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Standard Guide for

Conducting Static Short-Term Chronic Toxicity Tests Starting with Embryos of Four Species of Saltwater Bivalve Molluscs[1]

This standard is issued under the fixed designation E724; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This guide describes procedures for obtaining laboratory data concerning the acute effects of a test material on embryos and the resulting larvae of four species of saltwater bivalve molluscs (Pacific oyster, *Crassostrea gigas* Thunberg; eastern oyster, *Crassostrea virginica* Gmelin; quahog or hard clam, *Mercenaria mercenaria* Linnaeus; and the mussel species complex (*Mytilus spp.*) including the blue mussel, *Mytilus edulis* Linnaeus; the Mediterranean mussel, *Mytilus galloprovincialis* Lamark; and the Northern Bay Mussel, *Mytilus trossulus* Gould) during static 48-h exposures. These procedures will probably be useful for conducting static short-term chronic toxicity tests starting with embryos of other bivalve species (**1**)[2] although modifications might be necessary.

1.2 Other modifications of these procedures might be justified by special needs or circumstances. Although using procedures appropriate to a particular species or special needs and circumstances is more important than following prescribed procedures, results of tests conducted by using unusual procedures are not likely to be comparable to results of many other tests. Comparison of results obtained by using modified and unmodified versions of these procedures might provide useful information concerning new concepts and procedures for conducting 48-h acute tests starting with embryos of bivalve molluscs.

1.3 These procedures are applicable to most chemicals, either individually or in formulations, commercial products, or known mixtures. With appropriate modifications these procedures can be used to conduct acute tests on temperature, dissolved oxygen, and pH and on such materials as aqueous effluents (see also Guide E1192), leachates, oils, particulate matter, sediments, and surface waters. Renewal tests might be preferable to static tests for materials that have a high oxygen demand, are highly volatile, are rapidly biologically or chemically transformed in aqueous solution, or are removed from test solutions in substantial quantities by the test chambers or organisms during the test.

1.4 Results of toxicity tests with embryos of bivalve molluscs should usually be reported as the EC50 based on the total incompletely developed and dead organisms. It might also be desirable to report the LC50 based only on death. In some situations, it might only be necessary to determine whether a specific concentration is toxic to embryos or whether the EC50 is above or below a specific concentration.

1.5 This guide is arranged as follows:

[1] This guide is under the jurisdiction of ASTM Committee E50 on Environmental Assessment, Risk Management and Corrective Action and is the direct responsibility of Subcommittee E50.47 on Biological Effects and Environmental Fate.

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[2] The boldface numbers in parentheses refer to the list of references at the end of this guide.

1.6 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*Specific hazard statements are given in Section 6.

1.7 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[3]

E380 Practice for Use of the International System of Units (SI) (the Modernized Metric System)[4]

D4447 Guide for Disposal of Laboratory Chemicals and Samples

E729 Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians

E943 Terminology Relating to Biological Effects and Environmental Fate

E1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses

E1192 Guide for Conducting Acute Toxicity Tests on Aqueous Ambient Samples and Effluents with Fishes, Macroinvertebrates, and Amphibians

E1367 Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Estuarine and Marine Invertebrates

E1391 Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing and for Selection of Samplers Used to Collect Benthic Invertebrates

E1706 Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Freshwater Invertebrates

E1847 Practice for Statistical Analysis of Toxicity Tests Conducted Under ASTM Guidelines (Withdrawn 2022)[5]

3. Terminology

3.1 *Definitions:*

3.1.1 The words "must," "should," "may," "can," and "might" have very specific meanings in this guide. "Must" is used to express an absolute requirement, that is, to state that the test ought to be designed to satisfy the specified condition, unless the purpose of the test requires a different design. "Must" is only used in connection with factors that directly relate to the acceptability of the test (see 13.1). "Should" is used to state that the specified condition is recommended and ought to be met if possible. Although violation of one "should" statement is rarely a serious matter, violation of several will often render the results questionable. Terms such as "is desirable," "is often desirable," and "might be desirable" are used in connection with less important factors. "May" is used to mean "is (are) allowed to," "can" is used to mean "is (are) able to," and "might" is used to mean "could possibly." Thus the classic distinction between "may" and "can" is preserved, and "might" is never used as a synonym for either "may" or "can."

3.1.2 For definitions of other terms used in this guide, refer to Guide E729, Terminology E943, and Guide E1023. For an explanation of units and symbols, refer to Practice E380.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *embryo*—used herein to denote the stages between the fertilization of the egg and the trochophore **(2)** (Figs. 1 and 2).

3.2.2 *larva*—used herein to include the trochophore and the straight hinge stage **(2)** (Figs. 1 and 2).

4. Summary of Guide

4.1 Adult saltwater bivalve molluscs are brought into the laboratory, cleaned of detritus and fouling organisms, and identified to species. If the gonads are not ripe (see 10.4.5), the bivalves are conditioned to bring them into a suitable reproductive state. Bivalves with ripe gonads are maintained under conditions that keep the gonads ripe without inducing undesired spontaneous spawning or resorption of gametes. *Mytilus spp.* can be held for several months in a gravid state by keeping them in clean cold seawater without feeding. In order to start a test, spawning is induced by using one or more stimuli which may be physical (for example, temperature), biological (for example, heat-killed bivalve sperm), or chemical (for example, serotonin).

4.2 In each of two or more treatments, embryos and the resulting larvae of one species are maintained for 48 h. In each of one or more control treatments, the embryos and resulting larvae are maintained in dilution water to which no test material has been added in order to provide (*a*) a measure of the acceptability of the test by giving an indication of the quality of the organisms and the suitability of the dilution water, test conditions, handling procedures, and so forth, and (*b*) the basis for interpreting data obtained from the other treatments. In each of one or more other treatments, the

[3] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[4] Withdrawn. The last approved version of this historical standard is referenced on www.astm.org.

[5] The last approved version of this historical standard is referenced on www.astm.org.

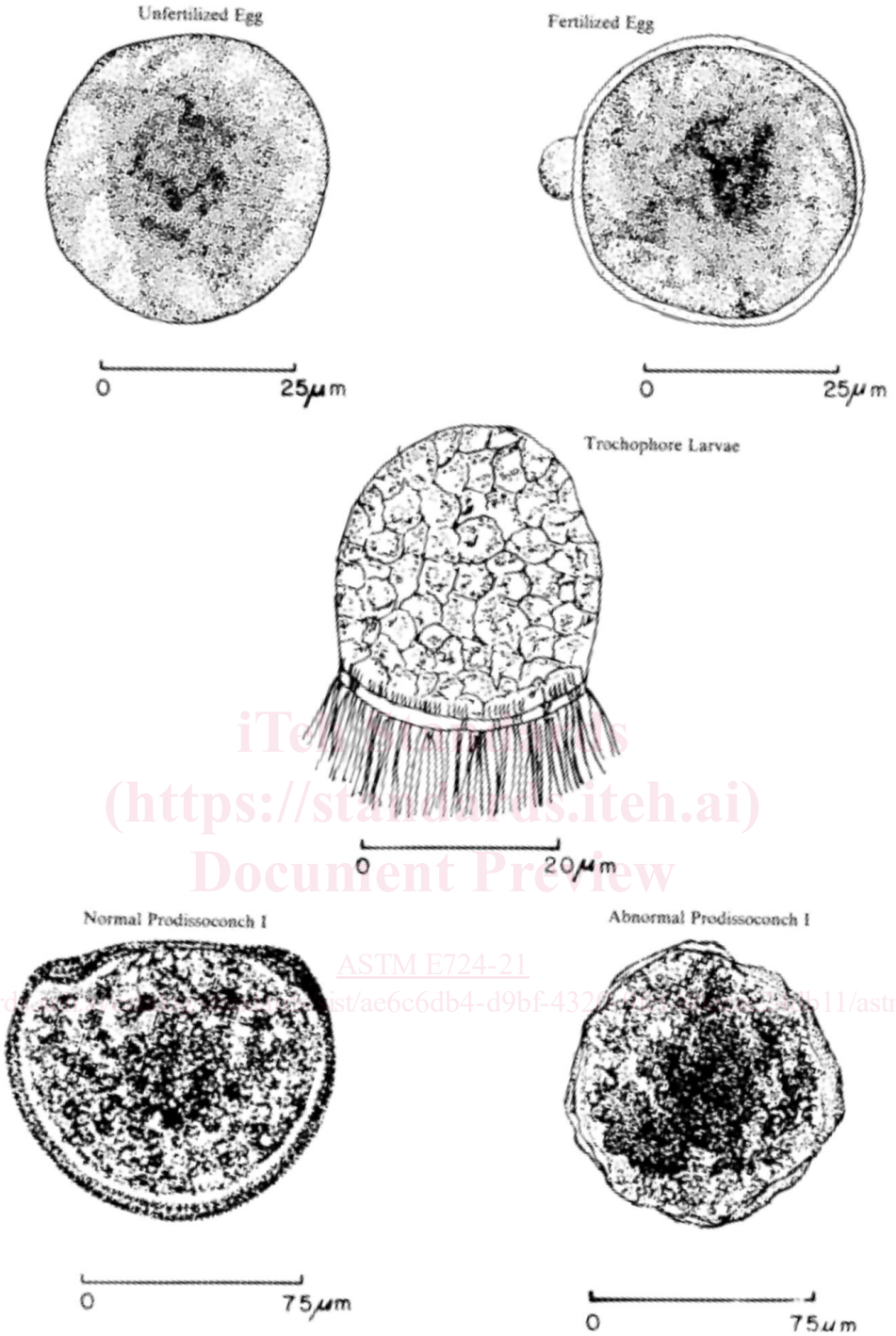

FIG. 1 Drawings Exemplifying Five Key Developmental Stages of Bivalve Larvae Occurring During the First 48 h of Development

embryos and resulting larvae are maintained in dilution water to which a selected concentration of test material has been added. The 48-h EC50 is calculated based on the proportion of live larvae with completely developed shells in chambers containing the test material to live larvae with completely developed shells in the controls at the termination of the 48-h test.

5. Significance and Use

5.1 An acute toxicity test is conducted to assess the effects of a short term exposure of organisms to a test material under specific experimental conditions. An acute toxicity test does not provide information concerning whether delayed effects will occur and typically evaluates effects on survival. A chronic

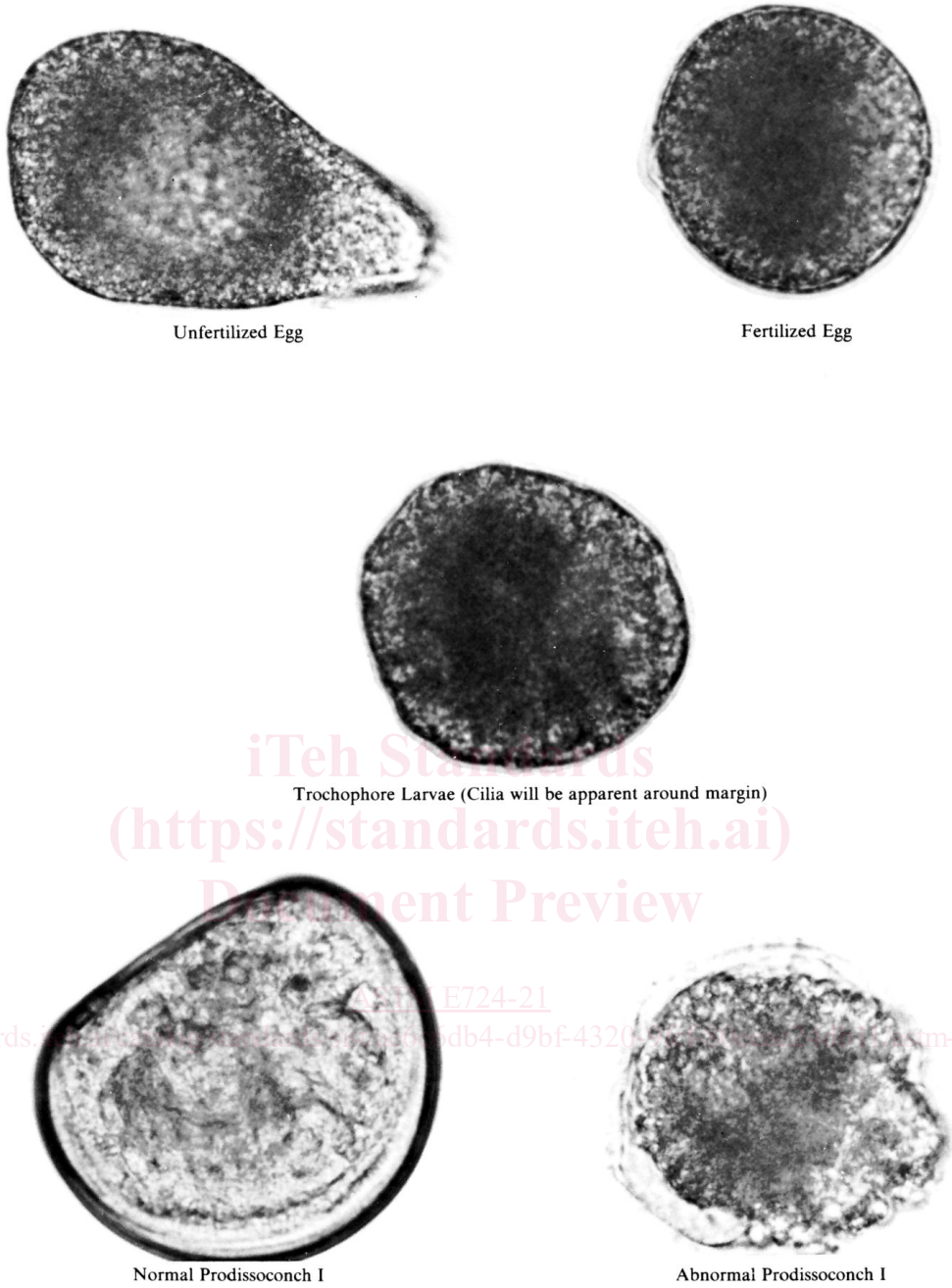

FIG. 2 Photomicrographs (×450) Exemplifying Five Key Developmental Stages of Bivalve Larvae Occurring During the First 48 h of Development

test is typically longer in duration and includes a sublethal endpoint to assess effects on a population that might occur beyond the exposure period. Because the bivalve embryo development test includes a sublethal endpoint, but is also short in duration, these tests are considered to be short-term chronic tests.

5.2 Because embryos and larvae are usually assumed to be the most sensitive life stages of these bivalve mollusc species and because these species are commercially and recreationally important, results of these acute tests are often considered to be a good indication of the acceptability of pollutant concentrations to saltwater molluscan species in general. Results of these acute toxicity tests are often assumed to be an important consideration when assessing the hazard of materials to other saltwater organisms (see Guide E1023) or when deriving water quality criteria for saltwater organisms (**3**).

5.3 Results of short-term chronic toxicity tests might be used to predict effects likely to occur to aquatic organisms in field situations as a result of exposure under comparable

conditions, except that toxicity to benthic species might depend on sorption or settling of the test material onto the substrate.

5.4 Results of short-term chronic tests might be used to compare the sensitivities of different species to different test materials, and to determine the effects of various environmental factors on results of such tests.

5.5 Results of short-term chronic toxicity tests might be useful for studying biological availability of, and structure activity relationships between, test materials.

5.6 Results of any toxicity test will depend on temperature, composition of the dilution water, condition of the test organisms, and other factors.

5.7 Results of short-term chronic toxicity tests might be used to predict effects likely to occur to aquatic organisms exposed to suspended particulates of dredged sediments disposed through the water column.

5.8 Results of short-term chronic toxicity tests might be used to predict effects likely to occur to aquatic organisms exposed to a bedded whole sediments.

6. Hazards

6.1 Many materials can adversely affect humans if precautions are inadequate. Therefore, skin contact with all test materials and solutions of them should be minimized by such means as wearing appropriate protective gloves (especially when washing equipment or putting hands in test solutions), laboratory coats, aprons, and glasses. Special precautions, such as covering test chambers and ventilating the area surrounding the chambers, should be taken when conducting tests on volatile materials. Information concerning toxicity to humans (**4**), recommended handling procedures (**5**), and chemical and physical properties of the test material should be studied before a test is begun. Special procedures are necessary with radiolabeled test materials (**6**) and with materials that are, or are suspected of being, carcinogenic (**7**).

6.2 Although in most cases disposal of stock solutions, test solutions, and test organisms poses no special problems, health and safety precautions and applicable regulations should be considered before beginning a test (see Guide D4447). Removal or degradation of the test material might be desirable before disposal of stock and test solutions.

6.3 Cleaning of equipment with a volatile solvent such as acetone should be performed only in a well-ventilated area in which no smoking is allowed and no open flame, such as a pilot light, is present.

6.4 **Warning**—An acidic solution should not be mixed with a hypochlorite solution because hazardous fumes might be produced.

6.5 To prepare dilute acid solutions, concentrated acid should be added to water, not vice versa. Opening a bottle of concentrated acid and adding concentrated acid to water should be performed only in a fume hood.

6.6 **Precaution**—Use of ground fault systems and leak detectors is strongly recommended to help prevent electrical shocks because salt water is a good conductor of electricity.

6.7 To protect hands from being cut by sharp edges of oyster shells, cotton work gloves should be worn over appropriate protective gloves (see 6.1), if necessary, when oysters are handled. When an oyster knife is used to open adults of any bivalve species, heavy rubber work gloves should be worn to protect hands from puncture.

6.8 Preservation of larvae to facilitate microscopic enumeration will be performed with a fixative agent such as buffered formalin, and biological stains (that is, Rose Bengal). Appropriate safety precautions should be taken when handling.

7. Apparatus

7.1 *Facilities:*

7.1.1 Flow-through troughs with appropriate trays (**8**) should be available for holding and conditioning the brood stock. The water-supply system should be equipped for temperature control and aeration (see 8.3), and should contain strainers and air traps. Air used for aeration should be free of fumes, oil, and water; filters to remove oil and water are desirable. Test chambers should be in a constant-temperature room, incubator, or recirculating water bath. A dilution-water tank or headbox, which might be used to prepare reconstituted water, is often elevated so that dilution water can be gravity fed into holding and conditioning troughs and test chambers. The facility should be well ventilated and free of fumes. To further reduce the possibility of contamination of brood stock and test organisms by test materials and other substances, especially volatile ones, holding and conditioning troughs should not be in a room in which toxicity tests are conducted, stock solutions or test solutions are prepared, or equipment is cleaned. During holding, conditioning, and testing, organisms should be shielded from disturbances with curtains or partitions to prevent unnecessary stress.

7.1.2 It is probably desirable to include some safeguards in the system that supplies water to holding and conditioning troughs. Monitors, possibly connected to auxiliary power supplies, might be designed to initiate aeration, sound alarms, or activate telephone auto-dialing alarms if water flow or temperature deviates from preset limits. If temperature becomes too high or low, corrective action should not cause the temperature of the water in holding and conditioning troughs to increase or decrease more than 2°C/day to reduce the chances of spontaneous spawning.

7.2 *Construction Materials*—Equipment and facilities that contact stock solutions, test solutions, or any water into which brood stock or test organisms will be placed should not contain substances that can be leached or dissolved by aqueous solutions in amounts that adversely affect test organisms. In addition, equipment and facilities that contact stock solutions or test solutions should be chosen to minimize sorption of test materials from water. Glass, Type 316 stainless steel, nylon, and fluorocarbon plastics should be used whenever possible to minimize dissolution, leaching, and sorption, except that stainless steel should not be used when testing metals. Concrete and rigid plastics may be used for holding and conditioning tanks and in the water-supply system, but they should be soaked, preferably in flowing dilution water, for a week or more before use (**9**). Brass, copper, lead, galvanized metal, cast-iron pipe,

and natural rubber should not contact dilution water, stock solutions, or test solutions before or during the test. Items made of neoprene rubber and other materials not mentioned should not be used unless it has been shown that embryos and resulting larvae of the test species do not show more signs of stress, such as discoloration, incomplete shell development, or death, when held for 48 h in static dilution water in which the item is soaking than when held in static dilution water that does not contain the item.

7.3 *Test Chambers:*

7.3.1 In a toxicity test with aquatic organisms, test chambers are defined as the smallest physical units between which there are no water connections. Chambers should be covered to keep out extraneous contaminants and bacteria and to minimize evaporation of test solution and material. Substantial concentrations of bacteria in the test solutions might severely reduce the survival of the embryos and resulting larvae, whereas differences in the amount of evaporation among test chambers will directly contribute to between-chamber variation in survival. All chambers in a test must be identical.

7.3.2 Tests are usually conducted in glass chambers that are 1 to 2 L in capacity. Very small test chambers, containing as little as 10 to 30 mL, and sealed test chambers **(10)** may be used if the survival and development of the embryos and resulting larvae in the control(s) are acceptable (see 13.1.6).

7.4 *Cleaning*—Test chambers and equipment used to prepare and store dilution water, stock solutions, and test solutions should be cleaned before use. New items should be washed with detergent and rinsed with water, a water miscible organic solvent, water, acid (such as 10 % concentrated hydrochloric acid), and at least twice with deionized, distilled, or dilution water. (Some lots of some organic solvents might leave a film that is insoluble in water.) At the end of the test, all items that are to be used again should be immediately (*a*) emptied, (*b*) rinsed with water, (*c*) cleaned by a procedure appropriate for removing the test material from the item (for example, acid for removing metals and bases; detergent or organic solvent for removing organic chemicals), and (*d*) rinsed at least twice with deionized, distilled, or dilution water. Acid is often used to remove mineral deposits. A hypochlorite solution, often recommended as a disinfection agent or to remove organic matter, should not be used due to the extreme toxicity of chlorine-produced oxidants to bivalve larvae **(11)**. Test chambers should be rinsed with dilution water just before use.

7.5 *Acceptability*—Before a test is started with embryos of a bivalve mollusc in new test facilities, it is desirable to conduct a nontoxicant test in which all test chambers contain dilution water with no added test material. This is desirable in order to determine (*a*) if embryos will survive and develop acceptably (see 13.1.6), (*b*) if the dilution water, handling procedures, and so forth, are acceptable, (*c*) if there are any location effects on either survival or development, and (*d*) the magnitude of between-chamber variance in the percentage of embryos that develop into live larvae with completely developed shells.

8. Dilution Water

8.1 *Requirements*—Besides being available in adequate supply, the dilution water should (*a*) be acceptable to adult bivalve molluscs and their embryos and larvae, (*b*) be of uniform quality, and (*c*) not unnecessarily affect results of the test except as in 8.1.4.

8.1.1 The minimal requirement for an acceptable dilution water for toxicity tests with embryos of oysters, clams and mussels is that at least 70 % 60 %, or, 90 % respectively, of the embryos resulting from eggs and sperm produced by appropriately conditioned adults result in live larvae with completely developed shells (normal in shape) while being maintained in the dilution water for 48 h. Natural salt water varies in quality enough that, even though it is usually acceptable, occasionally it might be toxic to embryos or larvae if, for example, certain toxic algae species are present such as some dinoflagellates **(12)**.

8.1.2 The quality of the dilution water should be uniform enough that the brood stock is held and conditioned and the test is conducted in water of the same quality. In particular, the salinity should always be between 18 and 34 g/kg or parts per thousand (ppt)**(13)** and within a test should not vary by more than 1 ppt among treatments or any renewals during a test. When a test is conducted on an effluent, brine, drilling mud, or other material that affects salinity when mixed with dilution water, it might be desirable to adjust salinity by adding sea salt (see 8.2.1.2) to raise the salinity or by adding distilled or deionized water (see 8.2.1.2) to lower the salinity. If salts are added, the adjusted material should be well mixed and allowed to equilibrate for minimum of 2 h with gentle aeration (24 h preferred) and salinity confirmed as salts dissolve. The addition of artificial salts can produce artifactual toxicity that is ameliorated with aging. If the salinity is adjusted, an additional salt control, consisting of dilution water diluted with distilled water to the salinity of the sample, and then adjusted back to the appropriate salinity with artificial salts should be tested.

8.1.3 The dilution water should not unnecessarily affect results of tests because of such things as sorption or complexation of test material. Therefore, except as per 8.1.4, concentrations of both total organic carbon (TOC) and particulate matter should be less than 5 mg/L in the dilution water. The concentrations of both TOC and particulate matter should be greater than 5 mg/L in the water in which the brood stock is held and conditioned in order to provide adequate food for the brood stock.

8.1.4 If it is desired to study the effect of an environmental factor such as TOC, particulate matter, or dissolved oxygen on the results of a test, it is necessary to use a water that is naturally or artificially high in TOC or particulate matter or low in dissolved oxygen. If such a water is used, it is important that adequate analyses be performed to characterize the water and that a comparable test be available or be conducted in a more usual dilution water to facilitate interpretation of the results obtained in the special water.

8.2 *Source:*

8.2.1 *Reconstituted Water:*

8.2.1.1 Use of a reconstituted water is often not worth the effort for tests starting with embryos of bivalve molluscs because of (*a*) the large volume needed for conditioning the brood stock, (*b*) the necessity of providing adequate food for the brood stock (see 10.4.8), and (*c*) frequently poor survival

TABLE 1 Reconstituted Salt Water (14)

NOTE 1—Add the following reagent-grade chemicals[6] in the amounts and order listed to 890 mL of water. Each chemical must be dissolved before the next is added.[A]

Chemical	Amount
NaF	3 mg[B]
$SrCl_2 \cdot 6H_2O$	20 mg
H_3BO_3	30 mg
KBr	100 mg
KCl	700 mg
$CaCl_2 \cdot 2H_2O$	1470 mg
Na_2SO_4	4000 mg
$MgCl_2 \cdot 6H_2O$	10780 mg
NaCl	23500 mg
$Na_2SiO_3 \cdot H_2O$	20 mg
$NaHCO_3$	200 mg

[A] If the resulting solution is diluted to 1 L, the salinity should be 34 ± 0.5 g/kg. The reconstituted water should be stripped of trace metals (15). If necessary, the water should be diluted to the desired salinity at time of use.
[B] The NaF should be omitted for tests with *C. gigas* and included for tests with *C. virginica*. Its value for or detriment to *M. mercenaria* and *M. spp.* is unknown.

and development of the embryos and resulting larvae. Commercially available sea salts have been used successfully in bivalve embryo testing given proper conditioning of the water. Be sure to only use salts that are fast-dissolving and closely match the ion mix found in natural seawater. A trial test with any new brand or batch of salt is recommended prior to any testing of samples when feasible. Additionally, commercially available sea salts should be free of ethylenediaminetetraacetic acid (EDTA) or other chelating agents that may remove or mask toxicity in a sample that is being tested.

8.2.1.2 Reconstituted water is prepared by adding a commercially avaiable sea salt or specified amounts of reagent-grade[6] chemicals to high quality water (**14**) with (*a*) conductivity approximately <1 µS/cm and (*b*) either TOC <2 mg/L or chemical oxygen demand (COD) <5 mg/L. A formula for reconstituted water acceptable for use with bivalves is given in Table 1. Acceptable water for dissolution of sea salts can usually be prepared by using a properly operated deionization, distillation, or reverse osmosis unit. Conductivity should be measured on each batch and TOC or COD should be measured at least twice a year and whenever substantial changes might be expected. If the water is prepared from a surface water, TOC or COD should be measured on each batch. Problems have been encountered with some species in reconstituted salt water, but sometimes these problems have been overcome by conditioning (aging) and aerating the reconstituted water.

8.2.2 *Natural Dilution Water*—If natural salt water is used, it should be obtained from an uncontaminated, uniform quality source. The quality of saline well water is usually more uniform than that of a saline surface water. If a surface water is used, it should be obtained from an area known to support a healthy, naturally reproducing population of bivalves. The water intake should be positioned (for example, about 1 m below the surface) to minimize fluctuations in quality and the possibility of contamination and to maximize the concentration of dissolved oxygen and healthy phytoplankton (see 10.4.8). A specially designed system is usually necessary to obtain salt water from a natural water source (see Guide E729).

8.2.3 Chlorinated water should not be used as, or in the preparation of, saline dilution water because chlorine-produced oxidants are quite toxic to embryos and larvae of bivalve molluscs (**7**). Dechlorinated water should be used only as a last resort because dechlorination is often incomplete. Sodium bisulfite is probably better for dechlorinating water than sodium sulfite and both are more reliable than carbon filters, especially for removing chloramines (**15**). Some organic chloramines, however, react slowly with sodium bisulfite (**16**). In addition to residual chlorine, municipal drinking water often contains high concentrations of copper, lead, zinc, and fluoride, and quality is often rather variable. The concentrations of most metals can usually be reduced by using a chelating resin (**17**), but use of a different dilution water might be preferable.

8.3 *Treatments:*

8.3.1 Dilution water should be aerated intensively for 24 to 48 h by such means as air stones, surface aerators, or column aerators (**18**) before addition of test material. To prevent contamination with undesirable bacterial species during aeration, the air used should be filtered through a 0.22-µm bacterial filter, the container should be covered, and aeration should not last for more than 48 h. Adequate aeration will bring pH and concentrations of dissolved oxygen and other gases into equilibrium with air and minimize oxygen demand and concentrations of volatiles. The concentration of dissolved oxygen in dilution water should be between 90 and 100 % of saturation (**19**) to help ensure that dissolved oxygen concentrations are acceptable in test chambers. Supersaturation by dissolved gases, which can be caused by heating the dilution water, should be avoided to prevent gas-bubble disease (**18, 20**).

8.3.2 The salinity and pH of dilution water may be adjusted by addition of appropriate reagent-grade chemicals,[6] sea salt (especially to prevent excessive decreases in salinity; see 8.1.2), acid, base, and deionized or distilled water, if it has been shown that the addition does not cause adverse effects on embryos, larvae, and adults of the test species at the concentration used.

8.3.3 Except possibly when holding and conditioning adult bivalve molluscs (see 10.4.6), filtration through bag, sand, sock, or depth-type (honeycomb) cartridge filters may be used to keep the concentration of particulate matter acceptably low (see 8.1.3) and as a pretreatment before ultraviolet sterilization or filtration through a finer filter.

8.3.4 Water that might be contaminated with facultative pathogens may be passed through a properly maintained ultraviolet sterilizer (**21**) equipped with an intensity meter and flow controls, or passed through a filter effective to 0.45 µm or less.

8.3.5 Water from a surface-water source should be passed through a graded series of filters, the finest effective to 1.0 µm or less to remove embryos and larvae of bivalve molluscs, parasites and predators. If bacteria are to be removed by filtration, a filter effective to 0.45 µm or less must be used.

[6] "Reagent Chemicals, American Chemical Society Specifications," Am. Chemical Soc., Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see "Analar Standards for Laboratory U.K. Chemicals," BDH Ltd., Poole, Dorset, and the "United States Pharmacopeia."

8.3.6 Filtration through activated carbon may be used to remove toxic algal exocrines and other organic chemicals.

8.4 *Characterization:*

8.4.1 The following items should be measured at least twice each year and more often if such measurements have not been made semiannually for at least 2 years or if a surface water is used: salinity (or chlorinity), pH, particulate matter, TOC, organophosphorus pesticides, organic chlorine (or organochlorine pesticides, plus PCBs), chlorinated phenoxy herbicides, ammonia, cyanide, sulfide, bromide, fluoride, iodide, nitrate, phosphate, sulfate, calcium, magnesium, potassium, aluminum, arsenic, beryllium, boron, cadmium, chromium, cobalt, copper, iron, lead, manganese, mercury, molybdenum, nickel, selenium, silver, and zinc.

8.4.2 For each method used (see 12.2), the detection limit should be below either (*a*) the concentration in the dilution water or (*b*) the lowest concentration that has been shown to unacceptably affect embryos, larvae, or adults of saltwater bivalve molluscs **(22)**.

9. Test Material

9.1 *General*—Test materials may include a range of sample types such as effluents, materials or products, specific chemicals, solvents, oils, surface waters, drilling fluids, stormwater, and sediments (see Guide E1023). For chemical or product testing studies the test material should be reagent-grade[6] or better, unless a test on a formulation, commercial product, or technical-grade or use-grade material is specifically needed. Before a test is begun, the following should be known about the test material:

9.1.1 Identities and concentrations of major ingredients and major impurities, that is, impurities that constitute more than about 1 % of the material,

9.1.2 Solubility and stability in the dilution water,

9.1.3 Measured or estimated toxicity to an aquatic species, preferably the test species or another bivalve mollusc,

9.1.4 Precision and bias of the analytical method at the planned concentration(s) of the test material, if the test concentrations are to be measured,

9.1.5 Estimate of toxicity to humans, and

9.1.6 Recommended handling procedures (see 6.1).

9.2 *Stock Solution:*

9.2.1 In some cases the test material can be added directly to the dilution water, but often it is dissolved in a solvent to form a stock solution that is then added to the dilution water. If a stock solution is used, the concentration and stability of the test material in it should be determined before the beginning of the test. If the test material is subject to photolysis, the stock solution should be shielded from light.

9.2.2 Except possibly for tests on hydrolyzable, oxidizable, and reducible materials, the preferred solvent is dilution water, although filtration or sterilization, or both, of the water might be necessary. If the salinity of the dilution water will not be affected, deionized or distilled water may be used. Several techniques have been specifically developed for preparing aqueous stock solutions of slightly soluble materials **(23)**. The minimum necessary amount of a strong acid or base may be used in the preparation of an aqueous stock solution, but such reagents might affect the pH of test solutions appreciably. Use of a more soluble form of the test material, such as chloride or sulfate salts of organic amines, sodium or potassium salts of phenols and organic acids, and chloride or nitrate salts of metals, might affect the pH more than use of the minimum necessary amount of a strong acid or base.

9.2.3 If a solvent other than dilution water is used, its concentration in test solutions should be kept to a minimum and should be low enough that it does not affect the test species. Because of its low toxicity to aquatic animals **(24)**, low volatility, and high ability to dissolve many organic chemicals, triethylene glycol is often a good organic solvent for preparing stock solutions. Other water-miscible organic solvents such as methanol, ethanol, and acetone may also be used, but they might stimulate undesirable growths of microorganisms, and acetone is also quite volatile. If an organic solvent is used, it should be reagent-grade[6] or better and its concentration in any test solution should not exceed 0.5 mL/L. A surfactant should not be used in the preparation of a stock solution because it might affect the form and toxicity of the test material in the test solutions. (These limitations do not apply to any ingredient of a mixture, formulation, or commercial product, unless an extra amount of solvent is used in the preparation of the stock solution.)

9.2.4 If no solvent other than water is used, only a dilution-water control must be included in the test. For oysters, at least 70 % of the embryos introduced into the control treatment must result in live larvae with completely developed shells (normal) at the end of the test and at least 90 % of all embryos introduced should be alive. For hard clams, at least 60 % of the embryos introduced must result in live larvae with completely developed shells at the end of the test, and at least 90 % of all embryos introduced should be alive. These values for live larvae with completely developed shells reflect apparently natural failure of up to 30 to 40 % of the larvae of oysters and hard clams to develop a shell. For mussels, at least 90 % of the embryos introduced into the control treatment must result in live larvae with completely developed shells (normal) at the end of the test and at least 50 % of all embryos introduced should be alive. These criteria values for mussels are consistent with USEPA guidance **(25)**.

9.2.5 If a solvent other than water is used and the concentration of solvent is the same in all test solutions that contain test material, a solvent control, containing the same concentration of solvent as the test solutions and using solvent from the same batch used to make the stock solution, must be included in the test. In addition, a dilution-water control should be included in the test. The number of embryos that result in live larvae with completely developed shells (normal) at the end of the test must be at least 70 % of the initial number in the solvent control for oysters and 60 % for hard clams (see 9.2.4). If a dilution-water control is included in the test, the number of embryos that result in live larvae with completely developed shells at the end of the test should be at least 70 % of the initial number in the dilution-water control for oysters and 60 % for hard clams.

9.2.6 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, 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 must be included in the test. The number of embryos that result in live larvae with completely developed shells at the end of the test must be at least 70 % of the initial number in the solvent control and in the dilution-water control for oysters and 60 % for hard clams (see 9.2.4).

9.2.7 If a solvent other than water is used to prepare a stock solution, it might be desirable to conduct simultaneous tests on the test material using two chemically unrelated solvents or two different concentrations of the same solvent to obtain information concerning possible effects of the solvent on the results of the test.

9.3 *Test Concentration(s):*

9.3.1 If the test is intended to allow calculation of an EC50, the test concentrations (see 11.1.1.1) should bracket the predicted EC50. The prediction might be based on the results of a test with the same or a similar test material and the same or a similar species. If a useful prediction is not available, it is usually desirable to conduct a range-finding test in which embryos and resulting larvae are exposed for a total of 48 h to a control and three to five concentrations of the test material that differ by a factor of ten. The greater the similarity between the range-finding test and the actual test, the more useful the range-finding test will be.

9.3.1.1 If necessary, concentrations above solubility should be used because organisms in the real world are sometimes exposed to concentrations above solubility. The use of concentrations that are more than ten times greater than solubility is probably not worthwhile. With some test materials, it might be found that concentrations above solubility do not kill or affect a greater percentage of test organisms than does the concentration that is the solubility limit; such information is certainly worth knowing.

9.3.1.2 In some situations, usually related to regulatory activities, it is only necessary to determine whether (*a*) a specific concentration of test material is toxic to embryos or larvae of the test species or (*b*) the EC50 is above or below a specific concentration. For example, the specific concentration might be the concentration occurring in a surface water, the concentration resulting from the direct application of the material to a body of water, or the solubility limit of the material in water. When there is only interest in a specific concentration, it is often only necessary to test that concentration plus a control (see 11.1.1.2), and it is not necessary to determine an EC50.

10. Test Organisms

10.1 *Species*—Whenever possible, either Pacific oysters (*Crassostrea gigas*), Eastern oysters (*C. virginica*), quahogs or hard clams (*Mercenaria mercenaria*), or blue mussels (*Mytilus edulis*) should be used as the test species. These species were selected on the basis of availability, commercial importance, past successful use, and ease of handling in the laboratory. Their use is encouraged to increase comparability of results and availability of much information about a few species rather than little information about many species. The species used should be identified by using an appropriate taxonomic key.

10.2 *Age*—The test must be begun with embryos within 4 h after fertilization when the embryos are in the 2-, 4-, and 8-cell stages.

10.3 *Handling*—Organisms should be handled as little as possible. When handling is necessary, it should be done carefully, gently, and quickly, so that organisms are not unnecessarily stressed. Adults that are injured during handling should be discarded. Gentle cleaning of the organisms is however recommended to remove other attached organisms and debris prior to sue for testing. Equipment used to transfer embryos of bivalve molluscs should be cleaned between uses by washing with detergent and rinsing with dilute acid and distilled or deionized water. Hands should be washed before and after handling brood stock.

10.4 *Brood Stock Source and Condition:*

10.4.1 For any one test or a series of related tests, all females and males in the brood stock must be collected from the same location, which should be known precisely. Brood stock may be obtained from a commercial source only if the original specific collection location of the bivalves can be identified. Aquacultured shellfish that forced into triploidy must not be utilized. To minimize the possibility of genetic or physiological adaptation to chemicals or aberrant water quality, organisms should be collected from a location that is not subject to obvious point or nonpoint source pollution and has water that is comparable in quality to the water that will be used for holding, conditioning, and testing. Gravid adult individuals with well developed normal shells should be obtained. Molluscs possessing high condition factors **(26)** (see Note 1), indicative of good food supply and water quality throughout the year, should be obtained because they are easier to hold and condition, and they yield higher quality gametes than molluscs with low condition factors. The investigator should be aware that sex ratios can differ substantially from 1:1. For example, Katkansky and Sparks **(27)** found that the percentage of females approached 95 % in some groups of 4-year-old *C. gigas* due to protandric hermaphroditism. A similar phenomenon might occur in some populations of *C. virginica.*

NOTE 1—The Condition Index (C. I.) for adult bivalves may be calculated as follows:

$$\text{C. I.} = 100\,(\text{tissue dry weight, g})/(\text{volume of shell cavity, mL})$$

The sex ratio of the sample should be reported.

10.4.2 Adults may be obtained from distant locations during periods of the year when animals with mature gonads cannot be obtained in the vicinity of the test laboratory. A preferable means of extending the availability of spawnable bivalves is to hold a population with mature gonads at an appropriate holding temperature (Table 2) after conditioning is complete. If done

TABLE 2 Recommended Temperature (°C)

Species	Holding Conditioning		Induction	Never to be Exceeded	Test
Crassostrea gigas	14–15	20	25–32	32	20
Crassostrea virginica	14–15	20–25	25–32	32	25
Mercenaria mercenaria	14–15	20–25	25–32	32	25
Mytilus spp.	8	12–15	12–18	25	15 or 18

correctly, this will prevent both undesired spontaneous spawning and resorption of gametes. Under suitable conditions, oysters can yield viable gametes for up to 4 months after conditioning.

10.4.3 During certain periods of the year, adult bivalve molluscs can be easily induced to spawn with a variety of biological, chemical, and physical stimuli, and it is essential to minimize these stimuli until spawning is desired. Accordingly, upon collection or purchase, adults should be transported without delay to the laboratory, cleaned of detritus and such fouling organisms as barnacles, and placed in flowing water with a salinity suitable to the species. Rough handling, extended periods of desiccation, or abrupt changes in temperature, salinity, or other water quality characteristics might induce spawning and reduce the value of, if not render useless, the stock for later controlled spawning. If unplanned spawning occurs in a trough, it is best to discard all individuals in the trough.

10.4.4 When brood stock is first brought into the laboratory, it should be changed to dilution water over a period of 2 or more days to prevent stress due to abrupt changes in water quality. Generally the temperature may be changed at a rate not to exceed 2°C/day, and the salinity at a rate not to exceed 5 g/kg/day. An abrupt increase in temperature might not only induce spawning, especially of males, but also seriously harm the gametes (**28**) and kill the adults.

10.4.5 The ripeness of the brood stock can be determined by sacrificing several animals and examining the gonads. One shell is removed and the mantle and gill are excised to reveal the tissue overlying the digestive gland. Depending on the species, a creamy white, pink, or orange color indicates ripe gonads. A sample of the gametes can then be obtained by making a small incision with a scalpel or razor blade and rinsing the wound with pallial fluid or saline water. A sample of the resultant suspension should then be examined under 100× or 400× magnification. Gametes from a ripe male are minute and rapidly become highly active when placed in salt water; those from a ripe female are large, initially teardrop shaped, and rapidly become spherical within a gelatinous matrix when placed in salt water, usually in less than 30 min.

10.4.5.1 If there are too few adults to permit sacrifice of animals, one might omit determination of ripeness of the brood stock and induce spawning after first preparing for use of the embryos in a test. This alternative is high risk and desirable only if the brood stock is too small to permit sacrifice of adults because of the low probability of a successful spawn in small populations.

10.4.6 If the brood stock contains ripe gonads, the adults should be placed in cool water (see Table 2). If not maintained in cool water, a brood stock with ripe gametes is usable for only 2 to 3 weeks after attaining maturity; thereafter the quality of gametes will begin to deteriorate or spontaneous spawning will occur. The adults may be held as described in 10.4.8 – 10.4.11 until it is desired to induce spawning.

10.4.7 If the brood stock does not contain ripe gonads, the brood stock should be conditioned prior to any attempt to induce spawning. To condition bivalves, the temperature and water should be gradually changed to the conditioning temperature (see Table 2) and the dilution water. It is important to condition the adult animals under proper conditions for an appropriate duration to promote gametogenesis and production of mature gametes. Spawning of adults before or after optimum maturation will usually result in unsatisfactory gametes.

10.4.8 During holding and conditioning, the brood stock should be furnished an adequate supply of acceptable food. Although cultured phytoplankton might be added to the water, it is usually advantageous to hold and condition the brood stock in natural salt water that contains as much natural phytoplankton as possible. When natural salt water is used, it should not be passed through an ultraviolet sterilizer or a filter effective to less than 50 µm.

10.4.8.1 If adults possess some glycogen reserves in the mantle, they can sometimes be held for six or more weeks without food and still produce acceptable gametes (**29**). Usually, however, adults should be provided an adequate supply of natural or cultivated phytoplankton (**29**) to prevent malnutrition (**30**). Adult bivalve molluscs should be provided enough water containing an acceptable food to support survival and growth. If the flow rate or the concentration of food, or both, is too low, a saltwater alga such as *Monochrysis lutheri, Isochrysis galbana,* or *Tetraselmis suecica* should be added to the water (**1, 2**). Algae may be cultured or purchased as concentrated paste or spat from commercial suppliers.

10.4.9 The flow rate during holding and conditioning should be high enough to prevent water quality degradation and provide adequate food. The concentration of dissolved oxygen should be maintained between 60 and 100 % of saturation (**19**). Supersaturation by dissolved gases should be avoided to prevent gas-bubble disease (**18, 20**). The flow rate does not need to exceed the total pumping capacity of the adults being held. The maximum pumping rate is about 30 L/h for adult *C. gigas* at 20°C (**31**), but it is between 4 and 15 L/h for adult *C. virginica,* 0.6 and 7 L/h for *M. mercenaria,* and 0.16 and 1.9 L/h for adult *Mytilus spp.*(**32**). A flow of at least 7 L/min has been recommended (**33**) for shallow, 13-L trays containing 15 adult (70 to 100 mm) Pacific oysters, that is, about 28 L/h for each oyster.

10.4.10 The brood stock should be carefully observed daily during holding and conditioning for signs of stress and mortality. Gaping molluscs that do not close when touched with a probe should be discarded. Molluscs that never open or do not deposit feces or pseudofeces also should be discarded.

10.4.11 Holding and conditioning trays should be drained and sprayed with fresh water at least once weekly to prevent accumulation of organic matter and bacteria. Dead bivalves

should be removed daily. If animals have begun to decompose, the troughs should be drained and sprayed with fresh water, and the trays should be cleaned with detergent and rinsed with fresh water. With enriched waters and elevated conditioning temperatures, more frequent cleaning might be appropriate.

10.5 *Spawning and Fertilization:*

10.5.1 If possible, the toxicity test should be designed to assess differences in sensitivity resulting from parentage. Ideally, the test should be conducted by subjecting progeny from each of at least three individual male-female pairings to each of the one or more control treatments and one or more concentrations of the test material. The separate testing of progeny from individual pairs allows the determination of differences between pairs, allows the calculation of an unbiased estimate of the mean for the population, and obviates the need for synchronous spawning because tests with the individual pairs need not be started at the same time. Alternatively, progeny from at least three females should be combined in equal proportions and exposed to each of the one or more control treatments and each of the one or more concentrations of the test material. This latter approach masks differences in sensitivity based on parentage and might bias the estimate of the population mean.

10.5.2 Females and males of all four species can usually be induced to spawn by raising the water temperature rapidly to 5 to 10°C above the conditioning temperature (see Table 2) and applying an additional stimulus. For mussels, the added stimulus is either (*a*) potassium chloride injected into the posterior adductor muscle of each specimen (**29**) or (*b*) thermal stimulation of adults previously held in moist bags at 5°C (**34**). For the other species, the added stimulus is heat-killed sperm from a sacrificed or preferably a naturally spawned male, gently dispensed immediately in front of the incurrent siphon so they are sucked into the pallial cavity. The sperm should be applied 1 to 2 h after the rise in temperature because bivalves usually will not spawn during the first h. Other techniques used to induce spawning, either alone or in connection with thermal stimulation, such as hydrogen peroxide (**35**), serotonin (**36**), and a high density of algae, are not reliable for producing high quality embryos.

10.5.3 In preparation for thermal stimulation of spawning, 10 to 50 animals should be selected from a population of bivalves with ripe gonads and placed singly or in small groups in spawning chambers, such as crystallizing dishes or glass baking pans. Byssus from mussels should be trimmed off using clean scissors as occasionally the byssus can contain extraneous material or other organisms that can affect the test results. The number of adults needed depends on the ease of inducing spawning, the ratio of males to females, and the design of the test. (If desired, each mussel should receive 1.0 mL of 0.5 *M* potassium chloride injected into the posterior adductor muscle immediately before being placed into a spawning chamber.) Chambers should be filled with dilution water at the conditioning temperature, and animals should be allowed to begin pumping before starting thermal stimulation.

10.5.4 The spawning chambers should be placed in a water bath thermostatically controlled at the appropriate spawning temperature, or if no such bath is available, in a water bath filled with hot water and then drained as soon as the spawning chambers attain a temperature 5 to 10 °C above the conditioning temperature (within 5 min). The temperature should never exceed 20 °C for *M. spp.*, and 32 °C for the oyster and clam species (**29**). Bivalves exhibit specific behaviors as they are about to spawn. Initially the animals should be actively pumping; as the temperature increases, pumping activity will increase. Oysters that are about to spawn will clap their valves together violently once or twice. Clams will extend their siphons to a greater extent than when simply feeding and curve them backward over the valve facing upward. Spawning can be recognized by the appearance of a whitish stream of gametes emerging from the animal (color may vary from white to orange). Upon spawning, each individual organism should be removed from the spawning tray and isolated in a beaker containing control water at the same spawning temperature. All of the shed gametes will be contained in the individual beaker and it allows for easy examination and grading of gametes shed by individuals form both sexes. While spawning is occurring, a sample of the released gametes should be examined to determine whether they are eggs or sperm and the container labeled. Experienced analysts may be able to determine this by sight. If no animals spawn within 60 min (30 min if the animals are pumping extremely actively), the water bath should be drained, refilled with water at the brood stock holding temperature, and the stimulation process repeated. “Cold Shocking” is also another technique used to induce nonspawning mussels to spawn. This is performed by placing nonspawning mussels back into cold seawater at ~4 °C for 30 minutes, or until the mussels begin siphoning. Then the mussels are placed back into water at the appropriate spawning temperature to induce spawning. Difficulty in inducing spawning of adults might be caused by insufficient conditioning and might result in diminished embryo quality. Otherwise, water quality or some other perturbation in the conditioning regimen should be investigated.

10.5.5 Fertilization is more likely to be successful if sperm is obtained from a natural spawn. If sperm cannot be obtained from a natural spawn, however, sperm may be stripped from males. Each animal should be carefully removed from the shell by cutting away the gill and mantle tissue. While the animal is held over a beaker containing dilution water, several incisions should be made over the region of the gonad on each side of the animal. The gametes should be gently squeezed from the animal and rinsed into the beaker with a gentle stream of dilution water from a squirt bottle. Use of eggs stripped from female bivalves is not recommended because it often results in an excess of poorly developed and malformed embryos.

10.5.6 Eggs should be passed through a 75-µm screen to remove clumps, feces, and other extraneous material. Alternatively a clean transfer pipette to suck up and remove this type of material is effective as well and is less apt to damage eggs than a screen. The concentration of eggs should be determined by counting a sample of the egg suspension. To ensure a homogeneous suspension of eggs, a perforated plunger should be used to suspend the eggs. (A plunger can be constructed by drilling holes in a disc of acrylic plastic or fiberglass of suitable diameter and attaching it to a PVC or acrylic rod of suitable