United States
Environmental Protection
Agency

Office of Water
(EN-336)

EPA 505/8-90-006a
September 1990

EPA

Mysids *(Mysidopsis bahia)* Survival, Growth, and Fecundity Toxicity Test

Supplemental Report For Saltwater Video Training Tape

EPA 505/8-90-006a

MYSID (*MYSIDOPSIS BAHIA*) SURVIVAL, GROWTH, AND FECUNDITY TEST

SUPPLEMENTAL REPORT

U.S. Environmental Protection Agency
Office of Water Enforcement and Permits
Permits Division
401 M Street, SW
Washington, DC 20460

EPA Contract 68-C8-0015
1990

FOREWORD

This report is a supplement to the video training tape "Mysid (*Mysidopsis bahia*) Survival, Growth, and Fecundity Test" (EPA, 1990a). The techniques illustrated in the tape and described in this report are based on the methods published in the EPA manual *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms* (EPA, 1988).

This report and the video tape it accompanies are part of a series of training tapes and reports produced by EPA's Office of Water Enforcement and Permits. The tape entitled "Culturing *Mysidopsis bahia* (EPA, 1990b) complements the material in this report by illustrating the methods used at EPA's Environmental Research Laboratory in Narragansett, Rhode Island for culturing *Mysidopsis bahia* (mysids) for use in marine toxicity tests. These tapes are available through The National AudioVisual Center, Capitol Heights, Maryland 20743. Other available tapes include the freshwater series:

"Culturing *Ceriodaphnia dubia*"
"Culturing Fathead Minnows (*Pimephales promelas*)
"*Ceriodaphnia* Survival and Reproduction Toxicity Tests"
"Fathead Minnow Larval Survival and Growth Toxicity Test".

This report has been funded wholly or in part by the Environmental Protection Agency under contract 68-C8-0015 to Technical Resources, Inc. It has been subject to the Agency's review, and it has been approved for distribution as an EPA document. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

ACKNOWLEDGEMENTS

This report was produced for EPA's Office of Water Enforcement and Permits (Laura Phillips, Work Assignment Manager) under contract to Technical Resources, Inc. (Lynn Bowler, Work Assignment Manager). Technical direction and expertise on the test methods were provided by Suzanne Lussier, Randy Comeleo, and other staff of the EPA Environmental Research Laboratory in Narragansett, Rhode Island. Several other individuals also have provided input to the techniques described for conducting the mysid survival, growth, and fecundity toxicity test, and while the list is not inclusive, these are George Morrison (EPA, ERL-Narragansett), Margarete Heber (EPA, Office of Water Enforcement and Permits), Bill Peltier (EPA, Region 4, Athens, GA), and Richard Montgomery (TRI).

INTRODUCTION

This report and the video "Mysid (*Mysidopsis bahia*) Survival, Growth, and Fecundity Test" (EPA, 1990a) were produced by the U.S. EPA to provide instructions for the conduct of the standard seven-day toxicity test using mysids. The standard methods are published in the EPA manual *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms* (EPA, 1988). The methods presented in this report and the video tape are based on the expert experiences and standardized practices developed at EPA's Environmental Research Laboratory (ERL) in Narragansett, Rhode Island.

The mysid survival, growth, and fecundity toxicity test is used by EPA for determining the toxicity of marine or estuarine discharges by measuring specified endpoints after a seven-day exposure period. Healthy animals and correct laboratory procedures are essential for accurate test results. Laboratory personnel should be very familiar with the test methods and with mysid handling techniques before conducting a test.

This report presents instructions for conducting the test according to the standard methods and additional tips provided by ERL-Narragansett for conducting the tests efficiently and accurately. The procedures presented in the first section of this report cover requirements for maintaining and feeding adult mysids. The second section explains methods used to produce enough juveniles of the same age for starting a test. The third section of this report provides instruction for starting, renewing, and terminating the test, including diagrams of the different sexes and life stages that are used as endpoints for statistical analysis. The glossary provides working definitions of some of the terms associated with culturing or testing mysids. Appendix A includes additional references on mysid testing that may provide further insight into the testing procedures and analysis of the results. Appendix B provides a listing of the apparatus and equipment needed to conduct the mysid toxicity test.

Culture Maintenance

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

Cultures should be maintained in glass aquaria supplied with flow-through or recirculating sea water (Lussier et al., 1988). The water temperature should be 25°C and salinity between 20-30 ‰. They should not fluctuate more than 2°C or 2 ‰, respectively. The light regime recommended for culturing is 16 hours light and 8 hours dark. The light should be phased in and phased out gradually so as not to startle the mysids.

Feeding

Mysids are fed 24-48 hours old *Artemia* nauplii twice daily. Feeding amounts should be adequate to provide live food at all times for the mysids to feed upon. Approximately 150 *Artemia* per mysid per day is the guideline used at ERL-Narragansett. *Artemia* supplies should be checked periodically for contamination and hatch rates.

Detailed instructions on culturing *Artemia* are presented in the video "Culturing *Mysidopsis bahia*", in the accompanying supplemental report, and in the EPA manual *Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms* (EPA, 1985).

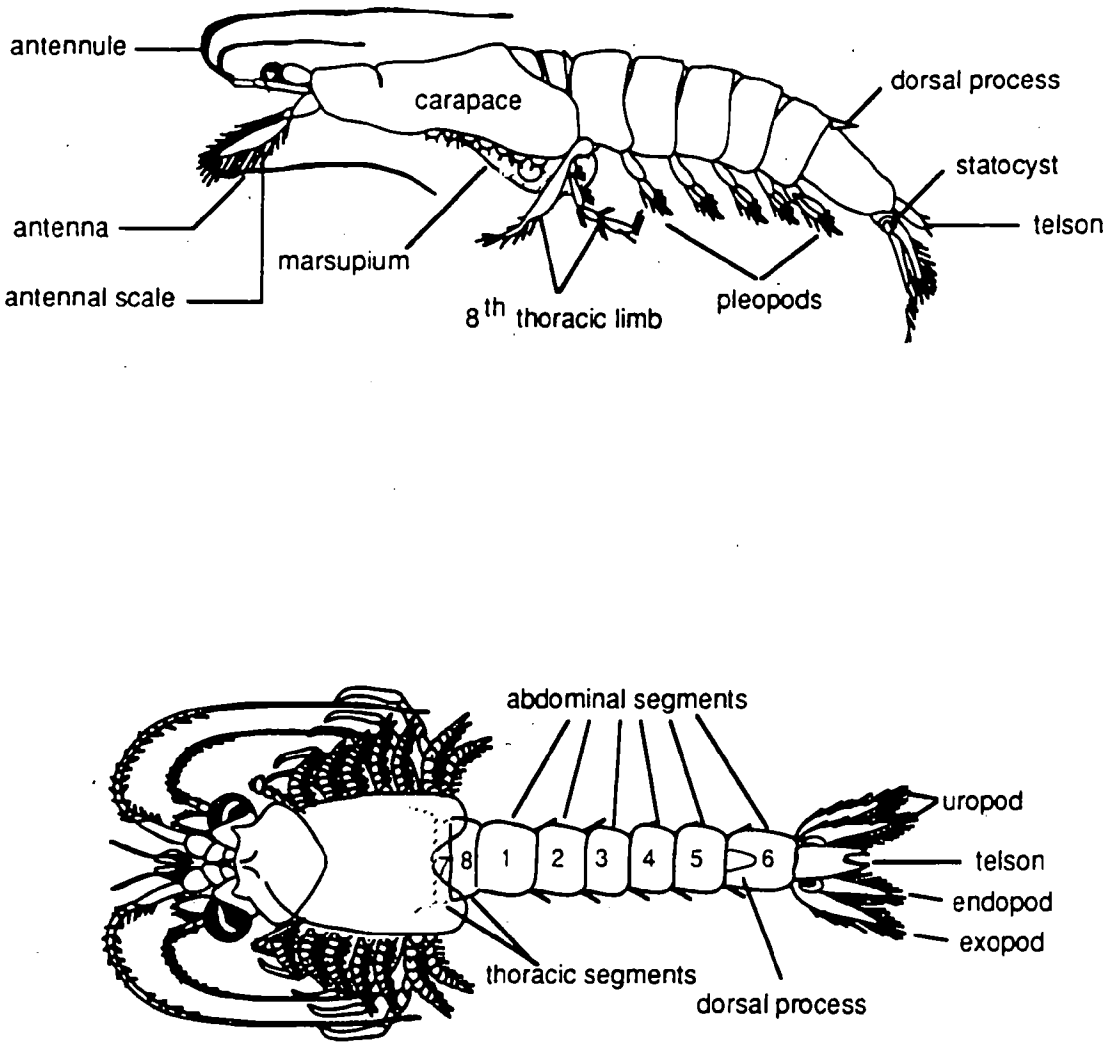

Figure 1. Lateral and dorsal view of a typical mysid. (From Stuck et al., 1979).

COLLECTING JUVENILES FOR TEST USE

The seven-day survival, growth, and fecundity test must be started with seven-day old mysids that are all within 24 hours age of each other. Seven-day old juveniles are needed in sufficient number to randomly select 5 juveniles for each replicate. For a test with 5 effluent concentrations and 1 control, with 8 replicates at each concentration, approximately 250-300 7-day old mysids should be available to choose from.

To collect juveniles and to be assured of their age range, a brood chamber is used (see Figure 2). At ERL-Narragansett the brood chamber is set up eight days before the start of the test. Gravid females selected from a minimum of three culture tanks are placed in a netted chamber inside a funnel. Gravid females are those ready to release their young and are identified by dark spots in their brood pouches. Because not all of the females will release young on the same day, an estimate of two juveniles per female per day should be used to determine the number of gravid females to be used.

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

Nutrition and temperature are also important factors in mysid development. During the seven-day holding period maintain the holding tanks at 26-27°C with a salinity similar to the culture/test water. If necessary, the salinity should be gradually adjusted (≤2 ‰/day) to the desired test salinity (20-30 ‰) during this holding period. Feed the juveniles 24- to 48-hour-old *Artemia* nauplii twice daily.

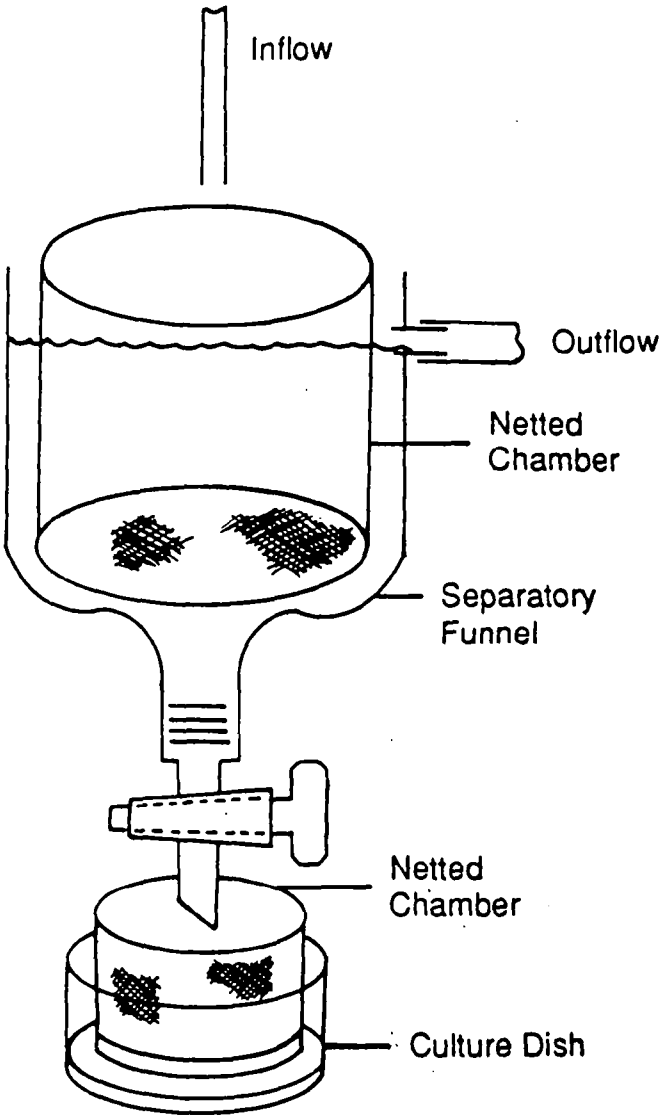

Figure 2. Apparatus for collection of postlarval mysids from gravid females. From Lussier et al., 1988.

CONDUCTING THE TEST

Effluent Preparation

Effluent and receiving water solutions must be prepared before starting a test. Once collected, a sample must be kept at 4°C until used for testing and it must be used for testing within 36 hours. Specific tests concentrations should be prepared and because the marine species used for testing are salinity sensitive, the effluent must be adjusted to the proper salinity.

Hypersaline brine is recommended for adjusting the effluent salinity while preparing the test concentrations. The EPA chronic manual (EPA, 1988) provides instructions for preparing the brine solution. To prepare test concentrations at the desired salinity, adjust the diluent (deionized water) with the hypersaline brine before adding it to the effluent. Using hypersaline brine instead of seawater allows the test to be run at higher effluent concentrations since less dilution will occur when adjusting to the proper salinity.

Effluent dilutions should be prepared each day of the test using <36-hour old effluent. A 0.5 dilution factor should be used to make 5 concentrations. Approximately 1 liter of test solution is needed at each concentration and for the control.

Once the test concentrations and the control are prepared, they should be warmed to 26°C in a temperature-controlled water bath. Each concentration and the control should be monitored for dissolved oxygen (DO), pH, salinity, and temperature. These parameters should fall within the recommended ranges for conducting the test and they should be recorded on the test data sheet. The recommended test conditions are presented in Table 1 and a sample test data sheet is shown in Exhibit 1.

Table 1. Summary of Recommended Test Conditions for *Mysidopsis bahia* Seven-day Survival, Growth, and Fecundity Test

Test type:	Static renewal
Salinity:	30 ‰ ± 2 ‰
Temperature:	26 - 27°C
Photoperiod:	16 hours light, 8 hours dark, with phase in/out period
Light density:	10-20 uE/m^2/s (50-100 ft.c.)
Test chamber:	8 oz plastic disposable cups, or 400 ml glass beakers
Test solution volume:	150 ml per replicate cup
Renewal of test solutions:	Daily
Age of test organisms:	7 days at start of test
Number of treatments per study:	Minimum of 5 treatments and a control
Number of organisms per test chamber:	5
Number of replicate chambers per treatment:	8
Source of food:	*Artemia* nauplii
Feeding regime:	Feed 150 24-hour old nauplii per mysid daily, half after test solution renewal and half after 8 - 12 hours
Aeration:	None unless DO falls below 60% saturation, then gently in all cups
Dilution water:	Natural seawater or hypersaline brine diluted with deionized water
Test duration:	7 days
Dilution factor:	Approximately 0.3 to 0.5
Effects measured:	Survival, growth, and egg development
Cleaning:	Pipette excess food from cups daily

Source: Adapted from EPA, 1988

TEST: _____

START DATE: _____

SALINITY: _____

	TRTMT	TEMP	SALINITY	D.O.	pH	TRTMT	TEMP	SALINITY	D.O.	pH	
DAY 1	REP										
	REP										
DAY 2	REP										
	REP										
DAY 3	REP										
	REP										
DAY 4	REP										
	REP										
DAY 5	REP										
	REP										
DAY 6	REP										
	REP										
DAY 7	REP										
	REP										

	TRTMT	TEMP	SALINITY	D.O.	pH	TRTMT	TEMP	SALINITY	D.O.	pH	
DAY 1	REP										
	REP										
DAY 2	REP										
	REP										
DAY 3	REP										
	REP										
DAY 4	REP										
	REP										
DAY 5	REP										
	REP										
DAY 6	REP										
	REP										
DAY 7	REP										
	REP										

Exhibit 1. Sample Test Condition Data Sheet

Test Set-up

The test chambers should be readied before the effluent concentrations are prepared. ERL-Narragansett uses disposable plastic drinking cups to conduct this test. The cups are presoaked in clean seawater and labeled with colored tape. Each concentration is indicated by a different color tape with the replicate number (1-8) written on it. The use of different colored tape makes renewals easier since all of the replicates of one concentration can be quickly identified.

Once the cups are prepared and the effluent solutions have been adjusted to within the proper parameter ranges, each test solution should be distributed to eight replicate cups. Each replicate should contain approximately 150 ml. The cups are placed in holding trays that are randomly placed in a temperature-controlled water bath. At ERL-Narragansett the holding trays are labeled with the same colored tape and replicate numbers as the cups which allows for easier collection and replacement of the randomized cups during renewals. The cups will stay in the same randomized positions for the duration of the test. Specific directions for test randomization are provided in the saltwater chronic methods manual (EPA, 1988).

The juvenile mysids are assigned to the test cups at a density of 5 mysids per cup. At ERL-Narragansett the mysids are randomly selected from the 7-day old juvenile pool and pipetted into small presoaked ampules, 2-3 at a time. This random selection and assignment is continued until all of the ampules contain 5 mysids. As the mysids are placed in these ampules, a minimum of water should be transferred with them so that the effluent concentrations are not diluted.

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

Once the test has been set-up, the mysids should be fed. The initial feeding rate is 0.5 ml of a food solution made from 4.0 ml concentrated *Artemia* nauplii in 80 ml uncontaminated, filtered seawater. This amount should provide live *Artemia* for the next 24 hours until test renewal. The food should be dispensed using an automatic pipet and the food solution should be swirled to ensure even distribution of the food. After feeding the mysids, cover the test chambers to prevent evaporation or contamination.

Renewals

Each day the mysids must be checked for mortality and the effluent solution must be replaced. If the test is to be conducted at the site of the outfall, effluent or receiving water samples should be collected daily. Off-site testing should be conducted using samples collected 24 hours before use. Although it is preferable to have freshly-collected effluent samples for each daily renewal, samples collected on days one, three, and five can each be used for renewals on 2 consecutive days. The lapsed time between sample collection and test use should not exceed 36 hours and in no circumstances should the time exceed 72 hours.

Effluent solutions should be prepared in the same manner as at the start of the test using effluent warmed to 26°C. Temperature and salinity should be carefully maintained at the EPA-recommended levels throughout the test period.

As a general rule, test chambers should be started, renewed, and terminated working from the lowest concentration, or control, to the highest concentration. For each concentration collect all of the replicates from the water bath and randomly select two of the replicates on which to perform the routine analyses. Measure and record the temperature, salinity, DO, and pH for at least two replicates of each concentration. A summary of the recommended test conditions was presented in Table 1. If the dissolved oxygen levels falls below 4.0 mg/l in any one of the exposure chambers, all of the chambers must be gently aerated. When conducting receiving water and effluent tests concurrently, the salinities should be similar throughout the test.

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

During this renewal the mysids in each chamber should be counted and the survival recorded on the test data sheets. Any dead animals should be discarded. A sample survival and fecundity data sheet is presented as Exhibit 2.

To add the new effluent solution to the cup, gently pour approximately 150 ml of the appropriate solution down the side of the cup avoiding as much turbulence as possible. After all of the concentrations have been renewed, the mysids should be fed. Immediately after renewal the feeding rate is 0.25 and the additional 0.25 should be fed 8-12 hours later. Detailed instructions for culturing *Artemia* are given in the tape "Culturing *Mysidopsis bahia*" and in its supplemental report.

TEST: ______________________

START DATE: ______________________

SALINITY: ______________________

Treatment Replicate	Day 1 # Alive	Day 2 # Alive	Day 3 # Alive	Day 4 # Alive	Day 5 # Alive	Day 6 # Alive	Day 7 # Alive	Females w/ Eggs	Females no eggs	Males	Immatures
1											
2											
3											
4											
5											
6											
7											
8											
1											
2											
3											
4											
5											
6											
7											
8											
1											
2											
3											
4											
5											
6											
7											
8											

Exhibit 2. Sample Survival and Fecundity Data Sheet

This renewal procedure must be repeated on days 2-6 of the exposure period. All data should be carefully recorded on the data sheets each day. If the survival rate in any replicate drops below 50%, the food provided to that replicate should be reduced by half.

TERMINATING THE TEST

On the last day of the seven-day exposure the replicates are checked for survival and fecundity and the animals are prepared for growth measurements. On the final day of the test the mysids should not be fed.

In preparation for the test termination, prepare small pieces (1 cm square) of clean, light-weight aluminum foil by labeling them with sequential numbers. After they are numbered, these pieces of foil should all be tared and their weights recorded on the growth data sheet. A sample growth data sheet is presented at Exhibit 3. Gloves should be worn or forceps should be used to handle the aluminum because oils from skin could increase final weight differences.

After the aluminum is prepared, collect the test chambers in the same manner as for conducting a renewal. That is, collect all of the replicates of one concentration at one time, starting with the control. Two replicates should be randomly chosen for final DO, temperature, salinity, and pH measurements. The dead mysids should be removed from the test chambers and the final survival count for each replicate should be recorded on the test data sheet. The minimum requirement for an acceptable test is 80% survival in the controls.

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

Using a stereomicroscope at 240X, the sexual development of each mysid should be determined and recorded on the test data sheet. Figures 2 through 5 show the sexual characteristics used to determine the maturity and fecundity of the mysids. Figure 2 is a mature female with eggs in the oviducts. This is most easily determined when viewed from above and is determined by large, dark, oval-shaped bodies in the mid-section of the thorax. A mature female with eggs in the brood pouch is characterized by the presence of dark pigmented spots on the lateral sides of the body. These can be seen both from above and from the side. This is shown in Figure 3. Females that have no eggs or embryos have an empty brood pouch and empty oviducts. These females can be identified by a single dark spot on each half of the brood pouch. These spots can be seen from both above and from the side, although from the top is easiest.

TEST: _____

START DATE: _____

SALINITY: _____

TREATMENT REPLICATE	PAN #	TARE WT.	TOTAL WT.	ANIMAL WT.	# OF ANIMALS	$\bar{X}$ WT./ ANIMAL
1						
2						
3						
4						
5						
6						
7						
8						
1						
2						
3						
4						
5						
6						
7						
8						
1						
2						
3						
4						
5						
6						
7						
8						

Exhibit 3. Sample Growth Data Sheet

Mature Female, Eggs in Oviducts

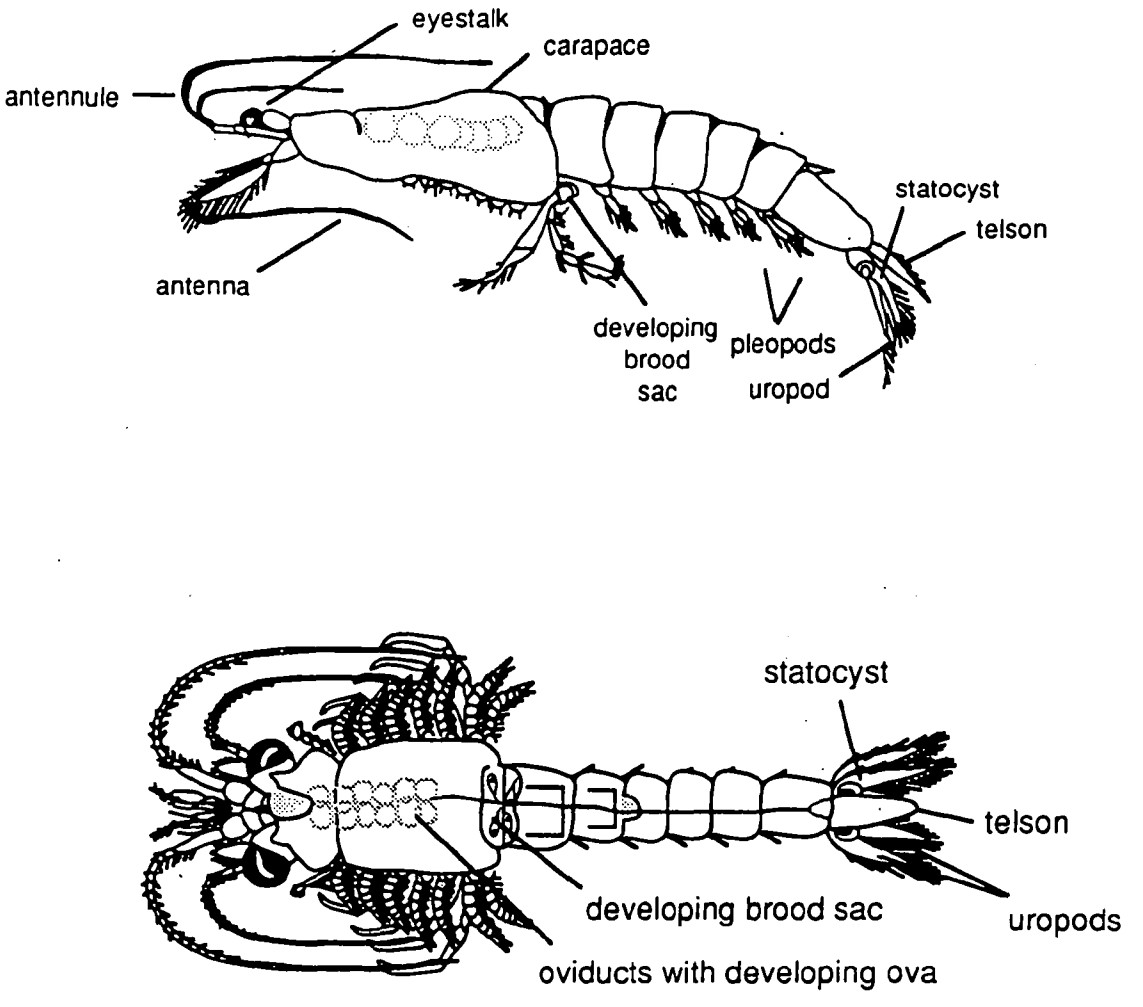

Figure 2. Mature female *M. bahia* with eggs in oviducts. Above: lateral view, Below: dorsal view. From Lussier, Kuhn, and Sewall, 1987.

Mature Female, Eggs in Brood Sac

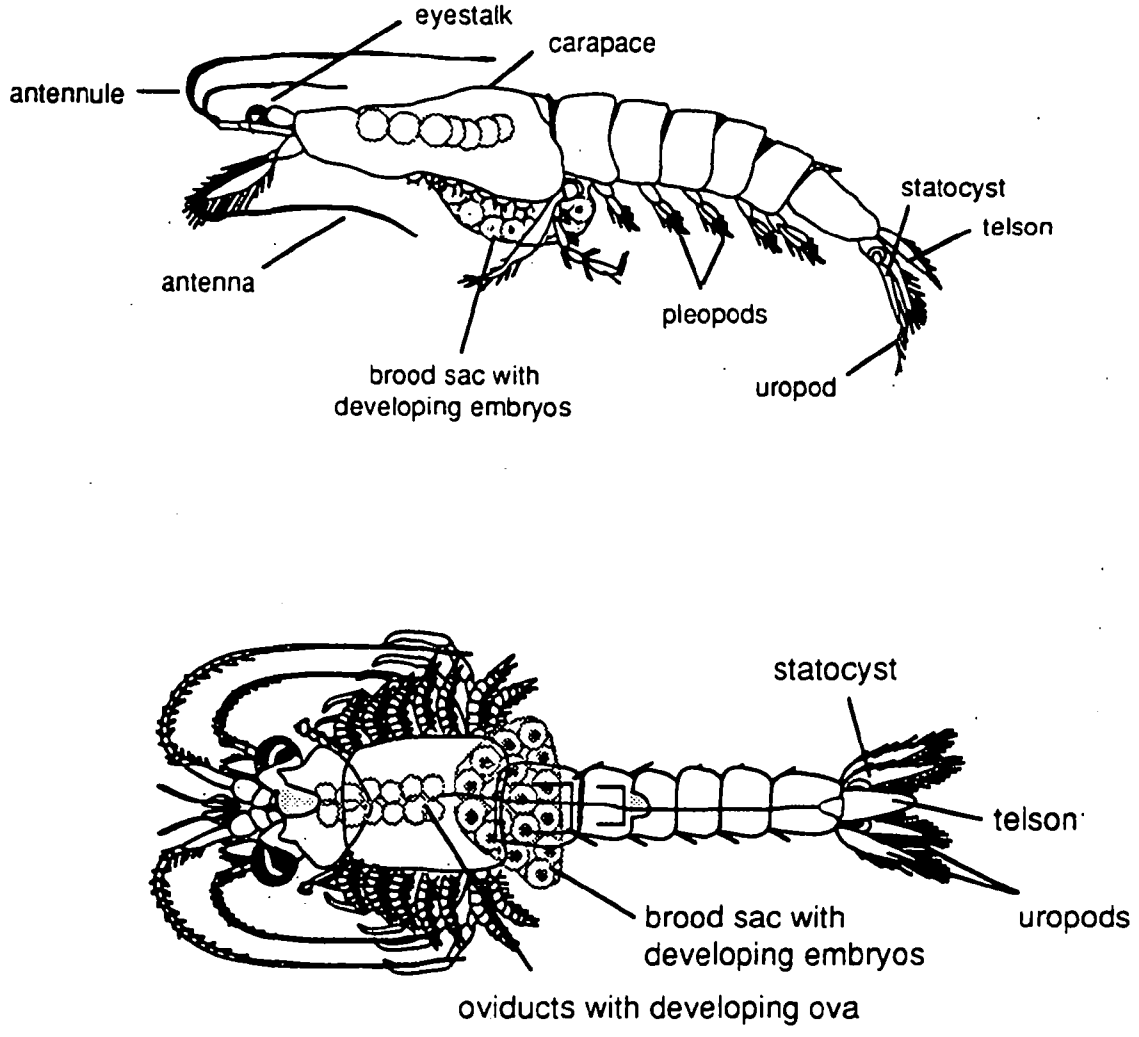

Figure 3. Mature female *M. bahia* with eggs in oviducts and developing embryos in the brood sac. Above: lateral view. Below: dorsal view. From Lussier, Kuhn, and Sewall, 1987.

Figure 4 presents a mature male mysid. Males are determined by the presence of gonads that appear either as clear circles, when viewing them from above, or as appendages at the junction of the thorax and abdomen when viewing them from the side.

Immature mysids are those that do not have characteristics that determine their classification as either mature males or females. Care must be taken, however, not to mistake a barren female for an immature mysid. Figure 5 presents a diagram of an immature mysid. As the sex of each mysid is determined it should be recorded on the survival and fecundity data sheet.

After the sex, maturity, and fecundity of each mysid from one replicate is determined, all of the mysids from the replicate should be placed on a Nitex® screen that rests on top of a beaker. These mysids are rinsed with deionized water to remove any salts that may interfere with the dry weights. After the animals are rinsed they are placed on the designated pre-tared piece of aluminum foil for that replicate. Note that all of the mysids from one replicate are placed on the same piece of foil. Once this process has been repeated for all of the replicates the mysids are dried in an oven at 60°C for 24 hours or 105°C for at least 6 hours. The mysids must be completely dried before they are weighed but they should not be overdried.

The mysids should be transported and stored in a desiccator when weighing them. This will prevent moisture from reabsorbing into the mysids. The mysids are weighed, one replicate at a time, to the nearest microgram (0.001 g.). The minimum requirement for an acceptable test is an average weight of at least 0.20 mg/mysid in the controls.

The analysis of this test compares the maturity, fecundity, growth, and survival of the exposed mysids to the control mysids. Because small differences in weight or appearance can easily change the results, it is critical to record observations and measurements clearly and accurately. The chronic methods manual (EPA, 1988) provides instructions for statistical analysis of the survival, growth, and fecundity data.

Mature Male

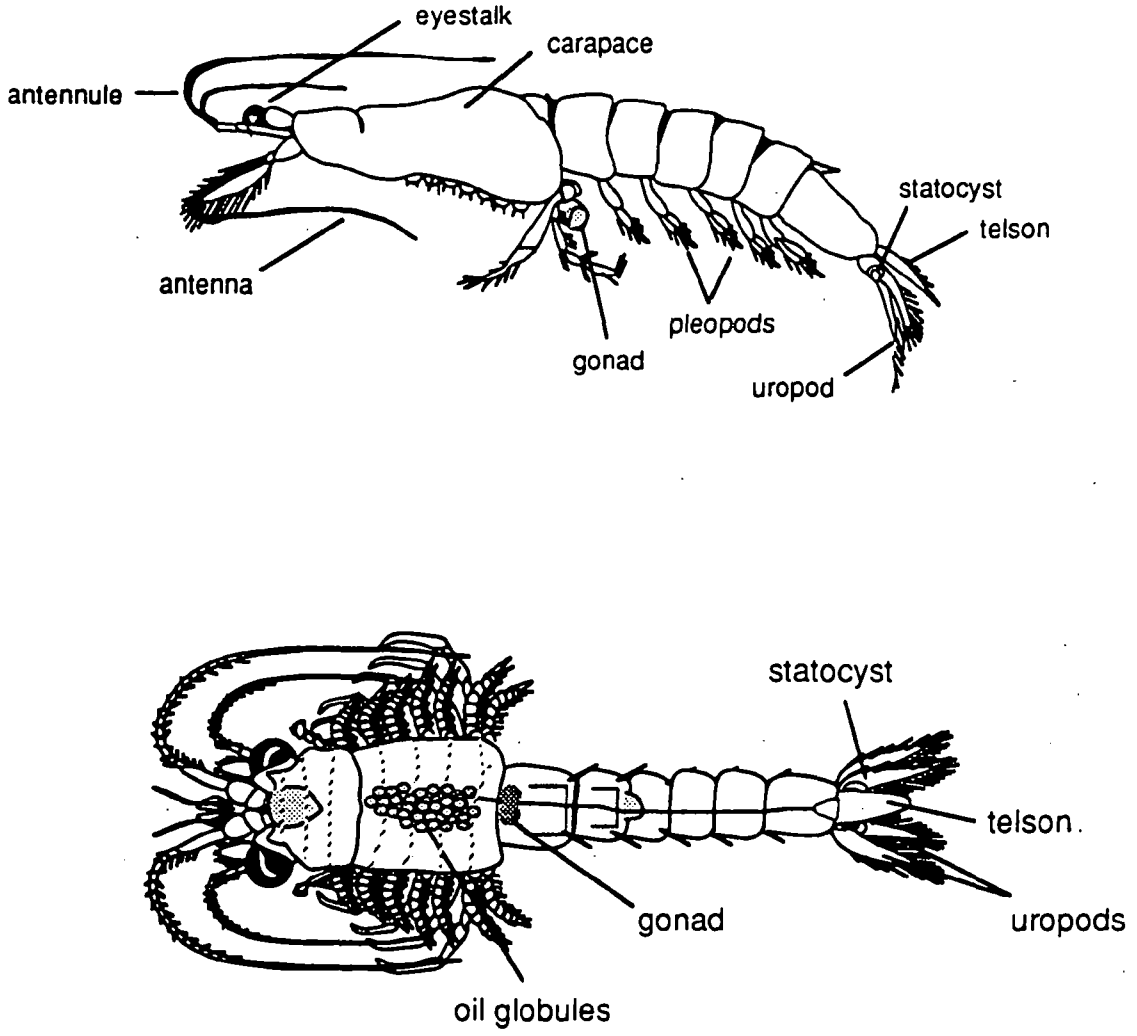

Figure 4. Mature male *M. bahia.* Above: lateral view. Below: dorsal view. From Lussier, Kuhn, and Sewall, 1987.

Immature

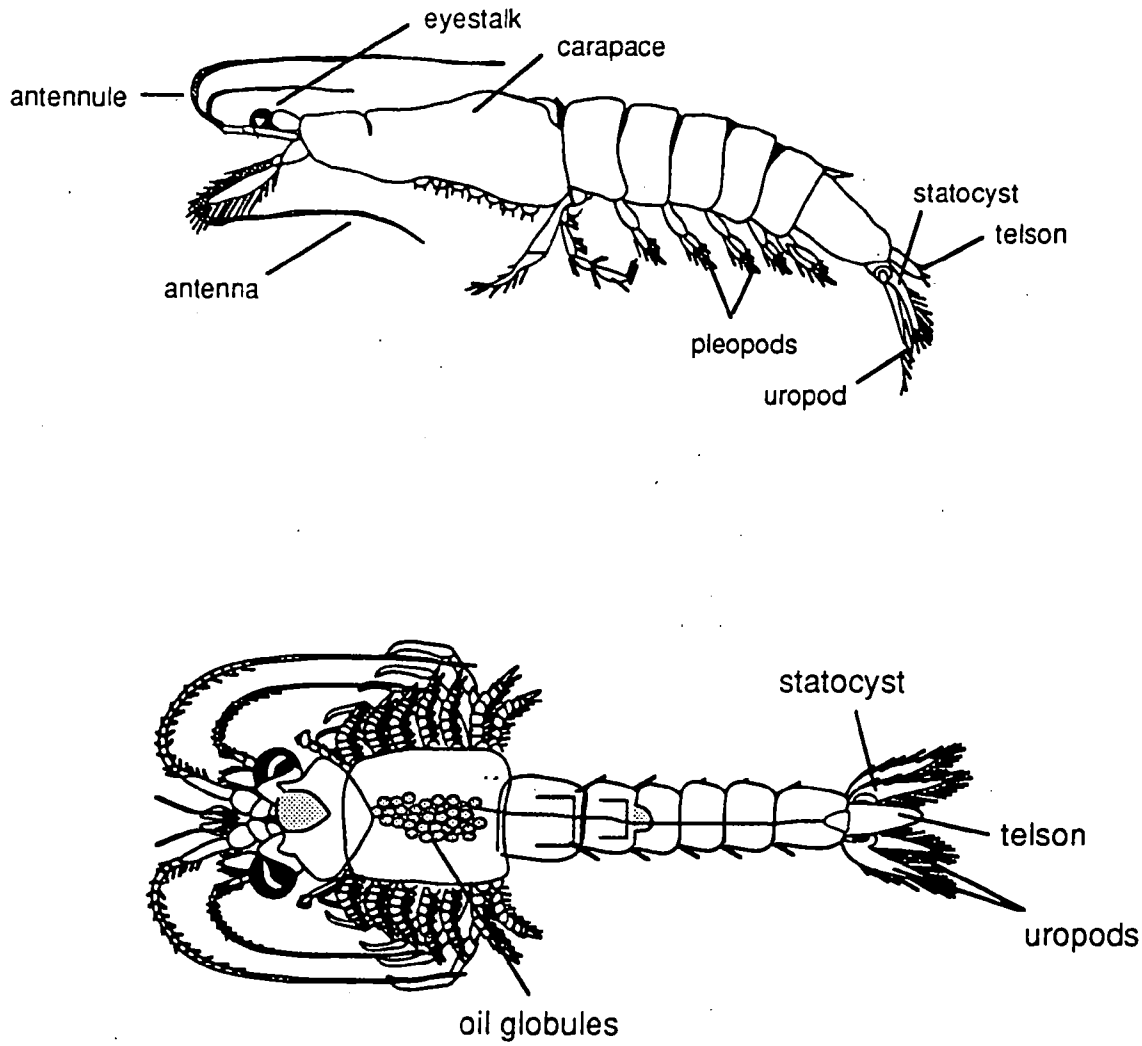

Figure 5. Immature *M. bahia.* Above: lateral view, Below: dorsal view. From Lussier, Kuhn, and Sewall, 1987.

GLOSSARY

Artemia - Brine shrimp used as food for mysid cultures, Brazilian or Columbian strains are preferable.

Cyst - The life stage of unhatched *Artemia*

Fecundity - Productivity or fertility.

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

Mysid (***Mysidopsis bahia***) - An epibenthic crustacean ranging 4.4 mm to 9.4 mm in length found in the Gulf of Mexico and along the Atlantic coast used in test procedures as an indicator species for aquatic toxicity.

Nauplii - The first stage of newly-hatched *Artemia*.

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.

APPENDIX A

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APPENDIX B

APPARATUS AND EQUIPMENT

Facilities for holding and acclimating test organisms.

Brine shrimp culture unit -- see Maintaining and Feeding Cultures above.

Mysid culture unit -- see Maintaining and Feeding Cultures above.

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

Environmental chamber or equivalent facility with temperature control (26 $\pm$ 1°C).

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

Balance -- capable of accurately weighing to 0.000001 g.

Reference weights, Class S -- for checking performance of balance.

Drying oven -- 105°C, for drying organisms.

Desiccator -- for holding dried organisms.

Air pump -- for supplying air.

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

pH and DO meters -- for routine physical and chemical measurements.

Tray -- for test vessels; large enough to hold 8 vessels at one time.

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

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

Light box -- for illuminating organisms during examination.

Refractometer or other method -- for determining salinity.

Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

Thermometer, National Bureau of Standards Certified (see USEPA Method 170.1, USEPA, 1979) -- to calibrate laboratory thermometers.

Test vessels -- 200 ml borosilicate glass beakers or 8 oz disposable plastic cups (manufactured by Falcon Division of Becton, Dickinson Co., 1950 Williams Dr., Oxnard, CA 93030) or other similar containers. Cups must be rinsed thoroughly in distilled or deionized water and then pre-soaked (conditioned) overnight in dilution water before use. Forty-eight (48) test vessels are required for each test (eight replicates at each of five effluent concentrations and a control).

Beakers or flasks -- six, borosilicate glass or non-toxic plasticware, 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.

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

Separatory funnels, 2-liters -- 2 - 4 for culturing *Artemia*.

Pipets, volumetric -- Class A, 1-100 ml.

Pipets, automatic -- adjustable, 1-100 ml.

Pipets, serological -- 1-10 ml, graduated.

Pipet bulbs and filters -- PROPIPET®, or equivalent.

Droppers, and glass tubing with fire polished edges, 4 mm ID -- for transferring organisms.

Forceps (fine tips such as jewelers' forceps) -- for transferring organisms to weighing boats.

NITEX® mesh sieves (150 μm and 100 μm) -- for concentrating organisms.

Depression glass slides or depression spot plates -- two, for observing organisms.