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8710 ARTHROPODS

Phylum Arthropoda is the largest group of animals; it comprises more than one million species, the majority of which are insects. Other arthropods include crustaceans, spiders, ticks, mites, and other less-known species. Arthropods are found in all environments, including both fresh and marine waters. Two classes of arthropods are used extensively in toxicity testing, the crustaceans and the insects. Test procedures are described for several different crustacean groups including *Daphnia* (8711), *Ceriodaphnia* (8712), mysids (8714), and decapods (8740). Representatives of the insect orders belonging to stoneflies, mayflies, caddisflies, and dipterans are the most commonly used groups in aquatic testing (8750).

Joint Task Group: Donald J. Reish (chair).

8711 *DAPHNIA**

8711 A. Introduction

Daphnia sp. (Figure 8711:1) are small freshwater crustaceans. They have been used for many years to assess the acute and chronic effects of single chemicals and complex mixtures.[1–3]

Daphnia are valuable as test organisms because of their sensitivity to toxic substances, ease of identification and handling, ubiquitous distribution, and extensive use in toxicity testing. *Daphnia* are fecund and reproduce parthenogenically, which allows for the establishment of clones with little genetic variability and with reproducible testing results.

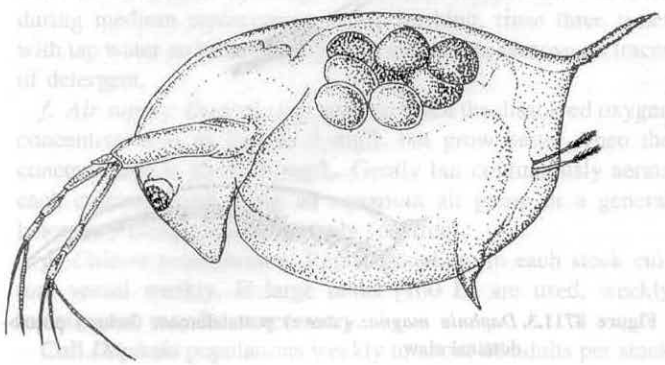

Figure 8711:1. *Daphnia* sp., adult female.

* Approved by Standard Methods Committee, 1997.
Joint Task Group: 20th Edition—Wayne G. Landis (chair), Colin E. Coggan, Joseph W. Gorsuch, Robert E. Morcock, Mark A. Palmieri.

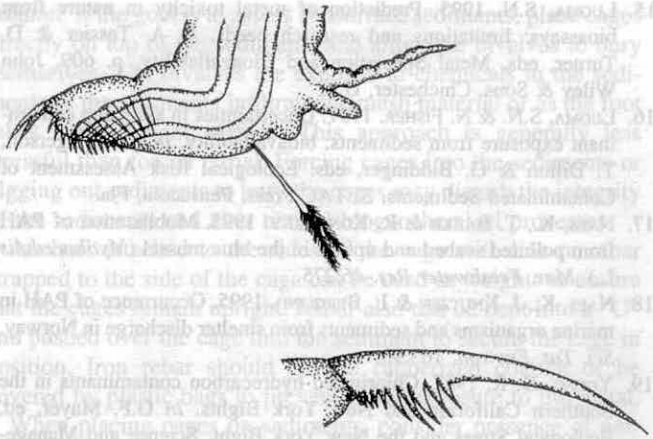

Figure 8711.2. ***Daphnia pulex:*** (above) postabdomen; (below) postabdominal claw.

1. Life History

D. pulex attains a maximum length of approximately 3.5 mm, whereas *D. magna* is much larger and attains a length of 5 to 6 mm. These species are differentiated with certainty only by determining the size and number of spines on the postabdominal claws when using a dissecting or compound microscope (see Figures 8711:2 and 3).[4]

The life span of *Daphnia*, from the release of the egg into the brood chamber until adult death, is highly variable and depends on species and environmental conditions.[4] Generally, it increases as temperature decreases. The average life span of *D. magna* is about 40 d at 25°C and about 56 d at 20°C. The average life span of *D. pulex* at 20°C is approximately 50 d. Four distinct life-cycle periods are recognized: egg, juvenile, adolescent, and adult. The adolescent period is a single instar between the last juvenile instar and the first adult instar; during this instar the first clutch of eggs reaches full development in the ovary. Under laboratory conditions, a clutch of 6 to 10 eggs (15 to 20 eggs in older animals) typically is released into the brood chamber. The eggs hatch and the juveniles, already similar in form to the adults, are released in approximately 2 d when the female molts. The time required to reach sexual maturity varies from 6 to 10 d and appears to depend on temperature. The growth rate is greatest during juvenile stages (early instars); body size may double during each of these stages. *D. pulex* has three to four juvenile instars, whereas *D. magna* has three to five juvenile instars. Each instar stage is terminated by a molt. Growth occurs immediately after each molt while the new carapace is still elastic.

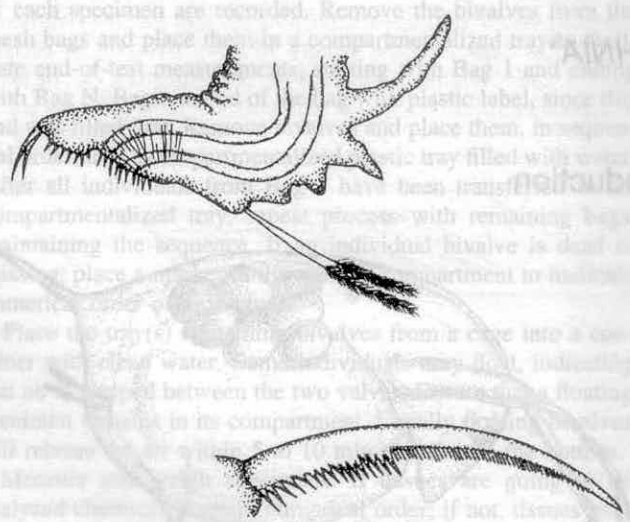

Figure 8711.3. ***Daphnia magna:*** (above) postabdomen; (below) postabdominal claw.

Populations of *Daphnia* consist almost exclusively of females during most of the year; males are abundant only in spring or autumn. For most of the year reproduction is parthenogenic, and only females produce young. Males are distinguished from females by their smaller size, larger antennules, modified postabdomen, and first legs having a stout hook used in clasping. Production of males appears to be induced principally by high population densities and subsequent accumulations of excretory products and/or a decrease in available food. These conditions, along with exposure to temperature extremes, may induce the appearance of sexual (resting) eggs in cases (ephippia) that are cast off during the next molt. The shift towards male and sexual egg production appears related to the metabolic rate of the parent. As a rule, males and ephippia will not be observed unless stock cultures are neglected or the culture experiences stress.

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8711 B. Selecting and Preparing Test Organisms

1. Obtaining and Selecting Test Species

Daphnia are widely available from many laboratories and commercial biological supply houses. Only 20 to 30 organisms are needed to start a culture. Some biologists prefer *D. pulex* to *D. magna* because it is more widely distributed and easier to culture. However, *D. magna* neonates (first instar) are larger and somewhat easier to use. Verify species used.

2. Culturing Organisms

a. Water supply: Although *Daphnia* cultures can be maintained successfully in some natural waters, preferably use a synthetic (reconstituted) water medium. Reconstituted water is easily prepared, is of known standardized quality, produces predictable results, and permits adequate growth and reproduction. Because daphniads are very sensitive to media hardness, reconstituted hard water (160 to 180 mg $CaCO_3$/L) is recommended for *D. magna*, whereas reconstituted moderately hard water (80 to 90 mg $CaCO_3$/L) is recommended for *D. pulex*.[1] See Table 8010:I for materials needed to prepare reconstituted water.

Dissolve salts in distilled or deionized water and aerate vigorously for several hours before use. Initial pH is approximately 8.0 but it will rise as much as 0.5 unit as the *Daphnia* population increases. Although *Daphnia* can survive over a wide pH range, the optimum is 7.0 to 8.6.[2] Because pH usually remains within this range, pH monitoring or adjustment during cultivation generally is unnecessary.

b. Food and feeding: Feed *Daphnia* either a mixture of green algae or a suspension of trout chow, alfalfa, and yeast.

1) Algae mixture—Food consisting of several species of algae is preferable.[3] For example, use three algae, *Ankistrodesmus falcatus, Selenastrum capricornutum,* and either *Chlamydomonas reinhardi* or *Chlorella* sp. To prepare the algal mixture, centrifuge algae, wash in filter-sterilized lake water (water passed through 0.22-μm filter), and centrifuge again. Transfer *Daphnia* to fresh culture water and feed using a sterile pasteur pipet by adding to *Daphnia* ≤ 9 to 10 d old, 2 drops of each alga per *Daphnia* culture beaker or to *Daphnia* 9 to 10 d old, 1 drop of each alga per 2 adults, rounding up when there is an odd number of adults.

At the end of a work week (e.g., Friday) add 1 extra drop of each alga per *Daphnia* culture beaker. Adjust algae feed so that the algae are almost cleared before *Daphnia* are transferred to fresh culture beakers. If only 2 of the 3 algae are available, add proportionately more of the two algae.

2) Trout chow suspension—Place 6.3 g trout chow pellets,* 2.6 g dried yeast,† and 0.5 g dried alfalfa‡ in a blender jar. Add 500 mL deionized water and mix at high speed for 5 min. Let settle in a refrigerator for 1 h. Decant and save top 300 mL; discard remainder. Freeze 30- to 50-mL portions in small (50- to 100-mL) polyethylene bottles with screw caps. Thaw portions as needed. After thawing, refrigerate but do not hold for longer than 1 week.

Feed 1.5 mL prepared food per 1000 mL of medium, three times per week. There may be excess food at this rate of feeding, but if the medium is aerated continuously and replaced each week, no problems should result.

3) *Selenastrum capricornutum*—The green alga *Selenastrum capricornutum* (Printz) can be used as a *Daphnia* food source.[4] Combinations of other green algae are also suitable [see three-algae mixture, ¶ 1) above]. The *Selenastrum capricornutum* culture procedure produces 7-d-old cultures containing four to five million algal cells per mL and 2- to 4-d-old cultures containing one to three million cells per mL. Prepare algal food and feed it three times per week to *Daphnia* as follows:

Combine volumes of 7-d-old and 3-d-old algal cultures in a ratio of two volumes to one, respectively. Centrifuge algal cells and resuspend in a volume of reconstituted moderately hard or hard water calculated to yield a combined algal culture containing approximately ten million cells/mL. Add sufficient volume of cell suspension to stock cultures daily to provide approximately 300 000 algal cells/mL of culture, e.g., add approximately 30 mL cell suspension to 1 L *Daphnia* stock culture.

c. Temperature: Protect *Daphnia* from sudden changes in temperature that may cause death or induce ephippial (sexual egg) production. Optimal temperature range is approximately 20° to 25°C. If laboratory temperatures are 20 ± 2°C, normal growth and reproduction of *Daphnia* can be maintained.

d. Lighting: Variations in ambient light intensities (538 to 1076 lux) and prevailing day/night cycles in most laboratories do not affect *Daphnia* growth and reproduction significantly. Provide a minimum of 16 h of light/d.

e. Culture vessels: Use culture vessels of clear glass or plastic to allow easy observation. A practical culture vessel is a 3-L glass beaker filled with approximately 2.75 L of medium.

Maintain at least five culture vessels to ensure backup cultures. A 3-L vessel stocked with 30 *Daphnia* will provide approximately 300 young/week.

Wash all culture vessels before use. After culture is established, clean each chamber weekly with distilled or deionized water and wipe with a clean sponge to remove accumulated food and dead *Daphnia*. Monthly, wash each vessel with detergent during medium replacement. After washing, rinse three times with tap water and then with culture medium to remove all traces of detergent.

f. Air supply: Daphnia can survive when the dissolved oxygen concentration is as low as 3 mg/L but grow better when the concentration is above 6 mg/L. Gently but continuously aerate each culture vessel using an aquarium air pump or a general laboratory compressed air supply (oil-free).

g. Culture maintenance: Replace medium in each stock culture vessel weekly. If large tanks (100 L) are used, weekly replacement may be unnecessary.

Cull *Daphnia* populations weekly to about 30 adults per stock vessel to prevent overcrowding, preferably during medium replacement. Transfer *Daphnia* with large-bore (15-mm-diam) glass pipet (with fire-polished end) or disposable plastic pipet.

* Conforming to U.S. Fish and Wildlife Service Specification PR(11)-78; obtainable at livestock feed stores.
† Fleishmann's or equivalent.
‡ Obtainable at most health food stores.

3. Selecting Test Organisms

Use *D. magna* or *D. pulex* neonates (first instar ≤ 24 h old), preferably from the second or third brood, to initiate tests. To obtain young for a test, remove females bearing embryos from the stock cultures 24 h before starting the test and place them in 400-mL beakers containing 300 mL medium and either 0.5 mL trout chow-yeast-alfalfa suspension (see B.2*b*) or 10 mL cultured algae. Use the young found in the beakers within 24 h. Five beakers, each containing 10 adults, usually will supply enough first instars for one toxicity test.

Because the appearance of ephippia is indicative of unfavorable conditions, do not use *Daphnia* from cultures producing ephippia.

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8711 C. Procedures

1. Short-Term Tests

a. Preparation of test materials and medium: Prepare test materials and concentrations, dilution water, and toxicant solutions as described in Section 8010F. Make up test solutions and controls in 100-mL quantities in 125-mL wide-mouth flint-glass bottles or equivalent vessels (Table 8711:I).

b. Performing tests: After preparing test solutions, segregate neonates that have been released from the mothers' brood chambers during the preceding 24 h at 20°C or 25°C and collect in one vessel (use neonates cultured at the test temperature). Introduce the same number of neonates (at least 10) into each test vessel and control. Use a plastic, disposable pipet with a 5-mm bore for collecting and transferring neonates. Alternatively, use a glass bulb pipet.

Introduce neonates to test solutions by releasing them below the surface of the solution. Observe animals regularly, ideally after 1 h and 4 h and daily thereafter. A 48-h exposure is generally accepted for a *Daphnia* acute toxicity test.[1] Record number of motile animals in each test vessel. Consider an animal nonmotile if it shows no independent movement even after gentle squirting with test solution from a pipet (nonmotile animals are not necessarily dead). At threshold concentrations of such substances as ethanol, acetone, and chlorobutanol, animals may show no movement and the heart may have ceased to beat but on transfer to dilution water they will recover. However, such animals maintained in the test medium will die. In addition to immobilization, note behaviors and features such as the number of *Daphnia* that are on bottom, lethargic, swimming, caught on the bottom or on debris, floating on surface, swimming erratically, or have a flared carapace.

Record conditions of the medium such as whether it is cloudy or if any particulate matter, precipitate, undissolved material, or film is present. Continue observations for a minimum of 48 h or as long as there is no more than 10% control mortality. Run tests in replicates of at least three.

Do not feed animals during tests. Longer-term tests require modifications of standard conditions.

TABLE 8711:I. SUMMARY OF SHORT-TERM AND LONG-TERM TOXICITY TESTS WITH *DAPHNIA* SPP.[1]

Parameter	Test Conditions	
	Short-Term	Long-Term
Species	*D. magna, D. pulex*	*D. magna, D. pulex*
Age of test animal	≤24 h	≤24 h
Test vessel type and size	125-mL wide-mouth flint-glass bottle	Glass or plastic container
Volume of test solution	100 mL	100 mL
Number of animals per test chamber	10	1
Test temperature	20 or 25°C	20 or 25°C
Photoperiod	16 h light/8 h dark	16 h light/8 h dark
Feeding regime	Do not feed	Feed second day and alternate days thereafter
Media change	Do not change	Three times a week for test duration
Number of replicates	Minimum of 3	Minimum of 2
Test duration	Maximum 48 h; longer only with control survivors	21 d at 25°C for *D. magna* for a set number of broods in control replicates
End points	Mortality or immobility upon stimulation	First generation survival, number of young produced, first appearance of broods, number of broods, dry weight of survivors/replicate

c. Criteria for test acceptability: An acceptable test will have no more than 10% mortality in the negative control. A clear dose-response must be apparent from a sample plot of the data. Ideally one concentration will have no effect (no more mortality than control) and the EC50 will be bracketed by concentrations producing mortality. Use a scatterplot of the 48-h data to identify outliers. Examine records for clerical or experimental errors when outliers exist.

2. Long-Term Tests

a. Determination of toxicity effect(s) on survival, growth, and reproduction: Sublethal effects may occur at lower toxicant concentrations than those causing acute toxicity. Precede long-term tests by acute (48-h) toxicity tests to establish the maximum concentration to be used.

b. Preparation of test medium: Prepare test medium as regular culture medium, but use water representative of that receiving the effluent discharge, or the dilution water used to culture the daphnids when testing chemicals. Prepare a series of 6 to 10 1-L quantities of medium to which graded amounts of effluents, mixtures, or chemicals have been added. Use as the highest concentration of chemical or effluent the equivalent to the 48-h LC50 or EC50 values. Reduce each successive concentration in a consistent progression (e.g., geometric). Use test dilution water as a negative control. Dispense each liter of test medium in 100-mL quantities to each of 10 glass or plastic chambers. Run tests in replicates of at least six (the minimum needed to detect statistical significance).

c. Performing tests: Preferably conduct test according to Good Laboratory Practice standards/regulations.[2–4] Segregate and collect 24-h-old neonate *Daphnia* that have been cultured at the test temperature. Introduce one neonate into each chamber randomly. On the following day and on alternate days thereafter, add an appropriate amount of food (see Section 8711B.2*b*). First-generation *Daphnia* (those animals used to begin the test) may be transferred to new media as necessary, but at least three times weekly. Make daily observations and note dead or immobilized animals. As animals grow and reproduce, remove young and record their number. Cover all test chambers loosely with plate glass or equivalent to minimize evaporation. Continue test for 21 d at 25°C. If desired, continue observations until a set number of broods, e.g., six, are reached in control animals; this may take 30 d at 20°C. Handle animals as in the individual cultures of stock animals. This test design may not be appropriate for highly volatile chemicals because of possible evaporation.

At the end of the exposure period, analyze results and test for statistically significant differences in the number of young produced, first-generation *Daphnia* survival, and, if appropriate, the dry weights of surviving animals in each treatment. Note appearance of first broods and number of broods.

d. Criteria for test acceptability: A test is invalid if control mortality exceeds 20% during exposure. The average brood production of the controls should be 60 ± 10 young over the duration of the test. If ephippia are produced in the controls, the test is invalid.

3. Statistical Analysis

Assemble, analyze, evaluate, and report data as described in Section 8010G.

4. Quality Assurance/Quality Control

Quality assurance (QA) practices for hazardous-waste toxicity tests consist of all aspects of the test that affect data quality,[5–7] including sampling and handling, source and condition of test organisms, condition of equipment, test conditions, instrument calibration, replication, use of reference toxicants, record keeping, and data evaluation.

Prepare a control chart for the reference toxicant. Plot and examine successive toxicity values (LC50) to determine whether the results are within prescribed limits. In this technique, a running plot is maintained for the toxicity values from successive tests with reference toxicant.

Run reference toxicant tests periodically. Suggested reference toxicants are $CdCl_2$ and sodium dodecyl sulfate. If the LC50 from a given test with the reference toxicant does not fall in the expected range for *Daphnia*, the sensitivity of the organisms and the overall credibility of the test system are suspect. In this case, examine test procedure for defects and repeat with a different batch of *Daphnia*.

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8712 *CERIODAPHNIA**

8712 A. Introduction

Ceriodaphnia is a genus of cladocerans that are smaller in size than their closely related and morphologically similar counterpart, *Daphnia*. *Ceriodaphnia* produce three to four broods per week under optimal conditions, whereas *Daphnia*, because of their larger size, do not reproduce until the fourth to sixth instar stage after hatching.[1,2] *Ceriodaphnia* were first used in effluent toxicity evaluations in 1984.[3] Several methods have been published.[4,5]

1. Life History

Ceriodaphnia have a life history similar to those of other daphnids and are believed to occur in limnetic areas all over the world.[2] *Ceriodaphnia* are pond- and lake-dwelling species that are usually common among the vegetation in littoral areas. The life span of *Ceriodaphnia*, from release of the egg into the brood chamber until death, is variable and depends on temperature as well as other environmental conditions. As with *Daphnia*, *Ceriodaphnia* life span usually is related to temperature; at 25°C and 20°C, the average life span for *Ceriodaphnia dubia* is 30 d and 50 d, respectively. The increase in life span at lower water temperatures is attributed to a lower metabolic activity.

Currently, no distinct developmental stages are recognized in the life cycle of *Ceriodaphnia*. The organism is referred to as a neonate during its first instar stage (when it is still less than 24 h old). The time to sexual maturity for *Ceriodaphnia dubia* varies from 3 to 5 d and probably depends on body size and environmental conditions, particularly temperature, water quality, and food availability. Typically, a clutch of 4 to 10 eggs is released into the brood chamber, but clutches with as many as 20 eggs occur. The eggs hatch in the brood chamber and the neonates are released in about 38 h, just before the adult female molts. The growth rate of the organism is greatest during the early instar stages and body size may double during each instar.

* Approved by Standard Methods Committee, 1997.
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Each instar stage is terminated by a molt. Growth occurs immediately after each molt while the new carapace is still elastic.

Ceriodaphnia populations consist almost exclusively of parthenogenic females during most of the year. Males appear primarily in the autumn and in the late spring. The factors responsible for the appearance of males are not fully understood.[1–2] Production of male eggs has been attributed partially to overcrowding of females, a decrease in available food, and a decrease in water temperature.[1–2] If continued, these same conditions appear to induce the production of sexual eggs. Gametogenic females are morphologically similar to parthenogenic females; however, sexual females produce only a few "resting eggs" and can copulate with males. When the fertilized eggs of these females pass into the brood chamber, the walls of the brood chamber become dark and thick and form an ephippium. Ephippia are embryos encased in a tough covering and are resistant to drying. The development of ephippia among cladocerans is an adaptation to adverse environmental conditions and allows populations to survive both drought and freezing conditions.

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8712 B. Selecting and Preparing Test Organisms

1. Obtaining and Selecting Test Species

Ceriodaphnia are available from many laboratories and commercial biological supply houses. A culture can be started with 10 to 20 organisms. Start cultures of test organisms at least 2 weeks before neonates will be needed for testing to ensure an adequate supply.

Only *Ceriodaphnia dubia* is now used in effluent toxicity tests. *Ceriodaphnia dubia*, toothed-pecten variety, is considered a morphological variant of *Ceriodaphnia dubia*. The distinguishing morphological characteristics between *Ceriodaphnia dubia* and *Ceriodaphnia dubia*, toothed-pecten variety, are shown in Figures 8712:1 through 3. Verify species of *Ceriodaphnia* before using.[1]

2. Culturing Organisms

a. Water supply and renewal: Culture water for *Ceriodaphnia* can be either an acceptable surface or well water source or a synthetic (reconstituted) water medium. Synthetic water usually is recommended because it is of known standardized quality, is easily prepared, and yields reproducible results. Ceriodaphnids

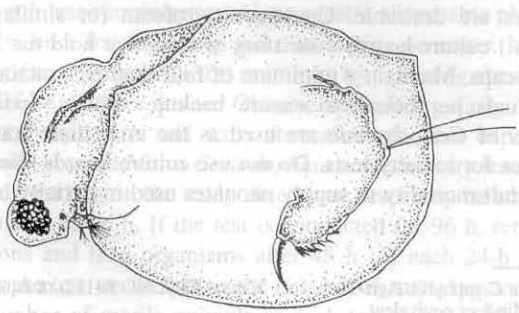

Figure 8712:1. ***Ceriodaphnia dubia.***

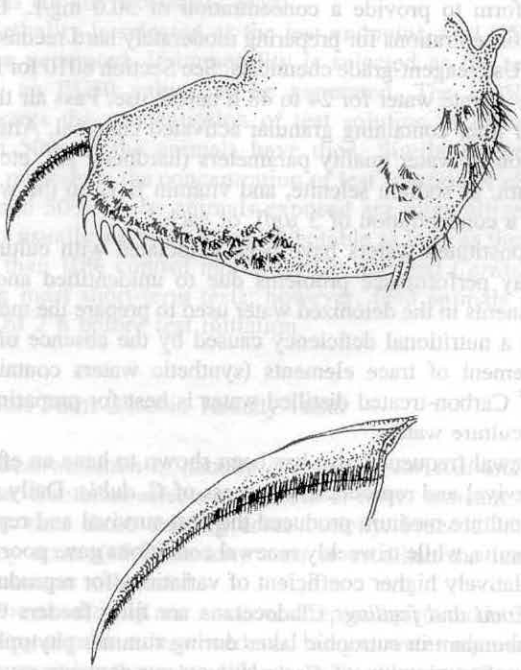

Figure 8712:2. ***Ceriodaphnia dubia:*** (above) postabdomen; (below) postabdominal claw.

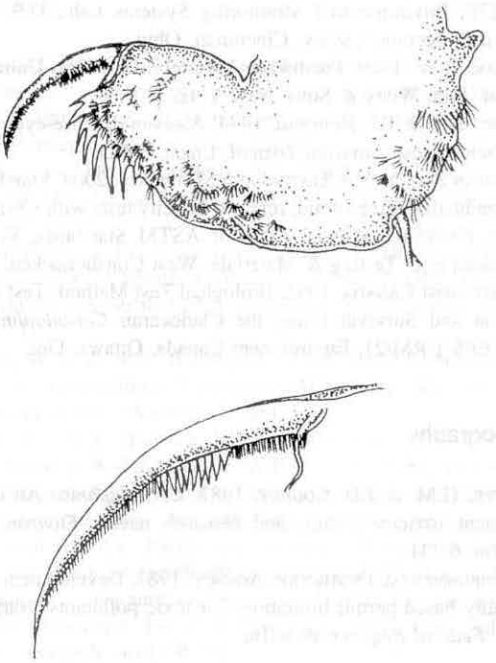

Figure 8712:3. *Ceriodaphnia dubia*, toothed-pecten variety: (above) postabdomen; (below) postabdominal claw.

are believed to perform better in moderately hard (80 to 100 mg $CaCO_3$/L total hardness) synthetic waters than in soft synthetic water (30 to 50 mg $CaCO_3$/L).

Prepare soft reconstituted water with 50-g/L stock solutions of $NaHCO_3$, $MgSO_4$, and KCl to provide 48.0 mg $NaHCO_3$/L, 30.0 mg $MgSO_4$/L, and 2.0 mg KCl/L. Add $CaSO_4 \cdot 2H_2O$ in powdered form to provide a concentration of 30.0 mg/L. Double these concentrations for preparing moderately hard reconstituted water. Use reagent-grade chemicals. See Section 8010 for further details. Aerate water for 24 to 48 h before use. Pass air through a water filter containing granular activated charcoal. After confirmation of water quality parameters (hardness, pH, etc.), add selenium, as sodium selenite, and vitamin B-12 to the water to obtain a concentration of 3 μg/L of each.[1]

Reconstituted waters have been associated with culture and bioassay performance problems due to unidentified and toxic components in the deionized water used to prepare the media[2] or due to a nutritional deficiency caused by the absence of a full complement of trace elements (synthetic waters contain four salts).[3] Carbon-treated distilled water is best for preparing synthetic culture waters.

Renewal frequency also has been shown to have an effect on the survival and reproductive success of *C. dubia*. Daily renewals of culture medium produced the best survival and reproduction results, while triweekly renewal conditions gave poor results and relatively higher coefficient of variations for reproduction.[4]

b. Food and feeding: Cladocerans are filter feeders that are most abundant in eutrophic lakes during summer phytoplankton blooms. Examination of *Ceriodaphnia* gut contents reveals algae, bacteria, and detritus.[1] *Ceriodaphnia* can be fed a combination food (known as YCT) consisting of yeast, dried cereal grass,* and commercially available flaked fish food† or trout chow. Supplement the diet with a 50:50 mixture of a green algal suspension of *Selenastrum capricornutum* and YCT.[5,6] *Selenastrum capricornutum* cultures can be established and maintained by following the algal culture procedures in 8111F.

Prepare food (YCT) for *Ceriodaphnia* as follows: Place 5.0 g of fish food flakes† or trout chow pellets in a blender containing 1 L synthetic water. Blend at high speed for 5 min. Transfer contents of blender to a 1-L separatory funnel and aerate continuously through spigot opening for 1 week at ambient laboratory temperature. Place mixture in a refrigerator and let settle for 1 h. Pour supernatant into a clean bottle and discard remainder.

Place 5.0 g dry yeast in 1 L synthetic water. Place in a blender at low speed for 5 min. Transfer to a bottle and refrigerate for 4 h. Mix well before combining for YCT.

Place 5.0 g dried cereal grass‡ in 1 L synthetic water in a blender. Mix at high speed for 5 min. Transfer to a bottle, refrigerate, and let settle for 4 h. Pour supernatant into clean bottle and discard the remaining solids.

Mix 300-mL volumes of each of the three components above. Filter mixture through a nylon§ 110-μm-mesh filter. Determine dry weight (i.e., total solids) concentration on each batch of YCT mixture. The food should contain 1700 to 1900 mg total solids/L. Adjust level of total solids by dilution with synthetic water if YCT dry weight is greater than 1900 mg/L. Place 30- to 50-mL portions in small polyethylene bottles with screw caps and freeze. Thaw portions as needed. Keep refrigerated and discard unused portions after 2 weeks.

Feed *Ceriodaphnia* mass cultures daily at the rate of 4 mL food/L medium. Feed individual cultures or test chambers daily at the rate of 0.1 mL YCT/d and 0.1 mL algae/d. The quality of each new batch of *Ceriodaphnia* food can be determined in a 7-d reproduction test with control water. Culture grid records also can be used to evaluate food quality.[1]

c. Temperature: Sudden changes of several degrees in temperature may cause death of *Ceriodaphnia*. Optimal temperature range is approximately 25 ± 1°C. Maintain cultures within this temperature range.

d. Lighting: Variations in ambient light intensities and prevailing day/night cycles in most laboratories do not affect *Ceriodaphnia* reproduction significantly. A light intensity of 550 to 1050 lux and a photoperiod of 16 h light and 8 h dark is recommended.

e. Culture vessels and maintenance: Culture individual adults in 30-mL plastic cups containing 15 to 20 mL culture medium. Feed organisms daily and transfer them to new culture media in new plastic cups at least three times per week; more frequent transfers are desirable. Construct styrofoam (or similar rigid material) culture boards consisting of slots that hold the 30-mL plastic cups. Maintain a minimum of four boards, containing 20 individuals per board, to ensure backup cultures. Individual cultures of *Ceriodaphnia* are used as the immediate source of neonates for toxicity tests. Do not use culture boards exceeding 20% adult mortality to supply neonates used in toxicity tests. A

* Such as Cerophyl®, Agri-Tech, Inc., Kansas City, MO 64112, or equivalent.
† TetraMin® or equivalent.
‡ Cerophyl® or equivalent.
§ Nitex® or equivalent.

healthy culture board will supply neonates for approximately 14 d.

f. Air supply: Ceriodaphnia can survive when the dissolved oxygen concentration is as low as 3 mg/L but reproduce better when the concentration is above 6 mg/L. As long as the culture water source is properly aerated, individual culture boards of *Ceriodaphnia* do not require aeration. Maintain DO levels within criteria for warmwater aquatic life.

3. Selecting Test Organisms

Use first instar neonate *Ceriodaphnia* less than 24 h old from a brood of eight or more from a female on her third or fourth brood. To obtain neonates for a test, transfer adult females to a new culture vessel containing fresh culture solution with a disposable, wide-mouth (approximately 4 mm) pipet. Keep tip of pipet under water surface when the *Ceriodaphnia* are released to prevent air from being trapped under the organism's carapace. Group cups of neonates together until enough are available to initiate a test.

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8712 C. Procedures

1. Acute Toxicity Tests

Prepare test materials and concentrations, dilution water, and toxicant solutions as described in Section 8010F. Make up test solutions and controls in 100-mL quantities in 125-mL wide-mouth flint-glass bottles or equivalent vessels. See Table 8712:I.

After preparing test solutions, segregate neonates that have been released from the mothers' brood chambers during the preceding 24 h at 25°C ± 1°C, and collect in one vessel. Use neonates cultured at the test temperature. At test initiation, organisms should be less than 24 h old, although in some circumstances, organisms less than 48 h old are acceptable. Introduce five neonates per replicate into each test solution vessel and control exposure.[1] Use a minimum of four replicates with each test concentration. Use a plastic, disposable pipet with a 4-mm bore for collecting and transferring neonates.

Introduce neonates to test solutions by releasing them below the surface of the solution. Observe animals regularly, initially after 1 h and 4 h and daily thereafter. A 48-h exposure is generally accepted for a *Ceriodaphnia* acute toxicity test.[1] However, depending on the study objectives, tests may be conducted for 24, 48, or 96 h. If the test is conducted for 96 h, renew test solutions and feed organisms after 48 h. At each 24-h interval during the test, record either the number of surviving animals or the number of motile animals in each test vessel. Consider an animal nonmotile if it shows no independent movement even after gentle squirting with test solution from a pipet (nonmotile animals are not necessarily dead).

If lethality is selected as the test endpoint, an LC50 value can be generated. If immobility is selected as the test endpoint, an EC50 value can be generated. The LC50 value represents the concentration of test solution or chemical at which 50% of the animals have died. Similarly, the EC50 value represents the concentration of test solution or chemical at which 50% of the animals exposed are immobilized. Test results usually are considered acceptable as long as there is no more than 10% control mortality. Do not feed *Ceriodaphnia* during most short-term tests; however, feed animals a minimum of 2 h before test initiation.

2. Short-Term Chronic Toxicity Tests

a. Determination of toxicity effect(s) on survival and reproduction: Sublethal effects may occur at lower toxicant concentrations than those causing acute toxicity. Precede long-term tests by acute (48-h) toxicity tests to establish the maximum concentration to be used.

b. Preparation of test medium: Prepare by methods used for preparing regular culture medium, but use water representative of that receiving the waste effluent, or the dilution water used to culture *C. dubia* when testing chemicals. Prepare a series of six to ten 250-mL quantities of medium to which log concentrations

TABLE 8712:I. SUMMARY OF ECOLOGICAL AND TOXICOLOGICAL TEST CONDITIONS USING *CERIODAPHNIA DUBIA*

Ecology/Test Condition	Description
Habitat	Fresh water
Length of life cycle	7–8 d
Type of test:	
Acute	7 d
Life cycle	7–8 d
Life stage tested	<24 h old
Test temperature	25 ± 1°C
Size of test chamber	30 mL
Volume of test solution	15 mL
Number of animals per container	1
Number of replicates	10
Dilution water	Moderately hard water + selenium; other waters determined by purpose of test
Water renewal	Daily
Nutrition	Feed 0.1 mL algae and 0.1 mg YCT*
Light cycle	16 h:8 h light/dark
Control mortality	Not to exceed 10%
End points:	
Acute	Death
Life cycle	Number of young and adults

* Algal concentration of 35×10^6 cells/mL; YCT (yeast, Cerophyll® and trout chow mix).

of effluents, mixtures, or chemicals have been added. Use as the highest concentration of chemical or effluent, the equivalent to the 48-h LC50 or EC50 values. Reduce each successive concentration in a consistent progression (e.g., geometric). Tests conducted with effluent generally use a 0.5 serial dilution such that the effluent concentrations tested are 6.25%, 12.5%, 25%, 50%, and 100% effluent. Use test dilution water as a negative control (i.e., 0% effluent). Dispense each 250 mL test medium in 15-mL quantities to each of ten glass or plastic 30-mL chambers. Preferably run tests in replicates of ten; a minimum of six is required for statistical significance.

c. Performing tests: Preferably conduct test according to Good Laboratory Practice standards/regulations.[2–5] Segregate and collect <24-h-old neonate *Ceriodaphnia* that have been cultured at the test temperature. Introduce one neonate into each chamber of the test and array randomly. At 24-h intervals, transfer the first-generation organisms (i.e., *Ceriodaphnia* used to begin the test) into fresh test solutions and add an appropriate amount of food to each test chamber (see Section 8711B.2*b*). Under some chronic test protocols, *Ceriodaphnia* may be transferred to new test media less frequently; however, feed organisms daily. Make daily observations and note number of dead animals. As animals grow and reproduce, remove young and record their number. Cover all test chambers loosely with plate glass or equivalent to minimize evaporation but not affect oxygen transfer. Continue test for 7 to 8 d at 25°C. Handle test animals as in the individual cultures of stock animals.

At end of exposure period, analyze results and test for statistically significant differences in the number of young produced, first-generation *Ceriodaphnia* survival, and reproduction (i.e., number of neonates produced) in each treatment. Note appearance of first broods and number of broods. Generally, chronic toxicity tests are considered acceptable if control mortality is less than 20%, surviving control females produce an average of 15 neonates per adult, and 60% of control females produce three broods or more.[6] The coefficient of variation (CV) for reproduction between replicates in the test control should be equal to or less than 20%. Determine CV by dividing the standard deviation for reproduction by the average number of young per surviving female and multiplying by 100. A CV greater than 20% indicates that conditions between replicates were substantially different. Substantial differences among control replicates may interfere with statistical detection of substantial differences between treatment exposures.

3. Criteria for Test Acceptability

An acceptable test will have no more than 10% mortality in the negative control. A clear dose-response must be apparent from a sample plot of the data. Ideally one concentration will have no effect (no more mortality than control) and the EC50 will be bracketed by concentrations producing mortality. Use a scatter plot of the 48-h data to identify outliers. Examine records for clerical or experimental error when outliers exist.

4. Statistical Analysis

Assemble, analyze, evaluate, and report data as described in Section 8010G.

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8714 MYSIDS*

8714 A. Introduction

1. Suitability for Toxicity Tests

Mysids are an important component of both the pelagic and epibenthic communities. They are preyed upon by many species of fish, birds, and larger invertebrate species, and they are predators of smaller crustaceans and larval stages of invertebrates. In some cases, they feed on algae. Mysids are sensitive to both organic and inorganic toxicants. The ecological importance of mysids, their wide geographical distribution, ability to be cultured in the laboratory, and sensitivity to contaminants make them appropriate toxicity test organisms.[1-8] Juvenile mysids used in these tests are taken from cultures shortly after release from the brood and exposed to varying concentrations of a toxicant in static or flow-through conditions. These procedures will be useful for conducting toxicity tests with other species of mysids, although modifications may be necessary. The tests are applicable to most chemicals, either individually or in formulations, commercial products, and known or unknown mixtures, and with appropriate modifications, can be used to conduct tests on factors such as temperature, salinity, and dissolved oxygen. These methods also can be used to assess the toxicity of potentially toxic discharges such as municipal wastes, oil drilling fluids, produced water from oil well production, and other types of industrial wastes.

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* Approved by Standard Methods Committee, 1997.
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8714 B. Selecting and Preparing Test Organisms

1. Selection of Test Species

Test species may be designated by a particular regulation (e.g., Federal Insecticide, Fungicide and Rodenticide Act; Toxic Substances Control Act). If the toxicity test is for nonregulatory purposes, any number of species can be tested if the test conditions are suitable for culturing the species. If it is desirable to use mysids that are not cultured routinely, it may be necessary to collect them from a single field source.

Select test species that meet the following criteria: *(a)* The species preferably occurs, or is closely related to a species that occurs, in the receiving water being studied; *(b)* the species is available in unbiased (i.e., not prescreened for resistant individuals by prior exposure to adverse conditions) numbers sufficient for the tests; *(c)* the species can be held in the laboratory in a healthy condition (i.e., active, feeding, free of lesion, etc.); and *(d)* the species represents an important trophic link or economic resource in habitats similar to that of the receiving water. If the data are available when selecting species, consider relative sensitivities of different species and life stages.

In accordance with the criteria listed in Section 8010E.1, the recommended test species include (but are not restricted to) the following:

a. Estuarine and freshwater mysids:
Neomysis mercedis (Figure 8714:1)
Americamysis almyra[1*] (Figure 8714:2)
b. Marine mysids:
Holmesimysis costata[2] [= *Acanthomysis sculpta*]† (Figure 8714:3)
Americamysis bahia[1*] (Figure 8714:4)
Americamysis bigelowi[1*] (Figure 8714:5)

* *Americamysis almyra, A. bahia,* and *A. bigelowi* are three of six species forming the group *Americamysis,* confined to the northwestern Atlantic and endemic to estuarine waters along the east coasts from New England to Colombia, and now distinguished from the group *Mysidopsis.* In previously published literature, they may be referred to as *Mysidopsis almyra, M. bahia,* and *M. bigelowi,* respectively.
† *Holmesimysis costata* is one of five species of the genus *Holmesimysis* which is present in the North Pacific Ocean. Confusion has existed about genus in which to place the mysid used in toxicity tests in California. Up to 1988 all authors referred to this mysid species as *Acanthomysis sculpta* (Tattersall). All known species of the genus *Acanthomysis* from the Pacific Coast of North America were placed in the new genus *Holmesimysis.*

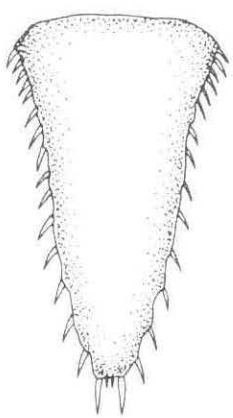

Figure 8714:1. ***Neomysis mercedes:*** telson.

2. Collecting and Handling Test Organisms

Collecting equipment and methods are described in Sections 8010E.2, 10200B, and 10500B. Handling and holding are discussed in Section 8010E.3 and 4. NOTE: Avoid subjecting mysids to unnecessary stress such as inappropriate capture and transport, temperature shock, or water quality change.

a. Estuarine and freshwater mysids: Neomysis mercedis (Figure 8714:1) is a Pacific coast species living in fresh and estuarine water to 18 g/kg salinity estuarine water; its temperature range is 6 to 22°C.[3] *N. mercedis* ranges from Prince William Sound, Alaska, to south of Point Conception, California.

Collect *N. mercedis* by hand dip nets or plankton tows in rivers and estuaries. Collection with a dip net (0.5- to 1.0-mm mesh) at night results in minimal mechanical damage[4] and yields many specimens in good condition and with little accompanying debris. Transfer specimens to a 100-L (30-gal) plastic container filled with site water and transport to the laboratory. Aerate with a portable air pump. Separate *N. mercedis* from other organisms and discard any specimen that is injured or does not appear to be in good condition. Specimens can be picked up by using a bulb pipet with a 5-mm bore or with a plastic spoon. Alternatively, collect organisms by towing a plankton net (0.5-mm mesh) from a boat in open water. This technique can result in high mysid mortality and much accompanying detritus. *N. mercedis* are abundant between February and July but scarce during the remainder of the year.[5]

Americamysis almyra[1] (Figure 8714:2) is an East Coast species often living sympatrically with *A. bahia,* but preferring lower salinities ranging from 10 to 20 g/kg at temperatures over 20°C.[6] It is found in inshore waters along the entire coast of the Gulf of Mexico and northward along the Atlantic coast to Patapsco River, Maryland.[1] Collect *A. almyra* with hand dip nets (350-μm mesh) or a 1.5-m beam trawl with a 0.9-mm mesh size pulled by hand in shallow areas of estuaries.[7] The dip net method yields many specimens in good condition and with little accompanying debris. Remove possible predator species, such as ctenophores, immediately. Transfer specimens to an insulated 4-L (1-gal) or larger plastic container filled with site water and transport to the laboratory. Aerate with a portable air pump. Separate *A. almyra* from other organisms and discard any specimen injured or not appearing to be in good condition. Specimens can be picked up by using a bulb pipet with a 5-mm bore. Alternatively, collect organisms by towing a plankton net (350-μm mesh) from a boat in open water at night. However, this technique can result in high mysid mortality and much accompanying detritus. *A. almyra* are abundant throughout the year in the southern latitudes.

b. Marine mysids: Holmesimysis costata [= *Acanthomysis sculpta*][2]† (Figure 8714:3) is the principal species of the genus in California marine waters. *H. costata* occurs abundantly offshore among the fronds of the giant kelp especially during the summer months.[8] Collect *H. costata* from a boat by passing a hand net (0.5- to 1.0-mm mesh) through the kelp canopy. Transfer spec-

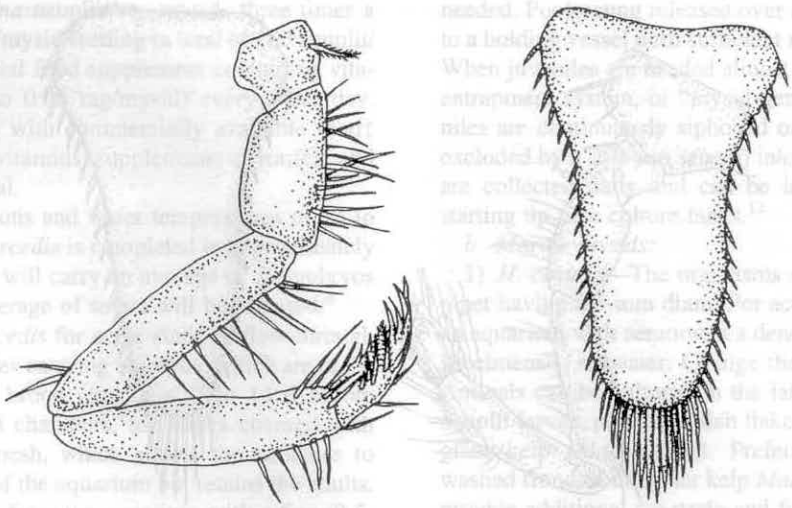

Figure 8714:2. ***Americamysis almyra:*** (left) endopod of thoracic leg 2; (right) telson.

imens to a 20-L (5-gal) bucket filled with seawater and transport to the laboratory. Pour contents of the bucket into one or more pans and separate *H. costata* from the other organisms. Discard any specimen that is injured or does not appear to be in good condition. Some specimens might be parasitized externally by a marine leech; do not use these specimens or place them in the laboratory stock colony. Mysids can be picked up by using a bulb pipet with a 5-mm diam.

Americamysis bahia[1] (Figure 8714:4), often sympatric with *A. bigelowi* and *A. almyra*, at temperatures over 20°C,[6] but preferring higher salinities ranging from 10 to 30 g/kg,[9] is found in inshore waters along the coast of the Gulf of Mexico, and northward along the Atlantic coast to Narragansett, R.I.[1] *Americamysis bigelowi*[1] (Figure 8714:5), is found on the Atlantic coast from Massachusetts (Georges Bank) southward to Florida. It often occurs sympatrically with *A. bahia*, with a salinity range from 30 to 35 g/kg[9] and in water temperatures from 2 to 30°C.

Collect *A. bahia* and *A. bigelowi* by using hand dip nets (350-μm mesh) in shallow areas of salt ponds and estuaries.[10] This method yields many specimens in good condition and with little accompanying debris. Remove possible predator species, such as ctenophores, immediately. Transfer specimens to an insulated 4-L (1-gal) or larger plastic container filled with site water and transport to the laboratory. Aerate with a portable air pump. Separate *A. bahia* and *A. bigelowi* from the other organisms and discard any specimen injured or not appearing to be in

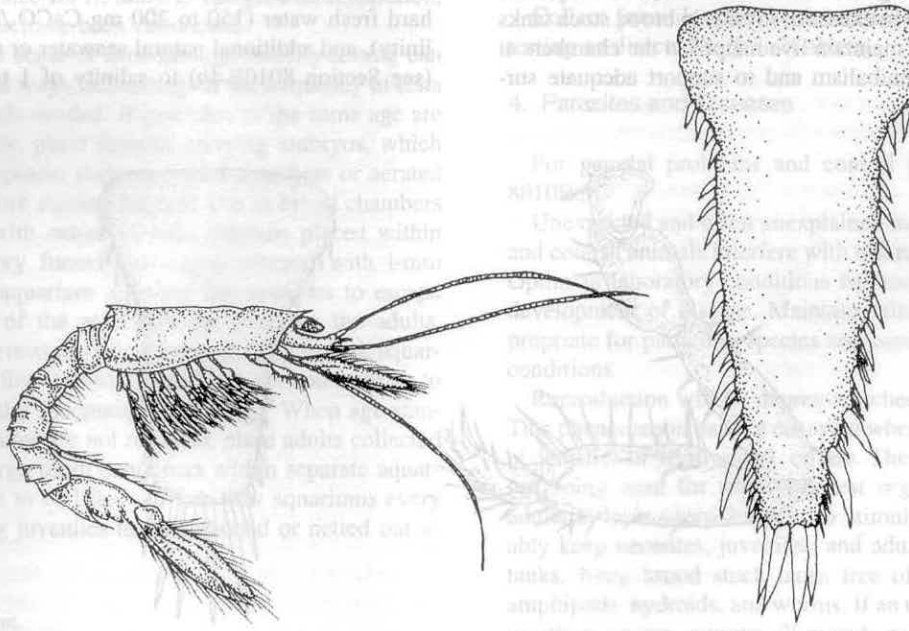

Figure 8714:3. ***Holmesimysis costata:*** (left) entire animal; (right) telson.

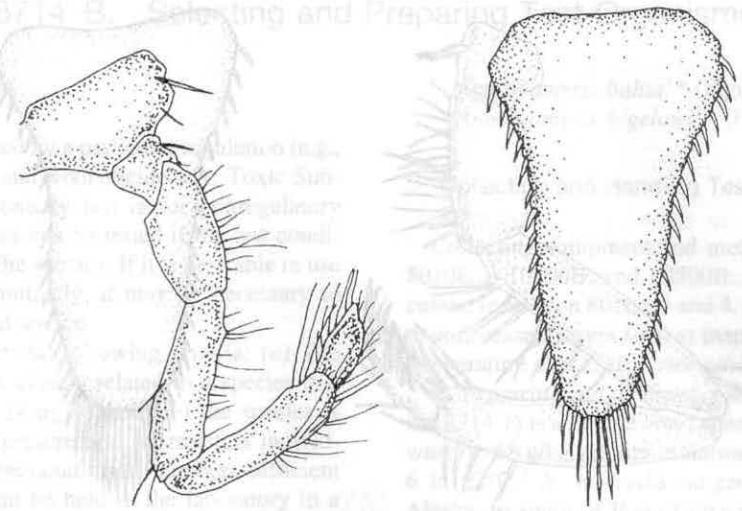

Figure 8714:4. ***Americamysis bahia:*** (left) endopod of thoracic leg 2; (right) telson.

good condition. Specimens can be picked up by using a bulb pipet with a 5-mm bore. Alternatively, collect by towing a plankton net (350-μm mesh) from a boat in open water at night. However, this technique can result in high mysid mortality and much accompanying detritus. *A. bahia* and *A. bigelowi* are abundant throughout the year in the southern latitudes, and from June through September in temperate latitudes in shallow water with a temperature above 20°C.

3. Holding, Acclimating, and Culturing Organisms

See Sections 8010E.3 and 4.

Keep mysids in tanks, aquariums, or screened enclosure depending on size and number. Use good quality dilution water (see Section 8010E.4*b*) for acclimation. Feed mysids live brine shrimp nauplii daily during acclimation. At least once daily, feed live brine shrimp nauplii in excess to mysids in brood stock tanks and in test chambers, to maintain live nauplii in the chambers at all times to prevent cannibalism and to support adequate survival, growth, and reproduction in the brood stock. Adjust ration in accordance with the number of mysids in the stock colony. A ration of 150 nauplii per mysid per day has been used successfully.[8] A regime of 75 nauplii per mysid twice a day or 50 nauplii three times a day might improve growth and reproduction in the brood stock. Use diets that are certified toxicant-free or test for toxic substances before use.

a. Estuarine and freshwater mysids:

1) *N. mercedis*—Culture in either a static or a flow-through system. For static system, use 75- to 114-L aquariums supplied with aeration and a subsurface filter of dolomite 3 to 5 cm in thickness. *N. mercedis* is extremely sensitive to nitrogenous wastes; clean aquariums daily to remove excess food. For a flow-through system, supply sufficient water for a minimum of two tank volumes per day. Successful cultures have been maintained at a temperature of between 15 and 19°C (optimum 17°C), hard fresh water (150 to 200 mg $CaCO_3$/L, hardness and alkalinity), and additional natural seawater or reconstituted seawater (see Section 8010E.4*b*) to salinity of 1 to 3 g/kg (optimum 2

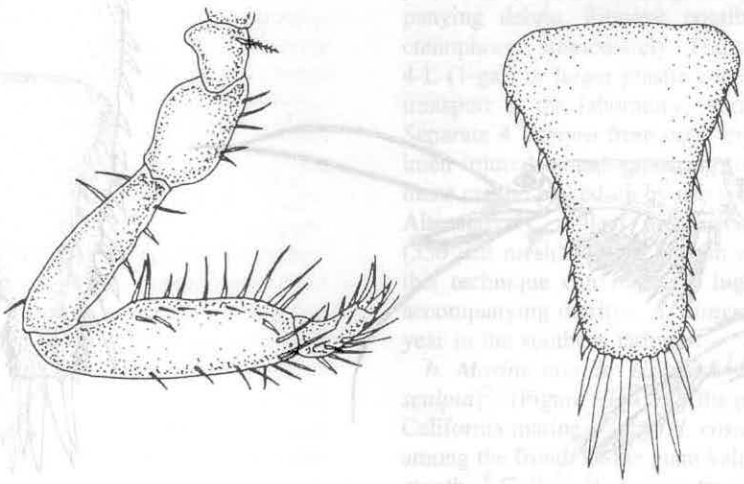

Figure 8714:5. ***Americamysis bigelowi:*** (left) endopod of thoracic leg 2; (right) telson.

g/kg).[4,8] Feed *Artemia salina* nauplii[11] to mysids three times a day at the rate of 50 nauplii/mysid/feeding (a total of 150 nauplii/mysid/d) and add an artificial food supplement containing vitamins and minerals (0.02 to 0.06 mg/mysid) every other day. Preferably supplement diet with commercially available food‡ having micronutrients and vitamins; supplements of rotifers and algae also may be beneficial.

Under laboratory conditions and water temperatures of 15 to 19°C, a life cycle for *N. mercedis* is completed in approximately 3 months. A gravid female will carry an average of 20 embryos in a brood, of which an average of seven will be released.[4]

To collect young *N. mercedis* for acute static or flow-through toxicity testing, place females carrying embryos, which are in the eye-development stage, in brood chambers, 7 to 14 d before starting the test. For brood chambers, use cages covered with 0.25- to 0.50-mm nylon mesh, which allows the neonates to escape into the main body of the aquarium but retains the adults. Remove neonates each day from the aquarium with a fine (0.5-mm) mesh dip net and transfer to a dish. Remove healthy specimens for testing. Pool the young released over a 2- to 3-d period and transfer them to a holding vessel until a sufficient number are obtained for a test.

2) *A. almyra*—Preferably culture in a flow-through system supplied with sufficient water for a minimum of two tank volumes per day for best control of salinity and nitrogenous wastes.[9] Alternatively, culture in static 76-L (20-gal) or larger aquariums supplied with aeration and a subsurface filter of dolomite 3 to 5 cm deep. Successful cultures have been maintained at a temperature of 25 to 27°C (optimum 26°C), in natural seawater or reconstituted seawater (see Section 8010E.4*b*) at salinity of 10 to 20 g/kg. Feed *Artemia* nauplii[11] to mysids twice a day at the rate of 75 nauplii/mysid/feeding (a total of 150 nauplii/mysid/d). Preferably supplement *Artemia* cultures with an enhancement product§ to ensure the amino acid content of the nauplii.[11] Supplementing the diet of *A. almyra* with rotifers and algae also may be beneficial. There is very little information regarding the life cycle and brood size for *A. almyra*, but growth, respiration, and energetics studies have been conducted.[7]

Juveniles for acute static or flow-through toxicity testing can be collected in several ways, depending on the frequency of tests and number of animals needed. If juveniles of the same age are required intermittently, place females carrying embryos, which are in the eye-development stage, in brood chambers or aerated finger bowls, 1 d before starting the test. Use as brood chambers either 4-L beakers with netted (1-mm) bottoms placed within wide-mouth separatory funnels, or cages covered with l-mm mesh‖ placed in an aquarium allowing the neonates to escape into the main body of the aquarium but retaining the adults. Remove neonates the next day from the separatory funnel, aquarium, or bowl with a fine (0.5-mm) mesh dip net and transfer to a dish. Remove healthy specimens for testing.[9] When age-standardized mysid juveniles are not required, place adults collected from cultures into large mesh containers within separate aquariums, allowing adults to be lifted out into new aquariums every few days and leaving juveniles to be siphoned or netted out as needed. Pool young released over a 2- to 3-d period and transfer to a holding vessel until sufficient number are obtained for a test. When juveniles are needed almost daily for testing, use a siphon entrapment system, or "mysid generator." In this system, juveniles are continuously siphoned out of an aquarium (adults are excluded by a 750-μm screen) into a collection vessel. Juveniles are collected daily and can be used either for testing or for starting up new culture tanks.[12]

b. Marine mysids:

1) *H. costata*—The organisms can be picked up with a bulb pipet having a 5-mm diam. For acclimation, place *H. costata* in an aquarium with aeration at a density of approximately 10 to 20 specimens/L seawater. Change the water if it becomes cloudy. Animals can be cultured in the laboratory on a diet of *Artemia* nauplii larvae, powdered fish flake food, and fresh fronds of the giant kelp (*Macrocystis*). Preferably add a fresh, carefully washed frond of the giant kelp *Macrocystis* to the brood stock to provide additional substrate and food for mysids.

H. costata can complete three or four life cycles a year under laboratory conditions. Females will produce more than one brood set under laboratory conditions. To obtain young mysids, place adult *H. costata* in a cage within an aquarium. Use a cage covered with nylon screening with a 0.25-mm mesh, which allows the newborn to escape into the main body of the aquarium but retains the adults. Remove newborn from the aquarium with a fine dip net or glass pipet and transfer to a dish where specimens can be observed and removed for testing.

2) *A. bahia* and *A. bigelowi*—Follow culturing instructions for *A. almyra* [¶ 3*a*2) above], but use salinity of 25 g/kg.

Under laboratory conditions with water temperatures of 25°C a life cycle of *A. bahia* and *A. bigelowi* is completed in 1 month or less, at which time the first eggs are laid. A gravid female will carry an average of eight embryos in a brood, all of which are normally released as healthy postlarvae; the first brood is released after 14 to 18 d and succeeding broods are released every 7 d. Productivity gradually declines in the last third of the 3- to 5-month life span.[9]

Collect juveniles for acute static or flow-through toxicity testing as directed in ¶ 3*a*2) above.

4. Parasites and Diseases

For general problems and control procedures see Sections 8010E.5.

Unexpected and often unexplained mortalities in experimental and control animals interfere with test results and interpretations. Optimize laboratory conditions for each species to prevent the development of disease. Maintain salinity and temperature appropriate for particular species and consistent with specified test conditions.

Reproduction will be depressed when culture density is high. This phenomenon has not occurred when cultures are maintained at densities of 10 mysids/L or less. Therefore, when cultures are not being used for supplying test organisms, remove enough adults at least every 2 weeks to stimulate reproduction. Preferably keep neonates, juveniles, and adults of mysids in separate tanks. Keep brood stock tanks free of other animals, such as amphipods, hydroids, and worms. If an outbreak of these animals or others occurs, remove all mysids and clean tank thoroughly. Examine mysids thoroughly before replacement, and discard any

‡ TetraMarin® or equivalent.
§ SELCO o r equivalent.
‖ Nytex® or equivalent.

having hydroids attached. Clean tanks with hot water and a 5% solution of hydrochloric or nitric acid.[9] Wash, dry, and autoclave substrate (i.e., dolomite or oyster shells), or discard it.

Handle mysids as little as possible. When handling is necessary, proceed gently, carefully, and quickly to reduce stress. Dip nets are best for removing gravid female mysids from brood-stock tanks. Such nets are commercially available or can be made from 350-μm mesh nylon netting, silk bolting cloth, plankton netting, or similar knotless material. Discard mysids that touch dry surfaces or are dropped or injured. Sterilize equipment used to handle mysids between uses by autoclaving. Wash new equipment with detergent and rinse with water, a water-miscible organic solvent, water, acid (such as 10% conc HCl), and at least twice with deionized, distilled, or dilution water. At the end of a test, clean all equipment by a procedure appropriate for removing the test material (e.g., acid to remove metals and bases; detergent, organic solvent, or activated carbon to remove organic chemicals), and rinse at least twice with deionized, distilled, or dilution water.[13]

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8714 C. Toxicity Test Procedures

1. Short-Term Procedures

a. General test procedures: Short-term testing can be used to determine relative toxicity of substances. Tests are made to determine LC50 or EC50 values and to estimate toxicant concentrations for intermediate- and long-term tests. Basic requirements for toxicity tests are described in Section 8010C.

Short-term tests may be static, static with renewal, or flow-through, depending on the objective of the test and the character of the toxicant or effluent (see Section 8010D). Acute static, static with renewal, or flow-through toxicity tests are conducted preferably with young mysids in accordance with other studies with this group.[1–3]

Collect young mysids of nearly uniform size in accordance with instructions given in Section 8714B.3. *H. costata* used in acute toxicity tests should be 3 to 7 d post-release from the brood sac, and *N. mercedis*, 1 to 5 d post-release. *Americamysis* species should be less than 24 h post-release from the brood sac. Use 10 to 20 mysids per toxicant concentration. Transfer mysids to each test chamber with a glass pipet. Feed mysids with brine shrimp larvae three times a day at the rate of 30 nauplii/mysid (a total of 90 nauplii/mysid/d) during the test period. Examine test chambers daily, record mortality, and remove all dead specimens and debris. Generally it is not necessary to consider mysid weight/L test solution given the low weight of mysids, but in flow-through tests use less than 10 g mysids/L test solution for tests at temperatures at or below 17°C and 5 g mysids/L test solution for tests at higher temperatures. For static testing, do not load above 0.8 g mysids/L at 17°C or less and 0.5 g mysids/L at temperatures above 20°C. Limit loading to ensure that concentrations of dissolved oxygen and test material do not fall below acceptable limits, concentrations of metabolic products do not exceed acceptable levels, and test mysids are not stressed because of cannibalism, aggression, or crowding.

b. Specific test procedures:

1) Freshwater and estuarine mysids

a) Equipment and physical conditions—Ensure that equipment and facilities that contact stock solutions, test solutions, or any water into which test organisms will be placed do not contain substances that can be leached or dissolved by aqueous solutions in amounts that adversely affect mysids. General requirements for toxicity test systems and materials are described in Section 8010F.1. In addition, choose equipment and facilities that contact stock or test solutions to minimize sorption of test materials from water. Use glass, Type 316 stainless steel, nylon, and fluorocarbon plastics to minimize dissolution, leaching, and sorption, but do not use stainless steel in tests on metals with salt water. Do not use cast iron pipe with salt water and preferably avoid its use in a fresh water-supply filter system because colloidal iron will be added to the dilution water and strainers will be needed to remove rust particles. Do not let brass, copper, lead, galvanized metal, or natural rubber contact dilution water, stock solutions, or test solutions before or during the test. Avoid items made of neoprene rubber or other materials not mentioned previously unless it has been shown that their use will not adversely affect survival, growth, or reproduction of mysids.[1,2]

Use test material of reagent grade or better unless a test of formulation, commercial product, or technical-grade or use-grade material is specifically needed. Before a test is begun, note the following about the test material: identities and concentrations of major ingredients and major impurities; solubility and stability in dilution water; precision and bias of the analytical method at the planned concentration(s) of the test material; and estimate of toxicity to humans.

Select temperature appropriate for the species being tested, and hold test temperature within ±2°C of mean test temperature during a 96-h test, or ±1°C for any 48-h period. Conduct tests with *Americamysis almyra* in a temperature range of 26 to 28°C,[2] and tests with *Neomysis mercedis* at 15 to 19°C.[2] Keep salinity within the tolerance range of the selected species. The optimum salinity for *A. almyra* is 10 to 20 g/kg and for *N. mercedis* is 1 to 3 g/kg.[2] If a test salinity other than the optimum is used, set up an additional control at the optimum salinity. Use ambient laboratory lighting with a photoperiod of 16 h light/8 h dark, preferably with 15- to 30-min dusk/dawn transition period to acclimate mysids to the test photoperiod.

Use dilution water from a surface source, well, or spring, or use reconstituted water (see Section 8010E.4). *N. mercedis* cultures have not been reported for media of reconstituted fresh water. Do not use chlorinated water as, or in the preparation of, dilution water, because chlorine-produced oxidants are toxic to mysids.[3] Establish a supply of dilution water that is available in adequate quantities, acceptable to test organisms, uniform in quality, and not likely to affect test results unnecessarily. An acceptable dilution water is one in which the test species will survive, grow, and reproduce satisfactorily. Maintain uniform quality of the dilution water so that the test organisms are cultured or acclimated, and the test conducted in water of the same quality.[1,2,4]

Use at least two test chambers (in which containers may be placed) for each concentration; these can consist of standard 57-L aquariums or can be constructed by gluing strong window glass with clear silicone adhesive. Because adhesives can sorb some organochlorine or organophosphorus pesticides, apply as little adhesive as possible. Finger bowls can be used for static acute toxicity test containers; for a 2-L bowl, use 20 animals; for a 350-mL bowl, 10 animals; each bowl constitutes a replicate. Flow-through toxicity tests can be conducted in a 2.5-L wide-mouth glass jar with a central standpipe. The test solution enters the compartment directly and flows through the standpipe into a drain. Cover standpipe with a 200- to 235-μm mesh nylon screen to avoid escape of the young mysids.[1,2,4]

For information pertaining to species selection, collection, holding, acclimation, disease control, and culturing see Sections 8010E and 8714B.

b) Test procedure

Range-finding test: If the approximate toxicity of test material is unknown, conduct an abbreviated range-finding test to determine the concentrations to use in definitive tests. Use three to five widely spaced toxicant concentrations (for example, a decade test having concentrations a factor of ten from each other). Static tests may be acceptable as would use of fewer mysids, e.g., five per container. Run this test for 24 to 96 h.

Definitive test: To determine LC50 or EC50 values, use a 96-h test period with a minimum of five toxicant concentrations (according to the results of the range-finding test) and a control. In some cases the test solution can be added directly to the dilution water, but usually it is dissolved in a solvent to form a stock solution that is then added to dilution water. If a stock solution is used, determine the concentration and stability of the test material in it and dilution water before beginning the test. If the test material is subject to photolysis, shield stock solution from light. Use a solvent control if dosing solutions are prepared in an organic solvent. Acceptable solvents are dimethylformamide, ethanol, methanol, acetone, and triethylene glycol (see Section 8010F.2). Limit concentrations of solvent to 0.1 mL/L of test solution. If a solvent other than water is used and the concentration of solvent is the same in all test solutions that contain test material, include at least one solvent control containing the same concentration of solvent and using solvent from the same batch used to make the stock solution, and also include a dilution water control. If a solvent other than water is used and the concentration of solvent is not the same in all test solutions that contain test material, include both a solvent control, containing the highest concentration of solvent present in any other treatment and using solvent from the same batch used to make the stock solution, and a dilution water control. The percentage of organisms that show signs of disease or stress, such as discoloration, unusual behavior, or death, must be 10% or less in the solvent control and in the dilution water control.

To establish definitive test concentrations, prepare solutions using a dilution ratio of 1.5 to 2 between successive concentrations (see Section 8010F.3).

c) Test initiation—On day of test, remove a sufficient number of mysids from the holding facility at one time to provide about one-third more animals than are needed. Select a set of test chambers [one test chamber for each test concentration plus control(s)] to be processed together to avoid possible selective bias during loading. Transfer mysids with a wide-bore (larger than the largest mysid) glass pipet with a smooth tip. Begin static tests by placing test organisms in the chambers within 30 min after the test material was added to the dilution water. Begin flow-through tests by placing test organisms in the chambers after the test solutions have been flowing through the chambers long enough for the concentrations of test material to have reached steady state.

d) Biological observations—Monitor survival by daily inspection (see Section 8010F.3). The criteria for death of mysids are opaque white coloration, immobility (especially absence of movement of respiratory and feeding appendages), and lack of reaction to gentle prodding. Count, record, and remove dead mysids daily. Count live animals at the beginning of the experiment and daily to account for cannibalism or death resulting from impingement on the sides of test compartments. Record missing or dead impinged animals. Do not stress live test organisms in an attempt to determine whether they are dead, immobilized, or otherwise affected. Prodding of organisms and movement of test chambers during test should be done very gently. Some organisms exposed to some organophosphorus compounds seem to be very sensitive to sudden changes in light intensity.

e) Chemical data recording—Analyze water in control and test chambers daily for pH, dissolved oxygen, salinity (for marine or estuarine species), and temperature (see Section 8010F.3*d*). Maintain DO concentrations at ≥60% saturation. When testing volatile substances, do not aerate test solution. However, take care that chemical substances that create a dissolved oxygen demand do not result in conditions inconsistent with the dissolved oxygen criterion of the test as well as of mysid health. As a last resort to maintain dissolved oxygen above the criterion, use aeration. If aeration is used, make frequent measurements to confirm test chemical concentrations and aerate all test chambers, including controls.

f) Verification of exposure—Before and during the test verify exposure concentrations of the test chemical (see Section 8010F.3*d*). In static and renewal tests, measure the concentration of test material, if possible, in at least the control and high, medium, and low concentrations of test material at the beginning and end of test. Measurement of degradation products may be desirable. Measure concentration of test material in flow-through test chambers as often as practical during test. Measure in all chambers concurrently at least once during test, preferably near the beginning; except for the control treatment, measure each test chamber (especially for those concentrations closest to the LC50) at least one additional time during the test on a schedule designed to give reasonable confidence in the concentration of the material in the test chambers during the entire exposure period, taking into account the flow rate and the number of independent metering devices; and measure at least one appropriate chamber whenever a malfunction is detected in any part of the metering system.

2) Marine mysids—The test procedures for marine mysids are essentially identical to those for freshwater and estuarine mysids except for the noted differences in biological and environmental requirements. Conduct tests with *H. costata*[2] at temperatures of 17 ± 2°C for south of Point Conception, California, and 15 ± 2°C for north of Point Conception, and with salinity of 30 to 35 g/kg. *H. costata* cultures have not been reported for media of reconstituted seawater. Conduct tests with *A. bahia* and *A. bigelowi*[1] at temperatures of 27 ± 1°C and salinity of 20 to 30 g/kg.

2. Life-Cycle Test Procedures for Marine and Estuarine Mysids

a. General test procedures: Life-cycle testing can be used to determine relative long-term toxicity of substances. Tests are conducted to determine changes in numbers and weights of individuals resulting from effects of the test material on survival, growth, and reproduction. Results may be used to predict long-term effects in field situations, compare chronic sensitivities of different species, and chronic toxicities of different materials.

Life-cycle toxicity tests are flow-through (see Sections 8010D.1 and 4) with a flow rate through each test container of at least 5 to 10 volume additions per 24 h. Start tests with young mysids in accordance with other studies with this group.[1,2,4] Mysids of *Americamysis* species used in life-cycle toxicity tests should be less than 24 h post-release from the brood sac; collect in accordance with instructions given in Section 8714B.3. Start test with 2 containers of 15 or 3 containers of 10 mysids each, in at least 4 to 8 true replicate chambers per concentration. Transfer mysids to each test chamber with a glass pipet. Feed mysids with brine shrimp larvae three times a day at the rate of 50 nauplii/mysid (a total of 150 nauplii/mysid/d) during test period.[5] Examine test chambers daily, record mortality, and remove all dead

specimens and debris. Generally it is not necessary to consider mysid weight/L test solution given the low weight of mysids, but in flow-through tests use less than 5 g of mysids/L of test solution at temperatures above 20°C. Limit loading to ensure that the concentrations of dissolved oxygen and test material do not fall below acceptable limits, concentrations of metabolic products do not exceed acceptable levels, and the test mysids are not stressed because of cannibalism, aggression, or crowding.[6]

b. Specific test procedures: Conduct *Americamysis bahia, A. bigelowi*, and *A. almyra* tests at a temperature of 27°C for approximately 28 d. Conduct life-cycle toxicity tests by two general methods, pairing and non-pairing.[4] The method using pairing may make it easier to collect population data for life table analysis. If the method with pairing is to be used, start the test with at least two containers of 15 randomly selected mysids each, in at least two true replicate chambers per concentration [see ¶ 3) below]. Pair mysids at sexual maturity (Day 12 to 14), with one female and one male in each test container. Preferably use at least 20 randomly selected pairs per treatment; transfer them between containers within a test chamber, but not from one test chamber to another, to create as many pairs as possible. Pair all mysids on the same day of the test. If the method without pairing is to be used, start test with at least three containers of ten randomly selected mysids each, in at least four true replicate chambers per concentration[6] [see ¶ 3) below]. Keep mysids in these containers throughout the test.

1) Equipment and physical conditions—For general guidance on equipment and materials, see ¶ 1*b*1)a). For the test material, in addition to the items noted in ¶ 1*b*1)a), ascertain acute toxicity to the test species, measurement or estimate of chronic toxicity to the test species, and recommended handling procedures.

Select temperature appropriate for the species being tested, and hold test temperature within ±1°C of mean test temperature. Conduct tests with *Americamysis* species in a temperature range of 26 to 28°C. Keep salinity within the tolerance range of the selected species. The optimum salinity for *A. bahia* and *A. bigelowi* is 20 to 30 g/kg and for *A. almyra*, 10 to 20 g/kg (see Section 8714B.2). If a test salinity other than the optimum is used, set up an additional control at the optimum salinity. Use ambient laboratory lighting with a photoperiod of 16 h light/8 h dark, preferably with 15- to 30-min dusk/dawn transition period to acclimate mysids to the test photoperiod.

For dilution water requirements, see ¶ 1*b*1)a).

Calculate minimum number of test chambers, test containers, and pairs of mysids per treatment, expected variance within test chambers, expected variance between test chambers within a treatment, and either the maximum acceptable confidence interval on a point estimate or the minimum detectable difference using hypothesis testing.[4,6]

Test solution can flow from one container to another but not from one chamber to another. Test chambers can be constructed by gluing strong window glass with clear silicone adhesive. Because adhesives can sorb some organochlorine or organophosphorus pesticides, apply as little adhesive as possible. Cover chambers to prevent contamination and reduce evaporation. Test solution may enter the container directly or containers may be oscillated in the test solution, or the water level in the test chamber may be varied by means of a self-starting siphon. Test containers used successfully include 250-mL glass beakers with holes drilled in the sides and covered with 250-μm mesh*, 90- or 140-mm-ID glass petri dish bottoms with collars made of 210- or 250-μm screen,[7] and 110- by 180- by 200-mm deep glass rectangular chambers partitioned into six containers with a 65-mm-high, 330-μm mesh nylon collar. Provide metering system that will accommodate type and concentration(s) of test material and necessary flow rates of the test solutions, mix test material with dilution water immediately before entrance to test chambers, and supply selected concentration(s) of test material reproducibly.[1] Ensure that mysids remain submerged and are not stressed by crowding or turbulence in exposure system. Use test containers that provide a surface area of at least 25 cm^2/mysid and a solution depth of at least 25 mm at all times.[4,6]

For information pertaining to species selection, collection, holding, acclimation, disease control, and culturing, see Sections 8010E and 8714B.

2) Test procedure—For general information on test procedures, see Section 8010F.3. If a life-cycle test is intended to allow calculation of an endpoint, include one or more control treatments and a geometric series of at least five concentrations of test material, each of which is at least 50% of the next higher one. Use results from range-finder or definitive tests to determine the appropriate range of concentrations. To determine whether a specific concentration reduces survival, growth, or reproduction, only that concentration and control(s) are necessary; however, two additional concentrations at about one-half and twice the specific concentration of concern are preferable.

While test solution sometimes can be added directly to dilution water, preferably dissolve it in a solvent (reagent-grade or better) to form a stock solution and add stock solution to dilution water in the metering system. If a stock solution is used, determine the concentration and stability of the test material in it before beginning the test. If test material is subject to photolysis, shield stock solution from light.

Use a solvent control if dosing solutions are prepared in an organic solvent. Acceptable solvents are triethylene glycol, methanol, ethanol, and acetone.[4] Limit concentrations of solvent to 0.1 mL/L test solution. Do not use surfactant in preparation of a stock solution. If a solvent other than water is used and solvent concentration is the same in all test solutions that contain test material, include at least one solvent control containing the same concentration of solvent and using solvent from the same batch used to make the stock solution, as well as a dilution water control. If a solvent other than water is used and the concentration of solvent is not the same in all test solutions that contain test material, conduct a solvent test to determine whether survival, growth, or reproduction of the test species is related to the concentration of solvent over the range used in the toxicity test if such a solvent test with the same dilution water and test species has not already been conducted. A life-cycle test is unacceptable if any treatment contains a concentration of solvent in the range of effect. If no effect on the test species using the same dilution water is found at the test concentration of solvent, a life-cycle test may be conducted using solvent concentrations within the tested range, but include in such tests a solvent control containing the highest concentration of solvent present in any

* Nytex® or equivalent.

other treatment using solvent from the same batch used to make the stock solution, and a dilution water control.

The percentage of organisms that show signs of disease or stress, such as discoloration, unusual behavior, or death, must be 30% or less in the solvent control and in the dilution water control.

To establish test concentrations, set highest concentration of life-cycle test to be equal to the lowest concentration that caused adverse effects in a comparable definitive acute test. Use a dilution ratio of 1.5 to 2 between successive concentrations.[1] For more information on experimental design, see Section 8010F.3*a*.

3) Test initiation—On the day that the toxicity test is initiated, remove a sufficient number of mysids from the holding facility at one time to provide about one-third more animals than are needed. Transfer mysids gently with a wide-bore (larger than the largest mysid) glass pipet with a smooth tip. Begin flow-through life-cycle tests by placing test organisms into randomly selected containers after test solutions have been flowing through the test chambers long enough for the concentrations of test material to have reached steady state.

4) Biological observations—See ¶ 1*b*1)d). Also count, record, and remove live young in each container daily. Record day of brood release.

Determine dry weight (dried at 60°C for 72 to 96 h or to constant weight) of each individual first-generation mysid alive at end of test to nearest microgram. Rinse mysids with deionized water to remove salt before drying. Weigh males and females separately to determine sex-specific effects.[8] Remove brine shrimp nauplii caught in female brood sacs before drying. Total body length (total midline body length from anterior tip of carapace to posterior margin of endopod of uropod, excluding setae) may be determined for mysids alive at the end of the test, but not for preserved mysids because of body curvature. Note any abnormal development or aberrant behavior for first- and second-generation mysids.

5) Chemical data—Analyze water in control and test chambers daily for salinity and temperature. Measure pH and dissolved oxygen (DO) at beginning, end, and at least weekly during the test in the control and pH at least once in the highest test concentration. Also measure DO whenever there is an interruption of the flow of test solution. For DO maintenance requirements, see ¶ 1*b*1)e).

6) Verification of exposure—Before and during the test verify exposure concentrations of test chemical. Measure concentration of test material in the flow-through test chambers at least weekly in each treatment, including the control(s) during the test. Measurement of degradation products may be desirable. If a malfunction that could alter the concentration of test material is detected in the metering system, take water samples immediately from affected test chambers and analyze as soon as possible.

3. Statistical Analysis

Assemble, analyze, evaluate, and report data as described in Section 8010G.

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8740 DECAPODS*

8740 A. Introduction

Decapod crustaceans are among the most commercially important invertebrates.

Larval, postlarval, or adult stages of several species of decapods may be found in large numbers in estuaries and rocky intertidal habitats near the shore, where they are vulnerable to various types of discharges. Because of their phylogenetic relationship to insects, and the fact that pesticides often are applied in watersheds draining to estuaries, use of decapods in the testing of pesticide toxicity is particularly relevant. Postlarvae of penaeid shrimp use the estuaries as nursery grounds until they are large enough to migrate offshore. Early life stages are particularly vulnerable.

There is considerable diversity among the decapods, with the basic separation of adult animals into the more active swimmers (Natantia), encompassing the shrimp and lobsters, and the more sedentary crabs (Reptantia). While there are exceptions and variations, shrimp are generally planktivores, while the lobsters and crabs are predators and scavengers. There is much greater similarity within the decapods at the larval and postlarval stages, when zooplankton is the primary food. Laboratory holding and testing is easier at these early stages because brine shrimp (*Artemia salina*) nauplii usually are an appropriate food.

* Approved by Standard Methods Committee, 1999.
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8740 B. Selecting and Preparing Test Organisms

Some species used frequently in toxicity studies are penaeid shrimp (*Penaeus* sp.) larvae, postlarvae, and juveniles,[1–3] which have high commercial value on the Gulf of Mexico coast, and larvae of the American lobster,[4] an equally important commercial species along the northeast coast of the U.S. The grass shrimp, *Palaemonetes* sp., is another species that has been used in many toxicity investigations,[5–12] because of the ease of collecting and holding, abundance in marshes and estuaries along the Gulf of Mexico and southeastern coast of the U.S., and sensitivity, especially to pesticides.

1. Selecting Test Organisms

Many of the species used previously in toxicity testing are listed below. Consult specific references to determine which life stages were tested and the methods used for collecting, holding, feeding, and exposing the animals. For information on selecting test organisms, as well as the handling, holding, and conditioning of the animals, see Section 8010E.1–3. Regional references to location and identification of decapods are given in Section 10900.

a. Marine and estuarine decapods:

1) Suborder Natantia

a) Section Penaeidea—Species of *Penaeus* are prominent commercial shrimp, harvested in the Gulf of Mexico.

Penaeus setiferus
Penaeus aztecus
Penaeus duorarum

b) Section Caridea

Crangon—cosmopolitan
Palaemonetes pugio—southeast coast of U.S., and Gulf of Mexico.
Palaemonetes vulgaris
Palaemonetes intermedius
Pandalus danae—Pacific Northwest, including Alaska.
Pandalus hypsinotus—Pacific Northwest, including Alaska.

2) Suborder Reptantia

a) Section Macrura

Panulirus (spiny lobster)—Point Conception south along coasts of southern California and Baja California.
Homarus americanus—northeast coast of U.S.
Petrolisthes—U.S. coast of Gulf of Mexico.
Rithropanopeus harrisii—U.S. coast of Gulf of Mexico.
Panopeus herbstii—U.S. coast of Gulf of Mexico.

Menippe mercenaria—rock or reef areas of Florida.
Cancer productus—Pacific coast of U.S.
Cancer oregonensis—Pacific coast of U.S.
Cancer magister (see Section 10900, Plate 12:M)—Pacific coast of U.S.
Callinectes sapidus (see Section 10900, Plate 12:N)—southeast and Gulf of Mexico coasts of U.S.
Uca pugilator—southeast coast of U.S.
b. Freshwater decapods:
Cambarus—43 species, between Blue Ridge Mountains and Mississippi River (see Section 10900, Plate 12:H).
Procambarus—97 species, New England and Great Lakes to Mexico.
Orconectes—59 species, Maine to Texas, most in Central Basin.
Macrobrachium ohione—Atlantic coastal plain from North Carolina to Georgia, along Mississippi River from St. Louis southward, and Texas.
Palaemonetes kadiakensis—west of the Alleghenies from southern Ontario and Great Lakes to Gulf coast of northeastern Mexico.

2. Collecting Test Organisms

a. Marine and estuarine decapods: In shallow estuarine environments and tidal flats, collect juvenile or adult decapods with seine nets. Hold the collection within the net in very shallow water while gently dipping individuals from the water to a water-filled ice chest. Cool the water to be used for transporting the animals to slow activity and to increase the initial levels of dissolved oxygen. Air pumps with battery packs may be necessary to provide sufficient oxygen during transport, particularly if animal density is high. This method of collection is also applicable to gravid adults of some species (blue crabs, *Palaemonetes*, etc.), to hatch the larvae in the laboratory and conduct larval toxicity testing.

Lobsters and blue crabs (*Callinectes*) are best obtained from traps, but a scientific collecting permit may be required to capture and retain gravid females. Penaeid shrimp, *Pandalus* species, and other decapods are best obtained by using short-duration (10-min) otter trawls, so that the animals are subjected to less stress from crowding in the cod end. Rapidly transfer the animals into cool, well-aerated water in an ice chest. Avoid excessive crowding during holding on shipboard and during transportation to the laboratory. Provide aeration during transport.

Some crab species (*Rithropanopeus, Petrolisthes, Menippe, Cancer,* etc.) are best collected by hand at low tide, but it will be difficult to obtain a sufficient number of individuals to conduct tests with adults. These species are best suited for tests with larvae.

b. Freshwater decapods: While adults may be tested when a sufficient supply of individuals is obtained, preferably collect gravid females and use the offspring in testing.

3. Holding, Acclimating, and Culturing Test Organisms

Guidelines for the culturing of test organisms, including decapods, are found in Section 8010E.4. The following sections provide guidelines for collecting, handling, and obtaining larvae from crayfish, crabs (e.g., *Cancer magister*), American lobster (*Homarus americanus*), and shrimp (*Penaeus* and *Palaemonetes*).

a. Water supply: See Section 8010E.4*b*.

b. Acclimating, holding, and maintaining stock cultures: See Sections 8010E.3 and 4. Risks in handling most adult crustaceans usually are not great because of their rigid exoskeleton and general durability. Both larval and adult forms of many species are cannibalistic and readily attack each other in the soft-shell stage. Hold juveniles and adults in individual compartments in long troughs or divided tanks. Form the compartments with perforated separators that slide into slots on the sides. Use stainless steel for freshwater forms and glass, acrylic, plastic, or plywood covered with fiberglass for marine forms. Provide rigid, transparent covers to prevent loss of the highly motile specimens. Use perforated separators to ensure a flow of water through each compartment to remove metabolic products and provide DO. The crustacean growth process, which involves a periodic ecdysis or sloughing of the rigid exoskeleton, imposes a lack of uniformity in test animals that is not readily detectable in advance. In the pre-ecdysis stage and during ecdysis animals are heavily stressed and more sensitive to unsatisfactory environmental conditions and toxicants.

1) Crayfish—Collect specimens from their natural habitat by trapping, seining, or by hand (see Section 8010E.2). General procedures for holding and acclimating are described in Sections 8010E.3 and 4. Because crayfish are cannibalistic, hold all but the young stages in separate compartments. Suitable holding, acclimating, and culturing chambers are stainless steel, glass, fiberglass-covered wood, or plastic troughs, 180 cm long, 30 cm wide, and 20 cm deep, with a divider down the center to make two long troughs. Make shallow channels on the sides and central divider every 15 cm into which separators can be slipped to make 12 compartments on each side, each approximately 15 × 15 cm square and 20 cm deep. This size is suitable for crayfish. The number and size of compartments depend on the size and number of organisms to be tested. To hold a large number of small crayfish, remove the separators to make a tank of the desired length. Provide separators with a large number of perforations so they operate as screens. Control water depth in test chambers by a standpipe in the last compartment of the trough. When cleaning the separators, temporarily raise them a short distance from the bottom to allow excess food and wastes to be washed out and remove the standpipe in the last compartment to insure strong flows. Clean routinely with a siphon and a brush to loosen materials from compartments, screens, walls, and bottoms. Supply water adjusted to the desired temperature and DO to the two head compartments by a siphon from a constant-head box. Use a minimum flow of 10 trough volumes/d. Adjust volume to maintain favorable water quality in each compartment. Required water depth depends on size of organisms; 15 cm is preferred. Provide each set of troughs with a transparent lid. For life-cycle studies beginning with eggs or newly hatched young, collect ovigerous females and place in flow-through troughs under natural water conditions. Begin acclimation to different conditions after 2 d. Hold animals in troughs until young hatch. Remove compartment dividers to provide freedom of movement of young. Clean as described in Section 8010E.4*d*. Use macerated fish food for juveniles and adults. Alternatively

use prepared dry fish food. Use very finely divided pieces of fish and commercial fish food pellets as food for the newly hatched.

2) Crabs—Static culture of brachyuran crab larvae has been achieved for several species of Atlantic coast crabs.[13–16] Long-term static or renewal bioassays with these species have been performed. Culture of dungeness crab, *Cancer magister*, larvae has been reported.[17] Culturing crab larvae requires a favorable water supply and control of competitors, predators, and disease. Filter water and disinfect it by UV light treatment. For unpolluted open ocean water, little or no treatment is required. If the supply is from an estuary receiving organic wastes, purify before use. Filter seawater for the flow-through system by gravity flow through a coarse, quartz sand filter and adjust to the desired salinity, approximately 25 to 30 g/kg, by adding fresh water. To remove other organisms, refilter under pressure through sequential layers of 40/60-mesh garnet, 20/30-mesh silica sand, and 0.3-cm hard charcoal. Follow by filtration through a polishing filter and treat with UV light. Use constant-level head boxes equipped with heating, cooling, and stirring devices, to deliver constant measured flows by siphons, selected nozzles, or constant and accurate delivery pumps.

Collect ovigerous females or purchase from fishermen and place in holding tanks or in flow-through troughs similar to, but larger than, those described for crayfish. Acclimate and condition as described in Section 8010E.3. When eggs are ready to hatch, transfer females to static tanks provided with aerated and UV-disinfected water at 30 g/kg and 13°C. As eggs hatch, dip out swimming first-stage larvae with beakers and transfer to culture beakers with large-bore pipets. Many crab larvae are positively phototactic. Collection of crab larvae may be simplified by applying an intense light source to one side of the hatching container.

Dungeness crab larvae have long delicate spines that make their culture in flowing systems difficult. Culture larvae to the fourth and fifth stage in 250-mL beakers that have a hole 15 mm in diameter blown through their sides near the bottom. Using silicone cement, fasten a plastic screen having 360-μm openings over this hole on the inside of the beaker and plastic screen with 210-μm openings over the hole on the outside. Because of the lip created by blowing the glass, the two screens are 3 to 4 mm apart. The larger-mesh screen on the inside is less likely to catch and damage spines of larval crabs while the smaller-mesh screen on the outside does not come in contact with larvae but does retain food organisms, brine shrimp nauplii. Set the 250-mL beakers in glass trays or aquariums large enough to accommodate 10 beakers and provide a depth of at least 10 cm. Supply trays with a constant flow of water by a tube that discharges near the tray bottom. Provide an automatic siphon at the outlet so there is continual filling and drawdown (Figure 8740:1). Construct the automatic siphon so that when the water reaches the high point and the siphon is activated, the beakers contain approximately 200 mL, and when the siphon is broken the beakers contain about 150 mL. The automatic siphon consists of a silicone rubber stopper drilled to receive an 8-mm-ID right-angle glass tube on one end and a 5-mm-ID right-angle glass tube on the other end. In a 1.3-cm-diam hole blown through side of tray, insert stopper with 8-mm hole on inside of tray. Insert tubes into stopper as shown in Figure 8740:1. Placement of hole inside of tray controls water level in beakers at 200 mL. The distance between the top of the inside hole in the stopper and the bottom of the inside siphon leg is equal to the difference in depth between 200 mL and 150 mL. Make siphon intake perfectly flat and smooth to prevent air from being drawn into the siphon. Adjust tube diameters to give a 15-min cycle, 10-min filling and 5-min drawdown.

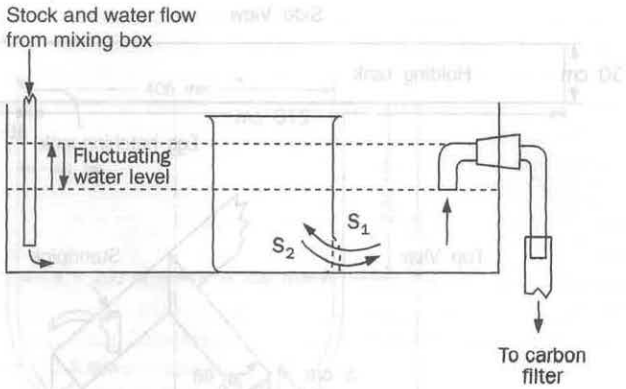

Figure 8740:1. Rearing and exposure beaker and automatic siphon for dungeness crab larvae. After BUCHANAN, D.V, M.J. MYERS & R.S. CALDWELL. 1975. An improved flowing water apparatus for culture of brachyuran crab larvae (unpublished).

When culture chambers are set up and functioning place 10 first-stage larvae in each beaker with a smooth large-bore pipet. The larvae can be fed nonliving food but preferably feed first-stage brine shrimp nauplii at the rate of 70 for each crab larva three times/week through the third stage, then 100 brine shrimp for each crab larva. The nutritional quality of brine shrimp will vary depending on source. This will affect the sensitivity of the larvae and the results of the test. Keep density of crab larvae low and that of food organisms high to minimize crab larvae contacts that may result in cannibalism. Before feeding, transfer larvae to clean chlorine-disinfected and rinsed beakers. Maintain a temperature of 12 to 13°C, pH 8, and a salinity of 25 to 30 g/kg. Adjust photoperiod to correspond with natural conditions or, if the cycle is off-season, to correspond to the normal annual cycle of light and dark. Exclude natural light and use fluorescent light (see Section 8010F.3*f*). Under these conditions, survival of 80 to 90% through the fourth zoeal stage has been attained. Larvae usually begin molting into the fifth zoeal stage by the 45th day. Mortalities then increase.

Juvenile and adult dungeness crabs are much less susceptible to disease than larvae. Older life stages are much less sensitive than larvae and will be more tolerant of conditions. With strict sanitation and unpolluted open-sea water, sand filtration alone provides sufficient water quality control. Hold juvenile and adult crabs in trough compartments similar to, but larger than, those used for crayfish. To allow sufficient space for each juvenile crab, use compartments 15 × 15 cm and 15 to 20 cm deep. For adult crabs use 30 × 30-cm or 40 × 40-cm compartments with a depth of about 30 cm. For large specimens use deeper water. For ease of supplying water, arrange troughs on stands having three shelves with space on each for two troughs. Feed cut-up or macerated fresh fish, clams, or mussels, or commercial dried fish foods to juveniles and adults. Remove unused food within 24 h to reduce fouling. Routinely clean sides and bottoms of compartments and remove wastes with vacuum or siphon cleaners.

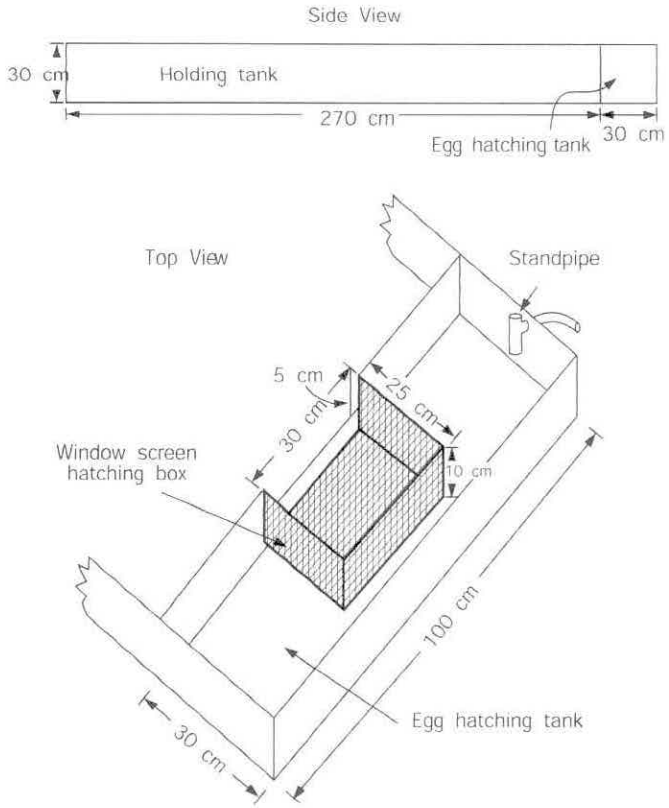

Figure 8740:2. Egg-hatching tank for lobsters.

Raise screen separators a few millimeters and flush as suggested for crayfish troughs.

3) American lobster, *Homarus americanus*—Obtain adult lobsters by trapping or purchase from lobster fishermen. Ovigerous females can be obtained most readily in early spring from lobster fishermen who have permits. Select females with brownish eggs because these eggs will hatch within a few weeks to a few months, depending in part on water temperature. Place each ovigerous female in a separate holding tank measuring at least 30 × 45 × 30 cm. The floor of the holding tank should have a raised grate that allows wastes to fall below the lobster; this helps prevent bacterial contamination of the egg mass. Fasten claws with elastic bands but not wooden pegs. Pass uncontaminated seawater continuously through tank at a rate that maintains DO at or above 80% saturation. Maintain salinity between 30 g/kg and that normal to seawater. Maintain temperature at 15°C. Feed brood lobsters once a week, preferably with crab or large krill, which are available frozen and provide the essential protein-binding carotenoid pigments. Other foods, such as commercial dry pelleted food, can be used for short periods of time. Use brine shrimp only for larvae and young juveniles.

When eggs are about to hatch, place ovigerous female in a hatching tank measuring 30 × 100 cm (Figure 8740:2). Place a partition across the lower end of the holding tank 30 cm from the end. Locate standpipe at one side of this box to maintain desired water level. Remove a piece 5 cm by 30 cm long from central portion of partition for outflow from hatching tank. Fit a screen box, 25 cm wide, 15 cm deep, and 39 cm long with a notch 5 by 30 cm in top of one side of the frame, against the notch in the partition. Use plastic window screen with 2-mm-square openings for the screen box. Supply with flowing seawater at a rate sufficient to maintain DO above 80% saturation. Maintain water temperature at 19 to 20°C.[18]

The larval development stages of *H. americanus* have been described.[19–22] The larval period extends from the time of hatching to the fifth molt or attainment of the fifth stage. Duration of the larval period depends somewhat on water temperature. During the first three stages, larvae are free-swimming, move toward light, and remain near the water surface. At the fourth stage the larvae are still photophilic but occasionally make excursions to the bottom from the surface and back. At the end of this stage the larvae become photophobic and become bottom-dwellers.

To rear larvae to various stages, construct from molded fiberglass a special 40-L culture tank for rearing larval stages with a combined water circulator and overflow device at the center (Figure 8740:3).[23] Only use flowing natural filtered seawater for culturing and testing lobsters.

The maximum concentration of lobster larvae is about 45/L. At higher concentrations there are more lobster-to-lobster contacts and cannibalism. As eggs begin to hatch, wash first-stage larvae over the 5 × 30-cm notch in the partition into the screen box. Dip larvae from this box with a small beaker and place in modified Hughes larvae rearing chamber by submerging the beaker and gently removing it.[22] Stock with less than 225 larvae. Because lobster larvae are cannibalistic, keep dispersed by currents and by providing many food particles for each larva. Best results are obtained by feeding newly hatched nauplii of brine shrimp to lobster larvae held in individual containers, under slow flow conditions. An automatic brine shrimp feeder has been described.[24] Techniques for rearing lobster to sexual maturity have been described;[25] such rearing is expensive and not recommended for routine toxicity testing.

By feeding live adult brine shrimp, a survival of 80 to 90% can be attained. When lobster larvae reach the fifth stage, place in individual compartments formed by placing separators in a trough as described for crayfish. For the fifth stage and juveniles, use 15- × 15- × 15-cm compartments. As lobsters grow use larger tanks. For those weighing 460 g or more, use a compartment 60 × 45 × 30 cm. Feed as recommended for ovigerous females. Ground, whole crabs improve coloration. During spring, summer, and fall, feed daily; during winter, feed once a week. After 24 h remove all unused food. Clean sides and bottom; siphon and flush out tanks. Growth rate depends not only on food and water quality but also on the holding tank size. Long before the lobster is physically restrained it reduces its growth in response to holding compartment size. During the first calendar year of life the lobster has an average of 10 molts. In nature, larval molting actively reaches a peak in the 15 to 20°C range; it seldom occurs below 5°C. Lobsters usually reach maturity at a weight of about 460 g. For mating, place a male in a compartment with a female immediately after she has molted and is in the soft stage. Success of mating decreases with time after molting. When temperatures of 22 to 24°C are maintained year round, lobsters reach maturity in 2 years. The rate of egg development depends in part on temperature. For extrusion of eggs, place females in a deeper tank because they need at least 45 cm of water over them. Provide the egg-laying tank with a rough or nonslip bottom that allows female to assume and remain in the egg-laying position until all eggs are laid and attached to the

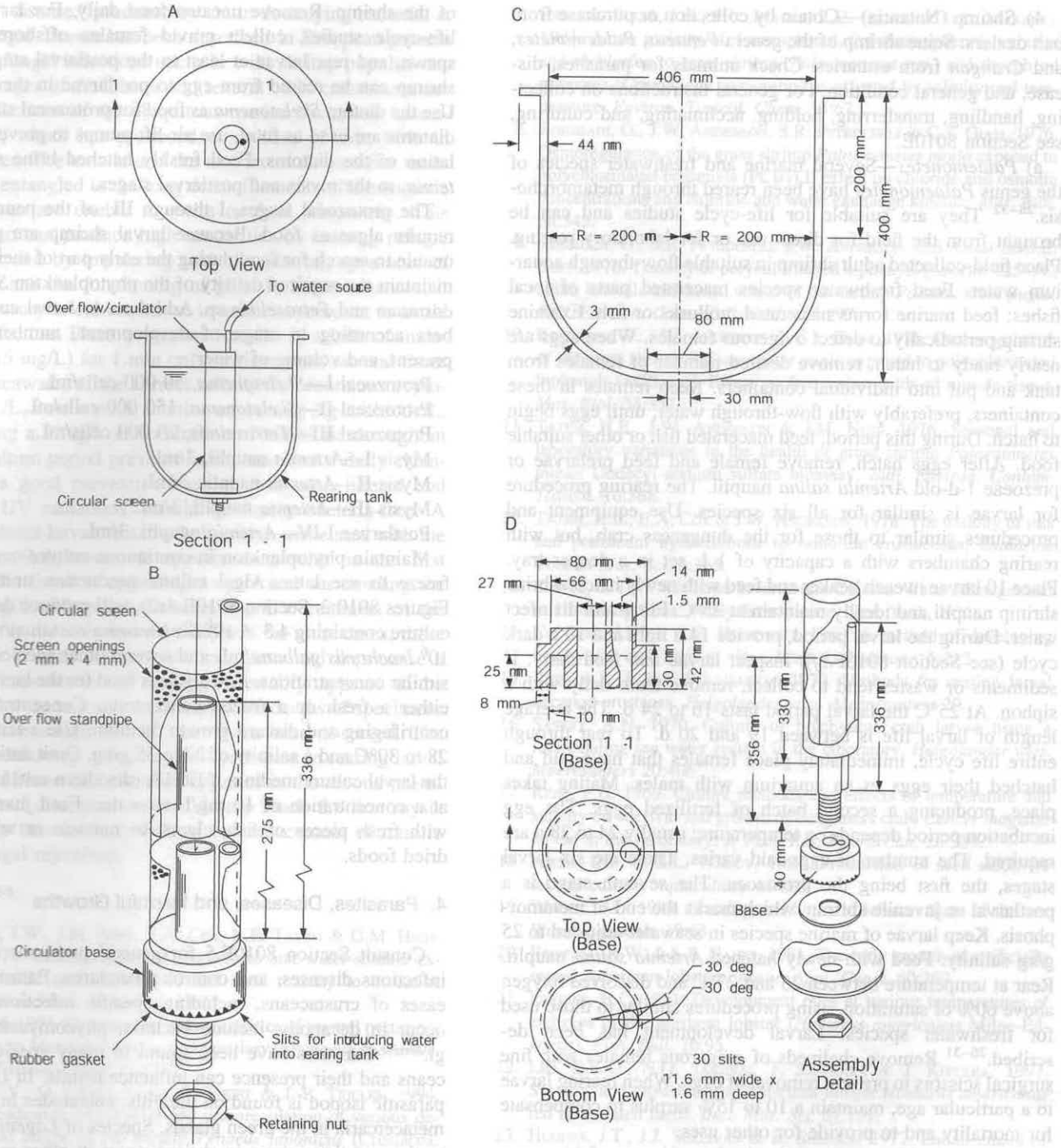

Figure 8740:3. Hughes lobster-rearing tank.[23] A—general views; B—views of overflow-circulator; C—details of rearing tank construction; D—construction and assembly details for rearing tank and overflow/circulator. This is a 40-L tank. For bioassays, scale to 5-L volume.

nonplumose hairs of the swimmerets. With stable temperatures, it should be possible to maintain larval cultures year round. Hold nonovigerous females and those bearing green eggs collected in the fall at low temperatures to retard development. Before eggs are needed, remove and gradually acclimate some females to egg-laying temperatures. Even when eggs have reached the brown stage, hatching can be spread out by different temperature regimes. Another method of producing larvae is to rear and mate lobsters in the laboratory at different times and under different temperature regimes. Although culturing in the laboratory is expensive it has certain advantages: larvae are produced on a year-round basis; larvae are of a known genetic constituency, which can reduce experimental variability; and complete-life-cycle tests can be conducted. A method is available to determine beforehand when lobster eggs will hatch. Once the eye pigment has been formed, monitor the course and rate of embryo development by measuring the eye periodically.

4) Shrimp (Natantia)—Obtain by collection or purchase from bait dealers. Seine shrimp of the genera *Penaeus, Palaemonetes*, and *Crangon* from estuaries. Check animals for parasites, disease, and general condition. For general instructions on collecting, handling, transferring, holding, acclimating, and culturing, see Section 8010E.

a) *Palaemonetes*—Several marine and freshwater species of the genus *Palaemonetes* have been reared through metamorphosis.[26–32] They are suitable for life-cycle studies and can be brought from the field for direct use or for laboratory rearing. Place field-collected adult shrimp in suitable flow-through aquarium water. Feed freshwater species macerated parts of local fishes; feed marine forms macerated mollusks or fish. Examine shrimp periodically to detect ovigerous females. When eggs are nearly ready to hatch, remove desired number of females from tank and put into individual containers. Keep females in these containers, preferably with flow-through water, until eggs begin to hatch. During this period, feed macerated fish or other suitable food. After eggs hatch, remove female and feed prelarvae or prezoeae 1-d-old *Artemia salina* nauplii. The rearing procedure for larvae is similar for all six species. Use equipment and procedures similar to those for the dungeness crab, but with rearing chambers with a capacity of 1 L set in a deeper tray. Place 10 larvae in each beaker and feed with newly hatched brine shrimp nauplii and ideally maintain at 25°C. Filter and disinfect water. During the larval period, provide 14 h light and 10 h dark cycle (see Section 8010F.3*f*). Inspect larvae and feed daily. If sediments or wastes tend to collect, remove them daily with a siphon. At 25°C the larval period lasts 16 to 24 d. The average length of larval life is between 19 and 20 d. To rear through entire life cycle, immediately place females that have laid and hatched their eggs in an aquarium with males. Mating takes place, producing a second batch of fertilized eggs. The egg incubation period depends on temperature; usually 24 to 28 d are required. The number of eggs laid varies. There are six larval stages, the first being the protozoea. The seventh stage is a postlarval or juvenile shrimp, which marks the end of metamorphosis. Keep larvae of marine species in seawater adjusted to 25 g/kg salinity. Feed with newly hatched *Artemia salina* nauplii. Rear at temperature between 23 and 27°C and dissolved oxygen above 60% of saturation, using procedures similar to those used for freshwater species. Larval development has been described.[26–31] Remove chelipeds of ovigerous females with fine surgical scissors to prevent removal of eggs. When rearing larvae to a particular age, maintain a 10 to 15% surplus to compensate for mortality and to provide for other uses.

b) *Penaeus*—The rearing and culturing of larvae of this genus have been described in several studies.[33–39] Hold shrimp in glass tanks of at least 30-L capacity. Provide each tank with flow-through water, 2 to 3 cm of sand over the bottom, and a screen over the top to prevent the shrimp from jumping out. Avoid overloading. Keep no more than 22 to 24 animals in a 30-L tank. For *Penaeus* spp., use a minimum flow of 7.5 L/g/d. Flows up to 22 L/g/d may be desirable to insure DO above 60% of saturation and the removal of metabolic products. Acclimate to laboratory test conditions for about 2 weeks. For short-term or medium-length tests with adults and juveniles, shrimp can be field-collected. Cut-up fish is a satisfactory food. Cut a fillet from mullet, grouper, or other abundant species into 1- to 2-cm pieces. Feed one piece per shrimp each 2 to 3 d, depending on the size of the shrimp. Remove uneaten food daily. For larval tests or life-cycle studies, collect gravid females offshore, let them spawn, and rear larvae at least to the postlarval stage. Penaeid shrimp can be reared from egg to postlarvae in the laboratory. Use the diatom *Skeletonema* as food for protozoeal stages. When diatoms are used as food, use air-lift pumps to prevent accumulation of the diatoms. Feed freshly hatched brine shrimp, *Artemia*, to the mysis and postlarval stages.

The protozoeal stages, I through III, of the penaeid shrimp require algae as food. Because larval shrimp are pelagic and unable to search for food during the early part of their life cycle, maintain the required density of the phytoplankton *Skeletonema costatum* and *Tetraselmis* sp. Add these to larval culture chambers according to stage of development, number of larvae present, and volume of water:

Protozoeal I—*Skeletonema*, 50 000 cells/mL
Protozoeal II—*Skeletonema*, 150 000 cells/mL
Protozoeal III—*Tetraselmis*, 20 000 cells/mL
Mysis I—*Artemia* nauplii, 3/mL
Mysis II—*Artemia* nauplii, 3/mL
Mysis III—*Artemia* nauplii, 3/mL
Postlarvae I-IV—*Artemia* nauplii, 3/mL

Maintain phytoplankton in continuous culture or harvest and freeze to use later. Algal culture production units shown in Figures 8010:2, Section 8010E.4*c*2), will produce daily 7.5 L of culture containing 4.3×10^6 *Skeletonema costatum*/mL or 7.0×10^6 *Isochrysis galbana*/mL and several other species of algae at similar concentrations. Add algae as food for the larval shrimp as either a fresh or a frozen concentrate. Concentrate algae by centrifuging and discard growth medium. Use a temperature of 28 to 30°C and a salinity of 27 to 35 g/kg. Omit antibiotics from the larval culture medium if EDTA (disodium salt) is substituted at a concentration of 10 mg/L seawater. Feed juvenile shrimp with fresh pieces of fish, clams, or mussels or with prepared dried foods.

4. Parasites, Diseases, and Harmful Growths

Consult Section 8010E.5 for general problems of parasites, infectious diseases, and control procedures. Parasites and diseases of crustaceans, including specific infections known to occur in decapods, include bacteria, phycomycetes, and fungi.[31–46] Parasites have been found in many species of crustaceans and their presence can influence results. In *Uca*, an ectoparasitic isopod is found on the gills, nematodes in the gut, and metacercaria in the green glands. Species of *Lagenidium* similar to the one that occurs in shrimp occur in other marine crustaceans. *L. callinectes* occurs in eggs and larvae of the blue crab. The blue crab has a barnacle (*Octolasmus lowei*) living in association with its gills and gill chamber, metacercariae in various organs, and the sacculinid *Loxothylacus taxanas* beneath its abdomen. *Saprolegia parasitica* attacks larvae of the shrimp *Palaemonetes kadiakensis*.

Adult shellfish in recirculated or flow-through systems are susceptible to biotoxins and pathogens. Remove metabolites and dead individuals from recirculating systems. Juvenile and adult lobsters, crabs, and shrimps are subject to bacterial and fungal infections. *Gaffkya*, a bacterial pathogen, is particularly prevalent in tank-held lobsters, while *Vibrio* disease occurs in tank-held postlarval adult shrimp. Most captive crustaceans are sub-

ject to "shell disease," produced by chitin-destroying bacteria. A systemic fungal disease has been described in European prawns and several fungal infections occur in wild shrimp populations. The larval stages of the lobster and several other crustaceans are prone to infections of the ubiquitous marine bacterium *Leucothrix mucor*, which has produced mortalities of over 90% in larval cultures. The exuvia and the new exoskeleton after molting become entangled in the long dense filaments of the bacteria and the larvae are unable to swim or feed adequately. This organism also can produce high mortalities by causing pelagic eggs to sink and by interfering with the filtering apparatus of larval forms and the functioning of gills. In some instances, it may be necessary to culture larvae in artificial seawater to avoid *L. mucor* infection. Place ovigerous females in a bath of malachite green (5 mg/L) for 1 min only or rinse them several times in artificial seawater of the correct salinity that contains streptomycin, 2 mL/L, from a stock solution containing 2 g antibiotic/L.

Maintaining a 1-mg/L concentration of antibiotic throughout the larval culture period prevents infections. Twice daily cleaning also is a good preventive method. Seawater, filtered and exposed to UV radiations, should be nearly bacteria-free. A disease of lobster larvae tentatively has been associated with the phycomycete *Haliphtorus*. It appears as a scab on the first segment of the thoracic appendages up to and surrounding the first row of gills. Thorough cleaning and UV treatment of the water supply is the only known treatment. In most cases, these scabs adhere to both old and new carapaces and thus cause a mechanical impediment to molting. Mortality appears to be restricted to larvae and young juveniles. No deaths of specimens with a carapace length over 27 mm have been observed. The fungus *Lagenidium* sp. causes serious problems in rearing larval shrimp. The disease first becomes apparent in the second protozoeal stage and disappears as the shrimp reach the first mysis stage. Shrimp become immobilized by replacement of muscle tissue by fungal mycelium.

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8740 C. Toxicity Test Procedures

A basic discussion of toxicity testing, including terminology and basic procedures, is presented in Section 8010. The types of tests are described in Section 8010C and the general recommendations for the type of facilities, equipment, water, and food are discussed in Section 8010E. In Section 8010F the appropriate materials for constructing a test system are described. Also important in this section is a discussion of the toxicant measurements necessary to assure that organisms are exposed to the calculated concentrations. Section 8020B describes the need for use of a reference toxicant as part of the test procedure. A reference toxicant is valuable for determining both the health of the test organisms and the ability of the analysts to reproduce the results of previous reference toxicity tests with the same species and conditions. Section 8020B also discusses the importance of controlling the exposure concentrations during toxicity testing.

Specific procedures for conducting toxicity tests with crayfish, crab larvae, adult, juvenile, or larval lobster, and adult or larval shrimp are described below.

1. Toxicity Test Procedures Using Larvae or Postlarvae of Crabs or Shrimp

a. General procedures: Read and understand the basic procedures and concepts described in Section 8010 before initiating tests. Conduct preliminary tests to become familiar with the specific organisms and test procedures given below.

1) Collecting adult decapods—Depending on the species, gravid adult decapods may be collected either by trap, otter trawl, seine net, or by hand at low tide (see Section 8740B.2). Small crab species may be found under rocks, while larger crabs may hide in dense marine grasses. Females with eggs (sponge)

may be observed with yellow to brown egg masses in a brood pouch (shrimp), or extending from the undersurface (crabs and lobsters). Because there are hundreds to millions of eggs per female, only a few gravid animals will be sufficient to conduct a toxicity test. Careful handling and decreased temperature will decrease the possibility of the females releasing the eggs during transport, before the exposures are ready.

2) Collecting larval or postlarval stages—Before attempting to raise larval decapods to postlarval stages, determine if this or a related species is being reared at a commercial or government facility for mariculture/aquaculture. Staff members at these facilities have the appropriate expertise to answer questions, and it is often possible to purchase or obtain the needed test organisms from a facility normally dealing with mass cultures. See Section 8740B.4 for procedures related to the acclimating, holding, and maintaining of stock cultures.

2. Static, Short-Term, Early-Life-Stage Test

As an example of a test system that may be used with several different species, the following is a toxicity test procedure that has been used with embryos of the blue crab, *Callinectes sapidus.*[1]

a. General procedures: Conduct preliminary tests to become familiar with the test procedures.

1) Collecting adult female *Callinectes sapidus* with sponge (embryos)—Collect *Callinectes sapidus* with sponge in crab traps or buy from local fishermen. They are available from March through October in the southeastern United States. Sponges that are bright yellow are preferred (Stage 3 embryos). Later stages include orange (Stage 6) and red-brown (Stage 7). Each sponge has 2 to 3 million embryos.

2) Collecting embryos from the sponge of *Callinectes sapidus*—Using forceps, remove pieces of sponge from a sponge-carrying female and shake pieces gently in a beaker of seawater (salinity between 18 and 33 parts per thousand). Take up embryos (Figure 8740:4) shaken from the sponge with a pipet and transfer to culture plates containing natural seawater. Embryos that are Stage 6 or younger can be kept in seawater at 3°C in the refrigerator for up to 1 month. When needed for toxicity tests, bring embryos to room temperature (20°C).

3) Exposure chambers—Use sterile 24-well polystyrene culture plates (well diameter 16 mm).

b. Conducting the toxicity tests: Add 10 embryos to each well. Add toxicants dissolved in water or 1 μL solvent (ethanol or acetone) but do not exceed aqueous solubility. For solvent controls use 1 μL solvent. Use five replicates for each concentration. Add 2 mL seawater to each well. Incubate plates at 28°C in the dark and examine each day until zoea (hatching stage) emerge from the egg sacs. Determine hatching by checking embryos each day under a dissecting microscope. Stage 3 embryos take approximately 7 d at 28°C to hatch. Stage 6 embryos take about 4 d to hatch. The zoea (Figure 8740:5) emerge over a 12-h period from Stage 9.

c. Interpreting results: Calculate EC50 values using probit analysis procedures, after counting the number of emerged zoea in control and toxicant treated wells. Hypothesis testing procedures (Dunnett's Test, etc.—see Section 8010G) may be used to estimate the NOEC and LOEC. An additional end point that may be of value is the concentration that produces a 25% inhibition in normal survival (IC25).

3. Long-Term Tests in Flowing Exposure Systems

a. General procedures: Become familiar with the test species and procedures described below before conducting a test with an effluent or specific toxicant.

b. Collecting and holding animals: See Sections 8740B.2 through 4 for information on collecting and holding the organism (crab, shrimp, lobster) selected for testing. Static systems (closed aquariums) can be used to hold decapods either before testing or to obtain larvae, but the water must be changed frequently, depending on the number of individuals and size of animals in each aquarium. Monitor ammonia levels in the water to determine when to renew the water. Avoid excess feeding, which leads to water cloudiness from bacterial growth. A flowing clean seawater system, where the water is completely replaced in 24 h (about 7 tank volumes/d), is ideal for holding decapods.

c. Preparation of exposure system: Figure 8740:1 shows apparatus that is also appropriate for use in the flowing-water exposure of fresh water or marine decapod larvae or postlarvae. These trays, which fill and empty as a function of the flow rate and the high and low levels of the automatic siphon, may be constructed to hold several partitions or several beakers with

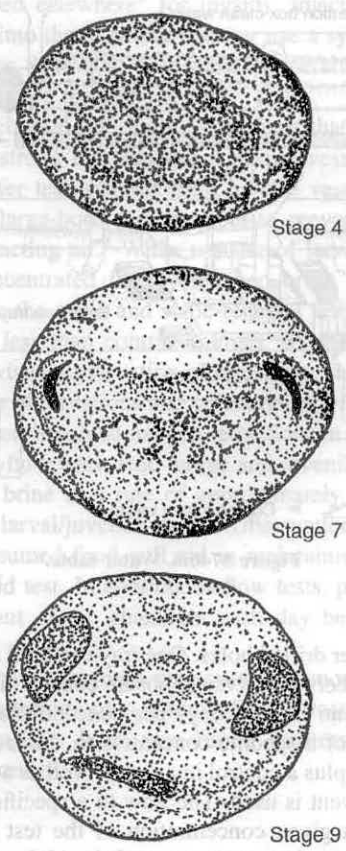

Figure 8740:4. Crustacean embryos.

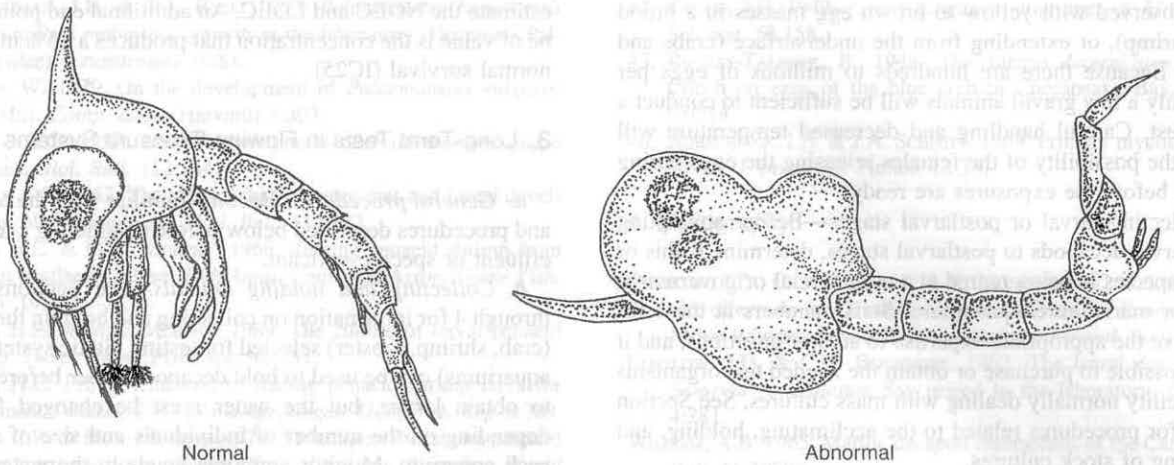

Figure 8740:5. Crustacean larvae.

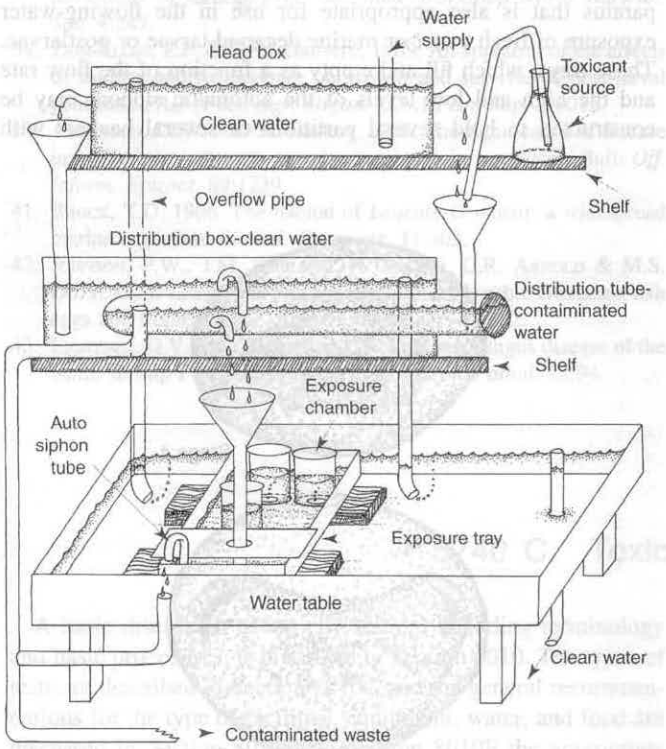

Figure 8740:6. Water table.

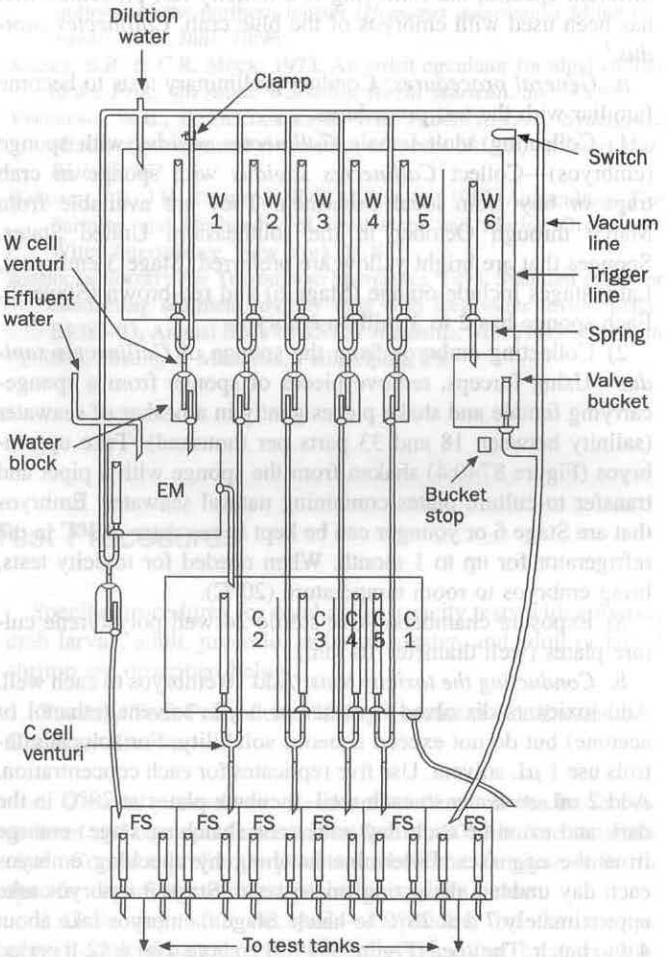

Figure 8740:7. Proportional diluter. Source: LEMKE, A.E., W.R. BRUNGS & B.J. HALLIGAN. 1978. Manual for Construction and Operation of Toxicity-Testing Proportional Diluters. EPA-600/3-78-072, U.S. Environmental Protection Agency, Duluth, Minn.

nylon mesh over drilled holes. One tray can hold all the replicate exposure chambers of a given exposure concentration, or if space permits there can be two trays per concentration, representing true replicates of a specific concentration. Include five toxicant concentrations plus a control in a test as well as a solvent control if a carrier solvent is used. The flow to a specific tray therefore must contain a given concentration of the test substance produced by mixing clean seawater and the high concentration of the toxicant (or full-strength effluent). Each tray (or set of

replicate trays) must receive one of the six concentrations of test material (including control), and the same total flow rate of water.

Two types of exposure systems have been used frequently. In a simple system, a Mariotte bottle of toxicant is placed above a head-box receiving a flow of clean water.[2] The head-box has an overflow tube to keep the head of water and flow rate stable, and the main flow from the box goes to the delivery box. As Figure 8740:6 shows, the slow drip from the bottle enters the flow of clean water so that mixing will occur before, and as, this water enters the delivery box. This long, narrow delivery box or tube receiving the high concentration of toxicant can be fitted with stoppers and glass tubing that exit the stopper and then make a 90° angle, paralleling the tube. The outer portion of the tubing then is bent to curve downward. When all stoppers and tubes are in place and the delivery box is receiving test solution, the tubes can be bent downward, such that the water begins to flow out the end. A funnel held in place over the exposure tray receives this water at a given rate from the test contaminated water supply, and a similar system (with larger tubing and higher flow rates) can be used to introduce the clean water to the same funnel. The flow rates of both systems can be regulated by the size of the tubing and the direction downward of the end of the tubing. The total flow rate in each tray must be the same (±15%), and the concentrations of the exposure dilutions should vary by a factor of approximately 0.5 (100%, 50%, 25%, etc.).

A second exposure system is the Brungs-Mount or Mount-Brungs diluter (Figure 8740:7). Another description and diagram of the system are found elsewhere.[3] Once constructed, this system will deliver six concentrations of contaminant to two replicate tanks, as long as needed.

d. Conducting the test: Expose postlarval decapods in beakers that are placed on trays, which receive the combined flow of clean and contaminated water. The total flow may be about 1 L/min, but the flow into and out of the beakers depends on the rise and fall of the water level in the tray. Each beaker will have a hole drilled by a diamond-hole drill near the bottom, with a nylon-mesh cemented (silicone) over the hole. Use a glass tube bent to form an automatic siphon, placed in the front of the tray, where the effluent can be captured and sent to the waste treatment system. Adjust tube length to govern the upper and lower levels of water in the tray and beakers; incoming total flow rate (funnel) will determine the number of fluctuations per day. For a standard test use two replicates (trays) of each inflowing concentration, and use at least four concentrations (preferably five) plus a control. NOTE: The beakers in a tray are not true replicates. If 10 or 12 trays are to be used, they must be relatively narrow and preferably hold only about 10 beakers. Depending on the size of the eggs, larvae, postlarvae, or juvenile, there may be from 1 to 5 animals per beaker. Use at least 10 animals per replicate to conduct a toxicity test. With this type of exposure system, the primary concerns are checking and regulating the flow rates of the clean and contaminated water about twice per day, and ensuring that inflow of the toxicant to the contaminated water is stable. Use these same time periods to count living and dead organisms and to observe any behavioral abnormalities. This type of test can proceed for 30 d or more if, for example, the purpose is to determine effects of a toxicant on the hatch, growth, and survival of larvae. The Mount-Brungs diluter system is another exposure method that can be run for 10 to over 30 d, given the supply of clean water and the volume of toxicant required. Procedures for more difficult conditions, such as studies of petroleum hydrocarbons, are available in the literature.[4–7]

4. Toxicity Test Procedures Using Larvae and Postlarvae of the American Lobster

a. General procedures: Conduct preliminary tests to become familiar with the test procedures given below.

Never hold lobster larvae and juveniles communally during a toxicity test. Cannibalism and wounds will affect the results of the test. Hold larvae and juveniles in individual glass* or inert plastic beakers or culture dishes for static or static/renewal tests. Choice of test container material will depend on the chemical makeup of the substance to be tested. Generally, glass is used for testing organics and inert plastic for inorganics. Use a seawater volume of 100 mL or more for each container at 28 to 32 g/kg salinity. Pre-dose containers with toxicant before adding test animals.[8] Keep test temperature within ±1°C of the temperature of the culture vessel seawater in which the test animals were reared or held. Ideal temperature is 20°C, which is the optimum seawater surface temperature for larval hatching and growth in nature. If necessary, hold individual test containers in an incubator or circulating water bath to maintain ideal temperature.

For continuous-flow testing, hold larvae and juveniles in individual nylon-screened† containers or perforated plastic tube containers in larger dosing boxes.[9] Use a testing system similar to that described elsewhere[9] for mysids. Inject toxicant from dosing pumps into the seawater lines or use a system of siphon diluters (Figures 8740:6,7). Use same temperature and salinity range recommended for static testing.

For testing, choose only vigorous animals that swim near the surface in the strong current of the culture vessel. For all test methods, transfer test animals from culture vessel to test containers with a large-bore capture pipet to prevent the animals' gills from contacting air.[8] When transfer of larvae is complete, add 1 mL concentrated freshly hatched brine shrimp nauplii (±500) daily to the static and static-renewal tests. Test animals generally feed less than control animals, so adjust feeding according to the visual observation of remaining nauplii in the test chambers. Such observations and adjustments will alleviate ammonia buildup or dissolved oxygen reductions in test containers. For continuous-flow tests, feed larvae and juvenile lobsters with thawed frozen brine at a rate of approximately 5 to 20/d, depending on the larval/juvenile state.[10] Observations of remaining or quickly consumed food will aid in maintaining healthy animals and a valid test. In continuous-flow tests, preferably pipet uneaten food out of the chambers each day before additional food is added.

Use no less than 10 animals per concentration with four or five toxicant concentrations plus control. Repeat tests at least three times, using animals from new brood lobsters each time to ensure correct results.

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* Pyrex or equivalent.
† Nytex or equivalent.

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8740 D. Data Evaluation

1. Calculating the Results

Section 8010G describes methods for calculating, analyzing, and reporting results of toxicity tests. In acute toxicity tests, the LC50 or EC50 values may be determined by probit, linear interpolation, or even graphical methods, which also may provide the 25% effects concentration (EC25). Computer programs* not only provide these values, but present the 95% confidence limits. Chronic tests may produce EC25 and EC50 values; also use hypothesis testing (Dunnett's, etc.) to produce NOEC and LOEC values. Most available computer programs will first test the data for assumptions of normality, and also test that the

* Such as ToxCalc, TOXSTAT, and EPA programs.

variances of the different treatment groups are homogenous. These criteria should be met before conducting hypothesis testing to determine NOEC and LOEC values. Transform percentage data by arcsine square-root transformation before using Dunnett's test. Transformations of quantitative data (log, square-root, etc.) as number of larvae, or weight of larvae often are useful in helping the data meet assumptions of normality and homogeneity of variance.

2. Reporting the Results

See Sections 8010G.2 and 8010H.

8750 AQUATIC INSECTS*

8750 A. Introduction

1. Ecological Importance

Aquatic insects are important components of lake and stream biota.[1–3] In trout streams, they comprise 50 to 90% of the macroinvertebrate species. Such groups as mayflies, stoneflies, caddisflies, and midges are major food items for many species of fish.[1,4] Aquatic insects may be more sensitive to certain pollutants than are fish.[5,6]

2. Suitability for Toxicity Tests

The wide variety of aquatic insects, their abundance in unpolluted streams, their sensitivity to low concentrations of pollutants, and the ease of maintaining many species under laboratory conditions make them useful test animals. Procedures using aquatic insects have been developed for determining acceptable environmental conditions or concentrations of toxicants.[2,7] Most studies have been short-term, but procedures are available for long-term tests.

Toxicants may interfere with survival, growth, reproduction, emergence, and metabolism of aquatic insects. Because effects of long-term exposure to sublethal concentrations of toxicants may be more relevant than effects of infrequent short-term exposure to higher concentrations, flow-through, long-term tests are recommended for these applications.

* Approved by Standard Methods Committee, 2000.
Joint Task Group: 20th Edition — Donald G. Huggins (chair), E. F. Benfield, Robert E. Morcock, Vincent H. Resh.

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8750 B. Selecting and Preparing Test Organisms

1. Species Selection

Use insects that are important food for fishes, readily available and abundant, relatively easy to keep and culture in the laboratory, and most sensitive to the materials under investigation.

a. Suggested test organisms:

1) Stoneflies (See Section 10900, Plate 13:A–D.)
Pteronarcys dorsata
Pteronarcys californica
Hesperoperla lycorias
Hesperoperla pacifica
2) Mayflies (See Section 10900, Plate 13:E–I.)

Hexagenia bilineata
Hexagenia limbata
Hexagenia rigida
Ephemerella subvaria
3) Caddisflies (See Section 10900, Plate 15:H–K.)
Brachycentrus americanus
Brachycentrus occidentalis
Clistoronia magnifica
b. Other species that have been used:
1) Stoneflies
Isogenus frontalis
Perlesta placida
Paragnetina media
Phasganophora capitata
Acroneuria californica
2) Mayflies
Ephemerella cornuta
Ephemerella grandis
Ephemerella doddsi
Ephemerella needhami
Ephemerella tuberculata
Stenonema ithaca
3) Caddisflies
Hydropsyche betteni
Macronemum zebratum
Arctopsyche grandis
Hydropsyche bifida
4) Diptera (See Section 10900, Plate 16:A–B.)
Chironomus plumosus
Chironomus attenuatus
Chironomus tentans
Chironomus californicus
Glyptochironomus labiferus
Goeldichironomus holoprasinus
Tanypus grodhausi
Tanytarsus (paratanytarsus) dissimilis

For each test, use early instar larvae or nymphs when possible, especially for growth studies. Of the listed species, only chironomids complete a generation in one summer. Use late instars for adult emergence tests. For all tests, insects that are cultured are preferable because their source and history are known.

2. Collecting Test Animals

When cultured animals are not available, collect all test specimens from clean, natural waters rich in aquatic insects (see Section 10500, Benthic Macroinvertebrates). Collect larger stream species from riffle areas of clean, well-aerated gravel rubble streams with hand screens or bottom samplers. Stir bottom and let current carry dislodged insects downstream into net.

Immediately after collection, gently place net contents in a 15- to 20-L insulated container partly filled with stream water. Transport to laboratory. Remove and discard larger rocks after it has been determined that they are free of insects. If transportation time exceeds 30 min, provide for aeration and temperature control. In laboratory, swirl water in containers and dip it out. Pour through a screen-bottom container (of a mesh that will retain insects required), held partly submerged in a tank of water. Wash screenings into a holding tank. If it is desired to separate insects, wash into a large white enamel pan containing 3 to 5 cm of water. Remove desired species with a large-bore pipet or small spoon-shaped screen and place in holding tanks. For riffle insects use oval or round flow-through tanks[1] provided with rocks for cover and paddle wheels to provide a current in dilution water.[1] Alternatively collect insects by gently picking up rocks, rubble, or gravel, and carefully washing or picking, then placing desired insects in insulated containers for transport to laboratory.

To obtain benthic insects, sample bottom materials with Eckman, Petersen, or Ponar dredges. Empty dredge into a large pail, add water, and swirl by hand. Partly submerge an appropriate mesh washing screen, pour a portion of swirling sample into it, and wash by moving up and down in the water. Place washed insects in an insulated container and continue until enough insects have been collected.

Chironomids probably will be the dominant insect species in silt bottom material. However, other important immature insects such as dragonflies, damselflies, several species of Diptera, beetles, and mayflies may be found in and on silt bottoms. The mayfly, *Hexagenia limbata*, is a large species often occurring in great abundance in soft, unpolluted muds rich in organic matter that occur in deep pools, ponds, lakes, and reservoirs. Obtain these by collecting top 8 cm of mud and washing as described previously.

3. Holding, Acclimating, and Culturing

a. General considerations: As soon after collection as possible, examine insects for injury. Place all uninjured specimens in holding chambers, supply them with food, and hold for at least 1 week for observation and acclimation to desired temperature. Acclimate stream species in flowing water. Keep in oval troughs that have a current of water or in stainless steel wire cages in running water.[1] In these troughs include flat stones covered with attached algae as cover and food for herbivorous species. Supply insects with materials to build larval and pupal cases. For caddisflies, use sand grains, small pieces of wood, and plant materials retained by a 16-mesh screen. Permit insects that construct tubes or cases to do so. Hold benthic species in aquariums provided with a 3- to 5-cm layer of unsterilized mud from the site where they were collected. *Hexagenia* require a substrate in which to burrow.[2] For chironomids use the highly organic ooze that overlies the bottom where they were collected. Alternatively, either silica sand or shredded paper toweling may be used as a substrate for chironomid larva.[3,4]

Provide water, DO, and other conditions as described in Section 8010E and F. Maintain final holding temperature within 3°C of temperature at which organisms were collected. For long holding periods, maintain natural seasonal temperatures. When aquatic insects are collected in winter at water temperatures of 1°C or lower, acclimate them to higher temperatures if they are to be used in short-term tests (Section 8010E.3).

Different species require different light intensities. Stoneflies require stones under which they can hide from direct light. Fix light cycle at a certain day length, or vary it seasonally to correspond with natural annual photoperiod. For *Chironomus plumosus*, use a 16-h photoperiod. Lamps and fixtures are described in Section 8010F.3*f*.

b. Food and feeding: Acroneuria, Isogenus, and *Paragnetina* are predators requiring live food. Feed to excess with small midges, blackfly larvae, mosquitoes, or small caddisfly larvae

from an unpolluted environment.[2] Feed *Pteronarcys* and *Ephemerella* to excess with coarse, chopped maple, birch, or aspen leaves that have fallen naturally and have been dried and then soaked in test water for at least 2 weeks before feeding. Feed *Hexagenia, Hydropsyche,* and *Arctopsyche* finely ground leaves and fish-food pellets. If the substrate is rich in organic matter, additional food may not be required for *Hexagenia.* Avoid overfeeding with fish food because it causes DO depletion. The larvae of some Hydropsychidae are highly carnivorous and cannibalistic; keep them well-fed with plankton, microcrustacea, blackfly larvae, and other organisms, collected from fish hatcheries, ponds, lakes, and streams with a net of No. 20 bolting silk.

Feed chironomids twice per week. Keep in jars supplied with algal culture medium [Section 8010E.4*c*1)a)] inoculated with algae including diatoms. Alternatively use a mixture of 5 g fish food plus 1 g powdered dried cereal grass* blended in 1 L of water. Add about 100 mL of this suspension to each culture per feeding. If there is no flow-through, remove 100 mL of test solution before feeding. Use 10-L culture jars containing 8 L or less of medium with a screen cover to retain adults.[5,6] Keep in a constant-temperature room at 21 to 24°C. For long-term studies follow natural temperature cycle of water from which chironomids were taken. Because the jars have a mud substrate, do not clean them or overfeed the organisms. Collect emerging adults for breeding in wire screen cylinders placed over the culture jars.[7,8]

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* Cerophyl®, Agri-Tech, Inc., Kansas City, MO 64112, or equivalent.

8750 C. Toxicity Test Procedures

1. General Procedures

Conduct tests as described in Section 8010D. If possible, use a minimum of 20 specimens for each toxicant concentration with an additional 40 animals for growth studies. Two species may be tested in the same tank if precautions are taken to avoid predation.

Do not use static testing with stream insects unless air stones or water movement can simulate natural water conditions. Use static tests with certain lake or reservoir species if required DO levels are maintained. For long-term tests, see Section 8010D.

a. Test tanks: Use glass and stainless steel aquariums of either 8-L or 20-L size for quiet-water species. For stream species, use round or oval, stainless steel or epoxy-painted troughs[1–3] (90 cm long, 15 cm wide, and 15 cm deep) in which natural stream flow is simulated. Set tanks side by side so paddle wheels on one long shaft can be used to circulate water in them all.[1] Jetted incoming water from the diluter also can maintain adequate flow.

b. Flow rate: Use flows to each tank of no less than 6 to 10 tank volumes/24 h. In aquariums without water-circulating devices use much higher flows for stream species to simulate stream flow. In oval test tanks use velocities near 0.5 cm/s. For quiet-water forms, such as *Hexagenia* and *Chironomus*, do not disturb mud substrate with water flow.

c. Aeration: Aeration is unnecessary; however, use if desired with nonvolatile toxicants to increase or control water movement, especially for tank tests with lake and reservoir species or if DO levels drop.

d. Cleaning: See Section 8010E.4*d*. Siphon out detritus on tank bottom weekly during long-term testing. If a mud substrate is used, no cleaning is necessary. Avoid overfeeding.

e. Substrate: For all stream riffle species use fine-mesh stainless steel screens formed into cylinders or cubes, which provide 10 to 15 cm^2/insect. Place cages in oval troughs or in glass cylinders.[1] For 30- to 90-d adult emergence tests, obtain clean rocks, 5 to 10 cm in diameter (one for every three insects) from

collection site for a substrate. Provide fine screen or sticks that protrude above water surface for adult emergence tests.

f. Light and photoperiod: See Section 8010F.3*f*. Use natural photoperiod at time of testing for locality in which test is conducted. Increase day length during adult emergence tests by 0.5 h every 2 weeks.

g. Temperature: See Section 8010F.1*b*. Use 10°C as a winter temperature. For trout stream insects, use summer temperatures near 15°C. Increase temperature during adult emergence tests by 1°C each week up to a maximum of 5°C above initial temperature. When using warm-water stream or lake insects, follow natural temperature cycle.

h. Time of year: Under natural conditions, most species emerge as adults in spring. Therefore start adult emergence tests no later than March 1st. *Hexagenia limbata* and most midge species are exceptions, emerging throughout summer in most localities.

2. Toxicant Preparation

See Sections 8010F.1 and 2*b*.

3. Test Procedures for *Hexagenia*

Use *Hexagenia* for short-term survival (96 to 168 h), survival for 5 to 60 d, adult emergence, or full-life-cycle tests (90 to 120 d). Use a minimum of 20 organisms per aquarium of not less than 8 L capacity. Use a water depth of 8 to 20 cm. Provide a fine organic ooze substrate 4 to 5 cm deep and as similar as possible to that where naiads occur naturally. When using newly hatched *Hexagenia* to start a test, use 50/tank. When *Hexagenia* eggs are used as a source of larvae, pipet them into petri dishes (about 200/dish) with 200 mL test water at about 20°C, and let hatch.

When substrate is mud, determine survival by counting number of dead animals that have left their burrows and/or by counting number of new burrows formed after disturbing mud surface sufficiently to destroy entrances to old burrows. If counts do not agree, use the latter. For acute toxicity tests, alternatively use an artificial substrate of epoxy resin to facilitate observation and monitoring of test animals. For growth or emergence tests, set up an additional set of containers so that naiads can be removed periodically for measurement. Remove 10 naiads from their burrows after 20 to 60 d to determine growth. Do not remove more than 50% of surviving animals before conclusion of these tests. Keep a record of total number removed. Use these animals to provide additional data on growth and emergence. Record body length, head capsule width, and live weight.

In acute toxicity tests, determine survival after 1.5, 3, 6, and 12 h and twice daily thereafter. As a sign of death, use failure of specimens to respond by movement to gentle probing or flash light illumination. In longer-term studies, check tanks daily to remove and record dead animals and cast naiad skins, which indicate successful molting.

For growth studies, determine initial range and mean of total length, head capsule width, and weight from specimens in holding tank. Kill all animals in warm water (40 to 50°C) before measuring. Take measurements twice during testing, using animals that are to be discarded. Obtain final measurements for all survivors. Make two counts: number of adults and cast skins; if different, use cast skins because some adults may have escaped.

Determine and record percentage of adults that emerge, sex, incidence of incomplete emergence (i.e., half-out of nymphal skin, wings unsuccessfully unfolded, etc.), adult length, weight, and head capsule width, and number of mature eggs.

4. Test Procedures for *Chironomus*

Follow procedures described in Section 8010F. For each concentration, use duplicate 20-L aquariums with mud or powdered dried cereal grass* substrate and screen covers. Maintain flow to each test container at about 2 L/h. Use a mud substrate similar to that for *Hexagenia*. Use lighting and photoperiod as described in Section 8010F.3*f*. Do not feed animals during short-term tests. Feed during 30-d and emergence tests as in Section 8750B.3*b*. If prepared food is used, add powdered dried cereal grass* or about 100 mL food suspension to each container twice per week.

For long-term tests, place 50 first-instar larvae (about 1.5 mm long and less than 24 h old) in each test aquarium. Transfer larvae with an eyedropper. Determine number of emerging adult males and females. Count both adults and pupal cases. If counts differ, use pupal case count. At 25 ±1°C emergence takes about 1 month. To determine success of fertilization of eggs, take 50 eggs and determine the percent hatchability. If it is impossible to separate and count eggs, hatch fertilized egg masses in beakers with same test water from which adults emerged. Count 60 larvae into a petri dish and examine for injured larvae. Transfer often will injure early instar larvae; if a correction for this is not made in the count, errors in percent survival result. After examination, count 50 larvae and return to test chamber and rear them to adult stage. End points for taking and analyzing data are emergence of adults, egg production, and hatching of young. Repeat complete test at least once.

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8750 D. Data Evaluation

Analyze, evaluate, and report data from various tests as described in Section 8010G.

8810 ECHINODERM FERTILIZATION AND DEVELOPMENT*

8810 A. Introduction

1. Background

The Phylum Echinodermata encompasses a widely distributed and diverse group of marine animals. The class Echinoidea includes sand dollars and sea urchins, organisms that are common inhabitants of rocky shores and ocean bottoms over all depth ranges. Many echinoid species are maintained easily in the laboratory and are responsive to simple methods of spawning inducement. Gametes and embryos are easily obtained and reared in the laboratory and have been the subject of scientific research for over 100 years.[1] Numerous species have been used and laboratory techniques have been developed that utilize various stages of the organism's life cycle.[2–6]

Toxicity tests utilizing the short-term exposure of gametes or embryos are of comparable or greater sensitivity to many contaminants than tests with other marine species and life stages.[7–11] Echinoid toxicity tests can be performed on small volumes (≥2 mL) over short time periods (1 to 96 h), and under static conditions without feeding. These tests have been used successfully to evaluate the toxicity of effluents, receiving waters, chemicals, and sediments, provided the salinity of the test samples is near typical ocean levels (28 to 34 g/kg). Recent adaptations of these test methods have expanded applications to include evaluation of genotoxic effects,[12] interstitial water,[13] and toxicity identification evaluation (TIE) studies.[14] Methods similar to these have been proposed or recommended as components of regulation programs.[13]

* Approved by Standard Methods Committee, 1997.
Joint Task Group: 20th Edition—Steven M. Bay (chair), Robert S. Carr, Paul A. Dinnel, Diane E. Nacci, Morris H. Roberts, Patti L. TenBrook, Jack Q. Word.

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8810 B. Selecting and Preparing Test Organisms

1. Selecting Test Organisms

In accord with the criteria listed in Section 8010E.1, the recommended test species include (but are not restricted to) the following:

Scientific Name	Common Name	Location	Approximate Spawning Season
Arbacia punctulata	Atlantic sea urchin	Atlantic coast Gulf coast	Summer Winter
Strongylocentrotus droebachiensis	Green sea urchin	Northern Atlantic and Pacific coasts	Winter
Stronglyocentrotus purpuratus	Pacific purple sea urchin	Pacific coast	Fall-spring
Dendraster excentricus	Pacific eccentric sand dollar	Pacific coast	Spring-summer

The use of the above species is encouraged to increase the comparability of results from different laboratories. Successful toxicity tests can be conducted with other species, such as the Hawaiian sea urchins *Echinometra* spp. and *Tripneustes gratilla*[1] or *Lytechinus* spp.[2] Use of alternate species may be advantageous in certain regions, but modifications to the test method may be necessary and the results may not be comparable.

2. Collecting Broodstock

Obtain test organisms (gametes or embryos) from broodstock collected from the field during their natural spawning season and held in the laboratory until needed. Collect in areas away from obvious sources of pollution and having water quality similar to that used for holding and testing. Organisms obtained from a commercial supplier may be used. *Dendraster excentricus* forms dense aggregations in intertidal or subtidal sandy areas; collect individuals by hand at low tide, by diving, or by dredge. Regular sea urchin species inhabit rocky or sandy areas of the intertidal and subtidal zones. Collect individuals by hand, either at low tide or by diving.

Discard any individuals damaged during collection or subsequent handling. Avoid sudden or extreme variations in temperature, salinity, or other environmental factors during collection and transport, because they may induce premature spawning. Animals may be shipped by overnight mail service in insulated containers containing an ice substitute. Do not ship animals submerged in water because the oxygen will be depleted rapidly and premature spawning may occur. Instead, wrap animals in seaweed or towels soaked in seawater to maintain high humidity.

3. Culture Techniques

Hold sea urchins and sand dollars in aquariums with either a flow-through seawater supply or recirculating filter system.[3] Feed sea urchins *ad libitum* brown macroalgae, such as *Macrocystis* spp. or *Egregia* spp. Substitute romaine lettuce[4] or commercial fish feed if fresh seaweed is unavailable. Rehydrated seaweed purchased from food markets also has been used successfully. Aquariums containing sand dollars should contain several centimeters of sand if animals are to be held more than a few days. Sand dollars feed on suspended or benthic materials (e.g., detritus or plankton); provide a source of unfiltered natural seawater or prepared food (e.g., powdered fish feed) if the animals will be held for long periods.

Holding temperature varies with the species and should be similar to that at the collection site. Recommended temperatures are 15 to 18°C for *A. punctulata*, 12 to 16°C for *D. excentricus*, 8 to 15°C for *S. purpuratus*, and 8 to 12°C for *S. droebachiensis*. Hold animals at 28 to 34 g/kg salinity.

4. Parasites and Diseases

A variety of commensal organisms (e.g., annelids and crustaceans) are often associated with sea urchins and sand dollars. These organisms are not harmful and are not essential to the survival of the broodstock.

Excessive microbial growth can result from the accumulation of feces in aquariums. These growths produce metabolites (e.g., hydrogen sulfide) that may be toxic or cause stress to the animals. Clean aquariums several times per week.

5. Gamete Preparation

Induce sea urchins or sand dollars to spawn just before the beginning of a test. Pool gametes from at least three individuals of each sex to provide a representative sample for testing. Females and males of most echinoid species usually can be induced to spawn by injection of 0.5*M* potassium chloride (KCl). Use a hypodermic syringe (20-gauge needle) to pierce the peristomial membrane surrounding the mouth and inject approximately 1.0 mL (0.5 mL for sand dollars) into the coelomic cavity. Use sterile needles and KCl to guard against disease if the animals will be returned to laboratory aquariums. Usually, two injections of 0.5 mL each are made on opposite sides of the mouth. Place sea urchins upright (oral side down) and observe for evidence of gamete release through the genital pores, located around the anus on the aboral surface. Sperm are milky white in color while eggs are orange to red, depending on the species.

Electrical stimulation is an alternate spawning method that has been used with success on some species (primarily *A. punctulata*). Place electrodes from a 12-V DC power source on either side of the anal pore of the urchin, and spawning occurs until the electrodes are removed.[4] This method has the advantage of permitting a check of gamete type and quality by applying the electrodes briefly. Neither spawning method kills the animal, which may be respawned in 30 to 60 d if held under appropriate conditions.

Invert females releasing eggs (oral side up) and place on a beaker filled to the brim with seawater of the appropriate temperature. The eggs will fall to the bottom of the beaker after extrusion. Collect sperm in the "dry" condition, without contact with seawater that activates the sperm. Remove sea urchin sperm from the gonopore area with a glass transfer pipet or automatic pipet (with enlarged tip) and place in a small conical test tube. Collect sand dollar sperm by inverting males over 5- to 10-mL beakers of seawater. The sand dollar sperm fall to the bottom with little dilution and can be removed easily by pipet. Use care to avoid transferring fecal material with the gametes.

Gametes for most species may be stored for several hours in an ice bath or refrigerator. Keep eggs from each female separate until evaluated for quality. Store *A. punctulata* eggs at the culture temperature. Examine subsamples of sperm and eggs from each animal under a compound microscope to evaluate their quality. Eggs should be round and a germinal vesicle (clear spot) should not be visible. The presence of a germinal vesicle indicates immature eggs. Viability also can be checked by removing a subsample, adding sperm and determining fertilization. Use eggs from a more mature female if more than a few percent of immature eggs are present. Evaluate sperm quality by diluting a subsample in seawater and checking for motility.

Pool equal volumes (at least 0.025 mL for sea urchins) of sperm from at least three males and store in a conical tube in an ice bath. Avoid additional dilution until just before test. Activate sperm by dilution in seawater and use within 30 min. Sperm have limited energy reserves and their viability declines exponentially within minutes of activation; carefully monitor holding times of activitated sperm and preferably standardize within the laboratory. Longer holding times (e.g., 60 min) can be used successfully, provided sperm are chilled on ice and higher sperm densities are used in the test. The final density of sperm needed for the test varies according to test type, species, and maturation stage. Determine this value from trial fertilization tests (see 8810C.4*d*) or previous experience.

Gently wash eggs once by centrifugation or let them settle in the spawning beaker and decant off excess seawater. Gently resuspend eggs in fresh seawater, pool, and let settle again. Use care when washing sand dollar eggs; they are surrounded by thick jelly coat that is easily disturbed (with adverse effects on fertilization).

Prepare a working stock solution of known egg density (dependent on test volume and test type). Check density by mixing stock solution well, removing a small subsample, diluting it 1:100 × with seawater, and counting number of eggs in a known volume on a Sedgwick-Rafter cell. Use of a perforated plunger (plastic disk containing numerous holes, attached to a plastic rod) is strongly recommended to provide a homogeneous suspension of gametes and embryos for this and other steps of the procedure. Adjust density by adding or removing seawater. Store the working stock at or below the exposure temperature (depending on species) and use within 2 h if possible.

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8810 C. Echinoderm Fertilization Test

1. General Procedures

Conduct exploratory tests (see Section 8010D) first if the concentration range to be tested is not known. Prepare dilution water and toxicant solutions and introduce them into test containers as described in Section 8010F.

Observe the following general precautions in procedures that involve handling of gametes. First, take stringent measures to avoid cross-contamination of egg and sperm solutions, e.g., use separate pipets and glassware for each sex. Seemingly minute amounts of sperm are sufficient to fertilize an egg stock prematurely and invalidate an entire test. Second, enlarge the opening of disposable pipet tips used for dispensing gametes. Trim tip with a razor blade to produce an opening of 1 to 2 mm when transferring eggs. Preferably also enlarge pipet tips used for concentrated sperm solutions to facilitate transfer of this highly viscous suspension. Modified pipet tips are not required for sand dollar sperm shed into seawater. Finally, avoid inadvertently warming gamete or embryo solutions during preparation steps, because this may greatly hasten degradation during storage. Use a temperature-controlled room or chilled water baths to ensure that all preparatory steps are conducted at or below test temperature.

2. Water Supplies

Maintain salinity of dilution water within 2 g/kg of the holding salinity. Adjust pH to 7.7 to 8.3, unless altered pH is an important factor in the experimental design. Dilution water quality should be sufficient to produce ≥70% fertilization in control samples.

a. Artificial seawater: See Section 8010E.4*b*2). Avoid commercial sea salt mixes because they are often toxic to echinoid gametes and embryos. However, some seawater formulations based on reagent-grade chemicals have been used successfully.[1,2] Conduct preliminary tests to determine suitability of each batch of artificial salts before use.

b. Natural seawater: Choose a source of natural seawater free of contamination and of uniform quality. Pass the water through a filter with an effective pore size ≤1.0 μm to remove parasites and predators. Additional treatment (e.g., aeration, additional filtration, sterilization, or activated carbon treatment) may be needed to obtain acceptable water quality, especially during storage. Avoid prolonged storage of seawater if possible, because aging (>24 h) natural seawater can produce potentially toxic metabolites.

c. Salinity adjustment: Echinoids have limited osmoregulation ability. Adjust salinity of test samples that deviate by more than 2 g/kg from the culture environment to eliminate potential interferences. Use hypersaline brine (HSB) or an artificial sea salt mixture for salinity adjustment. Exercise caution in selecting the material used to adjust salinity so that the toxicity of the sample is not altered by the introduction of chemicals such as chelators (e.g., EDTA) or toxic contaminants (e.g., heavy metals).

Partial freezing and thawing of seawater is a convenient method of preparing HSB in sufficient quantities for fertilization or embryo development tests.[3] Freezing (−10 to − 20°C) one or two 4-L containers (glass or plastic) overnight for 6 to 12 h will provide sufficient 80- to 100-g/kg brine for most tests. Evaporation also is an effective method of HSB preparation.[3] Salinity of the HSB should not exceed 100 g/kg.

Use the following formula to determine the volume of brine (V_B) to be added to the sample:

$$V_B = V_S \times (S_T - S_S)/(S_B - S_T)$$

where:

V_S = volume of test sample to be added, mL,
S_T = desired test salinity after adjustment,
S_S = initial salinity of sample, and
S_B = salinity of brine.

Check pH of adjusted samples. Add dilute hydrochloric acid or sodium hydroxide to adjust pH, if necessary.

3. Exposure Chambers

Conduct tests in glass culture tubes or vials of 10- to 20-mL capacity. Cover chambers loosely to prevent contamination during the test. Sealed chambers may be used provided that acceptable control performance is obtained. Disposable glass tubes, or scintillation or shell vials make convenient exposure chambers that can be discarded after the test. Ensure that all test chambers and equipment used to prepare test solutions are clean and noncontaminating. Disposable test chambers usually can be used straight from the box, although it is a good precaution to prerinse or soak them in distilled water or seawater. Avoid use of detergent and hypochlorite solutions in cleaning other equipment because of potential toxicity to test organisms. In multipurpose laboratories, use glassware dedicated solely to use in toxicity tests.

4. Conducting the Test

a. Setting up test chambers: Set up the test as described in Section 8010D. Prepare all solutions and equilibrate to test temperature before beginning to spawn animals. Prepare at least four replicates of each solution if hypothesis tests (e.g., Dunnett's test) are to be used to determine the NOEC or LOEC (see 8010B). Two or three replicates are adequate if point estimation techniques are to be used to determine values such as the EC50.

b. Duration and type of test: In the fertilization test, add a predetermined number of sperm to test solution and expose for 20 min (other times ranging from 5 to 120 min have been used). Then add eggs to produce a specific ratio of sperm to eggs and allow 20 min for fertilization to occur. Preserve samples by addition of formalin and examine under a compound microscope. Toxic effects are manifested by an impaired ability of the sperm to fertilize eggs, indicated by lack of an obvious fertilization membrane around the egg.

c. Test organisms: Fertilization tests can be conducted with all the recommended species.

d. Performing the test:

1) Preparation—Arrange replicate 5-mL samples of test solution in random order by assigning random numbers to individual test chamber numbers (i.e., replicate chambers of the first treatment group will have unrelated numbers such as 16, 31, and 4, instead of sequential numbers); then arrange the chambers in a rack in numerical order.

Other test volumes may be used if preferred (e.g., 2 mL or 10 mL), but adjustments to the following instructions will be necessary to maintain the desired sperm-to-egg ratios in the test chambers. Measure water quality of each test substance concentration on additional samples of test material. A single initial measurement is sufficient for most parameters unless the test material is highly unstable. Use of the proper sperm-to-egg ratio is critical to obtaining good test sensitivity and precision. Because a fixed number of eggs is used in the test, sperm-to-egg ratios are altered by varying the number of sperm added to the test chambers. The proper number of sperm is the least amount that produces >80% fertilization. Use of excessive (>2 × the optimal number) amounts of sperm may reduce test sensitivity.

2) Sperm density measurement—Use a portion of the concentrated pooled sperm to determine the density (be sure to reserve sufficient sperm to conduct the test and a possible trial fertilization). Use the following procedures to aid in accurately pipetting the highly viscous concentrated sperm: Enlarge pipet tip opening to about 2 mm, wipe off any sperm adhering to the outside of the pipet tip before delivery of the sample (take care not to wick away sperm from inside the tip), and repeatedly rinse pipet tip with dilution water after sample delivery until all sperm inside has been removed.

Add 0.025 mL sperm to approximately 180 mL seawater in a graduated cylinder. Bring mixture up to 200 mL using 10% acetic acid (kills sperm) to produce an 8000 × dilution. Cover the cylinder, mix well by inversion, and let bubbles dissipate. Add a sample of the mixture to each side of a hemocytometer. Alternate dilution volumes (e.g., 1 mL sperm solution in 100 mL) may be more suitable if a less dense sperm solution is used.

Let sperm settle for about 10 min. Examine a sufficient number of small squares on the slide so that about 100 sperm are counted. Examine the same number of squares on the opposite side of the hemocytometer. If the two counts are within 20%, use the mean to calculate the density according to the equation below. Reload hemocytometer and repeat counts if variability exceeds 20%.

$$\text{sperm/mL} = \frac{(\text{dilution factor})\,(\text{count})\,(4000 \text{ squares/mm}^3)\,(1000 \text{ mm}^3/\text{mL})}{\text{No. squares counted}}$$

Alternatively, use a ratio turbidimeter with a 1-cm-diam cuvette to determine sperm density rapidly.[4] The relationship between turbidity and sperm density is linear, but may vary with species, individual, season, or type of instrument. Use hemocytometer counts for initial calibration of turbidimeter and verification of method suitability.

3) Sperm-to-egg ratio selection—The sperm-to-egg ratio for the test usually is selected on the basis of the control performance of previous tests. The correct value may vary, depending on species and time of year. Conduct a trial fertilization test (just before the actual test) if adequate data are not available to determine the sperm-to-egg ratio [see ¶ 5) below]. For purple sea urchins, use sperm-to-egg ratios ≤500:1 if fertilization in controls is acceptable. Higher ratios may reduce test sensitivity and should only be used when prior experiments or trial fertilization results indicate the ratio is needed to obtain acceptable control fertilization (≥70%). Sperm-to-egg ratios above 3000:1 indicate unacceptable quality of purple sea urchin sperm; use additional animals or a different species (in better spawning condition). Optimum sperm-to-egg ratios are species-specific.[1,5]

4) Egg stock preparation—Add a sufficient volume of washed eggs to seawater to make 100 to 500 mL of a stock solution containing 2000 to 2500 eggs/mL. Determine density of eggs as directed in Section 8810B.5. Adjust density to proper range by adding or removing seawater. Verify that stock volume is sufficient for number and size of test chambers.

5) Trial fertilization—Determine density of pooled sperm as directed in ¶ 2) above. Prepare egg stock solution as directed in ¶ 4) above. Calculate volume of seawater needed to dilute 0.025 mL pooled sperm and produce a trial stock solution such that a sperm-to-egg ratio of 3000:1 will result when 0.1 mL of stock is added to test chamber (e.g., if 1000 eggs will be in each chamber and 0.1 mL sperm stock is added to the test sample, then a sperm stock density of 3.0×10^7 sperm/mL is needed). Prepare duplicate test chambers for each sperm-to-egg ratio to be tested (including 3000:1), each containing 5 mL seawater at correct temperature. Prepare trial sperm stock solution. Prepare several dilutions of stock that will produce desired range of sperm-to-egg ratios in test chambers.

A suggested dilution series is as follows:

Sperm-to-Egg Ratio	Trial Stock *mL*	Seawater *mL*
3000:1	No dilution	—
1304:1	5	6.5
545:1	2	9.0
231:1	1	12.0
100:1	0.5	14.5

Add 0.1 mL trial stock or dilution to appropriate test chambers. After 20 min, add 1000 eggs. Add formalin preservative after 20 additional min. Determine percent fertilized in a subsample of 100 eggs from each replicate. Select lowest sperm-to-egg ratio producing ≥90% fertilization. It may be necessary to determine the ratio by interpolation. Verify sperm density in stock dilution corresponding to the chosen sperm-to-egg ratio by a hemocytometer count.

6) Sperm stock preparation—Calculate sperm stock solution density required to produce desired sperm-to-egg ratio in the test chambers (e.g., a stock containing 2.5×10^6 sperm/mL is needed to produce a sperm-to-egg ratio of 250:1 when 1000 eggs are present). Remove 0.025 mL pooled sperm and dilute with seawater to produce a stock solution of the desired density. Mix the solution well and use within 30 min.

7) Sperm addition—Use an automatic pipet to add 0.1 mL sperm stock to each test chamber. Mix stock periodically during inoculations. Add sperm in a steady rhythm, with each addition at intervals of about 5 s.

8) Egg addition—Add eggs after a 20-min exposure period (other sperm exposure times of 5 to 60 min can be used for

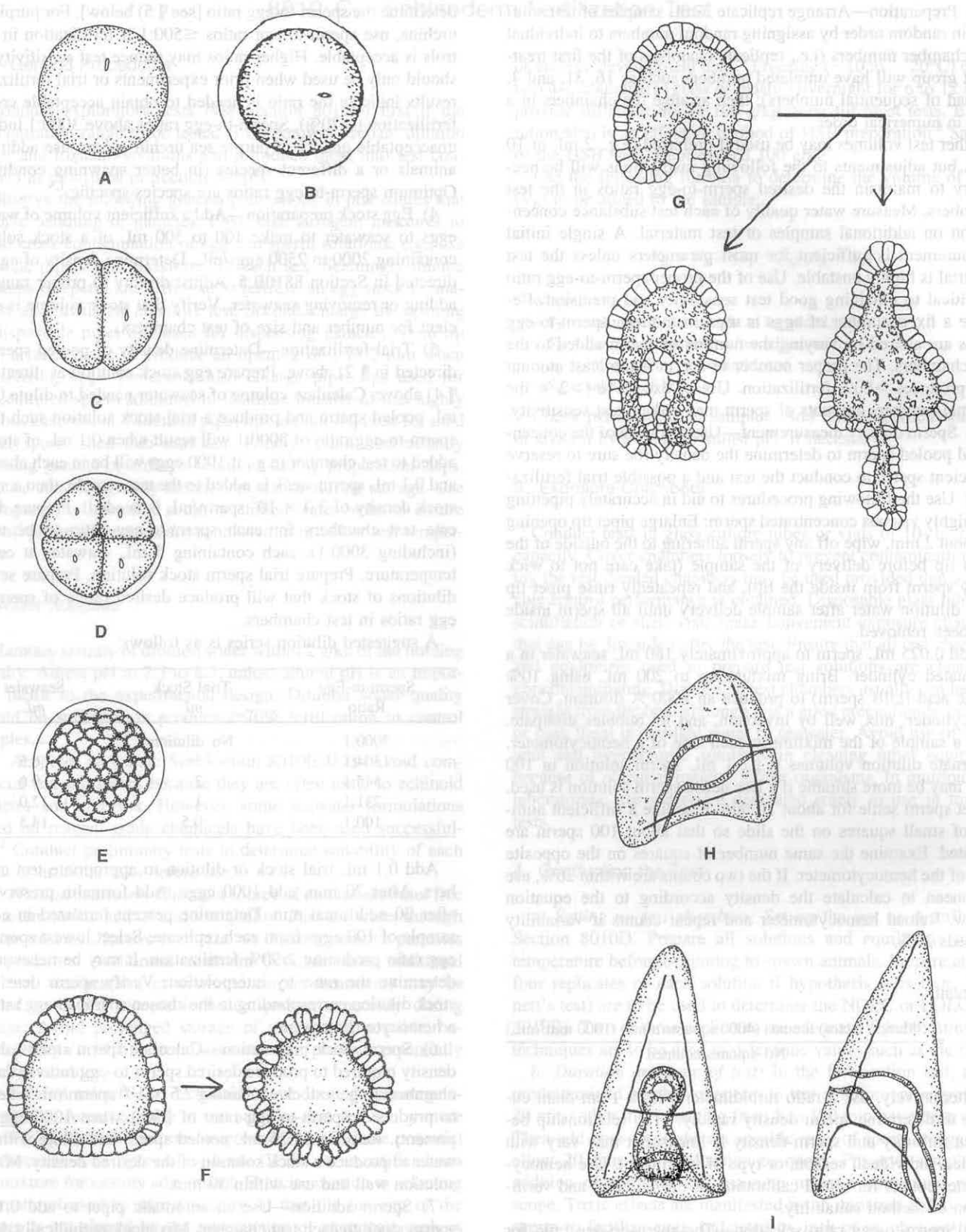

Figure 8810:1. Early developmental stages of sea urchins and sand dollars. A—unfertilized egg; B—fertilized egg; C, D, E—early cleavage; F—blastula with arrow indicating abnormal example; G—gastrula with arrows indicating abnormal examples; H—prism; I—frontal and lateral views of normal pluteus.

comparability with other laboratories). Use egg stock to add 1000 eggs (e.g., 0.5 mL stock) to each chamber using the same order and rhythm as for sperm. Use a perforated plunger or equivalent device to gently mix egg stock thoroughly during additions.

9) Test termination—Stop test by adding 0.25 mL concentrated formalin to each tube 20 min after egg addition (5% final concentration of formalin). Glutaraldehyde may be used as a preservative instead of formalin. Lugol's solution (Section 10200B) also is an effective preservative and has the advantage of being less toxic to the analyst. Cap test chambers securely, store at room temperature, and determine fertilization within 48 h, or as soon as practical. The samples can be stored indefinitely, but appearance of egg or fertilization membrane may change upon storage, making detection of the endpoint more difficult. Be extremely careful not to contaminate test equipment or laboratory furniture with preservative.

10) Sample evaluation—Examine eggs in exposure vial using an inverted compound microscope or transfer a representative subsample to a Sedgwick-Rafter cell for use with a conventional microscope. It is often convenient to concentrate the eggs before transfer by removing most of the overlying water with a pipet. Mix remaining sample well before transfer to counting chamber. Discard formalin-contaminated exposure chambers promptly (do not reuse chambers).

Examine at least 100 eggs (40 to 100 × magnification) from each replicate and score for presence or absence of an elevated fertilization membrane. Avoid bias by counting all eggs in subsamples transferred to counting chambers. Newly fertilized eggs usually have a completely elevated membrane around the egg (Figure 8810:1, A). The fertilization membrane may change in appearance with prolonged storage, partially collapsing or touching a portion of the egg. Consequently, count eggs showing any elevation of the fertilization membrane as fertilized. Exclude unusually small, immature, or abnormally-shaped eggs from counts.

Calculate percentage of fertilized eggs in each sample.

5. Statistical Analysis

Assemble, analyze, evaluate, and report data as described in Section 8010G.

6. Quality Assurance

Continued success in conducting this toxicity test depends on an overall effort to maintain and improve laboratory techniques and equipment.[3] Accurate background information regarding sample characteristics and test organism condition is necessary to enable correct interpretation of test results. Measure basic water quality parameters (pH, dissolved oxygen, salinity, temperature) on representative samples of controls and test materials. Because ammonia can be highly toxic to marine organisms, measure total ammonia with a sensitive method (e.g., 4500-NH_3.E) and report concentration of un-ionized ammonia (NH_3). Include additional controls or blanks in the experimental design to verify that special treatments (e.g., storage, centrifugation, pH adjustment, carrier solvent addition) do not produce unanticipated effects.

Use reference toxicant tests to provide a measure of test precision and possible organism condition.[3] The reference toxicant test usually consists of replicate exposures to three to five concentrations of a stable chemical (e.g., Cu, Cd, sodium dodecyl sulfate) that are sufficient to calculate a point estimate of effect (e.g., EC50). Preferably include a reference toxicant test with each experiment, or test at least monthly. Plot cumulative mean and confidence limits on a control chart to identify outlier values. Outliers indicate potential problems with the technique or test organisms.

7. References

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8810 D. Echinoderm Embryo Development Test

1. General Procedures

Conduct exploratory tests (see Section 8010D) first if the concentration range to be tested is not known. Prepare dilution water and toxicant solutions and introduce them into test containers as described in Section 8010F. Take precautions to avoid gamete cross-contamination and temperature stress (see 8810C.1). The procedure described below uses many of the same techniques described for the fertilization test (8810C) but has been optimized for use with embryos. Some laboratories conduct

both tests on a sample to gain additional information. The fertilization test also may be extended into an embryo development test by including additional replicate chambers. Such an approach requires modification of the test methods and may reduce precision of the embryo development results because of variable fertilization rates.

2. Water Supplies

Maintain salinity of dilution water within 2 g/kg of the holding salinity. Adjust pH to 7.7 to 8.3, unless altered pH is an important factor in the experimental design. Dilution water quality should be sufficient to produce ≥70% normal development (relative to initial number of embryos) in control samples.

a. Artificial seawater: See Sections 8010E.4*b*2) and 8810C.2. Avoid commercial sea salt mixes because they often are toxic to echinoid gametes and embryos.

b. Natural seawater: Choose a source of natural seawater free of contamination and of uniform quality. Pass water through a filter with an effective pore size ≤1.0 μm to remove parasites and predators. Additional treatment (e.g., aeration, activated carbon treatment) may be needed to obtain acceptable water quality.

c. Salinity adjustment: The sea urchin development test usually is more sensitive to deviations in salinity than is the fertilization test. Use the methods described in Section 8810C.2 to adjust salinity of samples that deviate by more than 2 g/kg.

3. Exposure Chambers

Preferably, use glass chambers of 10-mL to 1-L capacity. Maintain recommended density of test organisms regardless of volume. Cover chambers loosely to prevent contamination and reduce evaporation during the test. Sealed chambers may be used provided that acceptable control performance is obtained. Scintillation or shell vials make convenient exposure chambers that can be discarded after the test. Clean all equipment for preparing test solutions before use. Clean test chambers by soaking in fresh water or seawater. Avoid use of detergent or hypochlorite solutions because of potential toxicity to the test organisms.

4. Conducting the Test

a. Setting up test chambers: See 8810C.4*a*.

b. Duration and type of test: Various volumes of test solution (5 to 1000 mL) may be used. Add embryos to test solution and let develop under static conditions for 48 to 96 h until the pluteus stage is reached. Preserve subsamples (or the entire sample if vials are used) with formalin and examine under the microscope. Toxic effects are indicated by embryo mortality or abnormal development.

c. Test organisms: Embryo development tests can be conducted with all the recommended species.

d. Performing tests:

1) Preparation—Test preparation is the same as for the fertilization test [8810C.4*d*1)] with two exceptions. First, the test may be conducted in larger volumes (up to 1000 mL) if desired. Use of large volumes provides no distinct advantage in test sensitivity or precision, but does allow exposure chamber to be subsampled for water quality measurement or to determine effects at various times or developmental stages. Second, measure both initial and final water quality for each treatment group. Include one additional replicate test chamber in each treatment group for final water quality measurements when tests are conducted in small volumes (e.g., 10 mL).

Quantify toxic response by either complete count or relative count. For complete count, calculate percentage of normal pluteus larvae at the end of exposure, based on counts of all preserved test organisms and number of embryos added at the start. This method provides the most comprehensive assessment of effects because all instances of embryo mortality and aberrant development are included in the percentage. A relative count requires counts of only a subsample of organisms at the end of the test. Both embryo mortality and aberrant development are reflected in this method as well, but toxic effects may be underestimated if the test solution causes rapid decomposition of dead embryos and consequent failure to detect them during microscopic examination. Choose the evaluation method before the test is started so that all required information will be obtained.

2) Egg density adjustment—Add a sufficient volume of washed eggs to seawater to make 100 to 500 mL of a stock solution containing 1000 eggs/mL. It may be more convenient to prepare a more concentrated solution (e.g., 10 000 eggs/mL) if test volumes larger than 50 mL are used. Determine density of eggs as directed in Section 8810B.5. Adjust density to desired value by adding or removing seawater. Verify that stock volume is more than sufficient (approximately 50% greater) for the number and size of test chambers used.

3) Sperm stock preparation— Prepare a sperm stock by adding about 0.025 mL dry sperm to 50 mL seawater. If volume of stock solution needed to fertilize the eggs is not known from prior experience, determine density of the sperm stock with a hemocytometer (see 8810C.4*d*).

4) Egg fertilization—Add a sufficient volume of sperm stock to egg stock to produce a sperm-to-egg ratio of 200 to 1000:1. Mix well and examine a subsample after about 10 min to assess fertilization percentage. Add more sperm if less than 90% of the eggs are fertilized. A fertilization rate of less than 90% after the second addition of sperm indicates that the gametes are of poor quality. Spawn additional animals to obtain better gametes if possible. Add the embryos to the test containers as soon as possible (generally within 2 h but no later than 4 h after fertilization).

5) Embryo addition—Use an automatic pipet to add sufficient embryo stock solution to each test chamber to result in about 25 embryos/mL (e.g., 0.25 mL/10 mL of sample). It is important that the same number of embryos is added to each test chamber. Use a perforated plunger to mix the stock solution thoroughly during embryo addition.

If the complete count method will be used to evaluate toxic effects, add embryos to at least five additional test chambers containing control water. Intersperse these chambers throughout the experimental array and add embryos in the same manner used for the other chambers. Examine these additional chambers promptly to estimate actual number of embryos added.

6) Exposure—Loosely cover the test chambers and leave undisturbed for 48 to 96 h under static conditions. The optimum exposure length varies with species and test temperature. The exposure time should be long enough to allow the embryos to develop to the pluteus stage, yet short enough (≤96 h) that

internal food reserves are not exhausted. The following exposure conditions are recommended to provide consistency with results from other laboratories: *A. punctulata*, 48 h at 20°C; *D. excentricus*, 72 h at 15°C; *S. purpuratus*, 72 h at 15°C or 96 h at 12°C; and *S. droebachiensis*, 96 h at 12°C. These are target times; a few extra hours may be allowed to help assure that most (>90%) control larvae have attained the normal pluteus stage. Ambient laboratory light levels and photoperiods are adequate for all species.

7) Test termination—Preserve organisms for later microscopic examination by adding sufficient borax-buffered formalin (pH>7.0, see 10200B.2*a*) to produce a 5% concentration. Unbuffered formalin may be used provided that the samples are examined rapidly (within a few days), before skeletal components dissolve. Add formalin directly to test chamber if disposable vials or culture tubes are used. Otherwise, thoroughly mix test chamber contents, transfer a 10-mL subsample to a vial, and add formalin.

8) Test evaluation—Examine preserved embryos and larvae with a compound microscope at a magnification of 100 ×. Concentrate test organisms by removing overlying water from the storage vial and transfer to a Sedgwick-Rafter counting chamber for examination with a conventional microscope. Alternatively, use an inverted microscope to examine the organisms in the storage vial and eliminate losses due to transfer.

Normally developing embryos develop synchronously through a series of characteristic stages including early cleavage, blastula, gastrula, prism, and pluteus (Figure 8810:1, B-I). The appearance of pluteus larvae varies with species, but all normal plutei should have the following features: a pyramid shape supported by a framework of skeletal rods, an internal gut that is attached to the body wall at both ends and consists of three distinctive regions, and at least one pair of post-oral arms (Figure 8810:1, I). The length of the post-oral arms varies with species. Count as abnormal all grossly deformed pluteus larvae, deformed embryos, mostly normal-appearing embryos that have not attained the pluteus stage (inhibited development), and uncleaved fertilized eggs. Do not count unfertilized eggs.

Determine percentage of normal pluteus larvae for each replicate using either the complete or relative count method (below).

a) Complete count method—Count all embryos in preserved sample. Preferably use an inverted microscope to minimize counting errors. If a conventional compound microscope is used, use a consistent and efficient method to transfer embryos to counting chamber because lost embryos (remaining in vial or stuck to transfer pipet) are assumed to have died. Variability in recovery of larvae from the storage vial may introduce experimental error that reduces the ability to detect statistically significant effects. Calculate percentage of embryos developing to normal pluteus larvae, P_n, as follows:

$$P_n = 100(E_n/E_i)$$

where:

E_n = number of normal larvae at end of test, and
E_i = number of embryos at start of test.

b) Relative count method—It is easier and usually just as effective to determine percentage of normal development in a representative sample of at least 100 embryos and larvae at the end of the test. Calculate this value as follows:

$$P_n = 100[E_n/(E_n + E_a)]$$

where:

E_a = number of abnormal embryos/larvae, and other terms are as defined above.

5. Statistical Analysis

See Section 8010G.2 for general information.

Control performance may vary between tests because of factors such as variations in test temperature and gamete condition. Normalize response data to the control performance before statistical evaluation (e.g., EC50) and to facilitate comparisons between tests as follows:

$$P_{adj} = 100(P_n/M)$$

where:

P_{adj} = normalized value,
P_n = percent normal or fertilized for the sample, and
M = mean percent normal or fertilized for controls.

6. Quality Assurance

See 8810C.6.

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8910 FISH*

8910 A. Introduction

1. Background

Fish have been regarded as good test species for the assessment of aquatic toxicity because of their ecological and economic importance. While many different fish species may be used in toxicity studies, the selection of test species will depend on the objective of the test, the availability of the species, and the ease of culturing and handling individuals. Section 8910 provides guidance for species selection, culturing, and testing procedures for fish in aquatic toxicity studies.

Other methods for toxicity tests using fish are available.[1–4]

* Approved by Standard Methods Committee, 1997.
Joint Task Group: 20th Edition—James P. Swigert (chair), Mohamed Elnabarawy, Robert E. Morcock, J. Vincent Nabholz.

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8910 B. Fish Selection and Culture Procedures

1. Selection of Test Species

General guidelines for selecting test organisms are outlined in Section 8010E.1. Of prime consideration in the selection of a fish species is the purpose of the test. For example, test species may be designated by a particular regulation (e.g., FIFRA, TSCA), or, if the toxicity test is for non-regulatory purposes, any number of species may be tested provided that the environmental requirements of the species can be duplicated for the test. If it is desirable to use a fish that is not routinely cultured, it may be necessary to make collections from a single field source.

Select test species according to the following criteria: (*a*) the species should be available in unbiased (i.e., not pre-screened for resistant individuals by prior exposure to adverse conditions) numbers sufficient for the tests; and (*b*) the species should be capable of being held in the laboratory in a healthy condition (i.e., active, feeding, free of lesions, etc.) for at least 1 month. Consider relative sensitivities of different species and life stages if the data are available when selecting species.

2. Collecting and Handling Test Fish

Collecting equipment and methods are described in Sections 8010E.2 and 10600. Handling and holding are discussed in Section 8010E.3. It is extremely important to avoid subjecting fish to unnecessary stress such as inappropriate capture and transport, temperature shock, or water quality change.

a. Freshwater fish: Whenever possible, obtain routinely cultured species. Salmonid fish usually are available from private, state, and federal hatcheries. Obtain trout certified pathogen-free, if possible. When fish cannot be obtained from hatcheries, appropriate field collection is acceptable. Collecting permits usually are required by state agencies. Avoid fish from bait dealers or fishermen because information on source, handling, holding time, etc., usually is not available.

b. Marine and estuarine fish: Various life stages of marine fish may be collected from the field for laboratory tests. Vertical movement of early larval stages may necessitate nighttime collection. Many marine fish and most marine fish larvae are extremely fragile; handle carefully during collection, sorting, and transfer. For sorting and transferring larvae during and after collection, use a pipet appropriate to the size of the larval fish. Whenever possible, transfer larger larvae, juvenile, and adult fish by dipping or gently pouring. Fine-mesh dip nets also are suitable if transfers are made gently.

3. Holding and Acclimating

See Sections 8010E and F for additional discussion.

Keep fish stocks in tanks, small ponds, live boxes, or screen pens, depending on fish size and number. Use good-quality dilution water (See Section 8010E.4*b*) for acclimation. Feed fish natural or commercially available prepared foods daily during acclimation. Detailed information on handling, holding, care,

and feeding of fish is available.[1–4] Because food requirements vary with the species and size of fish, select an appropriate diet for the species. Fish obtained from a hatchery should be provided initially with food to which they are accustomed. Many fish can be maintained for long periods on dried food but live food supplements may be desirable. Do not overfeed. Diets should be certified toxicant-free or tested for toxic substances before use.

While maintaining fish during holding and acclimation, watch carefully for signs of disease, stress, physical damage, and mortality. Remove dead and abnormal individuals immediately. If mortality rate exceeds 10%, discard the entire stock.

Handle fish carefully and as quickly as possible.[1,2] For extensive handling such as weighing, measuring, or taking other data, anesthetize fish.[3]

For short-term tests, use fish of similar size and source. The length of the longest fish should not be more than 1.5 times the length of the shortest fish. Acclimate fish to laboratory conditions before the test. Standard acclimation periods range from 48 h to 14 d. However, when using early-life-stage fish in short-term tests, this may not be possible.

4. Parasites and Disease

a. Stress in relation to parasites and disease: Unexpected and often unexplained mortalities in experimental and control animals interfere with test results and interpretations. Optimize laboratory conditions for each particular species to prevent the development of disease.

When large numbers of organisms are retained in a relatively small space, microbial diseases or parasites may become epidemic. If the water is unpolluted and poor in nutrients, disease often can be prevented by strict sanitation. Disease may arise if the water is enriched with organic materials or if toxic substances are present. Pathogens and parasites that might be very rare in natural waters may become epidemic in intensive culture. Uneaten food and fecal material provide a potential source of bacteria, parasites, and toxic products. Filtration and/or sterilization of water, adequate feeding, regular cleaning of holding vessels, sterilization of equipment, and securing disease-free fish are the first lines of defense.

Organisms exposed to toxicants may become more susceptible to parasites and disease. Because various environmental factors may contribute to reduced resistance, pay careful attention to nutrition, oxygen supply, and water quality. To minimize accumulation of fecal material and hence dissolved oxygen demand, do not feed fish during the 2 d before initiating short-term tests for cold-water species and 1 d for warm-water fishes. Siphon daily from the holding tank any fecal material or uneaten food. In testing of young fish, feeding live brine shrimp nauplii may be desirable if starvation is a possibility during the test.

b. Control methods:

1) General—Ultraviolet light and ozonation have been used to control disease and parasites present in dilution and/or culture water. Antibiotics used in holding tanks reduce bacterial populations. To reduce mortality and to avoid introduction of disease into stock tanks, treat fish with a broad-spectrum antibiotic immediately after collection, during transport, or on arrival at the laboratory. Do not place treated organisms into holding tanks for 4 d after treatment or use treated organisms for tests until at least 14 d after treatment. Clean and disinfect tanks and containers with 200 mg sodium hypochlorite (NaOCl)/L for 1 h after removal of diseased fish. Dechlorinate with sodium thiosulfate and rinse with clear water before reusing tanks.

2) Recommended disease therapy—Treat freshwater fish to cure or prevent disease by the methods in ¶ *b*1) above and Table 8910:I. These methods have been found dependable, but their efficacy may be altered by temperature or water quality. If fish are severely diseased, destroy the entire stock. A number of good reviews of fish diseases and parasites and methods for their control have been published.[5–11]

Published information on related topics includes a summary of problems in marine fish larval culture,[12] a description of a larval culture system,[13] and a discussion of disinfection of water supplies.[14]

5. Culturing Test Fish

a. Freshwater fish: More than 30 species of freshwater fish have been reared for stocking fresh waters. The culture methods can be adapted to laboratory scale to produce various life stages of fish.[15–25] Methods are given below for three freshwater species commonly used in toxicity experiments: the rainbow trout, *Oncorhynchus mykiss*; the bluegill sunfish, *Lepomis macrochirus*; and the channel catfish, *Ictalurus punctatus*. For specifics regarding fathead minnow, *Pimephales promelas*, see Section 8921. These species are representative of test organisms that can be found in various freshwater habitats, ranging from free-flowing streams and rivers to ponds and lakes.

1) Rainbow trout, *Oncorhynchus mykiss*—Rainbow trout of various ages can be purchased from certified specific pathogen-free hatcheries. Life stages range from unfertilized eggs and sperm (fertilization can be performed in the testing laboratory) to juvenile fish. Preferably obtain eyed embryos, and grow these to testing size unless spawning, fertilization and early embryonic stages are important for the test.

Overnight courier shipment of hatchery-raised eyed trout embryos in special insulated cartons is standard. These cartons are adequate to maintain cold temperatures during shipping. Upon receipt, measure the ambient temperature of the egg mass and if necessary slowly temper the eggs (± 3°C/h) to the testing temperature or culture conditions. Maintain embryos at temperatures ± 2°C in a range from 8 to 12°C.[26]

To perform egg fertilization in the laboratory, obtain gametes in plastic bags from the supplier within 24 h of removal from the adult. Hold gametes in unopened bags and slowly acclimate to test or culture temperature. When eggs and milt are at the desired temperature, mix together with either ovarian fluid or a small volume of 0.75% saline solution. Gently stir and let stand for about 1 min while fertilization occurs. Pour off excess water and milt and replace with fresh dilution water. Repeat several times until the embryos are in clear water. It may be useful to let eggs rest for 1.5 to 2 h to harden before transferring to incubation cups or culture system.

8910:I. Recommended Prophylactic and Therapeutic Treatments for Freshwater Fish to Be Used for Experimental Purposes

Disease	Chemical	Concentration mg/L	Application
External bacteria	Hyamine 1662® or 3500®*	1–2 AI†	30–60 min in flow-through system‡
	Nitrofurazone (water mix)	3–5 AI	30–60 min in flow-through system‡
	Neomycin sulfate	25	30–60 min in flow-through system‡
	Oxytetracycline hydrochloride (water-soluble)	25 AI	30–60 min in flow-through system‡
Monogenetic trematodes, fungi, and external protozoa§	Formalin *plus* zinc-free malachite green oxalate	25 ± 0.1	1–2 h in static systems, 30–60 min in flow-through system‡
	Formalin	150–250	
	$KMnO_4$	2–6	1–2 h in static system, 30–60 min in flow-through system‡
	NaCl	14 000–30 000	5–10 min dip
		2000–4000	24 h minimum, but may be continued indefinitely
	Para-dimethyl aminobenzene-diazo sodium sulfonate (35% AI)‖	20	30–60 min in flow-through system‡
Parasitic copepods	Trichlorfon#	0.25 AI	Weekly for up to 4 weeks if necessary in static or flow-through systems. Do not use at >27°C.

*Benzalkonium chloride.
†AI = active ingredient.
‡Add concentrated stock solution to the inflowing water by a drip system or by the technique of Brungs and Mount.[5]
§One treatment usually is sufficient except for *Ichtyophthirius*, which must be treated daily or every other day until no sign of the protozoans remains. This may take 4 to 5 weeks at 10°C and 11 to 13 d at 15 to 21°C. A temperature of 32°C is lethal to *Ichthyophthirius* in 1 week.
‖Dexon® or equivalent.
#Masoten® or equivalent.

Place fertilized eggs (embryos) in incubation chambers until hatching. For small-scale cultures, such as those that may be manipulated experimentally during an early-life-stage toxicity test (Section 8910C.2), incubation cups may be constructed from 8-cm sections of 5-cm-OD plastic or glass tubing with nylon or stainless steel screening cemented over one end to retain the embryos. During incubation supply a flow of fresh, high-quality, well-oxygenated water over the embryos. Oscillate incubation cups (containing less than 250 embryos) in holding tank (or test) water by a rocker arm using a 2-rpm electric motor to supply the necessary flow. Incubation chambers for mass culture of trout generally consist of wire-screen trays with rectangular or oblong mesh (15- × 3.5-mm openings) stacked vertically in deep flow-through troughs; or spaced along horizontal troughs.[23,24] Agitate embryos (shocking) periodically. Every 2 d remove dead embryos that turn white. Maintain incubation chambers in the dark because exposure to light may result in premature hatching or death.[23] The rate of hatching is determined by the temperature regime; the optimum range, 7 to 10°C, produces hatching in 44 to 68 d.

After development to the free-swimming fry stage, provide at least one complete exchange of water per hour and use a 20-gal (75-L) aquarium per 100 fry. Maintain water pH between 6.5 and 8.5, dissolved oxygen above 5 mg/L, dissolved solids above 50 mg/L, and insure that the water is free of pollutants. Feed fry slightly to excess, as often as 10 times/d.[23,24] Daily rations generally average 7 to 9% body weight. Commercial dry food has proven successful in hatchery production; for laboratory culture, live brine shrimp nauplii are preferred for early stages.

When trout reach the fingerling stage, reduce quantity of fish in a tank to approximately 1 g fish/L flow /d.[23] Feed fish at a rate equivalent to 4 to 5% of body weight.[23,24] As fish grow, grade and sort them into separate tanks according to size to reduce size-dependent adverse hierarchical feeding dominance.

2) Bluegill, *Lepomis macrochirus*—Breeding and cultivating of bluegills may be carried out in a variety of ponds or tanks.[23–25] Adult bluegills average 100 to 150 g in weight (12 to 18 cm in length) and generally spawn when 1 year old. The breeding period is May to August and a given individual will spawn more than once during the season.

Stock spawning ponds with a 1:1 or, preferably, a 2:3 ratio of male to female adults. Although bluegills do not require the highly controlled environment required by trout, maintain adequate DO and water quality conditions (see Section 8010E.4*b*). Bluegills adapt readily to a wide variety of commercial feeds; feed to satiation.[25]

Provide spawning ponds with small piles of pea gravel in the shallow water (0.5 to 1.0 m deep) around the edges.[24] Male bluegills will use this material to build nesting areas. Space gravel piles at least 1 m apart to reduce aggression between males guarding territories.

If dense spawning for mass fry-production is desired, place spawning stalls side by side around the perimeter of the ponds.[24] Make stalls 1 m long and enclose on three sides by wood or concrete, place gravel on the bottom, and orient the open side toward the pond center. Hatching should take place within 5 d. To capture fry after hatching but before they have dispersed, slip

a screen over the open end of the stall after the females leave the nest. Stock fry in growing ponds at densities of 40 fry/m^2.

It is not recommended to rear fry in spawning ponds with adults, but if this is necessary reduce stocking densities of brood fish to limit losses due to predation by adults. For fairly high densities of fry (40/m^2) maintain only two or three pairs of spawning adults per 4000 m^2. If large fry are desired for harvest, use a lower stock density: one breeding pair per 4000 m^2 to produce 10 fry/m^2. Under intensive culture, periodically sort juvenile bluegills according to size to facilitate uniformity of specimens for toxicity tests.

3) Channel catfish, *Ictalurus punctatus*—To establish breeding stock, collect adult catfish 3 or more years old, preferred size 1 to 4.5 kg. Segregate adults by sex in holding ponds until spawning is desired. Feed adult catfish commercially available pellets supplemented with fresh or frozen cut fish and live minnows. Daily rations of dry feed should equal approximately 3% of the weight of the stock.[23,24]

When spawning season begins (April or May), increase the daily ration to 4% of body weight. Methods have been developed for spawning in ponds and pens,[23] but aquarium spawning is most efficient in terms of space and rates of successful spawning.[23] In this method, pair catfish according to size in troughs (23- to 240-L provided with flowing water and a spawning compartment (e.g., stainless steel milk can or similar structure). Inject females intraperitoneally with hormones: three doses of 10 mg acetone-dried fish pituitary material/kg female at intervals from 6 h in warm water to 24 h in cold climates or a single dose of 2200 IU/kg body weight of human chorionic gonadotropin.[23] Most injected fish will spawn within 16 to 24 h after the last injection. Fertilized eggs adhere to each other in oval gelatinous masses. New eggs are golden, later turning pink as the embryos develop. After spawning, remove spawners and eggs. Use the troughs for additional spawning.

Incubate embryos either in hatching troughs or in open-mouth hatchery jars. Hatching troughs may be of any convenient size but at least 25 cm deep and supplied with running water. Retain the egg mass in a wire-mesh basket suspended in the hatching trough and place a paddlelike agitator driven by an electric motor alongside each basket to insure mixing of the eggs. If hatchery jars are used, place each egg mass in a separate 6- to 8-L jar. Introduce a gentle flow of water just above the mass by a rubber tube to simulate the agitation provided in nature by the fanning activities of the male.

Catfish embryos hatch in 8 to 10 d at 24°C. The fry have light-colored bodies with pink yolk sacs. Remove fry from hatching troughs or hatchery jars near the time when the yolk sac disappears, but before complete absorption, approximately 3 to 5 d after hatching. Transfer fry by siphoning through a large-bore glass tube into rearing troughs.

Catfish fry can be reared according to the methods established for trout except that warmer temperatures (24 to 28°C) are necessary. For the first 4 to 5 d in the rearing troughs, feed fry sparingly with finely ground fish food 10 times/d. Siphon off uneaten food after 2 h. Increase daily rations until they equal approximately 4 to 5% of total body weight.

b. Marine and estuarine fish: Culture methods for marine and estuarine fish species are less developed than those for freshwater species. General information about marine fish culture methods can be found in several sources.[13,14,26–38] For any species, maintain adult brood stock, eggs, and larvae under conditions approximating those in the natural environment.

A method is described below for culture of the sheepshead minnow, *Cyprinodon variegatus*, which is routinely cultured and used in egg-to-embryo or embryo-larval toxicity tests.

The sheepshead minnow thrives over a wide range of salinities and temperatures. Acclimate adult fish ≥27 m standard length to laboratory conditions for at least 2 weeks at a salinity of at least 10 to 20 g/kg (‰, parts per thousand) and a temperature of 30°C. Hold photoperiod at 12 h light, 12 h dark. During this period feed liberally on fresh or frozen adult brine shrimp. Eggs from natural spawning may be obtained by placing a pair of adult fish in a spawning chamber about 12 × 18 × 10 cm high. Place spawning trays (2 cm deep, formed by 0.5-mm nylon screen attached to a frame and covered with 2-mm nylon screen) in the spawning chambers. Larger spawning tanks with several spawning pairs may be used, provided that each male has space to establish a territory. As embryos are deposited they fall through the screen into the trays, thus preventing predation by adult fish and allowing easy removal. Each pair may spawn a maximum of 10 to 30 embryos/d but average production is about 8/pair/d.

Alternatively, sheepshead minnows may be induced to produce eggs by hormone injection.[32] Inject each female intraperitoneally with 50 IU human chorionic gonadotropic hormone. Repeat after 2 d. On the third day most females can be readily stripped to obtain ripe eggs. Strip or dissect eggs into filtered seawater in a beaker and add macerated testes. The number of eggs produced per female by this method is 100 to 200, depending on fish size. This method has the advantage of producing eggs at specified times; however, the fish typically are sacrificed to obtain eggs and sperm, thus reducing brood stock.

Fertilized eggs may be hatched in flowing or static water systems. For a flowing water system, place embryos in a hatching chamber formed by gluing a 9-cm-high collar of 0.5-mm mesh nylon screen around a petri dish. Suspend hatching chambers in flow-through seawater aquariums with self-starting siphons. As the water level in the aquarium changes, water in the hatching baskets is exchanged gently. Alternatively, place embryos in separator funnels and aerate gently.[37,38] Sheepshead minnow fry hatch after 5 d at 30°C at a salinity between 15 and 20 g/kg. As embryos hatch, transfer to a rearing aquarium and immediately feed newly hatched brine shrimp (live or frozen) or a dry food. Supplement a dry-food diet occasionally with live organisms. Juveniles become sexually distinguishable when about 24 mm long and females may produce eggs within 3 months after hatching.

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8910 C. Test Procedures

1. Short-Term Tests

a. General test procedures: Short-term testing can be used to determine relative toxicity of substances. Tests are made to determine LC50 or EC50, and to estimate toxicant concentrations for intermediate- and long-term tests.

Short-term tests may be static, static with renewal, flow-through, or recirculating, depending on the objective of the test, the life stage being tested, and the character of the toxicant or effluent (see Section 8010D.1 and 2 and Section 8921).

Although any life stage may be used, short-term tests are performed most frequently with small species (less than 5 g body weight) or juvenile forms of large fish species (see Section 8910B.1). The life stage selected depends on test purpose, availability, and laboratory facilities (see Section 8910B.1). If culturing is necessary to obtain a particular life stage, see Section 8910B.5.

Select fish of near uniform size, with the longest no more than 1.5 times the length of the shortest. Use 10 to 20 fish per toxicant concentration. Additional replicates may be used to increase the number of test fish at each concentration. For juvenile and adult fish, terminate feeding 48 h before initiating tests. For all tests, limit fish weight/L test solution. This practice minimizes oxygen depletion, metabolic waste accumulation, and crowding-induced stress. In flow-through tests, use less than 10 g of fish/L of test solution for tests at or below 17°C or 5 g of fish/L at higher temperatures. For static testing, do not load above 0.8 g/L at 17°C or less and 0.5 g/L above 20°C at higher temperatures.

b. Specific test procedures:

1) Freshwater fish

a) Equipment and physical conditions—Use test equipment made of glass, No. 316 stainless steel, or perfluorocarbon plastics.* Use unplasticized plastics such as polyethylene, polypropylene, and polyvinylchloride† or silastic in the water delivery system. In static and flow-through systems with low rates of exchange, avoid certain types of TFE stoppers; pretest stoppers to insure absence of toxicity.

Select temperature appropriate for the species being tested, hold test temperature between ± 2°C of mean test temperature during a 96-h test, or ± 1°C during any 48 h. The photoperiod at the test site can be ambient laboratory lighting with a photoperiod of 16 h light/8 h dark. A 15- to 30-min dusk/dawn transition period is desirable to acclimate test fish to the photoperiod.[1,2]

Use dilution water from a surface source, well, or spring, or use reconstituted water.[3] If the source potentially is contaminated with pathogens, irradiate with UV before using. Analyze water in control and assay chambers daily for pH, dissolved oxygen, and temperature. Maintain DO concentration at ≥60% saturation. When testing volatile substances, do not aerate test solutions. However, take care that chemical substances that create a dissolved oxygen demand do not result in conditions inconsistent with the dissolved oxygen criterion of the test as well as of fish health. As a last resort to maintain dissolved oxygen above the criterion, use aeration. If aeration is used, it may be desirable to make frequent analytical measurements to confirm test chemical concentrations.

The length of a short-term test varies, generally from 24 to 96 h. Longer test periods may be used, depending upon characteristics and variability of the waste and the purpose of the test. Dilution factors in the receiving stream provide guidance in determining effluent concentrations for range-finding tests.

For information pertaining to species selection, collection, holding, acclimation, disease control, and culturing see Sections 8010E and 8910B.

b) Test procedure

Range-finding test: If the approximate toxicity of test material is unknown, conduct an abbreviated range-finding test to determine the concentrations that should be used in the definitive tests. Do this in tests at three to five widely spaced toxicant concentrations (for example, a decade test having concentrations a factor of ten from each other). For these tests, static tests may be acceptable as would use of fewer fish, e.g., five per chamber. Run this test for 24 to 96 h.

Definitive test: To determine LC50 or EC50, use a 96-h test with a minimum of five toxicant concentrations and a control according to the results of the range-finding test. Use a carrier control if dosing solutions are prepared in an organic solvent. Acceptable carriers are dimethylformamide, ethanol, methanol, acetone, and triethylene glycol. Limit concentrations of carrier to 0.1 mL/L of test solution. Carrier controls should not result in increased mortality. If more than 10% of the fish in a control system die, repeat the test. To establish definitive test concentrations, prepare solutions using a dilution ratio of 1.5 to 2 between successive concentrations.

2) Marine fish—The test procedure for marine species is essentially identical to that for freshwater fish. Additionally, determine salinity daily. Conduct toxicity tests requiring saline dilution water using natural or reconstituted seawater having a salinity appropriate to requirements of the test fish. Run stenohaline species with seawater having a salinity of 30 to 34 ‰ and euryhaline species at 10 to 25 ‰ (± 1 to 2 ‰) salinity.

Be aware that effluent testing often requires exposing fish to 50 to 100% effluent. This exposure may create a salinity-induced stress on stenohaline organisms that could invalidate test results unless salinity is controlled by adding appropriate amounts of sea salt or through addition of saline brine produced by evaporating natural seawater.

2. Early-Life-Stage Toxicity Tests

a. General test procedures: Start fish early-life-stage toxicity tests with newly fertilized eggs and expose them through their developmental stages to an early juvenile age.[4] Testing procedures for the freshwater rainbow trout and marine/estuarine sheepshead minnow are discussed below. For fathead minnow, see Section 8921. Other species can be used if the specific environmental requirements of the fish can be approximated in the laboratory.

* Teflon® or equivalent.
† Tygon® or equivalent.

Common to all these tests are end points such as time-to-hatch, survival during the different life stages, and growth. Also observe behavior to determine behavioral effects of the test compound. Histological, physiological, or biochemical end points relevant to the study objectives also can be measured.

1) Equipment and physical conditions—For a description of suitable diluter systems see Section 8010F. Set up a minimum of two replicate exposure chambers per test concentration. Construct egg hatching cups of glass tubing (8 cm diam × 10 cm long) with 40 mesh stainless steel or equivalent nylon‡ screen glued at one end with clear silicone sealant. Suspend egg cups from a rocker arm assembly and low-speed motor designed to oscillate the egg cups slowly up and down approximately 2 to 3 cm. Use a flow rate through the exposure chambers sufficient to replace 90% of the water in 8 to 12 h. A self-starting siphon tube in each exposure chamber can be substituted for the rocker-arm system to insure test solution exchange.

Provide light intensities over the chambers of approximately 400 to 800 lux and establish a 16-h light and 8-h dark photoperiod. Preferably provide a 15- to 30-min dusk/dawn transition period.

2) Chemical data recording—Periodically measure dissolved oxygen, pH, conductivity, temperature, hardness, and alkalinity. Typically measure hardness, alkalinity, and conductivity at least weekly. Record temperature at least daily or continuously in one chamber that is centrally located in the row of exposure chambers. Measure dissolved oxygen and pH at least weekly in each exposure chamber containing surviving fish.

3) Verification of exposure concentrations—Before and during the test verify exposure concentrations of the test chemical. This will confirm actual versus nominal concentrations.

Initially, analyze concentration in each replicate test chamber. If consistent concentrations are observed among replicates at each concentration, make subsequent measurements on composite samples from each replicate. However, make other checks of diluter function to ensure proper operating conditions.

It is not necessary that measured concentrations be within any specific percentage of the nominal concentrations. Indeed, such characteristics as hydrolysis rate and volatility may make it impossible. It is important to maintain consistent concentrations during exposure and to confirm concentrations by chemical measurements. Pre-exposure monitoring will confirm that the diluter system is operating properly and that fish exposure may begin. Fluctuating exposure concentrations may indicate an improperly operating diluter system, while slowing rising concentrations may indicate that system equilibration (e.g., volatility, adsorption to chamber surfaces, hydrolysis, etc.) has not been achieved.

Once concentrations have stabilized, start exposure. Measure exposure concentrations at least weekly until the test ends.

Dichotomous data have end points that fall within two categories such as mortality (alive-dead) and hatch (hatched-not hatched). These data can be analyzed with 2 × 2 contingency tables, logit, probit, and the chi-square statistic.[7]

b. Specific test procedures:

1) Rainbow trout—The rainbow trout early-life-stage test is conducted for 60 d post-hatch at 12 ± 2°C.

a) General considerations—Run the rainbow trout early-life-stage test beginning with newly fertilized embryos or eyed embryos. Commercial suppliers of eggs and sperm make it convenient to begin with male and female gametes and fertilize the eggs in the laboratory. Study designs may expose gametes before and/or during fertilization, as well as immediately after fertilization. Selection may depend on available time because use of newly fertilized eggs adds approximately 1 month to the duration of the test.

b) Equipment and physical condition—For a description of suitable diluter systems see Section 8010F. Use an exposure system similar to that described in 8910C.2*a*1) with the following changes. On bottom of the egg cup use 16 mesh stainless steel or nylon screen.‡ Incubate under little or no light. When eggs hatch, provide light intensities over the chambers of approximately 400 to 800 lux on a 16-h light and 8-h dark photoperiod. Preferably include 15- to 30-min dusk/dawn transition. Hold at 12 ± 2°C.

c) Test initiation—The test begins with distribution of newly fertilized eggs or eyed eggs to each incubation cup. Select number of embryos based on the desired discriminating power for the test and the number of replicates. At a minimum, apportion 60 embryos per experimental group among four incubation cups and place two cups in each of two replicate test chambers.

d) Biological observations—Monitor survival by daily inspecting embryos or hatchlings. Record observations on fish behavior, noting abnormal and normal individuals along with characteristics of any abnormalities.

During embryo incubation remove dead eggs to prevent fungal infections. Dead eggs can be distinguished from living eggs by their white color.

When hatching is greater than 95% complete, count post-hatch alevins and release them to their respective growth chambers. For approximately 2 weeks following hatching, alevins will feed from their yolk sacs. When the first few alevins begin to swim up, feed fry with brine shrimp nauplii in combination with a standard commercial fish food at least three times per day as needed.

Because hatching may occur over a 3- to 6-d period, use the time to obtain at least 95% hatch of control to establish the 60-d post-hatch growth period. Determine growth at 30 d post-hatch (midpoint of growth period) and 60 d post-hatch (end of test). Use a method of measurement that least stresses the fish. The photographic method[5] has been used successfully to estimate weight and lengths when fish are not to be sacrificed.

e) Chemical data recording—See Section 8910C.2*a*2).

f) Verification of exposure concentrations—See Section 8910C.2*a*3).

2) Sheepshead minnow—Run this test for 35 d at 25 ± 2°C.

a) Equipment and physical conditions—For a description of suitable diluter systems see Section 8010F. Use an exposure system similar to that described in 8910C.2*a*1) except with seawater salinity of 10 to 20 ‰.

b) Test initiation—See Section 8910B.5*b* for egg collection. The test begins with distribution of newly fertilized eggs to each embryo cup. Select a number of embryos based upon the desired discriminating power for the test and the number of replicates. At a minimum, distribute 60 embryos per experimental group among four incubation cups and place two cups in each of two replicate test chambers. Sheepshead minnows hatch in about 7 d

‡ Nitex or equivalent.

at 25°C.[6,7] Release larvae from hatching cup to the test chambers and start feeding. Initially feed a combination of live brine shrimp nauplii, then shift to brine shrimp and commercial food after 7 to 10 d.[6]

c) Biological observations—See Section 8910C.2*b*1)d).

d) Chemical data recording—In addition to those water quality factors described in Section 8910C.2*a*2), measure dilution water salinity at least weekly.

e) Verification of exposure concentrations—See Section 8910C.2*a*3).

3. Reproductive Toxicity Tests

a. General test procedures: Use newly spawned eggs, newly hatched larvae, juveniles, or sexually immature fish to start a test. The life stage selected depends on species, laboratory space and facilities, availability of the life stages, and test objective.

Expose enough fish to each concentration of toxicant to insure adequate numbers of each sex at maturity but low enough to prevent stress due to crowding. Additional fish may be introduced to tanks with similar treatment to provide specimens for histological examination, residue analysis, or selected physiological measures of condition. The exact number of fish required depends on life stage at start of test and test species (see Section 8910C.3*b*).

At start of test, measure total length and weight of all fish. Repeat measurement for any fish that die during the test. To prevent injury, anesthetize§ large fish before handling. Larvae and small fish may be measured by a photographic method.[5] At the end of a test, record length, weight, and, if possible, sex and gonadal condition of each fish.

For viability and hatchability tests, incubate eggs from each spawning at an optimum temperature in control water. Count live and dead eggs and remove dead eggs daily. Evaluate egg viability for all spawnings by incubating eggs until development clearly is observed (some defined stage of embryogenesis, e.g., eyeing, is reached). Determine hatchability for all spawnings in all exposure chambers or from a predetermined number of spawnings when the species tested is one that spawns continuously (many times per season). Count number of dead, deformed, and normal larvae hatched daily, using a dissecting microscope if necessary.

To evaluate larval growth and survival at each toxicant concentration, collect a uniform number of larvae (usually 20 to 50) at random from two or more successful hatches and place in chambers for that toxicant concentration. Determine length and number of larvae upon transfer to growth chambers, preferably by the photographic method. Determine total length of larvae at selected intervals and at end of test. Count, measure, and remove dead larvae daily.

For methods of toxicant mixing and delivery see Section 8010F.1*c*.

Use spawning tanks, exposure tanks, and growth chambers appropriate for the test species. Design each growth chamber so that test solutions can be drained down to 2.5 to 3 cm and the chamber transferred to a fluorescent light box provided with a millimeter grid for photographing fish.[5]

§ 3-aminobenzoic acid ethyl ester or equivalent.

Monitor fish and embryos maintained for physiological, biochemical, and histological tests carefully. As a minimum, report all pertinent data for each test container at the beginning, about a third of the way through, and at end of test. Include number and weight of individuals, number of spawnings, number of embryos, and total lengths of normal, deformed, and injured mature and immature males and females. Count and record all survivors and mortalities. Calculate mean incubation time for median spawning and hatch dates if known. The hatchability, fry survival, growth, and percent deformities also may be determined.

Measure toxicant concentration in all tanks at each concentration weekly. Composites of equal-volume daily grab samples for 1 week may be used if it has been shown that analytical results for the test compound are unaffected by storage. Include samples for assessing recovery (i.e., known additions) and blanks. Analyze enough samples throughout the test to determine whether toxicant concentrations are constant. If this is not possible, analyze enough samples weekly to establish variability of toxicant concentration (see Section 8010F.3*d*).

Record temperature continuously in a centrally-located tank. Measure oxygen levels periodically in each tank. Analyze water from the control and one exposure tank at least weekly for pH, hardness, alkalinity, and conductivity in freshwater systems and pH and salinity in marine systems. If any characteristic is affected by the toxicant, analyze that characteristic at least 5 d/week, rotating among tanks so that each is analyzed once every other week.

When possible, analyze mature fish and/or eggs, larvae, and juveniles for toxicant residues.

b. Specific test procedures: Procedures used for reproduction tests are described below for a freshwater and a marine species.

1) Brook trout, *Salvelinus fontinalis* (freshwater)—This test procedure extends over only a part of the life cycle because of the longevity of brook trout. It follows the life cycle from the yearling stage through spawning, egg hatching, and development for 90 d.

a) Equipment and physical conditions—For description of suitable diluter systems see Section 8010F. Set up duplicate tanks for each test concentration and control. Premix each concentration before delivery to duplicate spawning tanks and growth chambers.

Construct alevin-to-juvenile growth chambers with dimensions of 18 × 15 × 18 cm of glass or stainless steel with a glass bottom. Maintain water depth at about 13 cm. Design each chamber so that the water can be drained down to a depth of 2 to 3 cm to allow the chamber to be placed over a millimeter grid on a fluorescent light box for photographing fish for measurements of length.

Construct spawning tanks of No. 316 stainless steel, with dimensions of 80 × 30 × 40 cm. Use a 30-cm water depth. Place a spawning substrate or nest[6–8] in spawning tanks at the appropriate time. Use spawning nest, 28 × 33 × 7.5 cm, made of double-strength glass or stainless steel. Large fish may require a larger nest. Drill three 2.5-cm holes in each end, 2.5 cm from the bottom, and cover with 10-mesh stainless steel wire to allow water in the box to drain to a depth of 2.5 cm when the box is removed from the spawning chamber. Place a bottomless screen egg-retainer (27 × 32 × 1.3 cm with 2.5-cm square compart-

ments, constructed from 1.3-cm-wide strips of 7-mesh stainless steel screen) in the spawning box. Place 2-mesh stainless steel screen, 27 × 32 cm, to which 1.3- to 2.5-cm gravel is attached with silicone adhesive, on top of the screen egg retainer.[8] Use smooth gravel to prevent injury to active, spawning fish. This spawning box is readily removed from the spawning tanks to collect eggs for transfer to incubation cups. For spawning stocks, select yearling fish that will not grow too large for the spawning box. Fish weighing not more than 50 to 70 g at time of selection and 150 g at spawning are appropriate. If fish weigh more than 150 g, use a larger spawning box.

Provide a daylight period conducive to the spawning of brook trout. Ideally use 15- to 30-min dawn/dusk transition times and provide conditions described above in Section 8910C.1*b*1)a).

Provide water flow of 6 to 10 tank volumes/d to maintain oxygen levels above 60% saturation. Remove uneaten food and wastes from growth chambers daily. Brush interior surfaces to remove attached growths as needed.

b) Exposure procedures—To begin the test, collect juveniles from the field no later than March 1 and acclimate for at least 1 month or use cultured stock of equivalent age. Judge suitability of fish for testing on the basis of acceptance of food, apparent lack of disease, and occurrence of less than 2% mortality during acclimation and no mortality during the 2 weeks before the test.

Begin exposure by placing at least 12 acclimated yearling brook trout in each duplicate tank at each test concentration and suitable controls using a stratified random assignment (see 8010F.3*a*). This allows about a 4-month exposure to toxicant before the onset of secondary or rapid-growth phase of the gonads. Extra test animals may be included at the beginning so that fish can be removed periodically for special examination or for chemical analysis.

Use a particulate or pelleted trout food. Feed fish the largest particle or pellet they will take, at least twice daily. Base amount on a reliable hatchery feeding schedule.[9] Analyze each batch of food for pesticides.

Record mortalities daily and measure total length and weight of fish directly at initiation of tests and every 3 months thereafter. Do not feed fish for 24 h before weighing. Lightly anaesthetize them to facilitate measuring.

When secondary sexual characteristics are well-developed (approximately 2 weeks before spawning), separate males, females, and undeveloped fish in each tank and randomly reduce number of sexually mature fish to two males and four females per tank. Record number of mature, immature, deformed, and injured males and females in each tank and number from each category to be discarded. Thoroughly clean, sterilize, and rinse the spawning substrates and place one for each male in each spawning tank. As soon as spawning begins, set up incubation cups (as described in 8910B.5*a*1) or a suitable alternate system to receive embryos for hatching. Remove embryos from the substrate at a fixed time each day, preferably so fish are not disturbed during early part of the light period.

Randomly select 50 embryos from the first eight spawnings of 50 embryos or more in each duplicate spawning chamber and place in an embryo incubator cup. Count remaining embryos from the first eight spawnings and all embryos from subsequent spawnings and place them in separate incubator cups to determine viability as evidenced by development to a specific stage, e.g., formation of neural keel after 11 to 12 d at 9°C or eyeing. Remove and record number of dead embryos from each spawn. Never place more than 250 embryos in one incubator cup. Incubate all embryos to determine viability and discard after reaching some clearly distinguishable stage (development of neural keel or eyeing). Discarded embryos may be analyzed chemically or used for other measurements.

Obtain additional information on hatchability and alevin survival by transferring embryos from control tanks immediately after spawning (*a*) to tanks having test concentrations where spawning is reduced or absent and (*b*) to tanks where an effect is seen on survival of embryos or alevins, and by transferring embryos from those test concentrations to control tanks. Always reserve two growth chambers in each duplicate spawning tank for embryos produced in that tank.

Remove dead embryos daily from incubator cups. When hatching begins, record number of alevins hatching daily in each cup. On completion of hatching in any cup, transfer fish to a culture dish and randomly sample 25 alevins. Count dead or deformed alevins. Transfer 25 selected alevins to a growth chamber and place it over the light box to measure by the photographic method. After photographing, return alevins to incubator cup. Never net alevins, but transfer by gentle pouring or by large-bore pipets. Transport in growth chambers containing enough test solution to limit harsh contact with the bottom screening. Preserve unused alevins in formalin for subsequent histological examination. Record length and weight of discarded alevins separately from the data for fish kept for continued exposure.

For 90-d growth and survival exposures randomly select 20 alevins from each duplicate incubator cup for each test concentration and control. Because embryos from one spawn may hatch over a 3- to 6-d period, use the median hatch date to establish the start time of the 90-d growth and survival period. For growth tests select two groups of 20 alevins that are less than 3 weeks apart in age. Use any remaining groups only for hatchability testing. After photographing to determine length, preserve for weight determination. To equalize effects of incubator cups on growth, keep all groups selected for 90-d exposure in the incubator cups for 3 weeks after the median hatch date, then release into growth chambers. Begin feeding immediately. Keep the two groups from the same exposure chambers separate for replication of each test concentration. Record mortalities daily, total lengths at 30 to 60 d after hatching (by the photographic method), and total length and weight at 90 d after hatching. At the end of the test cease feeding juveniles for 24 h and then weigh. Terminate survival and growth studies after 3 months, at which time fish may be used for chemical analysis of tissue and physiological measurements of toxicant-related effects.

End exposure of all parental fish after 3 weeks in which no spawning occurs in any tank. Record mortality and weight, measure total length of parental fish, and check sex and condition of gonads (e.g., reabsorption, degree of maturation, spent ovaries).

Report, for each tank of a partial-life-cycle test, number and individual weights and total lengths of immature males and females at initiation of test, after 3 months, at reduction in numbers, and at end of test. Report individual weights and total lengths of normal, deformed, and injured fish, number maturing, number dying during test, number of spawnings and eggs, hatch-

ability and fry survival, growth, and deformities. Calculate a mean incubation time based on date of spawning and median hatch dates.

For additional information on the life cycle of brook trout consult other sources.[8–17]

2) Fathead minnow, *Pimephales promelas* (freshwater)—See Section 8921.

3) Reproduction tests with other freshwater species—Partial-life-cycle toxicity tests have been performed with the bluegill, *Lepomis macrochirus*, *Oryzias latipes*, and the flagfish, *Jordanella floridae*.[18–23] When culture techniques are available, other native freshwater species may be used as appropriate.

4) Sheepshead minnow, *Cyprinodon variegatus* (euryhaline)

a) Equipment and physical system—Use test apparatus similar to that used for freshwater fish with spawning aquariums at least 30 × 18 × 20 cm. Place a spawning chamber in each aquarium as described in 8910B.5*b*.

b) Exposure procedures—Begin with adult fish or preferably with embryos. Secure embryos either by natural spawning or by hormone-induced spawning (as described in 8910B.5*b*). Keep water temperature above 22°C, preferably at 30°C, with salinities above 15 mg/kg. When starting with adults, set up five or six spawning aquariums for each test concentration and controls. Use breeding fish, all from the same stock, that have been kept in holding tanks for at least 2 weeks, during which less than 2% mortality occurred. Feed fish a combination of frozen adult brine shrimp and dry trout food. Maintain water flow through spawning aquariums at 6 to 10 tank volumes/d. Use natural seawater filtered to remove planktonic larvae 15 μm and larger.

When embryos are produced, remove them from spawning chambers and place in hatching chambers for each concentration being tested as well as the controls. Start toxicant dosing in exposure chambers before the hatching chambers are placed inside them. Construct hatching chambers by cementing a 9-cm-wide strip of 500-μm nylon screen around a petri dish. Place the hatching chambers in 90- × 30- × 30-cm exposure chambers in 7 cm of water with flow-through of the toxicant. As embryos hatch, feed fry with newly hatched brine shrimp nauplii. Clean screens on incubation cups and chambers daily. Check and record daily survival of embryos and fry, which constitute the first filial (F_1) generation.

On the first day after hatching remove each chamber and count and measure fry photographically. During the first 2 weeks feed with newly hatched brine shrimp nauplii. During the following 2 weeks supplement this diet with dry trout pellets or dry mollie flakes. After 4 weeks count and measure fish by the photographic method and reduce the number to 50 for each test concentration and controls. Record length, weight, condition, and number of living, deformed, and dead fish remaining. Determine percent mortality and abnormality in each test concentration and controls. Preserve specimens for future tests or discard. Place the 50 selected fish, 25 each, in duplicate growth chambers having a glass bottom and provisions for drawing the water level down to 1 to 2 cm. Feed a mixed diet of brine shrimp and dry food twice daily and examine daily for dead specimens. At 8 weeks measure again by the photographic method. Twice daily, feed dry food supplemented with frozen adult brine shrimp until maturity. Check each batch of food for pesticides, PCBs, or other contaminants of concern. Clean all exposure aquariums and spawning and hatching chambers two to three times per week. Siphon out all wastes.

As fish approach sexual maturity, place separate pairs in spawning chambers, five pairs from each duplicate exposure chamber; i.e., 10 pair for each test concentration and controls, and continue exposure. Count, measure, and weigh all unused fish from each duplicate exposure chamber. Record number deformed and dead in each test concentration and controls, condition of fish, and other pertinent data. Preserve some fish for whole-body tissue residue analysis. As fertilized eggs are produced, remove at a specified time daily, count, and place 25 F_2 fish in a hatching chamber as for the F_1 generation. Record the total number of embryos produced in each chamber, time required to hatch, hatching success, and survival of embryos. Test those not placed in hatching chambers for fertility and record percent of fertile females.

Keep pairs in each spawning chamber until all needed embryos have been obtained. At termination, measure and weigh spawning pairs and record all other pertinent data. Preserve for toxicant analyses, if desired.

Expose embryos of F_2 generation in hatching chambers in their respective duplicate exposure chambers for each test concentration and controls as before. Count and measure by photographic method[5] as for the F_1 generation. Feed fish and record results. At the end of 4 weeks terminate the test. Weigh and measure all fish; record number of deformed fish and determine number that died. Preserve for histological examination and tissue analyses. Determine effects of each test concentration and calculate safe levels. During tests, record temperature daily, and oxygen concentration, pH, and salinity at least weekly. If possible, chemically analyze test water for toxicant at the beginning, at regular intervals (e.g., weekly) during exposure, and on completion of tests. Analyze lots of 10 fish from highest and lowest exposure concentrations and from controls for toxicant accumulation. Analyze dilution water for toxicant at beginning and end of test.

For additional information about the life cycle of *Cyprinodon variegatus*, and testing procedures, consult other sources.[22–26]

5) Life-cycle tests with other marine fishes—Life-cycle tests may be performed with other marine fishes such as *Fundulus heteroclitus* and *Menidia menidia*. For information on life cycle and culture of these species, consult other sources.[27–29]

4. Statistical Analysis

Analyze, handle, and report data as in Section 8010G.

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8921 FATHEAD MINNOW*

8921 A. Introduction

The fathead minnow, *Pimephales promelas* rafinesque, is a small, common, and widely distributed freshwater fish of the family Cyprinidae. This minnow is maintained easily in the laboratory and can be spawned year-round. These attributes have led to its widespread use in aquatic toxicology studies, particularly those utilizing early life stages (i.e., embryos and larvae), such as the short-term tests for measuring the chronic toxicity of effluents.

Other procedures for testing larval growth of fathead minnows are available.[1]

1. Description

Adult fathead minnows typically range in size from 43 to 102 mm, averaging about 51 mm (2 in.) in total length.[1,2] Young and nonbreeding adults are light in color with a distinct lateral band from caudal peduncle to head; males and females at this stage are difficult to differentiate, except that males are typically larger. In breeding condition (Figure 8921:1), males are distinguished from females by the presence of nuptial tubercles on the snout and by coloration. Mature males are dark in overall coloration with a saddle-like pattern behind the head, whereas females are quite drab.

2. Distribution, Biology, and Life History

The fathead minnow is tolerant of adverse conditions including high temperature and turbidity and low oxygen concentrations.[2] Because of this tolerance, the fathead minnow is found in a diversity of habitats and is widely distributed throughout central North America from Canada to northern Mexico.[2,3] It is most abundant in muddy streams, brooks, ponds, and small lakes.[2] This species is a popular bait fish and has been introduced to areas both within and outside of its native range because of the relative ease with which it is maintained and propagated.

The fathead minnow rarely lives beyond an age of 2 years. In warm, food-rich waters, it grows rapidly and may reach adult size and begin spawning in as little as 3 months. In cold waters, it make take a year to reach maturity.[3] It is omnivorous, with diet consisting at times of algae, organic detritus, aquatic insects, worms, small crustaceans, and planktonic organisms. Because it is highly prolific, can utilize many foods, and is widely preyed upon by other fish and fish-eating birds, the fathead minnow is considered an ideal forage fish and important bait species.

In the wild, fathead minnows begin spawning in the spring and often continue to spawn throughout the summer. Spawning typically begins when the water temperature reaches about 16 to 18°C, although this temperature may vary with the population and latitude.[2] Spawning usually occurs in the early morning in shallow water less than 1 m deep. The male selects a suitable substrate (e.g., underside of a log, branch, root, large rock, board) and herds a receptive female into position. Using her ovipositor, the female deposits her adhesive eggs (from 100 to 500 per spawn) on the substrate and is then driven off by the territorial male. The male aggressively guards the nest and often seeks out additional females to spawn in the nest. Time to egg hatching depends on water temperature. For example, eggs hatch in about 1 week at 22°C and between 4 and 5 d at 25°C. Newly hatched larvae are approximately 5 mm long and opaque white in color, and have large black eyes (Figure 8921:2).[2,4]

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* Approved by Standard Methods Committee, 1997.
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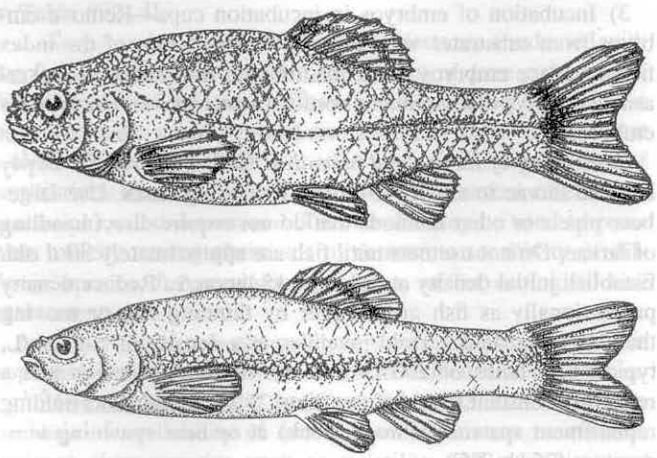

Figure 8921:1. Adult fathead minnows in breeding condition: (above) male; (below) female.

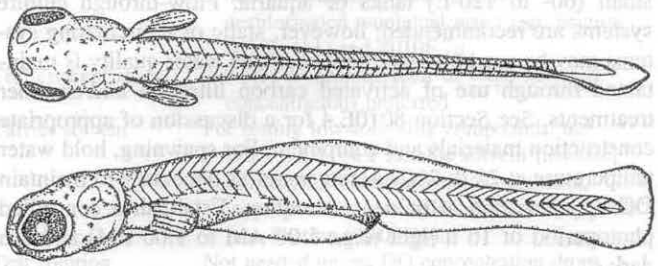

Figure 8921:2. Newly hatched fathead minnow larvae: (above) top view; (below) lateral view.

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4. Bibliography

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8921 B. Culture and Maintenance of Test Organisms

1. Obtaining Test Organisms

Organisms of various ages may be obtained for toxicity testing from commercial breeders, biological supply houses, or an in-house culture facility. An in-house breeding facility is recommended, and may be required, if testing is to be conducted with early life stages or fish of a specific age. Ensure that fish, particularly those from outside sources, are certified as to their identity, age, and freedom from disease. Preferably use sources that supply reference toxicant data with their shipments. In general, except as source of "new" genes, avoid using organisms from bait shops, hatcheries, or field populations because their suitability for use and disease status cannot be assured.

2. Culturing and Care of Test Organisms

Fathead minnows, including all life stages from egg to adult, may be successfully cultured in the laboratory using static, recirculating, or flow-through systems. Basic information on establishing and maintaining a culture facility is presented below. For additional information, see Sections 8010E.4 and 8910B, and other sources.[1–3]

a. Water supply and culture system: Supply cultures with good-quality water; reconstituted (synthetic) water, dechlorinated municipal water, and natural water (see Section 8010E.4*b*) are all acceptable. Natural water is preferred provided that its quality is relatively constant and it meets minimum acceptability criteria. Reconstituted water usually is recommended to be of moderate hardness (see Table 8910:I). Analyze all water supplies periodically for chlorine (free and combined), ammonia, toxic metals (e.g., Cr, Cd, Cu, Pb), organic compounds (e.g., pesticides, PCBs), and basic water quality factors (e.g., pH, DO, conductivity, hardness, alkalinity).

Choose a spawning unit designed to simulate natural spawning conditions (e.g., light, temperature). Typically, fish are bred in small (60- to 120-L) tanks or aquaria. Flow-through culture systems are recommended; however, static or recirculating systems may be used provided that adequate water quality is maintained through use of activated carbon filtration and/or other treatments. See Section 8010E.4 for a discussion of appropriate construction materials and equipment. For spawning, hold water temperature at 25 ± 2°C. Aerate water as necessary to maintain DO concentration at or near saturation. Establish a controlled photoperiod of 16 h light (e.g., 5:00 AM to 9:00 PM) and 8 h dark.

b. Establishment of breeding: Establish breeding units with 15 to 20 mature (>6-month-old) adults and two or three spawning substrates per tank. Construct spawning substrates from inverted halves (semicircular sections) of Schedule 40 PVC pipe (7.5 cm ID × 7.5 cm length) or other nontoxic material (e.g., glass, stainless steel). If the inverted surface is smooth, roughen it to aid embryo adhesion. At first it may be difficult to differentiate the sex of fish, but this process becomes easier over the next week as organisms develop their secondary sexual characteristics (Figure 8921:1). Remove excess males to maintain a sex ratio of approximately six females per male and no more than two males per tank.[1,2] As an alternative, pair females and males in divided tanks.

c. Embryo collection and incubation: Check spawning substrates daily in the late morning or early afternoon. Record the number of eggs per substrate and incubate embryos using one of the techniques described below.[1,2] Examine embryos daily and remove all that are dead (milky and opaque) or show fungal growth. Embryos maintained at 22 to 25°C will hatch in about 4 to 7 d.

1) Incubation of embryos on substrate—Place several substrates on end in a circular pattern (embryos on the inside) around a source of gentle aeration. Maintain a constant temperature and sufficient water depth to cover substrates.

2) Incubation of embryos in a separatory funnel—Remove embryos from substrates with a gentle rolling action of the index finger.[4] Incubate embryos in a 2-L separatory funnel containing approximately 1.5 L water. Hold separatory funnel in a constant-temperature bath and maintain constant gentle aeration from bottom of funnel.

3) Incubation of embryos in incubation cups—Remove embryos from substrates with a gentle rolling action of the index finger.[4] Place embryos in incubation cups attached to a rocker-arm assembly[5] that maintains constant water movement over the embryos.

d. Rearing of larvae and juveniles: Each day, transfer newly hatched larvae to small (10- to 60-L) rearing tanks. Use large-bore pipets or other methods that do not require direct handling of larvae. Do not use nets until fish are approximately 30 d old. Establish initial density at or below 15 larvae/L. Reduce density proportionally as fish grow larger by thinning fish or moving them to larger tanks. Hold juveniles at a density of ≤1 fish/L, typically in tanks of 200 L or more. Maintain aeration and a relatively constant temperature (20 to 25°C). Keep tanks holding replacement spawners (brood stock) at or near spawning temperature (25 ± 2°C).

e. Food and feeding: Feed larvae up to 30 d old two to three times a day with newly hatched brine shrimp (*Artemia salina*).

Culturing of brine shrimp is described elsewhere.[6] Supplement live food once or twice a day with commercial fish starter food. Feed older fish with frozen adult brine shrimp, commercial fish starter, and tropical fish flake food.[1,2] Fish may be fed *ad libitum* but avoid overfeeding because it increases tank maintenance, decreases water quality, and may increase stress and susceptibility to disease.

f. Parasite and disease control: Observe fish daily for disease and abnormal behavior. Parasites and disease will rarely be a problem if proper water quality and aeration are maintained in rearing tanks. If necessary, provide treatment as described in Section 8910B.4. Clean and disinfect tanks and related equipment on a regular basis and, in particular, before new fish are added or after any disease outbreaks. Avoid spread of disease by disinfecting dip nets before use.

3. Acclimating and Holding Test Organisms

When possible, quarantine and acclimate (preferably for at least 48 h) test organisms obtained from outside sources; however, no acclimation is necessary for tests initiated with fish early-life stages. During acclimation, protect fish from large changes in temperature or water quality (e.g., pH, hardness) and minimize handling. Avoid overcrowding and maintain sufficient DO concentrations. During acclimation, change water from 100% holding water to 100% dilution water. Keep all organisms in 100% dilution water for at least 48 h before use.

Observe fish daily for signs of stress and disease; remove dead or abnormal organisms promptly. Mortality of 5 to 10% is not unusual during the first 48 h of acclimation; however, do not use organisms in tests if mortality exceeds 5% in the 48-h period preceding test initiation. See Sections 8010E.3 and 8910B.3 for additional information.

4. References

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8921 C. Procedures

1. Short-Term (Acute) Test

Test procedures and conditions common to short-term tests on fathead minnows are summarized in Table 8921:I; additional procedures and conditions for specific short-term tests are shown in Table 8921:II.

a. Scope and application: Short-term tests are conducted with the fathead minnow to determine toxicity of pure compounds, formulations and mixtures, effluents, and receiving waters. Test populations are usually mixed sex. Mortality is the primary test endpoint (see Table 8921:II); other endpoints such as loss of equilibrium should be noted and an EC50 (median effect concentration) value determined. Test results may be used to compare toxicity among chemicals to determine sensitivity of different species, for regulatory purposes, or for ecological risk assessments.

b. General test procedures: Short-term (acute) test procedures applicable to the fathead minnow are described in several sources.[1–5] For test duration and types, see Table 8921:II. Choose type on basis of availability of test compound, mixture or effluent, toxicant characteristics such as volatility and solubility, and age/size of minnows used for testing (see Sections 8010D.1 and 2). Early life stages (i.e., larvae) or juveniles are preferred over

Table 8921:I. Test Conditions Common to Various Fathead Minnow Short-Term Tests

Test Condition	Type or Value
Light quality and intensity	Ambient laboratory levels; 550–1050 lux(50–100 ft-c)
Photoperiod	16 h light; 8 h dark
pH	6.0–9.0 (if outside this range, adjust to pH 7.0 and perform a parallel test without pH adjustment - see text)
Dilution water	High-quality fresh water. May consist of natural water, receiving water, moderately hard reconstituted fresh water, or dechlorinated municipal water (see Section 8010E.4*b*1) and 8010F.2*a*.
Test concentrations	≥5 plus a control; factor of ≥0.5 between concentrations preferred
Carrier solvent	For testing low-solubility compounds, use ≤0.1 mL/L of a suitable solvent (acetone, dimethylformamide, ethanol, methanol, isopropanol, acetonitrile, or ethylene glycol) (see Section 8010F.2*b*).
Test solution aeration	Not needed unless DO concentration drops below 4.0 mg/L; avoid supersaturation.

TABLE 8921:II. TEST CONDITIONS SPECIFIC TO VARIOUS FATHEAD MINNOW SHORT-TERM TESTS

Test Condition	Acute Test	Survival and Growth Test	Embryo-Larval Survival and Teratogenicity Test
Test type	Static, static-renewal, recirculating, flow-through	Static-renewal	Static-renewal
Duration	24, 48, or 96 h (typically)	7 d	7 or 8 d
Temperature	20°C ± 1°C or 25°C ± 1°C	25°C ± 1°C	25°C ± 1°C
Test chamber size	≥250 mL	≥500 mL	≥150 mL; ≥250 mL (preferred)
Test solution volume	≥200 mL	≥250 mL	≥70 mL; ≥200 mL (preferred)
Test solution renewal	After 48 h (minimum); after 24 h (preferred)	Daily	Daily
Age of test organisms			
Effluents and receiving waters	1–14 d (post hatching), ≤24-h range in age	<24-h-old larvae (post hatch); if larvae are not obtained from in-house cultures they should be <48 h old (<24-h range in age)	≤36-h-old embryos, ≤24-h range in age (maximum of 48 h if shipped)
Pure compounds and mixtures	1–14 d (post hatching), ≤24-h range in age or 30–60 d (post hatching); ≤24-h range in age		
Organisms per test chamber	≥10; 20–25 (preferred)	≥10; 15–25 (preferred)	≥10; 15–25 (preferred)
Replicate chambers per concentrations	≥2; 4 (preferred)	≥3; 4 (preferred)	≥3; 4 (preferred)
Organisms per concentration	≥20; 80–100 (preferred)	≥30; 60–100 (preferred)	≥30; 60–100 (preferred)
Feeding:			
Larvae (>2 d old)	0.2 mL *Artemia* (brine shrimp) nauplii concentrate before test initiation and 2 h before test solution renewal at 48 h	0.1 g newly hatched *Artemia* nauplii (<24 h old)	Not required
Juveniles	Feed before test initiation; do not feed during test.	Three times daily at 4-h intervals, or at a minimum, 0.15 g twice daily with 6 h between feedings. No feeding during final 12 h.	
Cleaning of test chambers	As required. Generally not required if test is conducted flow-through or solutions are renewed after 24 or 48 h.	Siphon daily, immediately before test solution renewal	Not required
Test endpoints	Mortality (normally LC50 and NOEC), behavior (activity, swimming, buoyancy, feeding, etc.)	Mortality and growth (weight)	Mortality and teratogenicity (deformed larvae)
Test acceptability	Mortality of control organisms ≤10%	Mortality of control organisms ≤20%; average dry weight per surviving organism in control chambers of ≥0.25 mg	Mortality of control organisms ≤20%

adults for use as test organisms because they are typically more sensitive and require smaller test solution volumes. Selection of larvae or juveniles depends on test purpose, availability, and laboratory facilities (see Section 8910B.1). Testing of effluents and receiving waters requires use of early life stages[1] (see Section 8921B.2).

Initially determine approximate toxicity of the test material in a range-finding test of 24 to 96 h. Use three to five widely spaced concentrations (e.g., dilution factor of 10) and, optionally, fewer organisms per concentration than normally recommended for definitive tests. Use results of the range-finding test to determine appropriate concentrations for use in the subsequent definitive test.

c. Specific test procedures:

1) Equipment and physical conditions—Always use materials that minimize sorption and leaching of toxic substances, such as tempered glass, perfluorocarbon plastics,* and No. 316 stainless steel (see Section 8010F.1). Clean and rinse all equipment, including new glassware, before use (see Section 8010E.4*d*). Flow-through tests may require use of a diluter system (see Section 8010F.1*c*) or continuous-flow-low-volume (e.g., peristaltic) pumps. Conduct testing in well-ventilated, temperature-controlled facility. Maintain test temperature and light conditions as directed in Tables 8921:I and II. Cover test vessels with clear plastic or glass allowing air circulation.

For dilution water requirements, see Table 8921:I. Prepare test solutions as described in Section 8010F.2*b*. Use a dilution factor of 0.5 or greater for determining test concentrations in the

* Teflon® or equivalent.

definitive test. For testing compounds of low solubility, follow carrier-solvent recommendation in Table 8921:I and use a solvent control. Analyze water in control and test chambers daily for pH, DO, and temperature. For renewal tests, make these measurements in new solutions and before renewing solutions, to see the range of conditions. Avoid aeration, particularly for volatile compounds, except to maintain DO concentrations at a minimum of 4 mg/L.

Outside pH range 6.0 to 9.0, the toxicity of metals and organics may be masked by the toxic effects of low or high pH. In such cases, preferably make two parallel tests, one with the pH adjusted to 7.0 and one without an adjusted pH. Adjust sample pH by adding 1*N* NaOH or 1*N* HCl dropwise, as required, being careful to avoid overadjustment.

2) Test initiation—Obtain suitable fathead minnow larvae (1 to 14 d old) or juveniles (30 to 60 d old) from an in-house culture or outside supplier. Larvae are often the most sensitive fish life stage and usually are required for compliance testing of effluents.[1] Distribute test organisms randomly between replicate test chambers containing test solutions. Use a minimum of 2 replicates with 10 organisms each. If sufficient numbers of test organisms are available, preferably test 80 to 100 organisms at each concentration (preferably 4 replicates each with 20 to 25 organisms). For static and static-renewal tests, use live weight loading in the test solutions below 0.65 g/L (20°C) or 0.40 g/L (25°C). For flow-through tests, use live weight loading below 5.0 g/L (20°C) or 2.5 g/L (25°C).

3) Solution renewal—Unless the supply of test material or effluent is limited, renew test solutions daily or every other day. Periodic renewal is especially important in tests of volatile compounds, chemicals with low solubilities, and compounds that degrade rapidly.

4) Feeding—See Table 8921:II. Avoid overfeeding because it may reduce toxicant concentrations and DO levels. Culturing *Artemia* is discussed elsewhere.[1,6]

5) Biological data and observations—Monitor and record mortality daily starting approximately 24 h after test initiation; remove dead organisms. Criteria for establishing death include lack of movement and no reaction to gentle prodding. Record general observations of fish appearance (coloration, deformities) and behavior (lethargy, lack of schooling, loss of equilibrium).

6) Chemical data recording—Measure conductivity, hardness, and alkalinity in freshly prepared solutions at test initiation, each test solution renewal, and at test termination in the highest concentration of test solution and in the dilution water. Measure pH, DO, and temperature at test initiation and daily thereafter in all test concentrations. In static-renewal tests, make measurements in both freshly prepared and 24-h-old solutions.

7) Verification of exposures—If resources are available for analyses, verify exposures by measuring concentrations of the test chemical in exposure solutions at test initiation and termination. Base statistical analyses and results (LC50, NOEC) on measured concentrations rather than nominal concentrations.

8) Test termination—End test after 24, 48, or 96 h. Before termination, record number of dead and abnormal fish in each test chamber.

2. Short-Term Methods for Estimating Chronic Toxicity

Several test methods are available for estimating the long-term (chronic) effects of a toxicant or effluent after a relatively short (7-d) period of exposure (see Section 8010D.5). Two of these tests, the larval survival and growth test and the embryo-larval survival and teratogenicity test, use fathead minnows as test organisms and are described below. These short-term tests were developed as cost-effective alternatives to long-term early-life-stage and life-cycle tests. They were designed primarily to evaluate effluent toxicity[7–10] and are often included as biomonitoring requirements in discharge permits. They also have been used successfully to estimate the potential chronic toxicity of pure compounds.[9,11–13] In addition, because embryos and larvae often are the most sensitive stages in a fish's life cycle,[14,15] studies with embryo-larval stages have been used for investigating teratogenesis and identifying developmental toxicants.[16–19]

a. Larval survival and growth test: This method estimates the chronic toxicity of effluents and chemicals using newly hatched fathead minnow larvae in a 7-d test. Test results are based on survival and weight of larvae. Test conditions and procedures are given in Table 8921:I and II. For testing effluents and receiving waters, see available literature.[7]

1) Equipment and physical conditions—For effluents and most chemicals, static-renewal exposures are typically used. Other types of exposure (e.g., static, flow-through) may be used if supplies of test materials are limited or if test compounds are volatile or degrade rapidly. Suitable test chambers include 500-mL or 1-L beakers made of borosilicate glass or nontoxic disposable plastic. For other requirements, see ¶ 1*c* above.

2) Test initiation—Obtain organisms and set up test chambers according to requirements of Table 8921:II. Begin tests with effluents as soon as possible, preferably within 24 h of sample collection. From a pool of larvae, randomly select and distribute one or two larvae at a time until each test chamber contains a minimum of 10, but preferably 15, larvae. Use a large-bore pipet or similar device for transferring larvae; do not use a dip net. During transfer, avoid adding excess water to test chambers because this will dilute exposure concentrations.

3) Solution renewal—Unless the supply of test material or effluent is limited or flow-through procedures are used, renew test solutions daily. Before renewal, remove uneaten and dead *Artemia*, dead larvae, and other debris by siphon. Take care not to accidentally remove or injure larvae. Use of a light box will enhance larval visibility and simplify this task. Add new test solutions to test chambers after removal of approximately 80 to 90% of old solutions. Add solutions slowly down the side of the test chamber to avoid injury to larvae.

4) Feeding—See Table 8921:II. Rinse *Artemia* with fresh water before feeding. Avoid overfeeding because this may reduce toxicant concentrations and DO levels. Do not feed during final 12 h of test. Refer to the literature for information on culturing of *Artemia*.[1,6]

5) Biological data and observations—See ¶ 1*c*5) above.

6) Chemical data recording—See ¶ 1*c*6) above.

7) Verification of exposures—See ¶ 1*c*7) above.

8) Test termination—Terminate test after 7 d. Before termination, record number of dead and abnormal fish in each test chamber (replicate). Prepare fish in each replicate for dry-weight determination. If necessary, preserve larvae in 70% ethanol or

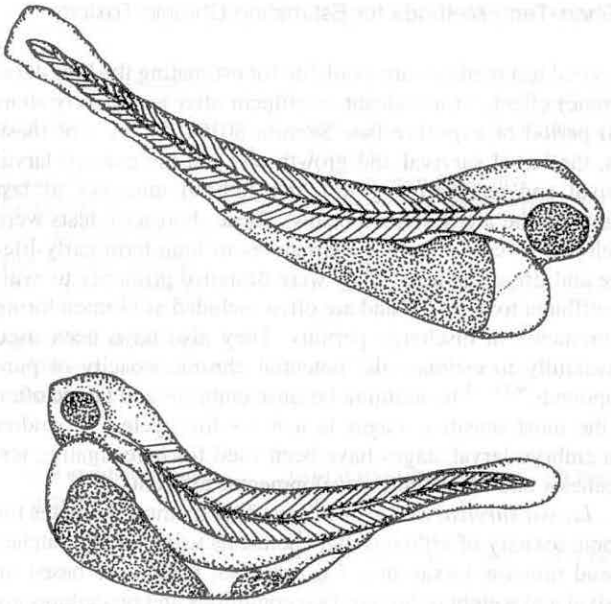

Figure 8921:3. Examples of abnormal fathead minnow larvae. Compare to Figure 8921:2.

4% formalin for up to 7 d before drying and weighing. Rinse each group of larvae with deionized water, transfer to a labeled and tared weighing boat, and dry at 60°C for 24 h (or at 100°C for 6 h). Let cool in a desiccator, weigh to nearest 0.01 mg, and record. For each replicate, determine mean individual dry weight per fish (using the number of original larvae) to the nearest 0.001 mg. For controls, calculate mean weight per surviving fish.

9) Test acceptability criteria—The test is considered acceptable if control survival is ≥80% and average dry weight per surviving larvae of fish in the control replicates is ≥0.25 mg.

10) Statistical analysis—Assemble, analyze, evaluate, and report data as described in Section 8010G and other references.[1,7] Calculate LC50 by a point estimation technique such as regression analysis. Obtain lowest-observed-effect concentration (LOEC) and no-observed-effect concentration (NOEC) values for survival and growth by using hypothesis-testing techniques such as Dunnett's Procedure or Steel's Many-one Rank Test.

b. Embryo-larval survival and teratogenicity test: This method estimates the chronic toxicity of effluents and chemicals by exposing fathead minnow embryo-larval stages in a 7- or 8-d test. Tests are initiated with fertilized embryos. Exposure is continued for several days after hatching of larvae (approximately 4 to 5 d after fertilization at 25°C), depending on age of embryos at test initiation. Test results are based on total frequency of both mortality and gross morphological deformities (terata). Examples of abnormally developed fathead minnow larvae are shown in Figure 8921:3. The test is useful in screening for teratogens (agents that produce terata) because organisms are exposed during early embryonic development when they are most susceptible. General test conditions and procedures are given in Tables 8921:I and II. For testing of effluents and receiving waters, see available literature.[7]

1) Equipment and physical conditions—For effluents and most chemicals, static-renewal exposures are typically used. Other types of exposure (e.g., static, flow-through) may be used if supplies of test materials are limited or if test compounds are volatile or degrade rapidly. Suitable test chambers include 250-mL or 500-mL beakers made of borosilicate glass or nontoxic disposable plastic. Chambers as small as 150 mL may be used when small solution volumes are used. For other requirements, see ¶ 1*c* above.

2) Test initiation—Use embryos, preferably less than 36 h old, to initiate tests. Obtain embryos from an in-house culture (see Section 8921B) or commercial supplier. Remove embryos from spawning substrates within 12 h of spawning. If organisms must be shipped from a supplier, embryos up to 48-h old may be used, provided all embryos are of the same approximate age. Set up test chambers according to requirements of Table 8921:II.

Preferably use larger volumes for testing compounds that are rapidly degraded, volatile, or have a low water solubility. Begin tests with effluents as soon as possible, preferably within 24 h of sample collection.

From a pool of embryos from three or more spawns, randomly select and distribute several at a time until each test chamber contains a minimum of 10, but preferably 15 to 25, embryos. Exclude abnormal and nonviable (milky-colored and opaque) embryos as well as any showing signs of fungal infection. A light box and stereoscopic microscope are recommended for examining and counting embryos. Use a large-bore pipet or similar device for transferring embryos. During transfer, avoid adding excess water to test chambers because this will dilute exposure concentrations.

3) Solution renewal—Unless the supply of test material or effluent is limited, or flow-through procedures are used, renew test solutions daily. Before renewal, remove dead embryos or larvae. Take care not to accidentally remove or injure embryos or larvae. A light table enhances fish visibility and simplifies this task. Add new test solutions to test chambers after removal of approximately 80 to 90% of old solutions. Add solutions slowly down the side of the test chamber to avoid injury to embryos and larvae.

4) Feeding—No feeding is required.

5) Biological data and observations—Each day, approximately 24 h after test initiation and before solution change, record number of dead (milky and opaque) and live embryos in each test chamber. After hatching begins, record each day the number of hatched, dead, live, and deformed larvae. Deformed larvae are those with gross morphological abnormalitites (Figure 8921:3) or other characteristics that preclude survival (Figure 8921:2 shows normal larvae). See other sources for detailed information on the embryology and development of the fathead minnow and identification of abnormalities.[14–20] Note that larvae typically do not become active for 1 to 2 d after hatching and may remain relatively immobile on the bottom of the test chamber during this time.

6) Chemical data recording—See ¶ 1*c*6) above.

7) Verification of exposures—See ¶ 1*c*7) above.

8) Test termination—Terminate test after 7 or 8 d. Before termination, record number of surviving, dead, and abnormal larvae in each test chamber (replicate).

9) Test acceptability criteria—The test is considered acceptable if control survival is ≥80%.

3. Early-Life-Stage Test

In this test, fathead minnow early life-stages, beginning with newly fertilized eggs, are exposed through embryo-larval development to an early juvenile age.[20–23] This test is run at 25°C for approximately 33 d (28 d post hatch) under flow-through conditions. Test endpoints include time-to-hatch, percent hatch, survival during different life stages, and growth. The intent is to determine the lowest effect and highest no-effect concentration of the test substance.

a. Equipment and physical condition: For a description of suitable diluter systems, see Section 8010F. Construct egg hatching cups (8 cm diam × 10 cm long) of glass tubing or other acceptable materials such as 316 stainless steel or TFE. Glue a 40 mesh nylon or stainless steel screen to one end of the cup using clear silicone sealant. Suspend egg cups from a rocker-arm assembly and low-speed motor designed to oscillate the egg cups slowly up and down approximately 2 to 3 cm. Use a low rate through the exposure chambers sufficient to replace 90% of the water in 8 to 12 h. A self-starting siphon tube in each exposure chamber can be substituted for the rocker-arm system to ensure test solution exchange.

Provide light intensities over the chambers of approximately 400 to 800 lux and establish a 16-h light and 8-h dark photoperiod, preferably with a 15- to 30-min dusk-dawn transition period. Hold temperature at 25 ± 2°C.

For dilution water requirements, see Table 8921:I. Prepare test solutions as described in Section 8010F.2*b*. For testing of compounds of low solubility, follow a carrier-solvent recommendation in Table 8921:I and use solvent control.

b. Test organisms: Use embryos, preferably less than 48-h old, to initiate tests. Obtain embryos from an in-house culture (see Section 8921B) or commercial supplier.

c. Test procedures: Use a minimum of five exposure concentrations and one control, and a dilution factor for determining test concentrations of ≥0.5. Set up a minimum of two (preferably four) replicate exposure chambers per test concentration. Use a range-finding test of 4 to 10 d conducted with juveniles to determine the test concentrations for the definitive study.

1) Test initiation—From a pool of embryos from three or more spawns, randomly select and distribute several at a time until 60 embryos are distributed per test concentration, divided among the replicate embryo incubation cups suspended in each test chamber. Exclude abnormal and nonviable (milky-colored and opaque) embryos as well as any showing signs of fungal infection. A light box and stereoscopic microscope are recommended for examining and counting embryos. Use a large-bore pipet or similar device for transferring embryos.

2) Feeding—Provide live brine shrimp (≤24-h old) three times daily during the first 5 d following hatching.[6] At about Day 7, supplement brine shrimp diet with a fine grade of commercial fish meal (fish starter). A slightly larger grade may be substituted as the fish grow.

3) Biological data and observations—Monitor survival by daily inspection. After hatching is complete, record number of live larvae, live embryos, dead embryos, and unaccounted-for embryos for each incubation cup, then release the larval fish from the incubation cup to the test chamber. Each day, record observations on fish behavior, noting abnormal and normal individuals and characteristics of any abnormalities. At test termination, measure standard length (to nearest 0.1 mm) and weigh fish, after blotting dry, to nearest 0.01 g.

4) Chemical data recording—Periodically measure DO, pH, conductivity, temperature, hardness, and alkalinity. Typically measure hardness, alkalinity, and conductivity at least weekly. Record temperature at least daily or continuously in one chamber that is centrally located in the row of exposure chambers. Measure DO and pH daily in random containers including a control and treatment as well as a minimum of every 7 d in each exposure chamber.

5) Verification of exposures—Before and during test, verify exposure concentrations of the test chemical. This will confirm actual versus nominal concentrations.

Initially, analyze concentration in each replicate test chamber. If consistent concentrations are observed among replicates at each concentration, make subsequent measurements on composite samples from each replicate. However, make other checks of diluter function to insure proper operating conditions. Although desirable, it is not necessary that measured concentrations be within any specific percentage of the nominal concentrations (e.g., ±20%). Such characteristics as hydrolysis rate and volatility may make it impossible. Maintain consistent concentrations by chemical measurements. Once concentrations have stabilized, start exposure. Measure exposure at least weekly until test ends. Base statistical analyses and results (LOECs, NOECs) on measured concentrations rather than nominal concentrations.

6) Test termination—Terminate test 28 d post-hatching.

7) Test acceptability—The test is acceptable if the survival of all control fish at the end of the test is ≥80% (based on the initial egg count) and survival is not less than 70% in any one control replicate.

4. Life-Cycle Reproductive Toxicity Test

This type of test uses newly spawned eggs or newly hatched larvae to start a test, continues through fish maturation and reproduction, and ends not less than 28 d after the hatching of the second generation. See Section 8910C.3 for a description of general test procedures for this test and other sources for additional details.[24–26]

a. Equipment and physical conditions: See Sections 8010F and 8910C.3. The physical systems are similar to those described for testing the brook trout (Section 8910C.3*b*).

Use one of the following two arrangements of test tanks (made of glass or stainless steel with viewing windows): The first consists of duplicate spawning tanks for each of the five or more test concentrations and controls, measuring 30 × 30 × 90 cm with a 30-cm-square portion at one end, screened off and divided in half to form two larval chambers for the progeny; deliver test water separately to the larval and spawning chambers of each tank, with about one-third of the water volume going to each larval chamber. Alternatively, use duplicate progeny tanks measuring 30 × 30 × 60 cm plus duplicate progeny tanks for each spawning tank. Use a larval tank with minimum dimensions of 30 × 30 × 30 cm, divided to form two separate larval chambers with separate standpipes, or separate 30 × 15 × 30 cm tanks. Supply test solutions and water for controls as in Section 8010F.1. Maintain a water depth of 15 cm in all tanks.

Flow rate, oxygen requirements, aeration, cleaning and operation are as described for the brook trout in Section 8910C.3*b*1).

Fathead minnows deposit eggs on the underside of submerged objects. For spawning substrates, use inverted semicircular sections of ceramic drain tile or PVC pipe (7.5 cm ID, 7 to 10 cm long), or equivalent (see Section 8921B.2*b*). If the inverted surface of the substrate is smooth, roughen it to aid embryo adhesion. Place substrate parallel to the long axis of the spawning tank so that each end is readily accessible to the fish. Fasten incubation cups, such as those described in 8921C.3, to a rocker arm with a vertical travel distance of 3 to 5 cm. For illumination, see 8010F.3*f*.

Use a 16-h light/8-h dark photoperiod, preferably with a 15- to 30-min dawn-dusk transition time.

Maintain temperature at 25 ± 2°C and record continuously.

b. Test initiation: Initiate tests with embryos or larvae from at least three females. Begin the life-cycle test by randomly selecting and distributing embryos or 1- to 5-d old larvae to each duplicate spawning tank for each test concentration. Extra fish may be added at the beginning so that some can be removed periodically for special examinations.

Exclude abnormal and nonviable (milky-colored and opaque) embryos as well as any showing signs of fungal infection. A light box and stereoscopic microscope are recommended for examining and counting embryos. Use a large-bore pipet or similar device for transferring embryos.

c. Feeding: Feed newly hatched larvae minimal amounts of live brine shrimp nauplii.[6] Avoid overfeeding. Continue feeding larval and juvenile fish twice daily with live brine shrimp nauplii for 30 to 60 d. Thereafter, frozen adult brine shrimp may be supplemented by pelleted and/or flake food. Feed quantitatively among all test groups.

d. Thinning and preparation for spawning: When test fish are 60 ± 2 d old, discard injured or deformed individuals and randomly reduce the number in each tank to 15. Record number, length, and weight of discarded and deformed fish. To obtain 15 fish per tank, it may be necessary to transfer or combine fish from duplicate tanks. Continue routine feeding and cleaning until fish mature and are almost ready to spawn. Place five spawning tiles in each duplicate spawning tank, separated fairly widely to reduce fighting among the territorial male fish. Place tiles so that their undersides and guard males can be seen from the tank end. When fish are fully mature (i.e., have well-defined secondary sexual characteristics–see Section 8921A and Figure 8921:1) and spawning is imminent, reduce the number of males to no more than four per tank. Reserve the fifth tile as cover for females. Do not remove males having established territories under tiles where a recent spawn has occurred.

e. Spawning and embryo incubation: Each day, check spawning tiles and remove those with newly deposited embryos, beginning about 6 h after start of the light period. Loosen embryos from spawning tiles and at the same time separate them from one another by lightly placing a finger on the egg mass and moving it in a circular pattern with increasing pressure until the embryos begin to roll. Wash groups of embryos into separate containers and return to spawning tanks. Count embryos, select those needed for incubation, and discard remainder. Check all embryos for different stages of development.[27] If more than one distinct stage is present, consider each stage as one spawning and handle separately as described below.

Each day, randomly select 50 unbroken embryos from a single spawn and place in an incubator cup to determine viability and hatchability. Count, record, and discard remaining embryos. Determine viability and hatchability on each spawn of at least 50 embryos until number of spawns (≥50 embryos) in each tank equals number of females in that tank. Subsequently, test for hatchability only on subsamples from every third spawning of at least 50 embryos. Remove spawns from tiles, count and record embryos, and discard.

If no spawning occurs for a week, cease testing of parental fish. Record total length and weight, sex, and gonadal condition of parental (F_0) fish, then discard.

Each day, record live and dead embryos in incubator cups, remove dead embryos, and clean cup screens. After larvae begin to hatch (about 4 to 5 d), cease handling or removing them from cups until all have hatched. At that time, if enough larvae are still alive, select 40 at random and transfer immediately to a larval growth chamber to determine survival and growth of the second (F_1) generation. Count and discard incubation groups not used for survival and growth studies.

f. F_1 generation larval-juvenile survival and growth: Select larvae for 30- and 60-d growth and survival exposures from early spawned embryos in each duplicate tank. Plan their distribution for hatchability tests so that a new group of larvae is ready to be tested as soon as possible after the previously tested group is removed from the larval chambers. Record mortality and larval lengths at 30 and 60 d after hatching. Weigh juveniles when exposures are terminated (60 d). Do not feed fish (larvae, juveniles, or adults) for 24 h before weighing.

g. Extended testing: Normally testing is concluded with the F_1 generation larval-juvenile exposures; however, an extended life-cycle test may be conducted through an additional generation, if desired. In this case, transfer 50 of the 60-d post-hatch F_1 fish from each growth chamber to the corresponding spawning chamber. Follow procedures used for the F_1 generation to determine survival of embryos, larvae, and juveniles of the F_2 generation. Cease testing adult fish on completion of spawning. Continue post-hatch study to 60 d.

h. Biological data and observations: Record the following data for each tank and the controls: total number and length of normal and deformed individuals at the end of 30 and 60 d for each generation; total length, weight, and number of each sex, both normal and deformed, at the end of the tests; mortality during tests; number of spawns and embryos produced in each and total embryo production by each generation; and percentage of larvae surviving and growth of juveniles as well as deformities produced.

Use fish and embryos obtained from the test for physiological, biochemical, histological, and other tests for toxicant-produced effects, as necessary.

i. Chemical data recording: Periodically measure dissolved oxygen, pH, conductivity, temperature, hardness, and alkalinity. See Section 8921C.3*c*4) for additional information.

j. Verification of exposures: Before and during the test, verify exposure concentrations of the test chemical. See Section 8921C.3*c*5). Base statistical analyses and results (LOECs, NOECs) on measured rather than nominal concentrations.

5. Statistical Analysis

Assemble, analyze, evaluate, and report data as described in Section 8010G.

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8930 AMPHIBIANS (PROPOSED)*

8930 A. Introduction

1. Significance

Amphibians are important organisms in freshwater ecosystems. Population declines in many species have been observed for several years.[1] There is also recent concern about a high incidence of malformations in some populations.[2] Because some of these declines and malformations probably result from exposure to aquatic toxic materials, an increased focus on amphibians in aquatic toxicology and the standardization of experimental protocols is imperative. The following summary outlines protocols for the use of anuran amphibians (Order Anura: frogs and toads). Anurans have been the most extensively studied and therefore represent the best avenue for standardization of methods. Salamanders (Order Caudata) have been used less frequently in toxicology studies and so will not be addressed specifically; however, modifications of the following methods can be made for salamanders as necessary.

This method is still under further development, but is currently the method of choice.

2. Test Organisms Characteristics

Two groups of anurans have been studied extensively in aquatic toxicology. The South African clawed frog, *Xenopus laevis* Daudin (family Pipidae), has been developed as a model for early-life-stage tests because of the ease with which it can be cultured and bred in the laboratory. Members of the genus *Rana* (family Ranidae) are used in studies when a native species is desired (See Section 10900, Plate 25:B). The two most frequently used species are the Northern leopard frog, *R. pipiens* Schreber, and the American bullfrog, *R. catesbeiana* Shaw. The distribution, life history, and biology of these amphibians can be found in the literature.[3–10]

Adult *Xenopus laevis* typically range in size from 70 to 125 mm snout-to-vent, with females usually larger. They are greenish-grey dorsally with darker mottling or spots and an unmarked ventral surface. The limbs are long and muscular, and the smaller forelimbs have claws on the three innermost digits. The eyes are located dorsally, and the entire body is flattened dorso-ventrally. Females can be distinguished by the presence of cloacal papillae, and males in breeding condition develop dark, sticky hairs on the digits and forearms. Larvae are transparent.[3]

Members of the genus *Rana* have long muscular hind limbs, narrow waists, and large, laterally placed eyes. Adult *R. pipiens* range from 50 to 90 mm snout-to-vent and are marked with large dorsal spots on a green or brown background and a pair of dorsolateral ridges. Sexes are difficult to distinguish, although females are usually larger. Adult *R. catesbeiana* range from 90 to 150 mm snout-to-vent and can be green, brown, or heavily mottled dorsally. Males have a bright yellow throat and an external tympanic membrane that is larger in diameter than the eye. Larvae of the two species are distinguished by features of the oral disk, coloration, and the larger size of *R. catesbeiana*.[4–10]

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* Approved by Standard Methods Committee, 2001.

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8930 B. Culture and Maintenance of Test Organisms

1. Obtaining Test Organisms

Xenopus laevis of various ages can be obtained for toxicity testing from commercial breeders, biological supply houses, or an in-house culture facility. An in-house breeding facility is recommended and may be required, because most assays will be conducted with early life stages. Ensure that specimens, particularly those from outside sources, are certified as to their identity, age, and freedom from disease. Examine animals upon arrival for skin lesions or red patches on the ventral surfaces. Quarantine new arrivals from the resident population for at least 2 weeks.[1,2]

Rana specimens at various life stages can be obtained from biological supply houses, hatcheries, and bait shops. Because these specimens probably will be field-collected, generally avoid them because of inaccurate species identity, the potential presence of disease from shipping, and lack of locality data. Preferably obtain specimens of *Rana* sp. by direct field-collection from habitats with known toxicant exposure history. Collection in this manner is subject to the constraints of the breeding season, but during this time a variety of life stages and sizes can be gathered easily and inexpensively as needed. Quarantine specimens before use in experiments. In-house breeding cultures are difficult to establish for *Rana* sp. because adults require live food and artificial fertilization of eggs.[3]

2. Culturing and Care of Test Organisms

Xenopus laevis, including all life stages from egg to adult, may be cultured successfully in the laboratory. Basic information on establishing and maintaining a culture facility is presented below. For detailed information, see other sources.[1,2,4] Separate life stages of *Rana* sp. can be cultured easily, but maintaining a continuous viable breeding facility for successful generations can be difficult for several reasons, as discussed below.

a. Water supply and culture system: Natural water is preferred for maintenance of adult *X. laevis*. Supplies from wells or springs usually are more uniform in quality than those from surface waters. Dechlorinated tap water can be used provided that residual chlorine is monitored and at low levels, because dechlorination can be incomplete (preferably dechlorinate with sodium bisulfite). The salt solution used for the Frog Embryo Teratogenesis Assay-*Xenopus* test (FETAX) and for breeding adults and culturing early stages (discussed below) also can be used for maintenance, but is suitable only for small colonies because of cost and formulation time. Maintain water temperature at 20±2 °C before addition of adults, and provide aeration if needed to maintain dissolved oxygen levels. Take quarterly measurements of the following items: pH, total dissolved solids (TDS), total organic carbon (TOC), organophosphate pesticides, organic chlorine (or organochlorine pesticides plus PCBs), chlorinated phenoxy herbicides, ammonia, bromide, beryllium, cadmium, chromium, copper, iron, lead, manganese, mercury, nickel, selenium, silver, and zinc. Physical and chemical limits on water include: pH 6.5 to 9.0, TOC <10 mg/L, alkalinity and hardness 16 to 400 mg/L as $CaCO_3$.[1,2,4,5] Water requirements for *Rana* sp. are similar to those for *X. laevis*.

The FETAX assay (8930C) requires the following salt solution for all experiments with *X. laevis*:

Sodium chloride, NaCl	625 mg
Sodium bicarbonate, $NaHCO_3$	96 mg
Potassium chloride, KCl	30 mg
Calcium chloride, $CaCl_2$	15 mg
Calcium sulfate, $CaSO_4 \cdot 2H_2O$	60 mg
Magnesium sulfate, $MgSO_4$	75 mg
Deionized or distilled water	1 L

The pH of the final solution should be 7.6 to 7.9. Use this solution for breeding, static or renewal assays, and flow-through experiments whenever possible. Other water sources must allow for embryonic growth at the same rate as this solution, and there should be acceptable levels of mortality and malformation rates.[1,2,4] Some types of test solutions will require modification of the above formulation by decreasing or increasing certain salts, or by using a more concentrated or more dilute solution.[5]

Maintain adult specimens of *X. laevis* in glass aquariums or fiberglass or stainless steel raceways at densities of 4 to 6/1800 cm^3. Water depth should be 7 to 14 cm. Use opaque tanks or shield the sides of the tank (e.g., with black plastic) so that specimens are not disturbed by outside activity. Use a photoperiod of 12 h day/12 h night.[1,2,4] For adult *Rana* sp., adjust tanks to provide a dry area because these species are not completely aquatic. This dry area can be created by tipping the tanks at one end so that water pools at the opposite side, or by adding platforms that are higher than the water level to allow specimens to climb out of the water. Keep adult *X. laevis* and *Rana* sp. in same-size groups to decrease cannibalism and competition for food.[3]

b. Establishment of breeding colonies: To establish breeding in *X. laevis*, use males that are at least 2 years old and 75 to 100 cm in snout-vent length, and females that are at least 3 years old and 100 to 125 cm in length. Breed males and females as single pairs. Fit a 20- or 40-L (5- or 10-gal) glass aquarium with a 1-cm mesh (nylon or plastic only) suspended about 3 cm from the bottom of the aquarium so that deposited eggs can lie undisturbed on the bottom of the aquarium. Shield the sides of the breeding aquarium (e.g., with black paper) and add an aerator if desired. Cover the top of the aquarium with an opaque porous material such as a fiberglass furnace filter.[1,2]

Breed adult *X. laevis* in the same dilution water in which the test is to be conducted. Hold water temperature at 20±2°C. To induce breeding, inject the male and the female with 250 to 500 and 500 to 1000 IU, respectively, of human chorionic gonadotropin into the dorsal lymph sac. The amount of human chorionic gonadotropin injected depends on the time of year and condition of the adults. The hormone concentration should be 1000 IU/mL in sterile 0.9 % NaCl. Use a 1-mL tuberculin syringe fitted with a 1.3-cm (½-in.) long, 26-gauge needle. Amplexus normally occurs within 2 to 6 h and egg deposition about 9 to 12 h after injection. The fertility rate should be > 75 %. Do not use eggs laid in "strings" or not perfectly round, because they develop abnormally.[1,2,4]

Eggs of *Rana* sp. and other North American anurans can be collected only during the breeding season, either directly from the field or from reproductively active adults. Obtain proper permits for collection of eggs or adults. Collect fertilized egg masses during the breeding season and treat as in ¶ *c* below. Reproduction in adult *Rana* sp. can be induced only in animals collected during the breeding season. Collect pairs that are in amplexus, and place them in an undisturbed holding tank with water; fertilized eggs can be collected in 24 h. Alternatively, artificially induce ovulation in gravid females collected from the field. The standard method of induction is by intraperitoneal injection of pituitaries from several adults into a gravid female. Extrude ova into a petri dish 24 to 48 h after injection by applying pressure to the female. Inseminate the ova by mincing testes, removed previously from a male, in buffer and applying the sperm suspension over the extruded ova.[3,6,7] Sperm and ova might also be collected by injections of synthetic human luteinizing hormone-releasing hormone (LHRH) in similar fashion to the methods described for *Xenopus* and chorionic gonadotropin (¶ *a* above). These techniques do not require destruction of animals but are not frequently used.[6–8]

c. Collection and maintenance of embryos: Dejellying of anuran embryos should begin immediately after egg laying for embryos needed for experimentation. Gently swirl embryos for 1 to 3 min in a 2 % w/v L-cysteine solution prepared in FETAX solution (¶ *a* above). Adjust pH of cysteine solution to pH 8.1 with 1*N* NaOH, and continue dejellying until just after all jelly is removed. Do not treat embryos too long because survival will be reduced. Place embryos in 60-mm glass or plastic petri dishes (25 embryos and 10 mL FETAX solution) and keep these in a constant-temperature room or a suitable incubator capable of holding 20±2°C. Renew solution every 24 to 48 h. Periodically identify developmental stage of embryos according to standard guides.[9–11]

d. Rearing of larvae and metamorphs: Transfer free-swimming larvae of *X. laevis* or *Rana* sp. to glass aquariums. Loading depends on size of larvae and use of static versus flow-through tanks. Determine developmental stages using standard guides. Transfer metamorphs and small adults *X. laevis* to tanks similar to those of adults. Transfer *Rana* larvae to adult tanks with dry areas upon forelimb emergence and initiation of tail reabsorption.[12,13]

e. Food and feeding: Feed *X. laevis* larvae salmon starter.*[14] *Rana* larvae can be fed a variety of foods including trout chow, boiled lettuce and spinach, and fish flakes; however, for comparison with *X. laevis* assays these species should be fed salmon starter. When larvae are to be used for experiments, feed early-stage larvae (actively feeding for < 20 d) up to the initiation of the test. Withhold food from later-stage larvae for 48 h up to the beginning of the test.[15,16]

Feed postmetamorphic and adult *X. laevis* salmon starter three times a week. Supplement food with liquid multiple vitamins and screen food for materials that will be tested.[1,2,4] Adult *Rana* sp. will not eat non-moving food. Feed them appropriate-sized crickets and other insects or earthworms. Dust food with a vitamin powder. If food animals, particularly crickets, are to be maintained for extended periods, feed diets fortified with calcium and vitamins. Anurans will absorb these supplements. Large *R. catesbeiana* will eat fish, crawfish, and small laboratory mice. Offer food on the dry area of the tank, but items that float or swim will be eaten from the water.[3,17–19]

f. Parasite and disease control: Observe animals in quarantine daily for signs of disease. Treat diseased frogs appropriately. Look for dull or dry skin, wounds or infection, poor posture, decreased body condition, and lethargy.[20,21] Withhold sick frogs from breeding for 4 to 6 weeks after treatment. Remove dead individuals and waste, and use separate cleaning and transfer equipment for each tank.

3. Acclimating and Holding Test Organisms

Use embryos immediately for embryo-larval stage tests.[1,2] Acclimate later-stage larvae before use by changing water from 100% holding water to 100% dilution water over 48 h. Hold subjects at 100% test water for 48 h.[15,16]

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* Available from Xenopus 1,® Dexter, MI.

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8930 C. Procedures

1. Frog Embryo Teratogenesis Assay–*Xenopus laevis* (FETAX)

FETAX is used to test toxicants for their effects on developmental processes. The assay is designed not only for amphibian research but also for identifying toxic effects in other species, including mammals. FETAX is therefore useful for screening substances that are potential teratogens in humans. The FETAX assay can be modified to accommodate species other than *X. laevis* with different developmental times or maintenance requirements. Details of the FETAX assay are presented in other sources.[1–3] The general procedure and modifications to the standard protocol are discussed below.

a. Scope and application: FETAX procedures can be used to test chemicals either individually or in formulations, as well as temperature, dissolved oxygen, pH, physical agents, aqueous surface and ground waters, leachates, aqueous extracts of water-insoluble materials, and solid-phase samples such as soils and sediments, particulate matter, sediment, and whole bulk soils and sediment. The assay uses *X. laevis* embryos and so requires no feeding and very little maintenance effort or laboratory space.

b. Range-finding test and selection of concentrations: Conduct range-finding tests to select proper concentrations for the definitive tests. Use at least seven concentrations that differ by a factor of ten. Conduct the test and calculate the 96-h LC5, LC16, LC50, LC84, and LC95 and the EC5, EC16, EC50, EC84, and EC95. If necessary, use the results of the first definitive experiment as another range-finder and readjust test concentrations.

c. Specific test procedures:

1) Facilities and equipment—Conduct tests in an incubator (see 8930B.2*c*) for housing the embryos. Ensure that all equipment and facilities that contact stock solutions, test solutions, or water in which embryos will be placed do not contain leachable or dissolvable substances at levels that would adversely affect embryo growth or development. For new test facilities, conduct a non-toxicant test in which all test chambers contain FETAX solution with no added test material. The embryos should grow, develop, and survive in numbers consistent with an acceptable test. For counting and evaluating abnormal embryos, obtain a binocular dissection microscope capable of magnifications up to 303, a darkroom enlarger to enlarge embryo images, and a map measurer or an ocular micrometer. Alternatively, count embryos with a digitizer interfaced to a microcomputer.

For toxicity tests of fluids, use 60-mm glass or plastic petri dishes as the exposure containers and modify these as necessary.[3] For aquatic sediment testing, use a 250-mL (9-oz.) specimen bottle with a glass tube/TFE* mesh insert. Add 35 g test soil in the bottle followed by insert and 140 mL FETAX solution. Place embryos on top of the insert.[4] For in situ field exposures,[5] particularly with native species, construct exposure chamber from a 2.5-cm-wide, 7.0-cm-diam circlet of plastic polyethylene mesh (1-mm mesh). Use a central nylon bolt/wing nut to hold the chamber together and line the chamber with TFE mesh to retain embryos. Anchor the chamber in the water with a stainless steel stake and mark the location with a fishing bobber. Equilibrate embryos by placing laboratory shipping container directly into sample location until temperatures are within 2°C. Use four to six exposure cages per on-site sample unit with 10 to 25 embryos in each cage.[5]

2) Test initiation—Induce breeding in three mating pairs and harvest clutches separately. Conduct three definitive tests using embryos from a single mating pair for each test. It is necessary to keep clutches separate because embryos from a particular mating pair might develop poorly although they initially appear acceptable. This would cause all the embryos to be discarded if embryos are mixed from different mating pairs. For each test concentration use two dishes each containing 25 embryos and 10 mL test solution. For each control, use four dishes of 25 embryos each. Maintain a temperature of 20±2°C throughout test to prevent malformations and allow proper growth of controls. For a positive control or reference toxicant, use 6-aminonicotinamide at 5 mg/L (the 96-h LC50) and 2250 mg/L (the 96-h EC50 for malformations), four dishes each.

* Teflon® or equivalent.

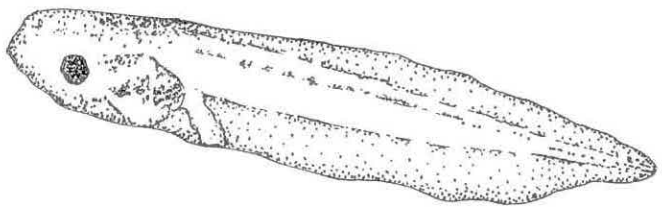

Figure 8930:1. Lateral views of a normal 4-d-old larva of *Xenopus laevis*.

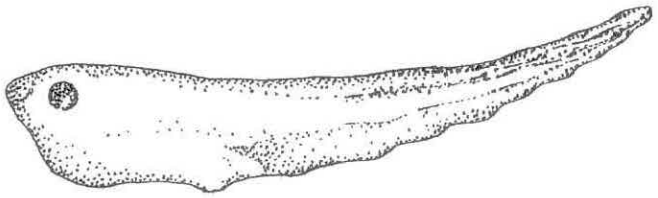

Figure 8930:4. Lateral view of abnormal 4-d-old larva of *Xenopus laevis*.

3) Solution renewal—Use the renewal procedure for the standard FETAX test by replacing test material every 24 h. Measure pH of control and highest test concentration during renewal. Remove test solution using a Pasteur pipet with an orifice that has been enlarged and fire-polished to accommodate embryos without damage in case the embryos are accidentally picked up. Conduct renewal as quickly as possible to minimize embryo desiccation.

4) Biological data recording—Measure mortality in test solutions by counting and removing dead embryos during solution renewal. Death at 24 h is determined by the embryo's pale skin pigmentation, decomposition, and lack of response to prodding. At 48 h, 72 h, and 96 h use the lack of heartbeat as an unambiguous sign of death. Record a final number of dead embryos at 96 h of exposure and the time to the first hind-limb buds in controls. Fix dead embryos and remaining live embryos in 3% formalin. Record developmental stage and malformations at the end of 96 h (Figures 8930:1 through 8930:4) according to standardized guides.[6–8] Compare exposed embryos to control embryos. Measure head-tail length data at the end of each test after embryos are fixed in 3% formalin.

5) Exogenous Metabolic Activation System (MAS)—Use an exogenous MAS when FETAX is used to evaluate developmental toxicity for human health hazard assessment, because early *X. laevis* embryos have limited xenobiotic metabolic capabilities, particularly cytochrome P-450. Make the MAS from rat liver microsomes and nicotinamide adenine dinucleotide as the generator system, and use Aroclor 1254 as the cytochrome P-450 inducing agent. Use MAS-only dishes as the negative control, and 4 mg/mL cyclophosphamide with and without MAS as the positive control. Make a P-450 control, to demonstrate that the cytochrome P-450 system is responsible for the observed bioactivation, by adding dithionite directly to the microsomes and bubbling carbon monoxide through for 3 min to inactivate P-450. To prepare 20 mL of test solution, place an appropriate volume of FETAX solution into a 50-mL flask, add MAS components and appropriate volume of test material stock to give desired concentration, then adjust final volume to 20 mL with FETAX solution. Divide this test mixture between replicate petri dishes to which the embryos will be added.

6) Duration of test—For *X. laevis*, run the FETAX assay for 96 h, at which point the control embryos should have hind-limb buds (stage 46[7]). If 90% of the controls do not show the presence of hind limb buds by 96 h, then extend the test another 3 h. For *Rana* sp. or other species, test duration may need to be modified to accommodate differences in developmental time. Report deviations from this standard exposure time as deviating from standard FETAX conditions.

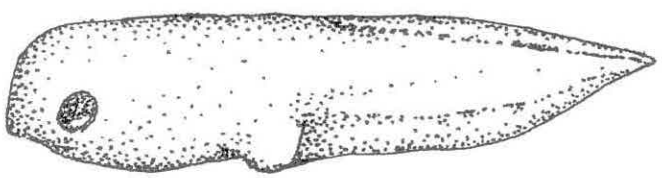

Figure 8930:2. Lateral view of abnormal 4-d-old larva of *Xenopus laevis*.

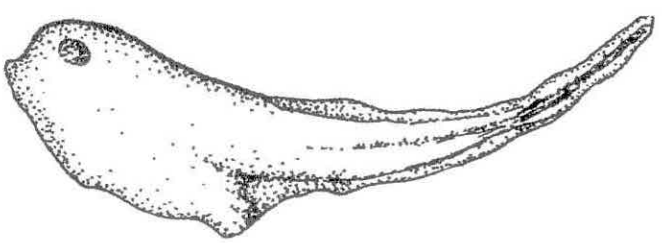

Figure 8930:3. Lateral view of abnormal 4-d-old larva of *Xenopus laevis*.

2. Short-Term Acute Tests for Later-Stage Larvae

Procedures for the testing of toxicants on postembryonic, free-swimming amphibian larvae (tadpoles) in an acute exposure can be designed similarly to tests for fish and macroinvertebrates. Details for standard methods in acute exposures have been described in detail [9,10] and are discussed in general below.

a. General procedure: Larvae are maintained for 2 to 8 d in each of two or more treatments in one or more test chambers. In each of the one or more control treatments, larvae are maintained in dilution water to which no test solution has been added. This procedure provides a measure of the acceptability of the test based on the quality of the test subjects, dilution water, test conditions, handling procedures, etc., and also provides the basis for interpreting data obtained from the other treatments. In each of the one or more other treatments, larvae are maintained in concentrations of test solution. Data regarding biological effects in each test chamber usually are obtained periodically during the test and analyzed to determine LC50s or EC50s for various lengths of exposure.

b. Types of experimental design: Two types of experimental design are appropriate in most cases. For an acute test intended to allow calculation of an LC50, EC50, or IC50, use one or more control treatments and a geometric series of at least five concentrations of test material. Except for the control(s) and the highest concentration, each concentration should be at least 60% of the next higher one. Alternatively, if it is only necessary to determine whether a specific concentration is acutely toxic to the

larvae or whether the LC50, EC50, or IC50 is above or below a specific concentration, only that concentration and the control(s) are necessary. Use two additional concentrations at about one half and two times the specific concentration to increase confidence in the results. For both types of tests at least one concentration of test material should kill or affect a percentage of larvae, other than 0 or 100%, near the percentage for which the LC, EC, or IC is to be calculated.

c. Setting up the test: In static and renewal tests, use loading in each test chamber of 0.5 to 0.8 g organism/L, with fewer animals at higher temperatures. In flow-through tests the loading in each test chamber should not exceed 1 g of organism/L /d. For consistency use temperatures similar to those in other assays such as FETAX. For static tests, maintain a dissolved oxygen level in each test chamber from 60 to 100% of saturation during the first 48 h of the test and 40 and 100% of saturation after 48 h. For renewal and flow-through tests, maintain the dissolved oxygen in each test chamber between 60 and 100% of saturation at all times during the test.

d. Specific test procedures:

1) Test initiation—Distribute test organisms randomly among test chambers. For static and renewal tests place test organisms in chambers within 30 min after test material is added. For flow-through tests place test organisms in chambers after test solutions have been flowing through chambers long enough for test material concentrations to have reached steady state.

2) Feeding—Do not feed larvae 24 to 48 h before nor during an acute toxicity test. The presence of food can add another route of uptake, and fecal matter and uneaten food will decrease the dissolved oxygen concentration and the biological activity of some test materials.

3) Physical and chemical data recording—For static and renewal tests, measure water quality parameters at the beginning and end of the test and at least every 48 h in between in the control and the high, medium, and low test concentrations as long as live organisms are present. Measure maximum and minimum temperatures daily in at least one test chamber. For flow-through tests, measure water quality parameters, including hardness, alkalinity, conductivity, dissolved oxygen, and pH, at the beginning of the test and measure temperature daily in at least one test chamber. To monitor test solution concentrations, take water samples from a point midway between the top, bottom, and sides of the test chamber. In static and renewal tests, measure the concentration of test material in at least the control and the high, medium, and low concentrations of test material at the beginning of the test. For flow-through tests, measure concentration of test material in the chambers as often as practical during test. In each treatment the highest measured concentration obtained during the test divided by the lowest should be less than 1.5.

4) Biological data recording—Measure the number of dead and affected organisms (lack of movement or response to gentle prodding, loss of equilibrium) in each test chamber every 24 h after the beginning of the test. Remove dead organisms at least once every 24 h if it can be done without stressing live organisms. Measure weights of test organisms after exposure is complete.

5) Test termination—Conduct test for at least 96 h or long enough to ensure that a time-independent toxicity level can be determined or estimated mathematically. When renewal or flow-through tests are conducted with organisms that will not be substantially affected by starvation for at least 8 d, conduct test for at least 8 d to determine whether additional organisms are affected or killed after 96 h. As an optional test, place remaining test organisms in dilution water that does not contain any added test material for 2 to 8 d and feed them to determine whether delayed effects occur.

3. Chronic Toxicity Tests

Few toxicology protocols have been developed to expose amphibians from embryo through the larval period to adulthood. Investigators have rarely attempted to expose amphibian larvae for the entire metamorphic period because this lasts several months for many species, and because larvae are extremely sensitive and difficult to keep alive at metamorphic climax. Examining toxicant effects during amphibian metamorphosis would be particularly valuable because certain critical stages of development might be sensitive to even low levels of toxicant. The following methods are useful in examining later-stage larvae under longer exposure times than a few days and as a compliment to FETAX assays of toxicants.

a. 10- and 30-d embryo-larval exposures: Transfer 4-d-old embryos (such as after FETAX assay) to glass aquariums and continue exposure through 10 d. Monitor same end points as in FETAX assay (change exposure water on Day 7).[11] If desired, extend exposure for 30 d.[12–16] Feed subjects as needed depending on tank size and renew exposure water every 48 h. After 30 d, measure length and weight and identify hind-limb abnormalities. Fix representative specimens in 3% formalin, pH 7.0.

b. Exposures through metamorphosis: Expose larvae in tanks containing 8 L treatment solution. Change solutions, measure water quality, and add food every 3 d. Observe mortality, deformities, abnormal pigmentation, and movements every 1 d or 3 d, depending on growth rate of species. Measure length in 6 to 10 randomly selected larvae every 6 to 9 d, depending on growth rate of the species. For semi-aquatic species such as *Rana* sp., transfer animals with emerged front limbs to plastic tubs that are tilted or have a float as a dry refuge. Add 1 L of treatment solution and withhold food. Change solutions every 3 d, note time to metamorphosis, and take size and tissue measurements as needed.[17,18]

c. Tail reabsorption/thyroid disruption test: This test is used to evaluate toxicants that potentially disrupt thyroid function or metamorphosis in general. Transfer active larvae (such as after FETAX assay) to glass aquariums and place on a regular feeding regime. When larvae reach the advanced hindlimb development stage, place 10 in each of four 5-L vessels containing a given concentration of exposure media. Renew solutions and note developmental stage, including tail length, every 96 h. Terminate exposure when tail has been reabsorbed and fix larvae in 3% formalin to observe gross effects on limb development and tail reabsorption.[13–16,19]

4. Performance and Behavioral Tests

Toxicity tests that use changes in performance or behavior as end points are more useful in some circumstances than examinations of mortality or gross abnormalities. Performance and behavioral responses are altered at sublethal concentrations and

after chronic exposure. Changes in performance or behavior can compromise an otherwise healthy individual's ability to find food, avoid predators, and locate mates. Avoidance of toxicants can lead to relocation in suboptimal habitat or decreased population densities. Published guidelines exist for examining behavioral alterations in amphibians that are similar to those designed for aquatic organisms in general.[20] The following assays have been developed specifically for amphibians to measure performance and behavioral parameters, and the utility of some of these tests in toxicology has been demonstrated with toxicant-exposed subjects. These tests can be conducted during or after toxicant exposure. Although direct observation of some of these activities is possible, usually remote camera and video recording equipment is necessary.

a. Swimming performance and activity: Evaluate swimming performance in amphibian larvae by placing a single subject in an appropriately-sized water-immersed track and prodding at the base of the tail. Measure responsiveness and speed.[21–23] Alternatively, measure unforced activity and distance traveled within a set time period (e.g., 5 min) to observe hyper- or hypoactivity in exposed subjects.[24] Elaborate on observations of activity by adding refuges or predators and measuring partitioning of time spent active, hiding, feeding, or performing other behaviors.[25]

b. Preference/avoidance of toxicants: Measure the preference/avoidance of toxicants by placing a subject in an appropriate chamber where it is presented with both control and test water and can move towards or away from either choice. One design for such a choice chamber consists of a Y-shaped apparatus in which two different water sources flow towards the subject. Measure the number of times the subject enters each stream or the amount of time the subject spends in either water stream.[26] Alternatively, construct an octagonal fluviarium with eight separate outputs into a circular swimming arena.[27–32] Each output should maintain a plume separate from the streams of the other outputs, such that the test toxicant output does not flow into control streams. The subject is placed in the central arena where it has equal access to all output streams. Give the subject 5 min without any test plume, 5 min with the test plume flowing, and then another 5 min without the test plume. Note the amount of time the subject spends in all the streams. For both exposure chambers, use either unexposed subjects or subjects previously exposed to a toxicant.

c. Examination of respiratory behavior in larvae: Place larvae in individual tanks, or mark individuals uniquely in group tanks.[33,34] With each individual, count the number of movements greater than one body length (activity) and the number of trips to the surface for 15 min. Count the number of times the buccal floor is elevated for 1 min. Perform these observations over 5 d. Larvae with compromised respiratory abilities will decrease their activity, increase their trips to the surface to gulp aerial oxygen, and increase their buccal pump rate.[35]

5. Statistical Analysis

Assemble, analyze, evaluate, and report data as described in Section 8010G.

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