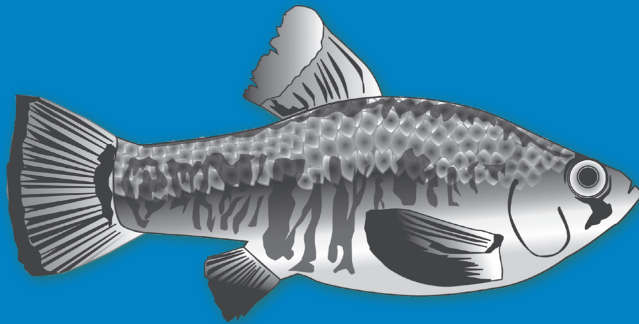
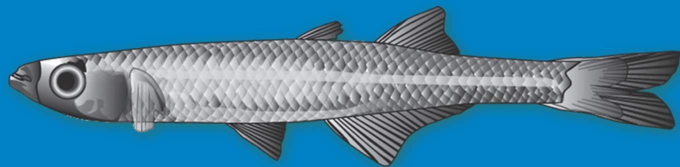
Sheepshead Minnow (*Cyprinodon variegatus*) and Inland Silverside (*Menidia beryllina*) Larval Survival and Growth Toxicity Tests

Supplement to Training Video





U.S. Environmental Protection Agency
Office of Wastewater Management
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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 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.

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[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.

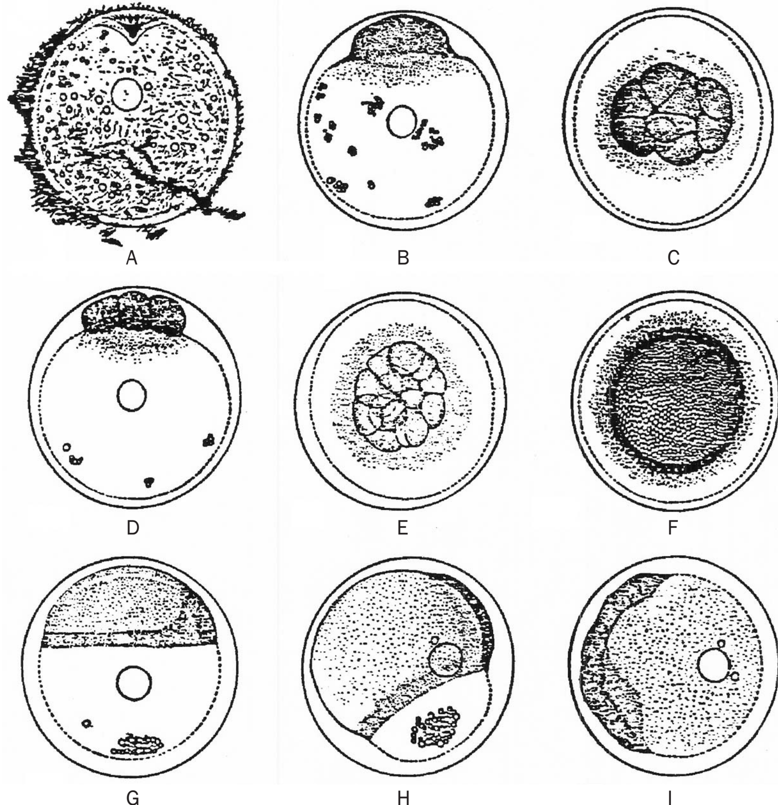
The larvae are fed newly hatched *Artemia* nauplii and crushed flake food, *ad libitum*, daily. The *Artemia* should be cultured in the laboratory in order to provide 24 – 48 hour old nauplii. Appendix B describes in detail how to culture *Artemia*.

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.

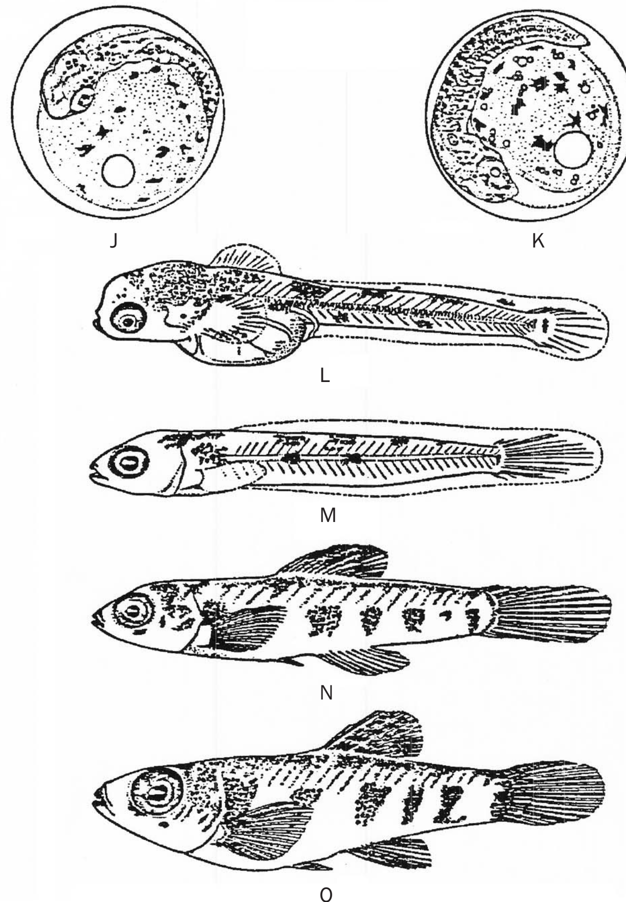
After collection, incubate the collected minnow embryos in seawater at 25°C, 20‰ – 30‰ salinity, and 16-hour light and 8-hour dark photoperiod for 5 – 6 days with aeration and daily water changes.

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 µE/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 25ºC.

Larvae will hatch in 6 – 7 days when incubated at 25ºC 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

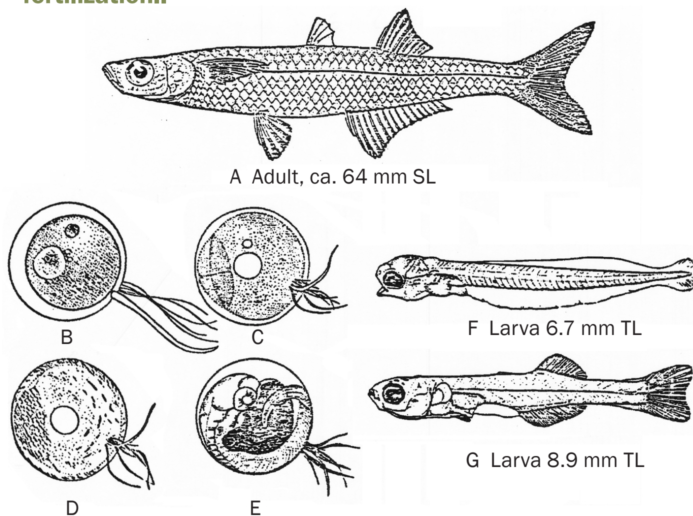
Effluent Sampling

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

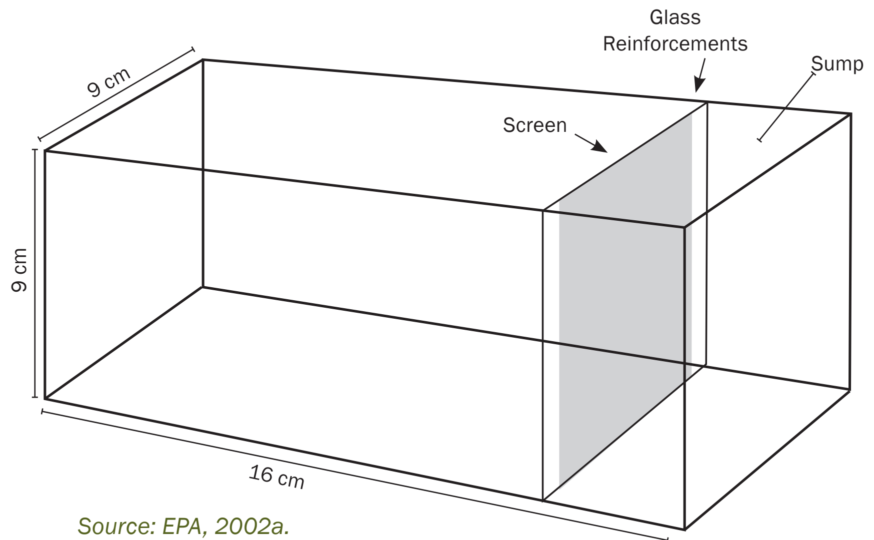
The type of dilution water used to make the test concentrations is dependent on the objectives of the test. Any specific requirements included in NPDES permits should be followed. The Saltwater Chronic Methods Manual (Section 7) provides the following guidelines:

- *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

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



Source: EPA, 2002a.

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.

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 25ºC 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 0º – 6ºC. 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 25ºC 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 25ºC ± 1ºC, 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.

Citations and Recommended References

Cripe, G.M., B.L. Hemmer, L.R. Goodman, and J.C.Vennari. 2009. Development of a Methodology for Successful Multigeneration Life-Cycle Testing of the Estuarine Sheepshead Minnow, *Cyprinodon variegatus*. Archives of Environmental Contamination and Toxicology. April 56(3):500-8.

EPA. 1985. *Aquatic Toxicity Testing Seminar Manual*. National Health and Environmental Effects Research Laboratory – Aquatic Ecology Division, Narragansett, RI. NHEERL-AED Contribution No. 796.

EPA. 1987a. Guidance manual for rapid chronic toxicity tests on effluents and receiving waters with larval inland silversides (*Menidia beryllina*). Contribution No. 792. Heber, M.A., M.M. Hughes, S.C. Schimmel, and D.A. Bengston. In: Schimmel, S.C. ed., Users guide to the conduct and interpretation of complex effluent toxicity tests at estuarine/marine sites. Environmental Research Laboratory, U.S. EPA, Narragansett, RI 02882. Contribution No. 796, 265 pp.

EPA. 1987b. Guidance manual for conducting complex effluent and receiving water larval fish growth-survival studies with the sheepshead minnow (*Cyprinodon variegatus).* Contribution No. x104. Hughes, M.M., M.A. Heber, S.C. Schimmel, and W.J. Berry. In: Schimmel, S.C. ed., Users guide to the conduct and interpretation of complex effluent toxicity tests at estuarine/marine sites. Environmental Research Laboratory, U.S. EPA, Narragansett, RI 02882. Contribution No. 796, 265 pp.

EPA. 1989. *Biomonitoring for Control of Toxicity in Effluent Discharges to the Marine Environment*. U.S. EPA Center for Environmental Research Information, Cincinnati, OH. U.S. EPA National Health and Environmental Effects Research Laboratory – Aquatic Ecology Division, Narragansett, RI. EPA/625/8-89/015.

EPA. 1991. *Technical Support Document for Water Quality-based Toxics Control*. U.S. EPA Office of Water Enforcement and Permits, Washington, D.C. EPA-505-2-90-001.

EPA. 2000a. Method Guidance and Recommendations for Whole Effluent Toxicity (WET) Testing (40 CFR 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.

EPA. 2002a. *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms, Third Edition (Saltwater Chronic Methods Manual).* Environmental Protection Agency, Washington, DC. EPA-821-R-02-014.

EPA. 2002b. *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, Fifth Edition.* (Acute Methods Manual). Office of Water, Cincinnati, OH. EPA-821-R-02-012.

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. Sheepshead Minnow (*Cyprinodon variegatus*) and Inland Silverside (*Menidia beryllina*) Larval Survival and Growth Toxicity Test. Supplement to Training Video. Whole Effluent Toxicity Training Video—Saltwater Series. March 2009. EPA 833-C-09-001

Johns, D.M., W.J. Berry, and W. Walton. 1981. International Study on *Artemia*, XVI. Survival, growth, and reproduction potential of the mysid, *Mysidopsis bahia* Molenock fed various geographical collections of the brine shrimp, *Artemia*. J. Exp. Mar. Biol. Ecol. Vol. 53, pp. 209–219.

Klein-MacPhee, G., W.H. Howell, and A.D. Beck. 1982. International Study on *Artemia*, XX. Comparison of a reference and four geographical strains of *Artemia* as food for winter flounder (*Pseudopleuronectes americanus*) larvae. Aquaculture. Vol. 29, pp. 279–288.

Leger, P., and P. Sorgeloos. 1984. International Study on *Artemia*, XXIX. Nutritional value of *Artemia* nauplii from various geographical origins for the mysid, *Mysidopsis bahia* (Molenock). Mar. Ecol. Prog. Ser. Vol. 15, pp. 307–309.

Norberg, T.J. and D.J. Mount. 1985. A new fathead minnow (*Pimephales promelas*) Subchronic toxicity test. Environ. Toxicol. Chem. 4(5):711-718.

Persoone, G., P. Sorgeloos, O. Roels, and E. Jaspers eds. 1980. The brine shrimp *Artemia*. Vols. 1–3. Proceedings of the International Symposium on the brine shrimp *Artemia salina*, Corpus Christi, Texas. 1979. Universal Press, Wetteren, Belgium.

Sorgeloos, P. 1981. Availability of reference *Artemia* cysts. Aquaculture. Vol. 23, pp. 381–382.

Vanhaecke, P., P. Lavens, and P. Sorgeloos. 1983. International Study on *Artemia*, XVII. Energy consumption in cysts and early larval stages of various geographical strains of *Artemia*. Ann. Soc. R. Zool. Belg. Vol. 113, pp. 155–165.

Vanhaecke, P., and P. Sorgeloos. 1983. International Study on *Artemia*, XIX. Hatching data for 10 commercial sources of brine shrimp cysts and reevaluation of the "hatching efficiency" concept. Aquaculture. Vol. 30, pp. 43–52.

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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 – 30ºC seawater to ensure adequate hatching. Add 10 cc brine shrimp cysts per liter, and aerate for at least 24 hours at 25ºC. (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:

<table>
<tr><td>Mix into 12-L deionized water:
Mix on a magnetic stirrer at least 1 hour or until all salts are dissolved.</td><td>180 g $NaNO_3$
12 g NaH_2PO_4
6.16 g EDTA</td></tr>
<tr><td>Add and stir again:</td><td>3.78 g $FeCl_3$•6 H_2O
(Solution should be bright yellow)</td></tr>
<tr><td colspan="2">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.</td></tr>
<tr><td colspan="2">Clean bottles or carboys thoroughly with soap and water, rinsing with deionized water between uses.</td></tr>
</table>

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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.

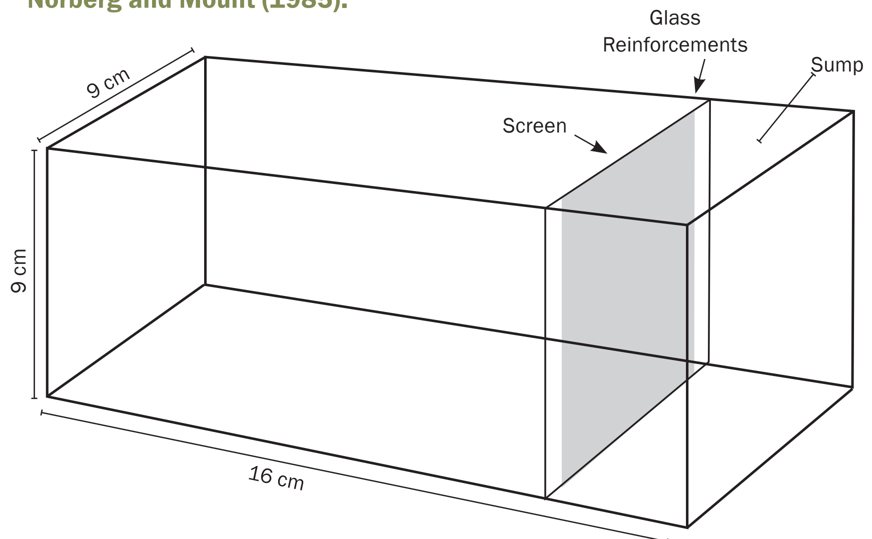
Sheepshead. Four chambers are required for each concentration and the control. Borosilicate glass 1000 mL beakers or modified Norberg and Mount (1985) glass chambers used in the short-term inland silverside test may be used. It is recommended that each chamber contain a minimum of 50 mL/larvae and allow 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/m^2/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/m^2/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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