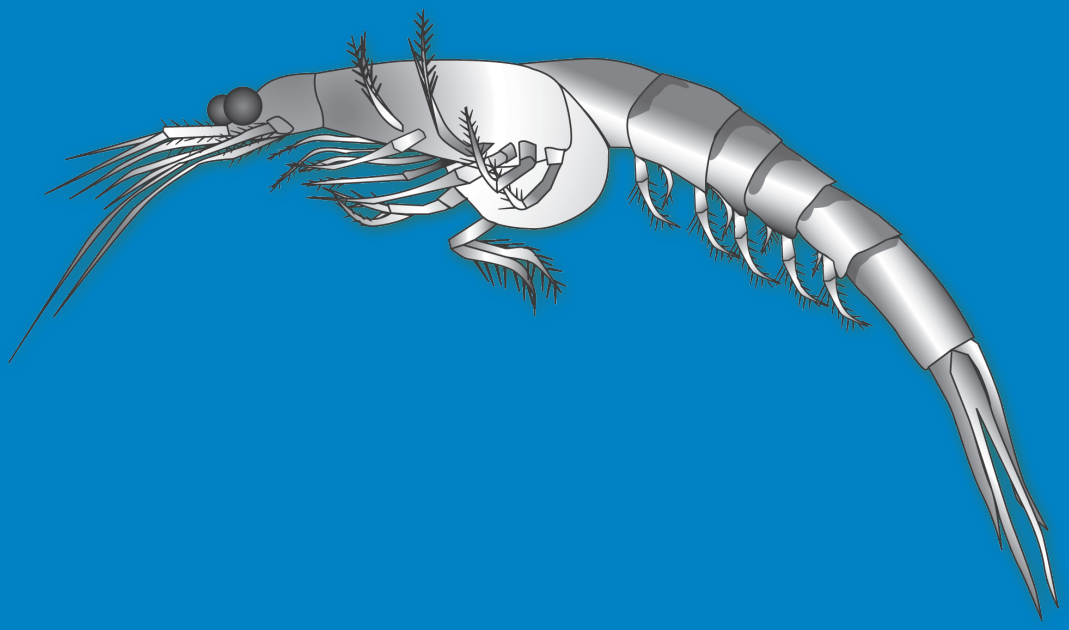
Mysid (*Americamysis bahia*) Survival, Growth, and Fecundity Toxicity Tests

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 "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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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.

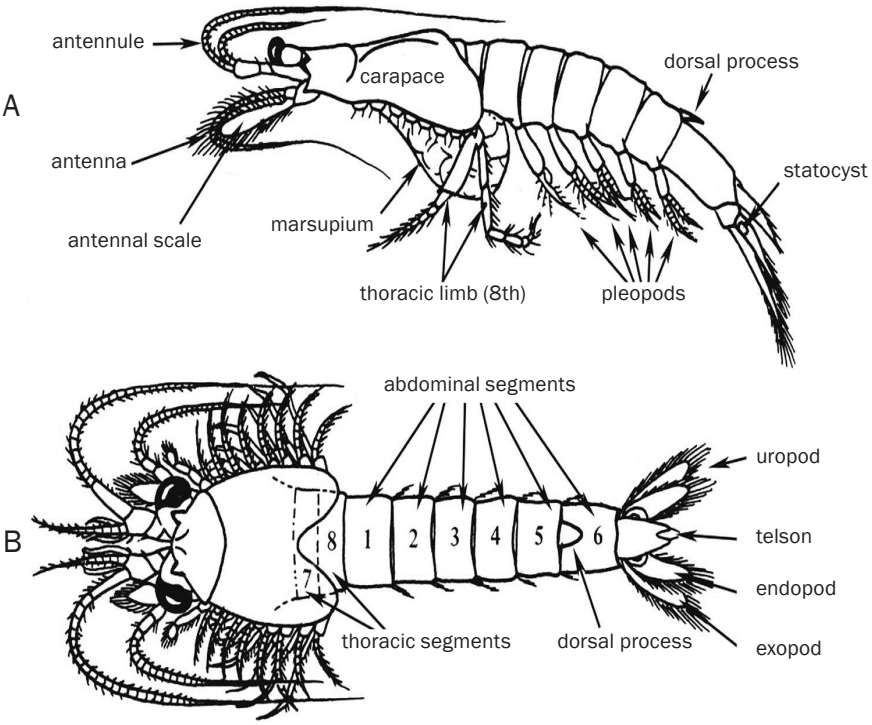
Cultures should be maintained in glass aquaria supplied with flow-through or recirculating seawater (Lussier et al., 1988). The water temperature should be 26°C and salinity between 20‰ to 30‰ and should not fluctuate more than 2°C or 2‰ per day, respectively. The light regime recommended for culturing 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



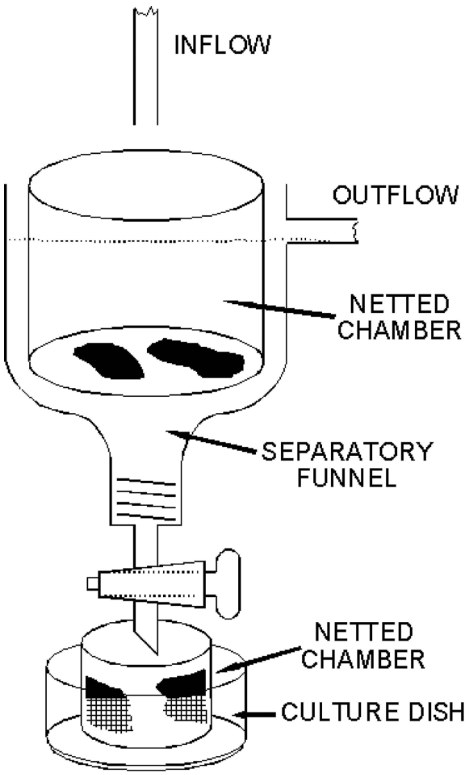
Source: Heard and Price, 2006 as modified from Stuck et al., 1979.

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

Parameter	Monitoring Frequency		
	Each New Sample	24-hr Exposure Period	
		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: _____

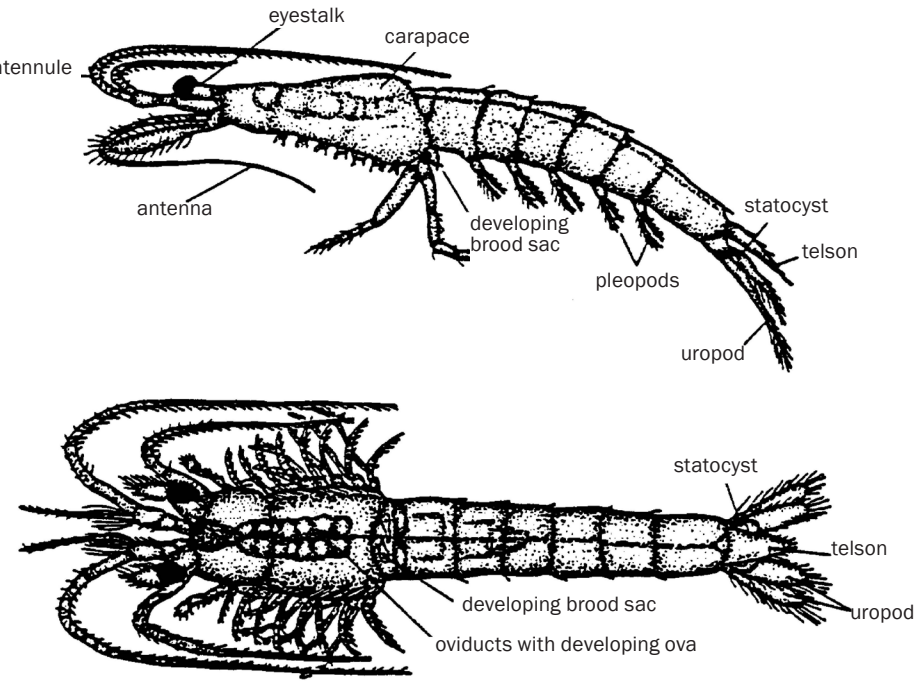
Treatment/ Replicate		Pan #	Tare Wt.	Total Wt.	Organism Wt.	# of Organisms	Wt./Organism
Control	1						
	2						
	3						
	4						
	5						
	6						
	7						
	8						
1	1						
	2						
	3						
	4						
	5						
	6						
	7						
	8						
2	1						
	2						
	3						
	4						
	5						
	6						
	7			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

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.

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



Source: Lussier, Kuhn, and Sewall, 1987.

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).

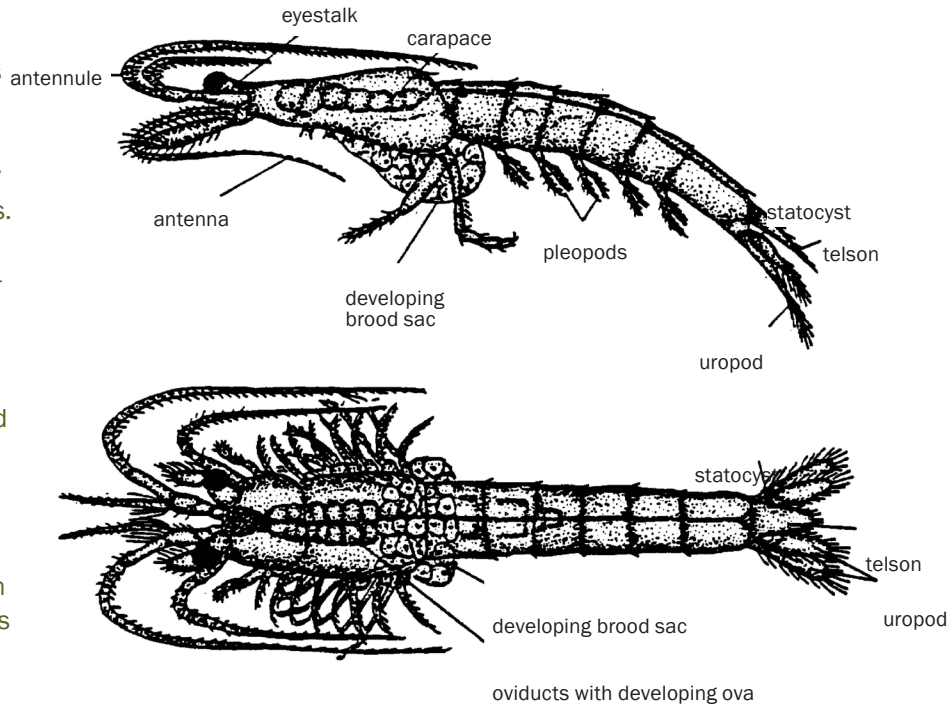
After the sex, maturity, and fecundity of each mysid from one replicate is determined, all of the mysids from that replicate should be placed on a Nitex® screen that rests on top of a beaker. Rinse the mysids with deionized water to remove any salts that may interfere with the dry weights. After the 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.

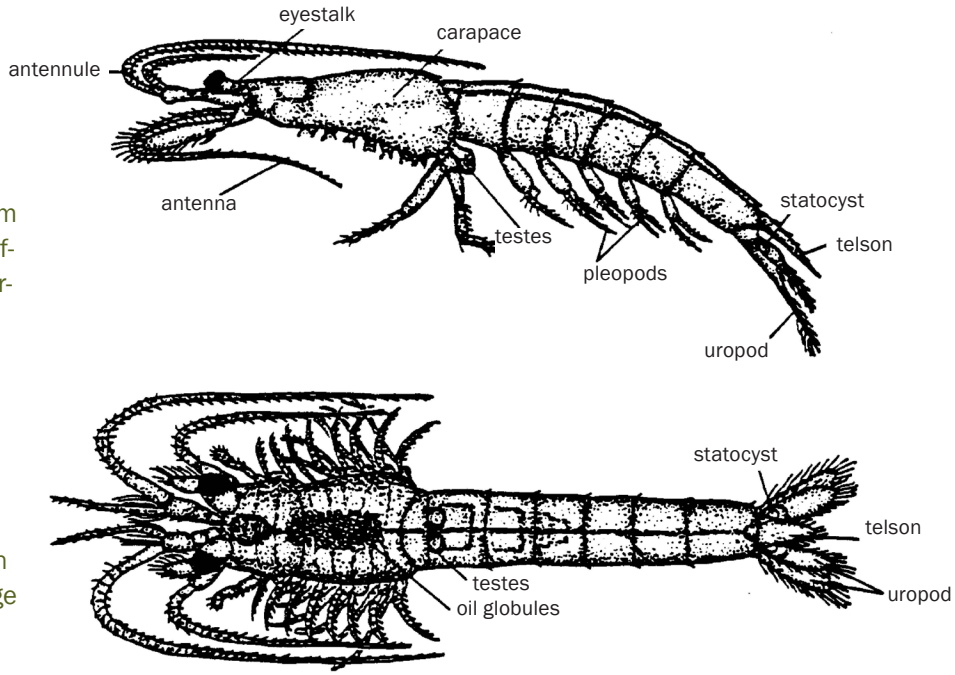
The analysis of this test compares the maturity, fecundity, growth, and survival of the

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)



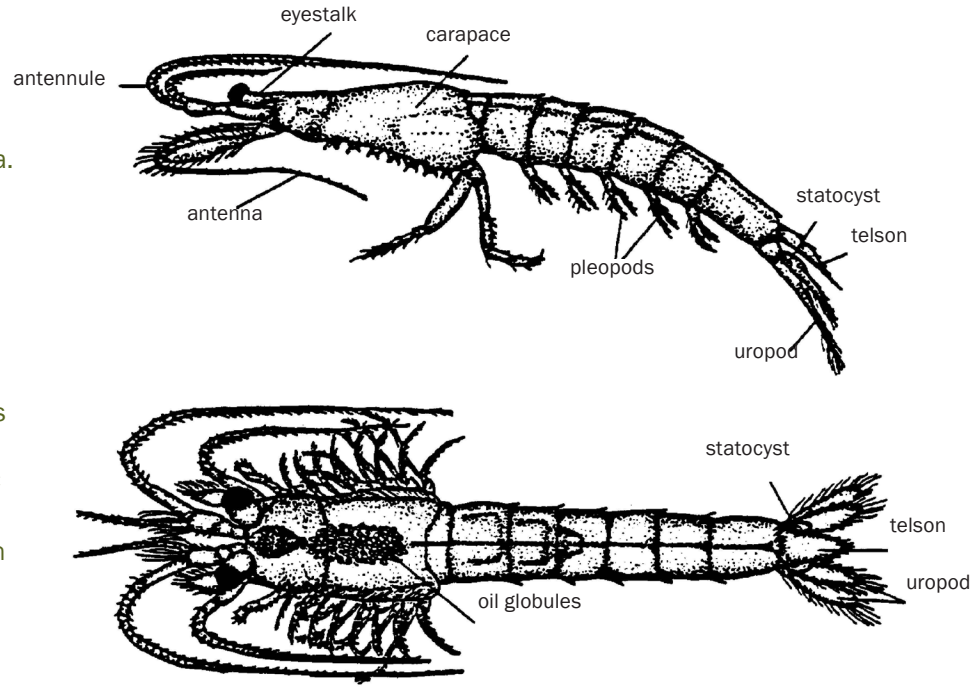
Source: Lussier, Kuhn, and Sewall, 1987.

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/m^2/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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