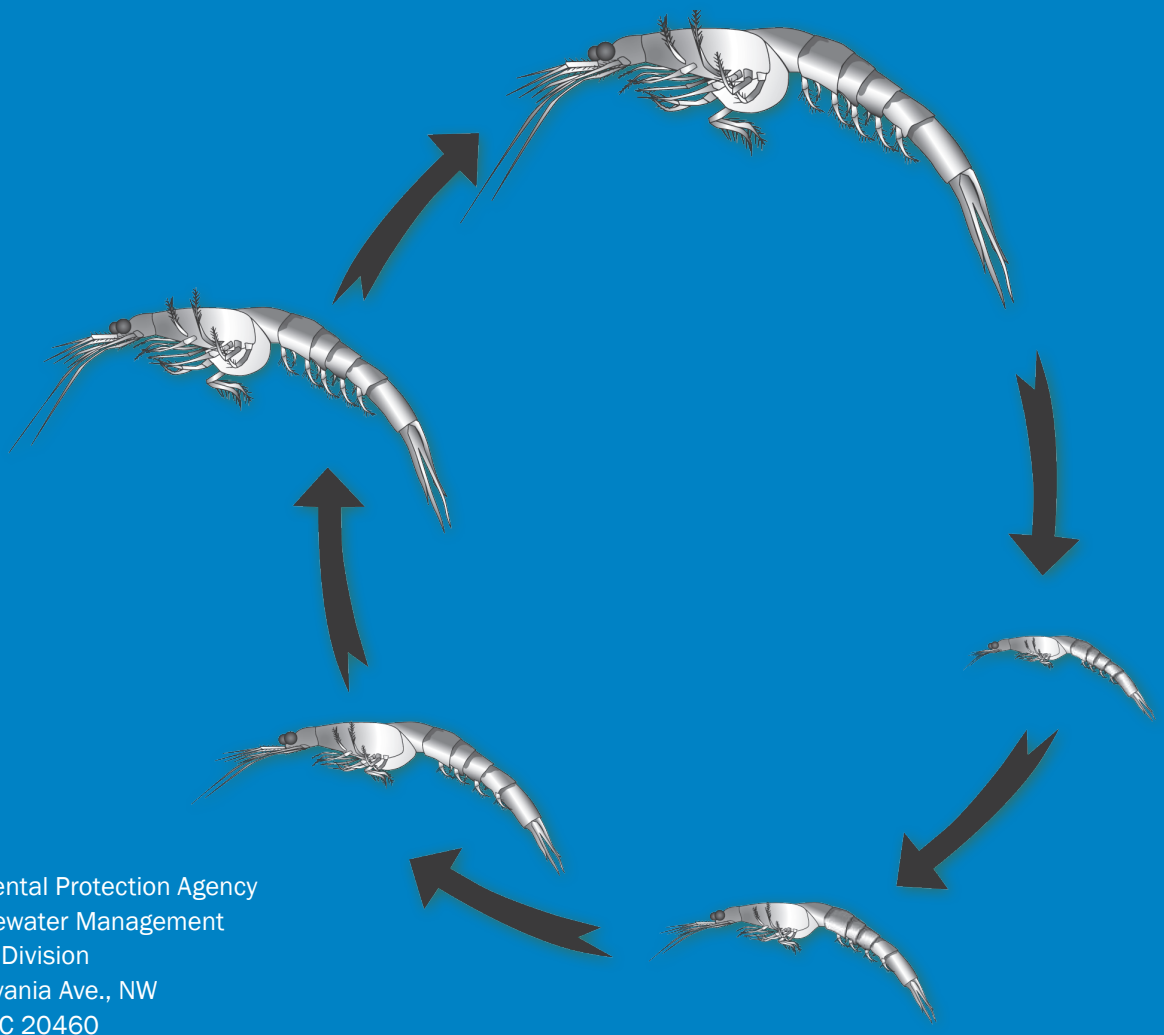
Culturing *Americamysis bahia*

Supplement to Training Video



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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 "Culturing *Americamysis bahia*" (EPA, 2009a). The methods illustrated in the video and described in this supplemental guide support the methods published in the U.S. Environmental Protection Agency's (EPA's) *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, Fifth Edition* (2002a), referred to as the Acute Methods Manual. The video and this guide provide details on culturing of mysids for the use in conducting tests based on the expertise of personnel at the following EPA Office of Research and Development (ORD) laboratories:

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

NHEERL – Gulf Ecology Division in Gulf Breeze, Florida

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

This guide and its accompanying video are part of a series of training videos produced by EPA's Office of Wastewater Management. The video entitled "Mysid (*Americamysis bahia*) Survival, Growth, and Fecundity Toxicity Tests" (EPA 2009b) complements the material in this video by explaining the 7-day short-term chronic toxicity test method using mysids. This Saltwater Series includes the following videos and guides:

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

"Culturing *Americamysis bahia*"

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

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

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

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

"*Ceriodaphnia* Survival and Reproduction Toxicity Tests"

"Culturing of Fathead Minnows (*Pimephales promelas*)"

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

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

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Contents

Figures

Table

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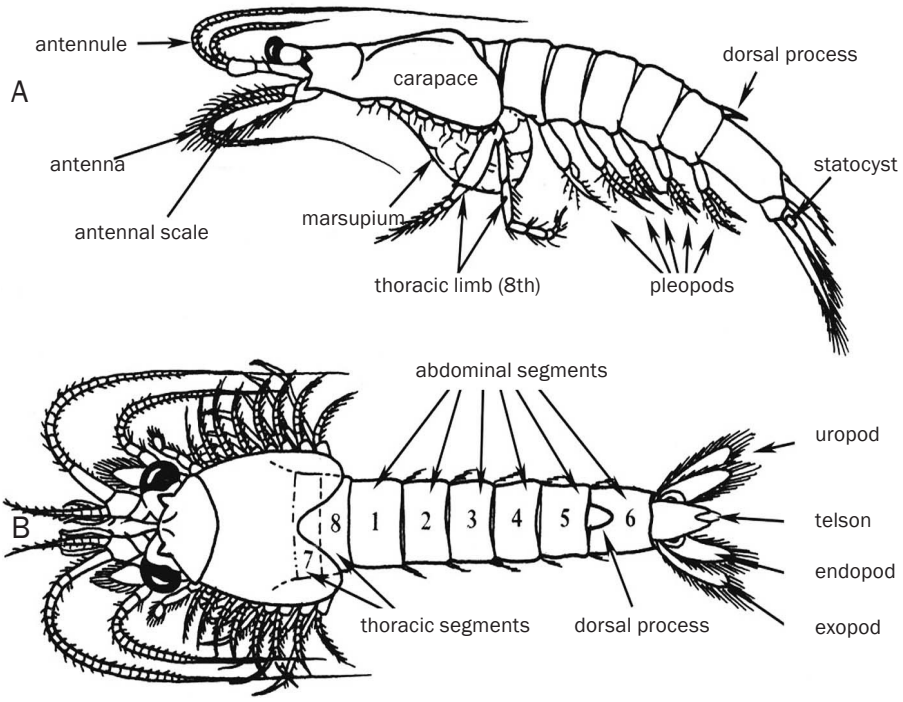
Introduction

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

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

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

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



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

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

Water and Light

Culture Water

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

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

Table 1. Recommended Culture Conditions for *Americamysis bahia*

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

Source: Lussier et al., 1988.

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

Photoperiod

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

Culture Vessels

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

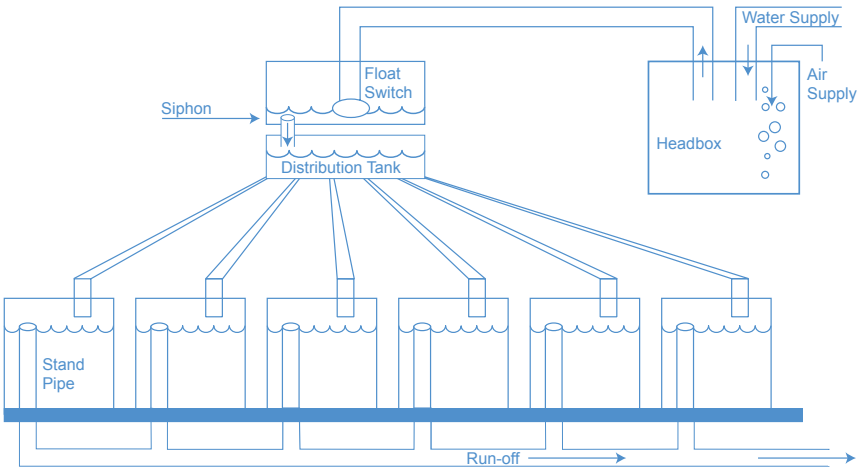
Water Delivery Systems

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

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

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

Figure 2. Intermittent Flow-Through Water Delivery System



Source: EPA 2002b.

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

Culture Start Up and Maintenance

Starting Cultures

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

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

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

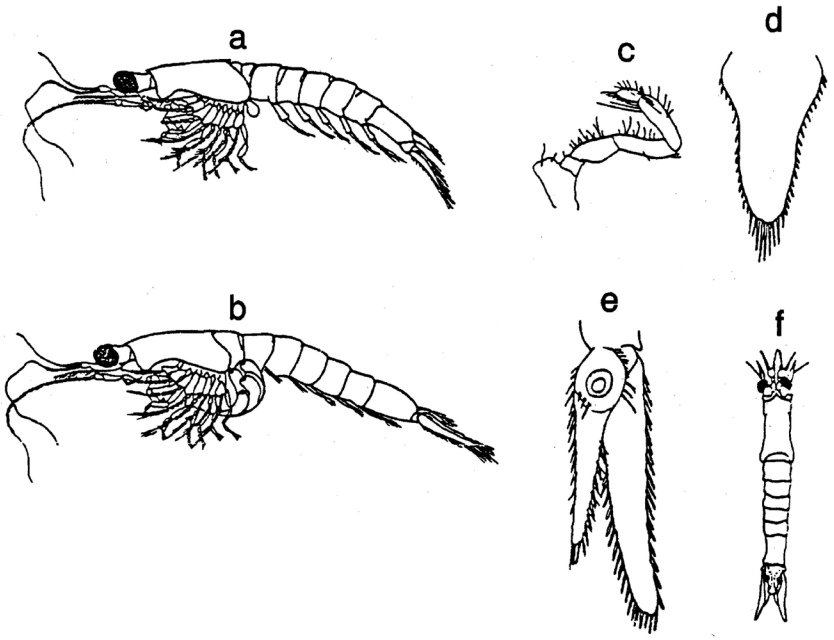
For optimum growth and reproduction, the stocking density for adult mysids should be approximately 20 mysids per liter. Juveniles can be stocked at higher densities than adults. A healthy, unstressed culture should have at least 70% of the females carrying eggs in their brood pouch.

TAXONOMY

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

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

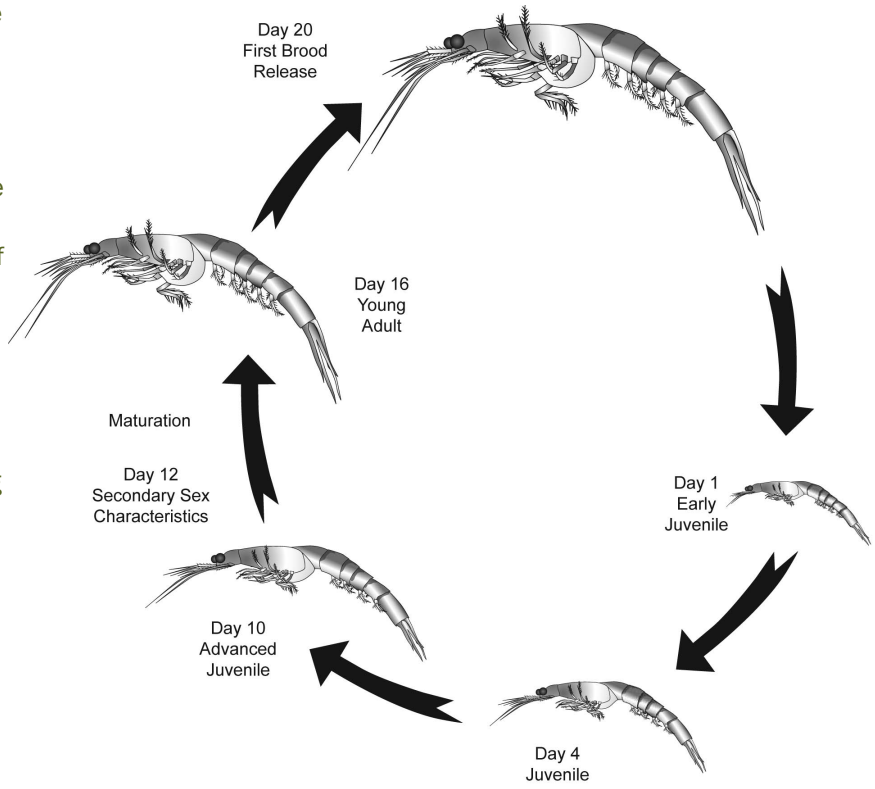
Figure 3. Morphological Characteristics Used in Mysid Identification



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

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

Figure 4. Life Cycle of a Mysid



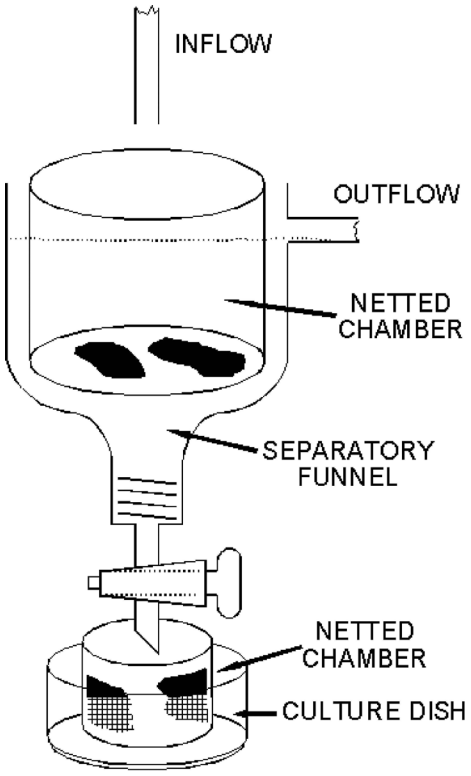
Collecting Test Organisms

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

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

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

Figure 5. Illustration of Mysid Brood Chamber



Source: Lussier, et al., 1987.

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

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

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

Tank Cleaning

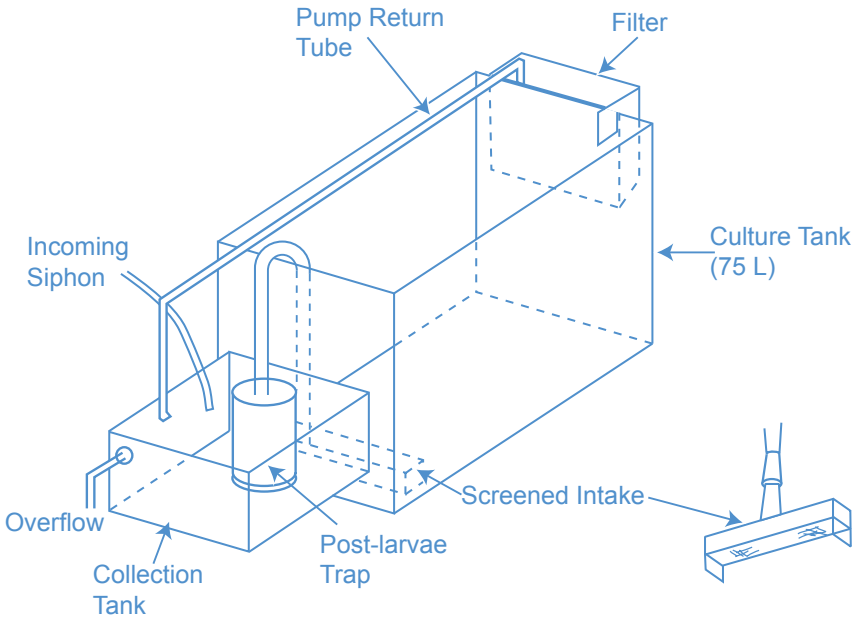
Culture tanks should be cleaned at least once each month. The sides of each tank should be scraped to remove any algal growth and the gravel should be stirred to dislodge the accumulated debris, which will clear the dolomite filter.

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

Record Keeping

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

Figure 6. Illustration of Mysid Generator



Source: Lussier et al., 1988.

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

Figure 7. Data Form for Mysid Cultures

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

Food Preparation

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

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

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

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

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

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

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

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

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

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www.epa.gov/npdes/wqbasedpermitting.

Glossary

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

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

Cyst. The life stage of unhatched *Artemia*.

Epibenthic. Pertaining to the area just above the sediment.

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

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

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

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

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

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

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

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Appendix A
Apparatus and Equipment List

Air line and air stones. For aerating cultures, brood chambers, and holding tanks, and supplying air to test solutions with low DO.

Air pump. For oil-free air supply.

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

Beakers or flasks. Six, borosilicate glass or non-toxic plasticware, 2 – 3 L for making test solutions.

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

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

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

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

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

Facilities for holding and acclimating test organisms.

Light box. For illuminating organisms during examination.

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

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

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

Pipet bulbs and fillers. Propipet®, or equivalent.

Pipets, automatic. Adjustable, 1 – 100 mL.

Pipets, serological. 1 – 10 mL, graduated.

Pipets, volumetric, Class A. 100 mL.

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

Refractometer or other method. For determining salinity.

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

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

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

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

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

Volumetric flasks and graduated cylinders. Class A, borosilicate glass or non-toxic plastic labware, 50 – 2000 mL for making test solutions.

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

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

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