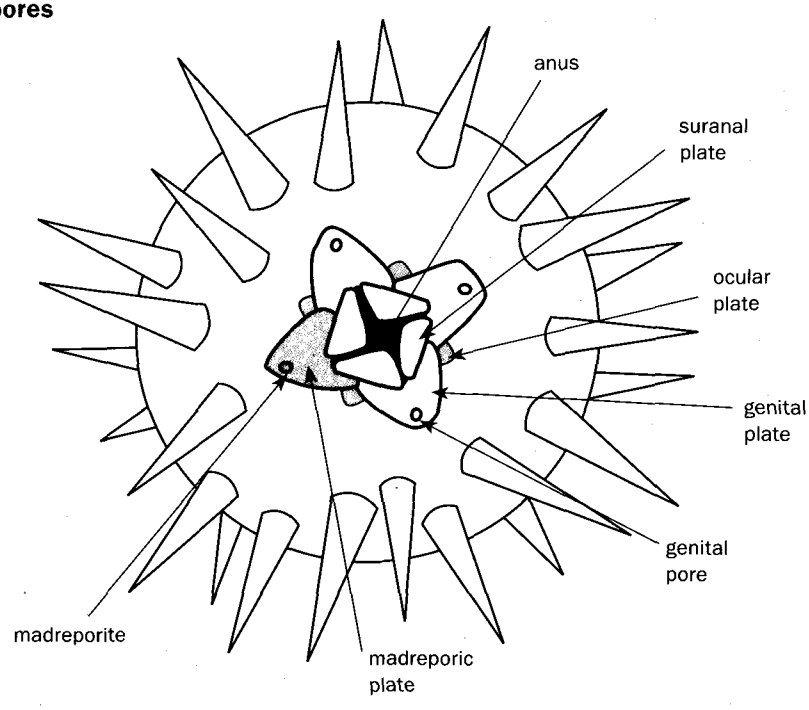
Obtaining and Maintaining Sea Urchins

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‰ ± 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).

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



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 E/μm²/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°C ± 3°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.

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. 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.

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

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 10^4.

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

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

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

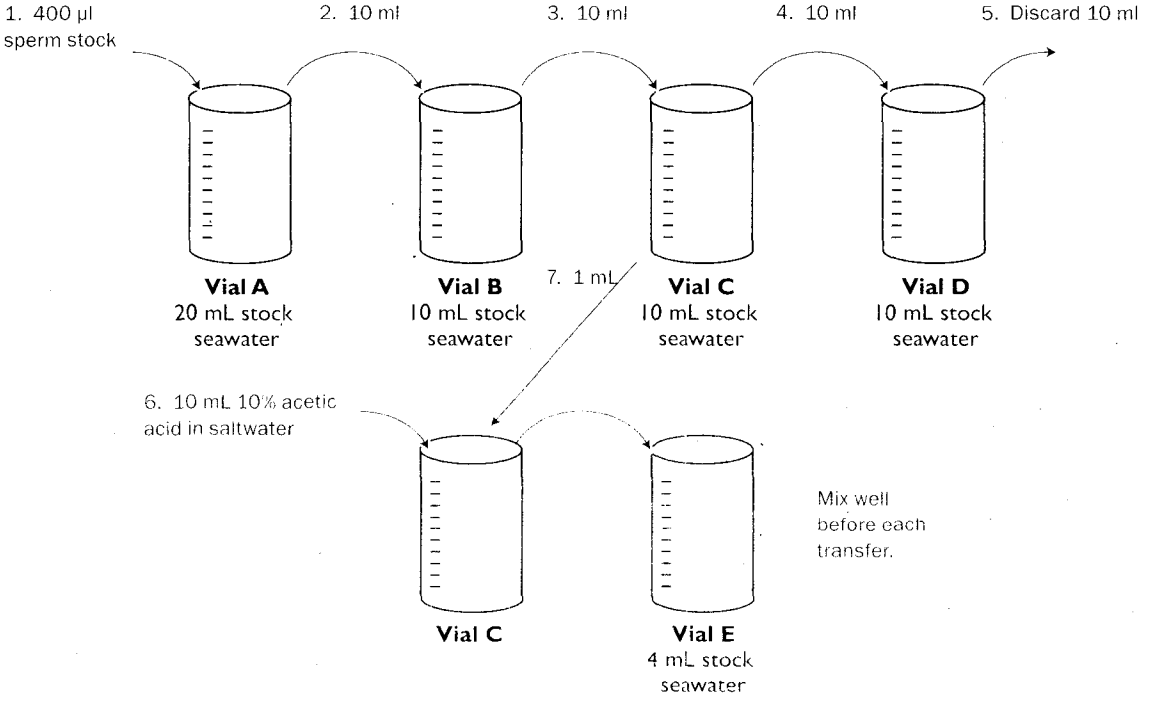
To prepare the sperm suspension for the test, select the vial containing an SPM greater than 5 x 10^7 SPM. To determine the dilution needed for the test:

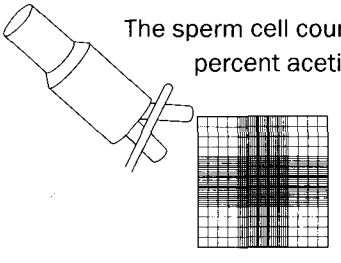
$$\frac{\text{The calculated SPM}}{(5 \times 10^7)} = \text{Dilution Factor (DF)}$$

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

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

1.	Add 400 µL 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 ± 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:

(# of eggs counted) - 200 = 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 ± 20 eggs/mL. The test requires 24 mL of egg test stock for a control and five exposure concentrations.

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.*

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°C – 6°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‰ ± 2‰. 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°C ± 1°C and maintain that temperature while preparing the test concentrations. To test a series of decreasing concentrations of effluent, use a dilution factor of ≥ 0.5. When starting with effluent that has 0‰ salinity and using HSB, the maximum effluent concentration that can be prepared at 30‰ is 70 percent effluent. Table A-1 presents the volumes needed for the test concentrations using HSB.

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_{50}, IC_{25}) analyses are not used. EPA specifies the PMSD must be calculated when NPDES permits require sublethal 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. 2000a. Method Guidance and Recommendations for Whole Effluent Toxicity (WET) Testing (40 CFR Part 136). Office of Water, Washington, D.C. EPA 821-B-00-004.

EPA. 2000b. Understanding and Accounting for Method Variability in Whole Effluent Toxicity Applications Under the National Pollutant Discharge Elimination System Program. Office of Wastewater Management, Washington, D.C. EPA 833-R-00-003.

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EPA. 2002c. Final Rule. 40 CFR Part 136. Guidelines Establishing Test Procedures for the Analysis of Pollutants; Whole Effluent Toxicity Test Methods. 67 FR 69952-69972, November 19, 2002.

EPA. 2009. Sperm Cell Toxicity Tests Using the Sea Urchin (*Arbacia punctulata*). Supplement to Training Video. Whole Effluent Toxicity Training Video Series, Saltwater Series. March 2009. EPA 833-C-09-001.

EPA references are available online at **www.epa.gov/npdes**.

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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_{25} (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_{50} (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_{50}).

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-Karber. 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 µm 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.[1]

		Solutions To Be Combined	
Effluent Solution	**Effluent Concentration (%)**	**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
Total			**2,080**

[1]*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°C ± 1°C).

Fume hood. To protect from formaldehyde fumes.

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

Hemacytometer, 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®, 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 µm 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 ($CdCl_2$), copper sulfate ($CuSO_4$), sodium dodecyl sulfate (SDS), and potassium dichromate ($K_2Cr_2O_7$), 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 ± 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)[1]

Test type	Static, non-renewal *(required)*
Salinity	30‰ ± 2‰ of the selected test salinity *(recommended)*
Temperature (C°)	20°C ± 1°C *(recommended)* Test temperatures must not deviate by more than 3°C during the test (i.e., max. temp – min. temp ≤ 3°C) *(required)*
Light quality	Ambient laboratory light during test preparation *(recommended)*
Light intensity	10 – 20 μE/m²/s, or 50 – 100 ft-c (Ambient laboratory levels) *(recommended)*
Test chamber size	Disposable (glass) liquid scintillation vials (20 mL capacity), pre-soaked in control water *(recommended)*
Test solution volume	5 mL *(recommended)*
Number of sea urchins	Pooled eggs from 4 females and pooled sperm from 4 males per test *(recommended)*
Number of eggs and sperm cells per chamber	About 2,000 eggs and 5,000,000 sperm cells per vial *(recommended)*
Number of replicate chambers per concentration	4 *(required minimum)*
Dilution water	Uncontaminated source of natural seawater; deionized water mixed with HSB or artificial sea salts *(available options)*
Test concentrations	Effluents: 5 and a control *(required minimum)* Receiving waters: 100% receiving water *(or minimum of 5)* and a control *(recommended)*
Dilution factor	Effluents: ≥ 0.5 *(recommended)* Receiving Waters: None or ≥ 0.5 *(recommended)*
Test duration	1 hour and 20 minutes *(required)*
Endpoint	Fertilization of sea urchin eggs *(required)*
Test acceptability criteria	70% – 90% egg fertilization in controls *(required)*
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. *(required)*
Sample volume required	1 L per test *(recommended)*

[1]*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 10^4 = 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 10^7 SPM): _____

Dilution: SPM/(5 X 10^7) = _____ 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 10^7 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:

Figure E-2. Sperm Cell Toxicity Test, Sample Data Sheet #2 – Raw Data

Test ID:_____ Time:_____
Performed by: _____ Date:_____

Egg Counts at End of Test

	Replicate 1		Replicate 2		Replicate 3		Replicate 4	
Conc. (%)	Total	Unfert	Total	Unfert	Total	Unfert	Total	Unfert

Statistical Analysis:

Analysis of variance: _____

Control: _____

_____

Different from Control (P): _____

_____

Comments: _____

_____

_____

_____

_____

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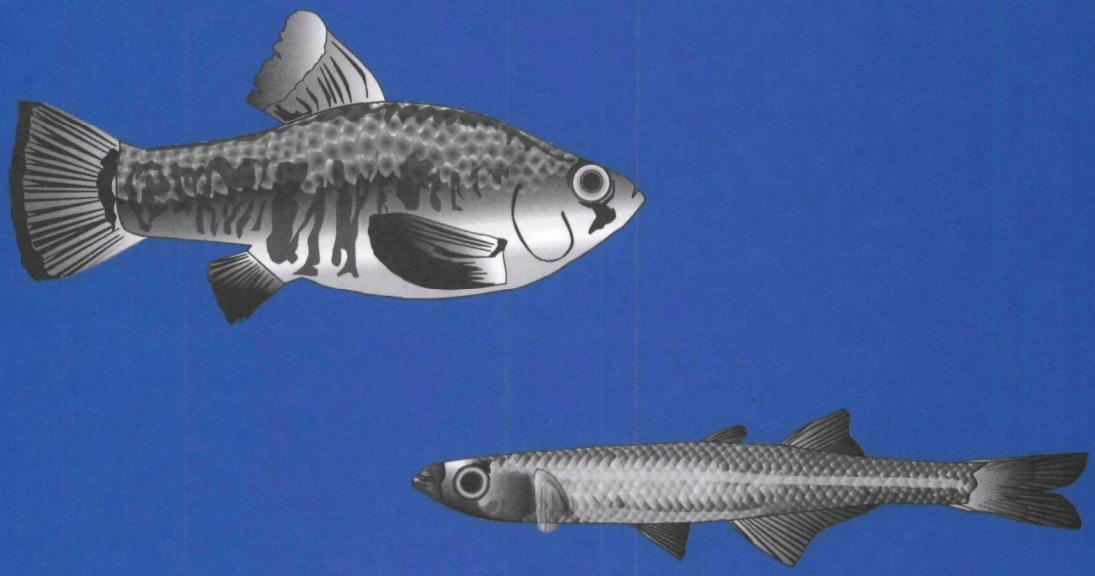
Sheepshead Minnow (*Cyprinodon variegatus*) and Inland Silverside (*Menidia beryllina*) Larval Survival and Growth Toxicity Tests

Supplement to Training Video



U.S. Environmental Protection Agency
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NOTICE

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Foreword

This supplemental guide serves as a supplement to the video "Sheepshead Minnow (*Cyprinodon variegatus*) and Inland Silverside (*Menidia beryllina*) Larval Survival and Growth Toxicity Tests" (EPA, 2009). The methods illustrated in the video and described in this 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 guide accompanies the Environmental Protection Agency's (EPA's) video training for conducting sheepshead minnow (*Cyprinodon variegatus*) and inland silverside (*Menidia beryllina*) larval survival and growth toxicity tests (EPA, 2009). The test methods are found in *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms, Third Edition* (EPA, 2002a). The tests were developed by EPA's Office of Research and Development's (ORD's) National Health and Environmental Effects Research Laboratory – Aquatic Ecology Division (NHEERL-AED) in Narragansett, Rhode Island. 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 whole effluent toxicity (WET) permit limitations regulate pollutant discharges on a whole effluent effect basis rather than solely by a chemical specific approach.

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 sheepshead minnows and inland silverside 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 guide summarizes methods developed at ORD for measuring effects on larval survival and growth of the sheepshead minnow *Cyprinodon variegatus* and the inland silverside *Menidia beryllina* after exposure to complex effluents in marine or estuarine environments. These short-term tests span an exposure time of 7 days to estimate the chronic toxicity of effluent or receiving water on newly-hatched larvae in a static renewal exposure system. The methods described in this guide and demonstrated in the accompanying video are detailed in the EPA methods manual, *Short-term Tests for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms, Third Edition* (EPA, 2002a)[1].

Care and Feeding of Adults and Larvae

SHEEPSHEAD MINNOWS

Adult sheepshead minnows (*Cyprinodon variegatus*) can be field collected from Atlantic and Gulf of Mexico coastal estuaries south of Cape Cod using near-shore nets, purchased from commercial biological supply houses, or raised from young fish to maturity in the laboratory. To minimize inbreeding, use of feral brood stocks or first generation laboratory fish is recommended. Fish that are field-caught should be held for a minimum of 2 weeks before use in testing to determine that they are healthy and not injured.

[1] *The methods for these two species are presented together in the video and this guide because they are conducted in a very similar manner. The complete methods in the Saltwater Chronic Methods Manual are presented in Section 11 (Sheepshead Minnows) and Section 13 (Inland Silverside).*

Culture Water

The quality of water used for test organism culturing and for dilution water in toxicity tests is extremely important. Water for these two uses should come from the same source. Holding and rearing tanks and any area used for manipulating live sheepshead minnows should be located in a room or space separated from that in which toxicity test are to be conducted.

The salinity of the culture systems should between 20‰ and 30‰. Water temperature for the brood stock should be maintained at 24°C – 26°C. The holding and rearing tanks should be aerated so that the dissolved oxygen is not less than 4.0 ppm.

Replace approximately 10% of the culture water every 2 weeks, or 25% monthly. The culture water should be clear. If the water appears cloudy of discolored, replace at least 50% of it. Replacement water should be well oxygenated and at the same temperature and salinity as the existing culture water. Salinity is maintained at the proper level by adding deionized water to compensate for evaporation. Artificial seawater is prepared by dissolving artificial sea salts in deionized water to a salinity of 20‰ – 30‰ (see Appendix A for preparation of hypersaline brine solution [HSB]).

Photoperiod

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

Culture Vessels

Holding tanks are kept at ambient laboratory temperature (25°C) until the fish reach sexual maturity (3 – 5 months post hatch) at which time they can be used for spawning. Mature sheepshead minnows have an average length of approximately 27 mm for females and 34 mm for males. Once mature, males will begin to exhibit sexual dimorphism and initiate territorial behavior. Once sexually mature, hold the adults in water reduced to 18°C – 20°C.

To avoid excessive build up of algal growth, periodically scrape the walls of the culture system. Some of the algae will serve as a supplement to the diet of the fish. A partial activated carbon "charcoal" change in the filtration systems should be done monthly or as needed. The detritus (dead brine shrimp nauplii and cysts, adult brine shrimp, other organic material accumulation) should be siphoned from the bottom of rearing and holding aquaria or tanks each week or as needed.

Water Delivery Systems

Adult sheepshead minnows (>1 month) are kept in natural or artificial seawater in a flow-through or recirculating aerated glass aquarium that is equipped with an undergravel or outside biological filter, or cartridge filter. Static systems are equipped with an undergravel filter. Recirculating systems are equipped with an outside biological filter constructed in the laboratory using a reservoir system of crushed coral, crushed oyster shells or dolomite and gravel, charcoal, floss, or a commercially available cartridge filter or an equivalent system.

Food Preparation

The adult sheepshead minnows are fed flake food three to four times daily, supplemented with frozen adult brine shrimp.

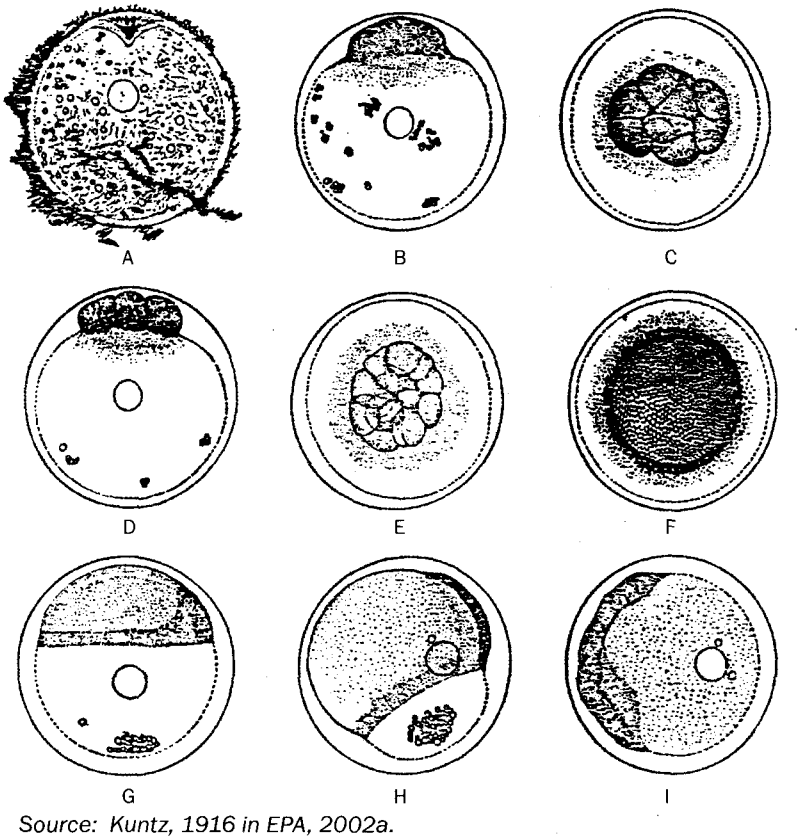
The larvae are fed newly hatched *Artemia* nauplii and crushed flake food, *ad libitum*, daily. The *Artemia* should be cultured in the laboratory in order to provide 24 – 48 hour old nauplii. Appendix B describes in detail how to culture *Artemia*.

Obtaining Larvae for Toxicity Tests

For the sheepshead larval survival and growth toxicity test, larvae that are less than 24 hours old are needed at the start of the test. To have the appropriate age larvae at the start of a test, induce the minnows to spawn by raising the system temperature to 25°C approximately 1 week before the start of the test. This gradual temperature increase is started in the morning. By afternoon, transfer the adults (at least five females and three males) to a spawning chamber, or basket made from 3 – 5 mm NITEX® screen, within an aquarium outfitted with a mesh screen (150 – 250 µm mesh) under the basket or on the bottom. The fish will begin to spawn within 24 hours and the eggs will fall through the basket onto the mesh collecting screen.

To keep the egg collecting screens clean, feed the spawning fish while the collecting screen is removed for egg collection.

Figure 1. Embryonic development of sheepshead minnow, *Cyprinodon variegatus*: A. Mature unfertilized egg, showing attachment filaments and micropyle, X33; B. Blastodisc fully developed; C/D. Blastodisc, 8 cells; E. Blastoderm, 16 cells; F. Blastoderm, late cleavage stage; G. Blastoderm with germ ring formed, embryonic shield developing; H. Blastoderm covers over ¾ of yolk, yolk noticeably constricted; I. Early embryo. (Continued, J – O on page 4).



Source: Kuntz, 1916 in EPA, 2002a.

Collect eggs daily by washing the eggs off of the screen into a large tray. Roll the eggs gently on the screen during collection, pressing any food or waste through, leaving the eggs on top of the screen. Embryos will tend to stick together due to the presence of adhesive threads. After embryos have been manipulated, wash them by placing them in a 250-µm sieve and rinsing them with seawater from a squeeze bottle. This should reduce any fungal contamination of the embryos.

Females also can be induced to spawn artificially by intraperitioneal injection with human chorionic gonadotrophin (HCG) hormone. Natural spawning is preferable because repeated spawnings can be obtained from the same brood stock. Additional details on forced spawning are provided in section 11.6.15 of the *Saltwater Chronic Methods Manual* (EPA, 2002a).

The collected embryos should be checked under a dissecting microscope to identify any abnormal or unfertilized eggs. The embryos should be in stages C – G as illustrated in Figure 1.

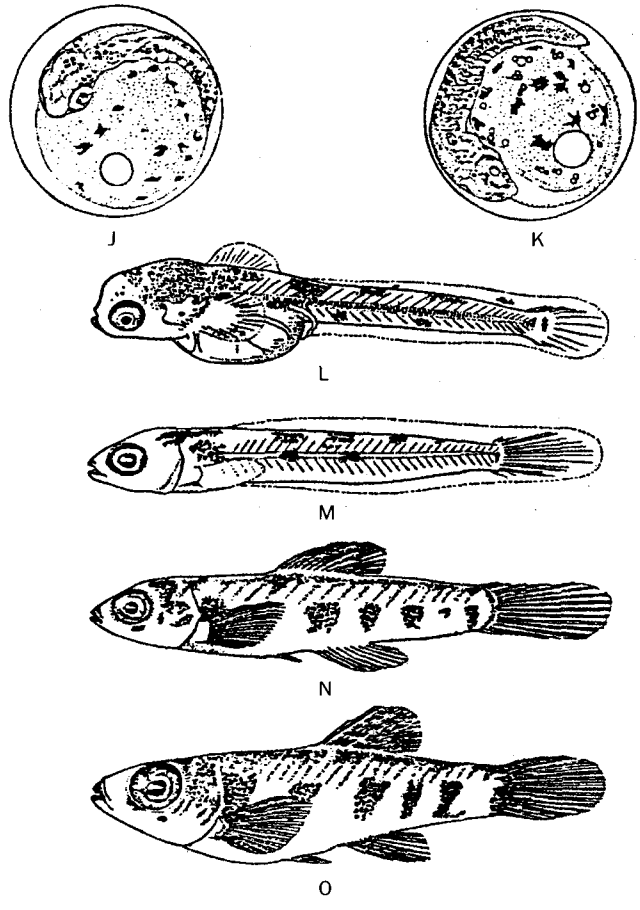
After collection, incubate the collected minnow embryos in seawater at 25°C, 20‰ – 30‰ salinity, and 16-hour light and 8-hour dark photoperiod for 5 – 6 days with aeration and daily water changes.

At 48 hours after collection, check the embryos under a dissecting microscope and discard any abnormal or unfertilized eggs. At this time, the embryos should be at stages I or J as illustrated in Figure 1. To conduct one

test with four replicates of 15 larvae and five effluent concentrations plus a control, collect approximately 400 viable embryos for incubation at this stage. Reducing the salinity, raising the temperature, or changing the water can help induce hatching. If culture dishes are used, they should be covered to reduce evaporation which could increase salinity.

For the sheepshead minnow growth and survival toxicity test, use larvae that hatch less than 24 hours before the start of the test. If some embryos hatch earlier than 24 hours prior to the test start, remove them but keep them to supplement the younger larvae in case there are not be enough larvae at the start of the test. If this is done, larvae should not be more than 48 hours old and should all be within 24 hours of the same age. Selection of the older larvae should be randomized by placing them back into the pool before selection.

Figure 1 (continued). Embryonic development of sheepshead minnow, *Cyprinodon variegatus*: J. Embryo 48 h after fertilization, no segmented throughout, pigment on yolk sac and body, otoliths formed; K. Posterior portion of embryo free from yolk and moves freely within egg membrane, 72 h after fertilization; L. Newly hatched fish, actual length 4 mm; M. Larval fish 5 days after hatching, actual length 5 mm; N. Young fish 9 mm in length; O. Young fish 12 mm in length.



From Kuntz, 1916 in EPA, 2002a.

INLAND SILVERSIDE

Inland silversides (*Menidia beryllina*) also can be obtained by beach seine from Atlantic and Gulf of Mexico coastal estuaries, from biological supply houses, or by raising young fish in the laboratory. Gravid females can be found in low salinity waters along the Atlantic coast during April to July. If beach seines (3 mm – 6 mm mesh) are used, silversides should not be landed onto the beach as they are very sensitive to handling and should not be removed from water by net — only by bucket or beaker. Several species of silversides may be included in field caught specimens (e.g., *M. beryllina*, *M. menidia*, and *M. peninsulae*); care should be taken to identify and separate the species.

If fish are collected from the field, record the temperature and salinity at each collection site so that the conditions can be maintained in the culture tanks. After transfer to laboratory culture tanks, slowly introduce laboratory water (maximum change of 2°C/day and 5‰ salinity/day) to bring the water up to 25°C and 20‰ – 32‰.

CULTURE WATER

Only natural seawater is recommended for the culture and maintenance of the more sensitive silverside brood stock. Maintain holding and spawning tanks at a temperature of 25°C and a salinity of 20‰ – 32‰.

PHOTOPERIOD

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

CULTURE VESSELS

Adult inland silverside should be stocked in tanks of a minimum volume of 150L at a density of 50 fish/tank. Detritus should be siphoned off from the bottom weekly, or as needed.

WATER DELIVERY SYSTEMS

Adult inland silversides are kept in a flow-through or recirculating aerated glass aquarium that is equipped with an undergravel or outside biological filter, or cartridge filter. Static systems are equipped with an undergravel filter. Recirculating systems are equipped with an outside biological filter constructed in the laboratory using a reservoir system of crushed coral, crushed oyster shells or dolomite and gravel, charcoal, floss, or a commercially available cartridge filter or an equivalent system.

FOOD PREPARATION

Feed silverside larvae the rotifer *Brachionus plicatilis* until 4 – 6 days post-hatch, and the smallest *Artemia* nauplii available (<12 hour old) beginning on day 5. After day 7, feed the larvae with *Artemia* only and increase the size to 12 – 24 hours old. Food preparation instructions are provided in Appendix B.

The adult inland silversides should be fed flake food or frozen brine shrimp twice daily and *Artemia* nauplii once daily.

The larvae are fed newly hatched *Artemia* nauplii and crushed flake food, *ad libitum*, daily. The *Artemia* should be cultured in the laboratory in order to provide 24 – 48 hour old nauplii. Appendix B describes in detail how to culture *Artemia*.

OBTAINING LARVAE FOR TOXICITY TESTS

Inland silversides are sexually mature after 1 – 2 months. In the wild, eggs are adhered to submerged vegetation. In the laboratory, silversides are encouraged to spawn by placing polyester aquarium filter fiber in the tanks. The fiber (~ 15 cm x 10 cm x 10 cm) is suspended on a string 8 cm – 10 cm below the surface of the water and in contact with the side of the tank. These should be placed into the tank 14 days prior to the beginning of a test. Place the floss directly above an airstone to keep it aerated, and weigh it down to keep it from floating on the surface.

When the fish spawn into the fiber, the hard, light yellow embryos (~0.75 mm in diameter) can be separated from the fibers by hand, or the eggs and fiber can be placed together into a 10-gallon aquarium. The floss should be suspended 8 cm – 10 cm below the surface of the water and should be stretched to keep the embryos from being crowded. Lightly aerate the tank and hold the temperature at 25°C.

Larvae will hatch in 6 – 7 days when incubated at 25°C and maintained in seawater ranging from 5‰ – 30‰. The larvae will free themselves from the fibers at which time they are easily identified and should be removed. The newly hatched larvae will range from 3.5 mm – 4.0 mm in total length. Figure 2 illustrates the life stages of the inland silverside.

For the inland silverside larval survival and growth toxicity test, use 7- to 11-day-old larvae. For one test using 15 larvae for each of four replicates and five test concentrations plus a control, approximately 400 larvae are needed.

Test Method

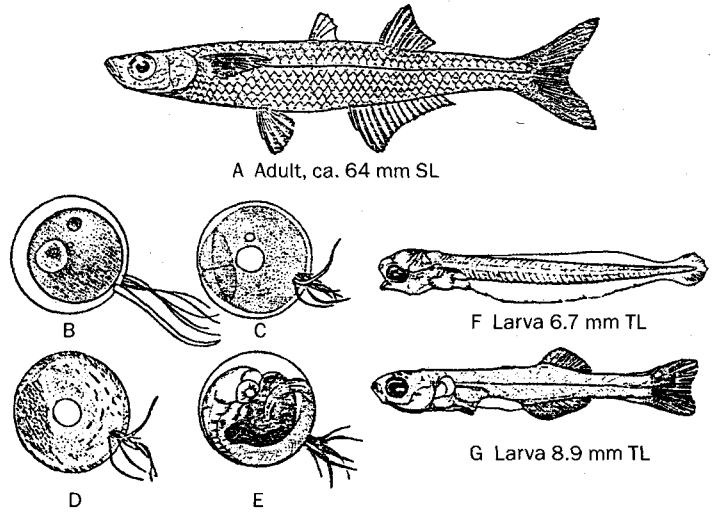
Effluent Sampling

For both species, handle effluent and receiving water samples in the same manner. Store effluent or receiving waters in an incubator or refrigerator at 0°C – 6°C until the tests begin, but not longer than 36 hours if being used for compliance for a NPDES permit. Prepare dilutions of the effluent sample using a 0.5 dilution factor (e.g., 6.25%, 12.5%. 25%, 50%, and 100%). If a high rate of mortality is observed during the first 1 – 2 hours, additional replicates in the lower ranges of effluent concentration should be added.

The tests require about 5 – 6 L of each effluent or receiving water sample each day, enough for renewing four replicates of each concentration plus the control and for performing chemical analyses.

It is essential to maintain constant salinity among treatments and treatment replicates throughout the test. Use concentrated seawater or hypersaline brine (HSB) to keep the salinity of the solutions between 20‰ and 30‰ for the sheepshead minnows, and between 5‰ and 30‰ for the inland silversides. Before adding the solutions to the test chambers, warm the samples to 25°C in a water bath. Keep the temperature constant (25°C ± 1°C) for the duration of the test.

Figure 2. Inland silverside, *Menidia beryllina*: A. Adult, ca. 64 mm SL; B. Egg (diagrammatic), only bases of filaments shown; C. Egg, 2-cell stage; D. Egg, morula stage; E. Advanced embryo, 2½ days after fertilization..



From Martin and Drewry, 1978 in EPA, 2002a.

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:

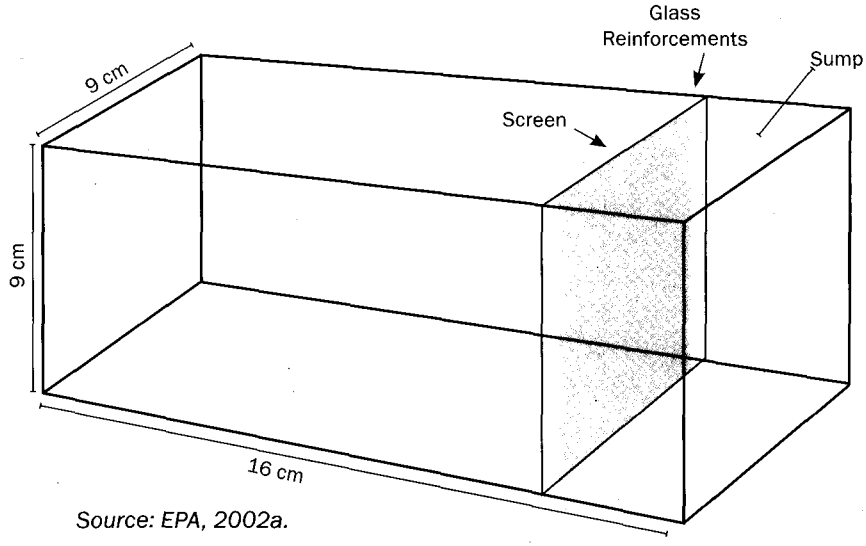
- *If the test is conducted to estimate the* ***absolute chronic toxicity of the effluent****, synthetic dilution water should be used. If the cultures were maintained in different water than used for dilution water, a second set of control replicates should be conducted using the culture water.*
- *If 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.*

Dilution Preparation

Set out the test chambers. Typically, there will be at least five dilutions plus one control, and a minimum of four replicates. For both species NHEERL-AED uses glass chambers equipped with a screened-off sump area (see Figure 3). One

thousand mL glass or disposable plastic beakers also can be used as test chambers. Add a small amount of clean seawater to each chamber, enough to cover the bottom to a depth of about 1 cm.

Figure 3. Glass test chamber with sump area. Modified from Norberg and Mount (1985).

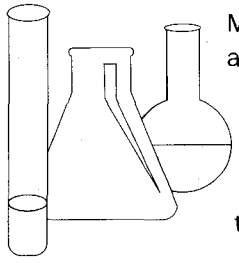


Source: EPA, 2002a.

Pipet two or three larvae at a time into each chamber, adding larvae to all chambers; then start again, adding more until each chamber contains the required number of larvae — a minimum of 10. Use a minimum amount of seawater to deposit the animals into the containers to avoid diluting the effluent samples further. Using a white background or a light table facilitates counting the larvae in the chambers. Since clean seawater is in all of the chambers, larvae can be exchanged among test chambers until all contain the correct number. Because the inland silverside larvae are sensitive to handling, it may be best to distribute them into chambers containing control solution 1 day before the start of the exposure period.

Randomly apply colored labels to the chambers to indicate treatment and replicates. Fill each chamber with approximately 750 mL of the appropriate test solution, pouring through the sump area or down the side. Each test chamber should contain a minimum of 50 mL of test solution/larvae and a depth of at least 5 cm.

Routine Chemistries



Measure the initial temperature, salinity, and dissolved oxygen (DO) in each chamber. Record all measurements on the test data sheet. Copies of the data sheets used at NHEERL-AED are provided in Appendix F.

When all measurements have been taken and recorded, place the chambers in a 25°C water bath according to a random numbers table. Keep the chambers in those same positions for the duration of the test.

Renewals

Each day, the test and control solutions must be replenished. Prepare new dilutions daily from effluent stored at 0° - 6°C. When tests are performed on site, effluent and receiving water should be collected daily. Off-site toxicity tests are often performed with effluent collected on days 1, 3, and 5 of the exposure period. Again, do not store the effluent samples longer than 36 hours before use. Warm the solutions to 25°C in a water bath just before adding to the chambers.

Temperature and salinity should be maintained under carefully controlled conditions across all test concentrations and replicates throughout the test. Each day before changing the solutions, measure and record the temperature in each chamber. Maintain the chambers at 25°C ± 1°C, and supply 16 hours of ambient laboratory light and 8 hours of darkness each day for both species. Measure and record the salinity from each chamber every day as well, before renewing the test solutions. Note that there should be no more than a 2‰ salinity difference between any two chambers on a given day. If receiving water and effluent tests are conducted concurrently, the effluent salinity should be adjusted to match the receiving water

sample if possible. Monitor DO concentrations each day and record the data on the data sheet. If DO falls below 40% saturation in any one of the exposure chambers, all chambers must be aerated.

Before changing the test solutions, count and record the number of live larvae in each replicate, discarding any dead animals. Then remove any uneaten *Artemia* from the chamber using a siphon or a large pipet. To avoid removing test animals along with uneaten food, set the chambers on a light box or light table to better observe the larvae. Besides making the larvae more visible, the light also serves to concentrate the nauplii on the bottom of the chamber. Siphon the water and remaining *Artemia* into a large beaker or white plastic tray. Individual larvae that are accidentally removed can be seen easily in the beaker, and should be returned to their respective test chambers. Note the accidental siphoning of any larvae in the test records. Once the solution in the test chamber is emptied to a depth of 7 – 10 mm, slowly and carefully add approximately 500 – 750 mL of new test solution, pouring down the side of the chamber or into the sump area to avoid excessive turbulence. After changing all the solutions, return the chambers to their same randomized positions in the water bath and feed the larvae.

Feeding

Proven quality *Artemia* nauplii should be used to feed the larvae daily throughout the test. Two concentrations of prepared nauplii are used sequentially during the exposure period. Detailed instructions for culturing *Artemia* are included in Appendix B. The first food solution used for day 0 – 2 consists of 4 mL concentrated *Artemia* nauplii in 80 mL seawater. Feed each replicate 2 mL of this solution on the first 2 days of the test. The 2 mL volume should yield approximately 0.10 g wet weight of *Artemia* nauplii. Care should be taken to swirl the solution to maintain a constant distribution of *Artemia* and each 2 mL portion should be drawn individually to avoid differences in feeding rates due to the settling of *Artemia* in the dropper.

For days 3 – 6 of the test, feed the larvae 2 mL per replicate of a more concentrated solution of 6 mL of concentrated *Artemia* in 80 mL of seawater. This 2 mL volume should yield approximately 0.15 g wet weight *Artemia* nauplii. Uneaten *Artemia* should be siphoned out of the chambers each day so that the larvae eat newly hatched *Artemia* and to avoid depletion of DO within the chamber. On day 7, the larvae are not fed.

It is important that all chambers receive the same amount of food throughout the test. If the survival rate in any chamber falls below 50%, reduce the amount of food supplied to that chamber by ½ for the remainder of the test. Cover the chambers between feedings to reduce evaporation.

Test Termination

At the end of the test, on day 7, the larvae are counted to determine survival rate. Working with groups of replicates, remove any dead larvae from the chambers, carefully recording the number of surviving animals. Record the final temperature, salinity, and DO for each chamber.

Pour the contents of each chamber through a 500-µm mesh screen over a large beaker. Quickly submerge the screen in an ice and deionized water bath. The cold will immobilize the fish, and swirling the screen in the deionized water will wash away uneaten *Artemia* and salts that may interfere with the weight determination. Dry the animals for immediate weighing or preserve them for later drying in separate scintillation vials containing 4% formalin or 70% ethanol. To dry the surviving animals, place all of the fish from each replicate into a labeled, pre-weighed aluminum weighing boat, and dry the fish at 60°C for 24 hours, or at 105°C for 6 hours. Gloves should be worn or forceps should be used to handle the aluminum weighing boats because oil from skin could affect weight differences.

After drying, and until they are weighed, place the dried larvae directly into a desicator to prevent moisture from the air adsorbing to the samples. Weigh each sample to the nearest 0.01 mg. Because small differences in weight or appearance can easily change the test results, it is critical to record observations and measurements clearly and accurately. Determine the weight of the larvae alone by subtracting the weight of the weigh boat. Divide the final dry weight by the number of larvae in the sample to determine the aver-

age dry weight of the surviving larvae. This average weight is then compared statistically to the control animals' average weight to identify any effluent effects on the fishes' growth.

Test Acceptability and Data Review

Test data are reviewed to verify that EPA's WET test methods' test acceptability criteria (TAC) requirements for a valid test have been met. For the test to be considered acceptable, control survival must be ≥ 80% for both species. The average dry weight of unpreserved control larvae must be ≥ 0.60 mg for the sheepshead minnow, and ≥ 0.50 mg for the inland silverside. Minimum dry weights for preserved animals are ≥ 0.50 mg for the sheepshead minnow and ≥ 0.43 mg for the inland silverside.

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, 2000a).

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_{50}, IC_{25}) 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.

Other Procedural Considerations

- Keep careful records throughout the test.
- Record any deaths and whether any larvae were accidentally siphoned out of their chamber.
- Take special note of any behavioral changes that the larvae may exhibit, or any physical abnormalities.
- Note the results of the chemical and physical measurements taken during the test.

These data should be carefully compiled and are considered important clues to how the effluent may affect marine animals. The methods manual, *Short-term Methods for Estimating Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms, Third Edition* (EPA, 2002a) details the procedure for data analysis.

The larval survival and growth toxicity tests described here are currently used to assess the potential toxic effects of complex chemical mixtures on marine and estuarine organisms. Used in conjunction with chemical-specific methods, these tests can provide a comprehensive and effective approach to assessing the impact of complex effluents discharged to marine and estuarine environments.

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

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Glossary

Acute toxicity. An adverse effect measured 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.

Algae. Rotifers are fed the algae *Tetraselmus suecica* or *Chlorella* sp.

Artemia. The marine invertebrate (referred to as brine shrimp) used as the recommended food source for culture and test species; Brazilian or Colombian strains are preferred because the supplies are found to have low concentrations of chemical residues and nauplii are of suitably small size.

Average mean dry weight. All the fish exposed in a given test chamber (replicate) are weighed together. The total dry weight is divided by the number of surviving fish in the replicate to obtain the average mean dry weight.

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

Crash. Sudden (overnight) death of cultured organisms in a tank.

Cyprinodon variegatus. The scientific name for the fish species, sheepshead minnow. The sheepshead minnow is a short, deep-bodied, compressed fish. It has large scales and a dark marginal band on its tail. It occurs in hypersaline lagoons and connecting channels, and is found on muddy bottoms in turbid waters from North and South America: Massachusetts, USA to northeastern Mexico; also West Indies; northern coast of South America, Bahamas, Antilles, Gulf of Mexico, Yucatan and Venezuela. It is omnivorous, consuming organic detritus and algae, as well as microcrustaceans, and dipteran larvae. Sheepshead minnows are very abundant and easily reproduced in captivity.

Cyst. The life stage of unhatched *Artemia*.

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

Effluent sample. A representative collection of a NPDES permitted facility's discharge that is to be tested.

Effluent concentration. Different dilutions, or concentrations, of an effluent used to determine the biological effects on test organisms (i.e., inland silversides or sheepshead minnows).

Flow-through water delivery system. An open water flow system that delivers fresh water or seawater to culture tanks, which 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_{25} (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.

Larvae. Post-hatch fish that are not free-swimming and are morphologically immature (i.e., <24 hr-old).

LC_{50} (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_{50}).

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.

Menidia beryllina. The scientific name for the fish species, inland silverside. It is a marine/estuarine species that ascends rivers. In fresh water, inland silverside usually occurs at the surface of clear, quiet water over sand or gravel. It feeds on zooplankton and is found in coastal waters from the Western Atlantic: Massachusetts to southern Florida in the USA and around the Gulf of Mexico to northeastern Mexico.

Nauplii. Free-swimming microscopic larvae stage characteristic of copepods, ostracods, barnacles, etc. typically only with three pairs of appendages.

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-Karber. 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.

Rotifer. The rotifer, *Brachionus plicatilis* is fed to newly-hatched inland silverside larvae until they are large enough to be fed *Artemia*.

Static renewal. The daily replacement of effluent medium in the test chamber.

Static water system. An enclosed system contained within one culture tank. The water is filtered through an underground or charcoal filter and is delivered back to the same tank.

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 µm 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 gives volumes needed to make 20‰ test solutions from effluent (0‰), deionized water, and 100‰ HSB. Quantities of effluent, deionized water and a HSB of 100‰ (only) needed for conducting daily renewals of test solutions at 20‰ salinity. The highest concentration achievable is 80% effluent at 20‰ salinity and 70% effluent at 30‰.

Table A-1. Preparation of Test Solutions at a Salinity of 20‰ Using HSB for a Final Test Concentration Volume of 4000 mL.

Exposure Concentration	*Effluent (0 ‰ mL)*	*Deionized Water (mL)*	*HSB (100‰) (mL)*
80	3200	—	800
40	1600	1,600	800
20	800	2,400	800
10	400	2,800	800
5	200	3,000	800
Control	—	4,000	0

Appendix B:
Preparing Brine Shrimp and Rotifers for Feeding

INTRODUCTION

The brine shrimp (*Artemia* sp.) is used to feed larval *Menidia beryllina* and *Cyprinodon variegatus* in the 7-day effluent toxicity tests. However, just after hatching, *M. beryllina* are too small to ingest *Artemia*, and must be fed rotifers (*B. plicatilis*). Preparation and culture of *Artemia* and rotifers are described below.

CULTURING *ARTEMIA*

Brine shrimp are highly suited to this testing protocol because: 1) the naupliar stages are nutritionally acceptable to these species; 2) they may be obtained from cysts within 24 hours after immersion in seawater; and 3) the cysts are readily available and can be stored for prolonged periods of time. There are some disadvantages to keep in mind, as well. For example, it may be difficult to obtain large quantities of cysts. In addition, the shrimp's nutritional quality may vary considerably from batch to batch because they are obtained from diverse geographical areas.

Rates of fish growth and survival differed when fed strains of brine shrimp from various geographic locations (Klein-MacPhee, et. al., 1982; Johns et al., 1981; Leger and Sorgeloos, 1984). Therefore, reference brine shrimp have been recommended for use in toxicity testing or as a standard for comparison against other geographic strains of brine shrimp (Sorgeloos, 1981).

Brine shrimp normally hatch after incubation for 24 – 48 hours at room temperature. Different geographical strains may differ somewhat in time-to-hatch (Vanhaecke and Sorgeloos, 1983) and may diminish in nutritional quality after 48 hours (Vanhaecke et al., 1983). Therefore, it is important to harvest the nauplii as soon as possible after approximately 90% have hatched.

A batch of cysts should be started every 24 hours (for feeding the following day) with the same proportion of cysts to seawater so that consistent densities of nauplii are obtained daily (Persoone et al., 1980).

1. Fill a 2- to 4-liter separatory funnel (or other appropriate container) with enough 25 – 30°C seawater to ensure adequate hatching. Add 10 cc brine shrimp cysts per liter, and aerate for at least 24 hours at 25°C. (Two separatory funnels are recommended, started on alternate days, since it may require more than 24 hours to hatch certain strains of brine shrimp.)

2. Nauplii will hatch from brine shrimp cysts within 24 – 48 hours, but before nauplii are fed to the fish, they should be separated from the cysts by taking advantage of their phototactic response or by straining the culture. After removing the source of air, the nauplii's phototactic response is stimulated by covering the top of the funnel with a dark cloth or paper towel for 5 minutes. The nauplii will concentrate at the bottom. However, leaving nauplii longer than 5 minutes without aeration may cause mortality. Another way to stimulate phototactic response is to rinse the nauplii into a beaker (500 mL) or a black separator box (15 x 8 x 8 cm high), place a light source at one end, and leave for no more than 10 – 15 minutes. After live nauplii migrate toward the light, they can be pipetted or siphoned out of the container, leaving the unhatched cysts behind. The nauplii can also be separated from the cysts using a sieve.

3. Pour the nauplii onto a nylon screen (mesh <150 µm), rinse with filtered control seawater, and drain off most of the water.

4. On days 0, 1, and 2, weigh 4 g (wet weight) or pipette 4 mL of concentrated, rinsed *Artemia* nauplii from the quantity of *Artemia* on the screen. On days 3 – 6, weigh 6 g (wet weight) or pipette 6 mL nauplii from the quantity of *Artemia* on the screen. Resuspend the *Artemia* in 80 mL of sea water in a 100 mL beaker. For days 0 – 2, the final suspension yields 0.10 g wet weight of *Artemia* nauplii whereas for days 3 – 6, the final suspension yields 0.15 g wet weight of *Artemia* nauplii.

Aerate or swirl the *Artemia* to equally distribute the nauplii; then withdraw and dispense individual 2 mL portions of *Artemia* to each test chamber using a pipette or adjustable syringe. Uniform distribution of food to all replicates is critical to minimize the variability of larval weight, which is important for successful tests. If the replicate chambers are subdivided, divide the 2 mL equally among the compartments; if the survival rate of any replicate on any day falls below 50%, reduce the volume of *Artemia* dispensed to that replicate by ½.

Some live *Artemia* should remain overnight in test chambers. However, excessive *Artemia* can decrease DO concentrations to below the acceptable limit. Siphon the uneaten *Artemia* from each chamber prior to test solution renewal to ensure that the fish larvae mainly eat newly-hatched nauplii.

Brine Shrimp Quality Control

At a minimum, each batch of purchased brine shrimp should be tested to ensure that they provide the nutrients necessary for adequate fish growth. Before use, individual lot numbers of cysts are fed to the test organisms in 7-day studies to confirm that the diet is adequate for the purposes of the test. The shelf-life of an opened container of cysts may be affected by humidity and temperature, so they should be tested each time a test is started. As long as more than 90% of the cysts hatch in 24 – 48 hours and the control responses are acceptable, the cysts may be used (refer to the EPA manual, *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters in Marine and Estuarine Organisms* [EPA, 2002a] for acceptability parameters).

Preparing Rotifer Cultures (*Brachionus plicatilis*)

Newly hatched *Menidia beryllina* larvae are too small to ingest *Artemia* and must be fed rotifers (*Brachionus plicatilis*). *B. plicatilis* can be cultured continuously in the laboratory when fed algae or yeast in 10- to 15-L Pyrex carboys at 25°C – 28°C, 25‰ – 35‰ salinity. Four 12-L culture carboys, with an outflow spout near the bottom, should be maintained simultaneously to optimize production.

Fill clean carboys with autoclaved seawater. (Alternatively, heat filtered seawater by placing an immersion heater in the carboy, and maintain the temperature to 70°C – 80°C for 1 hour.) When the seawater has cooled to 25°C – 28°C, aerate and add a start-up sample of rotifers (50 rotifers/mL) and food (about 1 L of a dense algal culture or 0.1 g yeast per liter of seawater). Yeast should be dissolved in a minimum of tap water or deionized water before adding it to the culture.

Check the carboys daily to ensure that adequate food is available and that the rotifer density is adequate. If the water appears clear, add yeast (0.1 g/L) or remove 1 L of water and replace it with algae. Remove the water via the bottom spigot, filtering it through a ≤60 µm mesh screen. Return any rotifers collected on the screen to the culture.

Keep the carboys away from light to reduce the amount of algae that attaches to the carboy walls. If detritus accumulates, populations of ciliates, nematodes, or harpacticoid copepods that may have been inadvertently introduced can rapidly take over the culture. If this occurs, discard the cultures.

If a precise measure of the rotifer population is needed, resuspend rotifers collected from a known volume of water in a smaller volume, preserve them with formalin, and count them in a Sedgwick-Rafter chamber. As the density exceeds 50 rotifers/mL, the amount of food per day should be increased to 2 L of algae or 0.2 g/L of yeast. The optimum density, 300 – 400 rotifers/mL, will be reached in about 7 – 10 days and should then be cropped daily. This density is sustainable for 2 – 3 weeks. Once that is attained, the rotifers should be cropped daily.

These rotifers are fed to *M. beryllina* larvae after hatching until about 5 days old. About 5 days after hatching, the larvae can begin feeding on newly hatched *Artemia* nauplii. They are fed *Artemia* daily throughout the 7-day test.

ALGAL CULTURES

Algae for feeding the rotifers, *Tetraselmus suecica* or *Chlorella* sp., can be cultured in 20-L plastic water bottles. Autoclave the bottles (at 110° for 30 minutes) after adding filtered seawater. Cool the bottle to room temperature and place them in a temperature controlled chamber at 18°C - 20°C. Each bottle or carboy should contain 1 L of *T. suecica* or *Chlorella* sp. starter culture and 100 mL of nutrients.

The nutrient formula for the algal culture is:

Mix into 12-L deionized water: Mix on a magnetic stirrer at least 1 hour or until all salts are dissolved.	180 g $NaNO_3$ 12 g NaH_2PO_4 6.16 g EDTA
Add and stir again:	3.78 g $FeCl_3 \cdot 6\ H_2O$ *(Solution should be bright yellow)*
Aerate the algal culture vigorously by inserting a pipette through a foam stopper at the top of the bottle or carboy. A dense algal culture will develop in 7 – 10 days and should be used by day 14. For continuous supply of algal cultures for rotifer feeding, new cultures should be started every 1 or 2 days. For four 12-L rotifer cultures, 6 – 8 continuous algal cultures are needed.	
Clean bottles or carboys thoroughly with soap and water, rinsing with deionized water between uses.	

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Appendix C: Apparatus and Equipment – Sheepshead Minnow and Inland Silverside Tests

Air lines and air stones. For aerating water containing embryos or larvae, 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, six Class A. Borosilicate glass or non-toxic plasticware, 1000 mL for making test solutions.

Brine shrimp, *Artemia*, culture unit.

Crystallization dishes, beakers, culture dishes (1 L), or equivalent. For incubating embryos.

Desiccator. For holding dried larvae.

Dissecting microscope. For checking embryo viability (Sheepshead test only).

Droppers, and glass tubing with fire polished edges, 4 mm ID. For transferring larvae.

Drying oven. 50–105°C range, for drying larvae.

Environmental chamber or equivalent facility with temperature control (25 ± 1° C).

Facilities for holding and acclimating test organisms.

Forceps. For transferring dead larvae to weighing boats.

Inland Silverside culture unit. The test requires approximately 400, 7 – 11 day old larvae. It is preferable to obtain the test organisms from an in-house culture unit. If it is not feasible to culture fish in-house, embryos or larvae can be obtained from other sources by shipping them in well oxygenated saline water in insulated containers.

Light box. For counting and observing larvae.

Meters: pH and DO. For routine physical and chemical measurements.

NITEX® or stainless steel mesh sieves (≤ 150 µm, 500 µm, 3 – 5 mm). For collecting *Artemia* naupili and fish embryos, and for spawning baskets, respectively.

Pipet bulbs and filters. PROPIPET®, or equivalent.

Pipets, automatic. Adjustable, 1 – 100 mL.

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

Pipets, serological. 1 – 10 mL, graduated.

Reference weights, Class S. For checking performance of balance. Weights should bracket the expected weights of the weighing pans and the expected weights of the pans plus fish.

Refractometer. For determining salinity.

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

Separatory funnels, 2 L. Two to four for culturing *Artemia* naupili.

Sheepshead minnow culture unit. The maximum number of larvae required per test will range from a maximum of 360, if 15 larvae are used in each of four replicates, to a minimum of 240 per test, if 10 larvae are used in each of four replicates. It is preferable to obtain the test organisms from an in-house culture unit. If it is not feasible to culture fish in-house, embryos or newly hatched larvae can be obtained from other sources if shipped in well oxygenated saline water in insulated containers.

Siphon with bulb and clamp. For cleaning test chambers.

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

Test chambers.

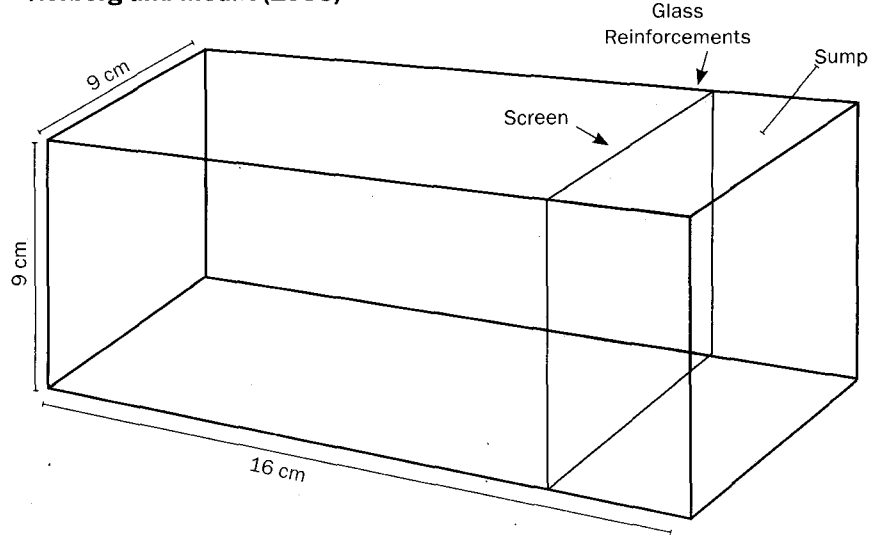
Sheepshead. Four chambers are required for each concentration and the control. Borosilicate glass 1000 mL beakers or modified Norberg and Mount (1985) glass chambers used in the short-term inland silverside test may be used. It is recommended that each chamber contain a minimum of 50 mL/larvae and allow adequate depth of test solution (5.0 cm). To avoid potential contamination from the air and excessive evaporation of test solutions during the test, the chambers should be covered with safety glass plates or sheet plastic (6 mm thick).

Inland Silverside. Four chambers are required for each concentration and the control. The chambers should be borosilicate glass or nontoxic disposable plastic labware. To avoid potential contamination from the air and excessive evaporation of test solutions during the test, the chambers should be covered with safety glass plates or sheet plastic (6 mm thick).

Each test chamber for the inland silverside should contain a minimum of 750 mL of test solution. A chamber such as the one in Figure C-1 constructed of glass and silicone cement has been used successfully for this test. This chamber holds an adequate column of test solution and incorporates a sump area where test solutions can be siphoned and renewed without disturbing the fragile inland silverside larvae.

When constructing the chamber it is recommended that the screen be a 200-µm Nitex® screen (rather than stainless steel) and thin pieces of glass rods be silicone cemented to the screen to reinforce the bottom and sides of the screen to create the sump area. A minimum of silicone should be used while sill ensuring that the

Figure C-1. Glass test chamber with sump area. Modified from Norberg and Mount (1985).



Source: EPA, 2002a.

larvae cannot get trapped or drawn into the sump area. All new chambers should be soaked overnight in seawater (preferably in flowing seawater) to cure the silicone cement before use.

Other types of glass chambers can be used such as 1000 mL beakers. However, each chamber should contain a minimum of 50 mL of test or control solution per larvae and allow adequate depth of test solution (5.0 cm).

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

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

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

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 embryos from substrates and containers, and for rinsing small glassware and instrument electrodes and probes.

Water purification system. Millipore® Milli-Q®, deionized water (DI) or equivalent.

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Appendix D:
Reagents and Consumable Materials

Buffers, pH 4, pH 7, and pH 10. (Or as per instructions of instrument manufacturer). For standards and calibration check (see EPA 2002a).

Data sheets (one set per test). For data recording.

Ethanol (70%) or formalin (4%). For use as a preservative for the fish larvae.

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

Markers, waterproof. For marking containers, etc.

Membranes and filling solutions for DO probe, or reagents. For modified Winkler analysis (see EPA 2002a).

Sample containers. For sample shipment and storage.

Tape, colored. For labeling test chambers.

Vials, marked. Twenty-four per test, containing 4% formalin or 70% ethanol, to preserve larvae (optional).

Reference toxicant solutions. Reference toxicants such as sodium chloride (NaCl), potassium chloride (KCl), cadmium chloride ($CdCl_2$), copper sulfate ($CuSO_4$), sodium dodecyl sulfate (SDS), and potassium dichromate ($K_2Cr_2O_7$) are suitable for use in the NPDES Program and other Agency programs requiring aquatic toxicity tests.

Reagent water. Defined as distilled or deionized water that does not contain substances which are toxic to the test organisms.

Weighing pans, aluminum. Twenty-four per test (one for each replicate.)

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Appendix E: Summary of Test Conditions and Test Acceptability Criteria

Table E-1. Summary of Test Conditions and Test Acceptability Criteria for the Sheepshead Minnow, *Cyprinodon variegatus*, Larval Survival and Growth Test with Effluents and Receiving Waters (Test Method 1004.0)

Test type	Static, with 24-hr renewal (*required*)
Salinity	20‰ – 32‰ (maintained at ± 2‰ of the selected test salinity) (*recommended*)
Temperature (C°)	25°C ± 1°C (*recommended*). Test temperatures must not vary by more than 3°C during the test (*required*)
Light quality	Ambient laboratory (covered, soft white) light (*recommended*)
Light intensity	10 – 20 μE/m²/s (ambient laboratory: 50 – 100 ft-c) (*recommended*)
Photoperiod	16 hr light/8 hr dark (*recommended*)
Test chamber size	600 mL – 1 L containers (*recommended*)
Test solution volume	500 – 750 mL/replicate (loading and DO restrictions must be met) (*recommended*)
Renewal	Daily (*required*)
Age of test organisms	Newly hatched larvae (less than 24-hr old; within 24-hr age of each other) (*required*)
Number of larvae per test chamber	10 (*required minimum*)
Number of replicate chambers per concentration	4 (*required minimum*)
Number of larvae per concentration	40 (*required minimum*)
Source of food	Newly hatched *Artemia* nauplii (less than 24-hr old) (*required*)
Feeding regime	Feed once per day 0.10 g wet weight *Artemia* nauplii per replicate on days 0–2; feed 0.15 g wet weight *Artemia* nauplii per replicate on days 3–6 (*recommended*)
Cleaning	Siphon daily, immediately before test solution renewal and feeding (*required*)
Aeration	None, unless DO concentration falls below 4.0 mg/L, then aerate all chambers. Rate should be less than 100 bubbles/min. (*recommended*)
Dilution water	Uncontaminated source of natural seawater, artificial seawater, deionized water mixed with HSB or artificial sea salts (*available options*)
Test concentrations	Effluent: Five and a control (*required*). Receiving waters: 100% receiving water (or minimum of five) and a control (*recommended*)
Dilution factor	Effluents: ≥0.5 (*recommended*) Receiving waters: None, or ≥0.5 (*recommended*)
Test duration	7 days (*required*)
Endpoints	Survival and growth (weight) (*required*)
Test acceptability criteria	80% or greater survival in controls, average dry weight per surviving organism in control chambers must be 0.60 mg, if unpreserved *or* 0.50 mg or greater average dry weight per surviving control larvae after not more than 7 days in 4% formalin or 70% ethanol (*required*)
Sampling requirement	For on-site tests, samples collected daily and used within 24 hr of the time they are removed from the sampling device. For off-site tests, a minimum of three samples (e.g., collected on days 1, 3, and 5) with a maximum holding time of 36 hr before first use. (*required*)
Sample volume required	6 L per day (*recommended*)

Source: EPA, 2002a. Saltwater Chronic Methods Manual.

Table E-2. Summary of Test Conditions and Test Acceptability Criteria for the Inland Silverside, *Menidia beryllina*, Larval Survival and Growth Test with Effluents and Receiving Waters (Test Method 1006.0)

Test type	Static, with 24-hr renewal (*required*)
Salinity	5‰ – 32‰ (maintained at ± 2‰ of the selected test salinity) (*recommended*)
Temperature (C°)	25°C ± 1°C (*recommended*). Test temperatures must not vary by more than 3°C during the test (*required*)
Light quality	Ambient laboratory (covered, soft white) light (*recommended*)
Light intensity	10 – 20 μE/m²/s (ambient laboratory: 50 – 100 ft-c) (*recommended*)
Photoperiod	16 hr light/8 hr dark (*recommended*)
Test chamber size	600 mL – 1 L containers (*recommended*)
Test solution volume	500 – 750 mL/replicate (loading and DO restrictions must be met) (*recommended*)
Renewal	Daily (*required*)
Age of test organisms	7 – 11 days post-hatch; within 24-hr age of each other (*required*)
Number of larvae per test chamber	10 (*required minimum*)
Number of replicate chambers per concentration	4 (*required minimum*)
Number of larvae per concentration	40 (*required minimum*)
Source of food	Newly hatched *Artemia* nauplii; survival of 7–9 day old *M. beryllina* larvae improved by feeding 24-hr old *Artemia* (*required*)
Feeding regime	Feed 0.10 g wet weight *Artemia* nauplii per replicate on days 0–2; feed 0.15 g wet weight *Artemia* nauplii per replicate on days 3–6 (*recommended*)
Cleaning	Siphon daily, immediately before test solution renewal and feeding (*required*)
Aeration	None, unless DO concentration falls below 4.0 mg/L, then aerate all chambers. Rate should be less than 100 bubbles/min. (*recommended*)
Dilution water	Uncontaminated source of natural seawater, artificial seawater, deionized water mixed with HSB or artificial sea salts (*available options*)
Test concentrations	Effluent: Five and a control (*required*). Receiving waters: 100% receiving water (or minimum of five) and a control (*recommended*)
Dilution factor	Effluents: ≥ 0.5 (*recommended*). Receiving waters: None, or ≥ 0.5 (*recommended*)
Test duration	7 days (*required*)
Endpoints	Survival and growth (weight) (*required*)
Test acceptability criteria	80% or greater survival in controls, 0.50 mg average dry weight of control larvae where test starts with 7-day old larvae and dried immediately after test termination, or 0.43 mg or greater average dry weight per surviving control larvae, preserved not more than 7 days in 4% formalin or 70% ethanol (*required*)
Sampling requirement	For on-site tests, samples collected daily and used within 24 hr of the time they are removed from the sampling device. For off-site tests, a minimum of three samples (e.g., collected on days 1, 3, and 5) with a maximum holding time of 36 hr before first use. (*required*)
Sample volume required	6 L per day (*recommended*)

Source: EPA, 2002a. Saltwater Chronic Methods Manual.

Appendix F: Data Sheets

Figure F-1. Data Form for the Sheepshead Minnow and Inland Silverside, Larval Survival and Growth Toxicity Test. Daily Record of Larval Survival and Test Conditions.

Test Dates: _____ Species _____

Type Effluent: _____ Field _____ Lab _____ Test _____

Effluent Tested: _____

Concentration:																															
	Replicate:								*Replicate:*								*Replicate:*								*Replicate:*							
Days	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7
# Live Larvae																																
Temp (°C)																																
Salinity (‰)																																
DO (mg/L)																																
# Larvae/Dry Wt.				Mean Weight/ Larvae (mg) ± SD					# Larvae/ Dry Wt.				Mean Weight/ Larvae (mg) ± SD				# Larvae/ Dry Wt.				Mean Weight/ Larvae (mg) ± SD				# Larvae/ Dry Wt.				Mean Weight/ Larvae (mg) ± SD			

	Replicate:								*Replicate:*								*Replicate:*								*Replicate:*							
Days	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7
# Live Larvae																																
Temp (°C)																																
Salinity (‰)																																
DO (mg/L)																																
# Larvae/Dry Wt.				Mean Weight/ Larvae (mg) ± SD					# Larvae/ Dry Wt.				Mean Weight/ Larvae (mg) ± SD				# Larvae/ Dry Wt.				Mean Weight/ Larvae (mg) ± SD				# Larvae/ Dry Wt.				Mean Weight/ Larvae (mg) ± SD			

	Replicate:								*Replicate:*								*Replicate:*								*Replicate:*							
Days	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7
# Live Larvae																																
Temp (°C)																																
Salinity (‰)																																
DO (mg/L)																																
# Larvae/Dry Wt.				Mean Weight/ Larvae (mg) ± SD					# Larvae/ Dry Wt.				Mean Weight/ Larvae (mg) ± SD				# Larvae/ Dry Wt.				Mean Weight/ Larvae (mg) ± SD				# Larvae/ Dry Wt.				Mean Weight/ Larvae (mg) ± SD			

Time Fed								

Comments:

Source: EPA, 1987a.

Figure F-1 (continued). Data Form for the Sheepshead Minnow and Inland Silverside, Larval Survival and Growth Toxicity Test. Daily Record of Larval Survival and Test Conditions..

Test Dates: _____ Species _____

Type Effluent: _____ Field _____ Lab _____ Test _____

Effluent Tested: _____

Concentration:																															
	Replicate:								*Replicate:*								*Replicate:*								*Replicate:*							
Days	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7
# Live Larvae																																
Temp (°C)																																
Salinity (‰)																																
DO (mg/L)																																
# Larvae/Dry Wt.				Mean Weight/ Larvae (mg) ± SD					# Larvae/ Dry Wt.			Mean Weight/ Larvae (mg) ± SD					# Larvae/ Dry Wt.			Mean Weight/ Larvae (mg) ± SD					# Larvae/ Dry Wt.			Mean Weight/ Larvae (mg) ± SD			

	Replicate:								*Replicate:*								*Replicate:*								*Replicate:*							
Days	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7
# Live Larvae																																
Temp (°C)																																
Salinity (‰)																																
DO (mg/L)																																
# Larvae/Dry Wt.				Mean Weight/ Larvae (mg) ± SD					# Larvae/ Dry Wt.			Mean Weight/ Larvae (mg) ± SD					# Larvae/ Dry Wt.			Mean Weight/ Larvae (mg) ± SD					# Larvae/ Dry Wt.			Mean Weight/ Larvae (mg) ± SD			

	Replicate:								*Replicate:*								*Replicate:*								*Replicate:*							
Days	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7
# Live Larvae																																
Temp (°C)																																
Salinity (‰)																																
DO (mg/L)																																
# Larvae/Dry Wt.				Mean Weight/ Larvae (mg) ± SD					# Larvae/ Dry Wt.			Mean Weight/ Larvae (mg) ± SD					# Larvae/ Dry Wt.			Mean Weight/ Larvae (mg) ± SD					# Larvae/ Dry Wt.			Mean Weight/ Larvae (mg) ± SD			

Time Fed							

Comments:

Source: EPA, 1987a.

Figure F-2. Data Form for the Sheepshead Minnow and Inland Silverside, Larval Survival and Growth Toxicity Test. Summary of Test Results

Test Dates: _____ Species: _____

Effluent Tested: _____

Treatment						
No. Live Larvae						
Survival (%)						
Mean Dry Wt/Larvae (mg) ± SD						
Signif. Diff. from Control (o)						
Mean Temp. (°C) ± SD						
Mean Salinity ‰ ± SD						
Ave. DO (mg/L) ± SD						

Comments:

Source: EPA, 1987a.

Figure F-3. Data Form for the Sheepshead Minnow and Inland Silverside, Larval Survival and Growth Toxicity Test. Dry Weights of Larvae.

Test Dates: _____ Species: _____

Pan. No.	Conc. & Rep.	Initial Wt. (mg)	Final Wt. (mg)	Diff. (mg.)	No. Larvae	Avg. Wt./ Larvae (mg)

Source: EPA, 1987b.

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