Designation: E724 – 98 (Reapproved 2012)

Standard Guide for

Conducting Static Acute 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 blue mussel, *Mytilus edulis* Linnaeus) during static 48-h exposures. These procedures will probably be useful for conducting static acute 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 acute 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 acutely 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.

2. Referenced Documents

2.1 *ASTM Standards:*[3]

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

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

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, 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. 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 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 obtain information concerning the acute 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.

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

[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.

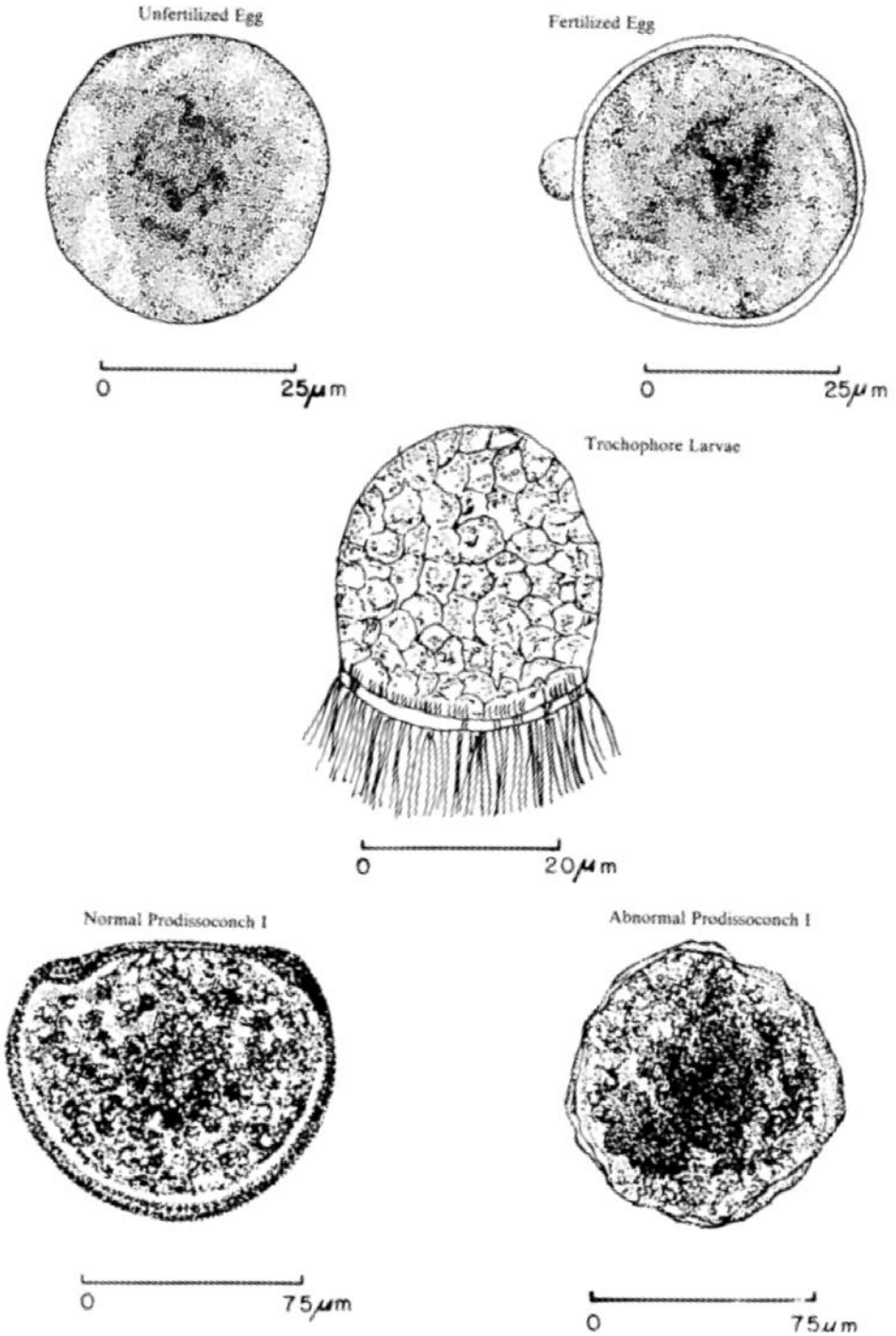


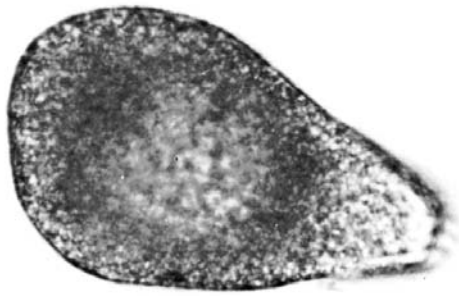
FIG. 1 Drawings Exemplifying Five Key Developmental Stages of Bivalve Larvae Occurring During the First 48 h of Development

saltwater organisms (see Guide E1023) or when deriving water quality criteria for saltwater organisms **(3)**.

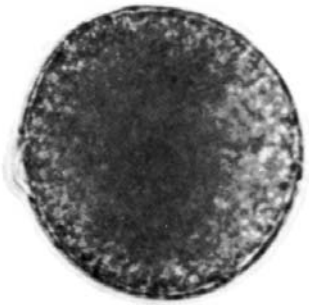
5.3 Results of acute toxicity tests might be used to predict acute effects likely to occur to aquatic organisms in field situations as a result of exposure under comparable conditions, except that toxicity to benthic species might depend on sorption or settling of the test material onto the substrate.

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

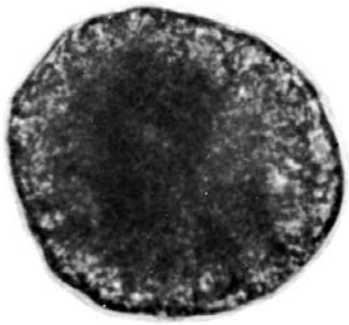
5.5 Results of acute toxicity tests might be useful for studying biological availability of, and structure activity relationships between, test materials.



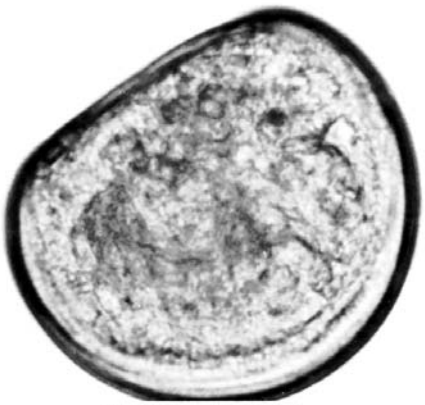
Unfertilized Egg



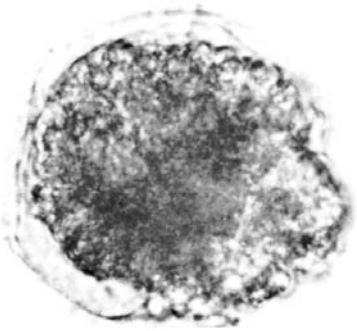
Fertilized Egg



Trochophore Larvae (Cilia will be apparent around margin)



Normal Prodissoconch I



Abnormal Prodissoconch I

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

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

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. 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.

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 acute toxicity tests starting with embryos of oysters or hard clams is that at least 70 % or 60 %, respectively, of the embryos resulting from eggs and sperm produced by appropriately conditioned adults result in live larvae with completely developed shells while being maintained in the dilution water for 48 h. Information is not presently available to provide similar guidance for the mussel. 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, dinoflagellates are present (**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 32 g/kg (**13**) and within a test should not vary by more than 1 g/kg 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.

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 specal water.

TABLE 1 Reconstituted Salt Water (14)

NOTE 1—Add the following reagent-grade chemicals[5] 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. edulis* is unknown.

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 10.5.8), and (*c*) frequently poor survival and development of the embryos and resulting larvae.

8.2.1.2 Reconstituted water is prepared by adding a sea salt or specified amounts of reagent-grade[5] chemicals to high quality water (**14**) with (*a*) conductivity <1 μΩ/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

[5] "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."

possibility of contamination and to maximize the concentration of dissolved oxygen and healthy phytoplankton (see 10.5.8). 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 (**16**). Some organic chloramines, however, react slowly with sodium bisulfite (**17**). 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 (**18**), 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 (**15**) 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 (**15, 20**).

8.3.2 The salinity and pH of dilution water may be adjusted by addition of appropriate reagent-grade chemicals,[5] 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 10.5.6), 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.

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*—The test material should be reagent-grade[5] 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 acute 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 usually 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[5] 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 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. These stipulations are species-specific and might be too high or too low for the blue mussel as well as bivalves not covered in this guide.

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 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 acutely 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.

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 edulis	8	12–14	15–20	20	16

10.3 *Source*—Embryos used to start a test should be obtained from females and males that had been maintained for at least 2 weeks in the dilution water in the laboratory before they were stimulated to spawn.

10.4 *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. 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.

10.5 *Brood Stock Source and Condition:*

10.5.1 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. 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. Large individuals with well developed normal shells should be obtained. Molluscs possessing high condition factors **(25)** (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 **(26)** 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.

10.5.2 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 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.

10.5.3 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.

10.5.4 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 **(27)** and kill the adults.

10.5.5 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 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.

10.5.5.1 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.

10.5.6 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 10.5.8-10.5.11 until it is desired to induce spawning.

10.5.7 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.

10.5.8 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.

10.5.8.1 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 (**28**). Usually, however, adults should be provided an adequate supply of natural or cultivated phytoplankton (**28**) to prevent malnutrition (**29**). 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**).

10.5.9 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 (**15, 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 (**30**), 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 *M. edulis* (**31**). A flow of at least 7 L/min has been recommended (**32**) for shallow, 13-L trays containing 15 adult (70 to 100 mm) Pacific oysters, that is, about 28 L/h for each oyster.

10.5.10 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.

10.5.11 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.

10.6 *Spawning and Fertilization:*

10.6.1 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.

10.6.2 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 bay mussels, the added stimulus is either (*a*) potassium chloride injected into the posterior adductor muscle of each specimen (**28**) or (*b*) thermal stimulation of adults previously held in moist bags at 5°C (**33**). 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 (**34**), serotonin (**35**), and a high density of algae, are not reliable for producing high quality embryos.

10.6.3 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. 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 bay 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.

10.6.4 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. edulis,* and 32°C for the oyster and clam species (**28**). 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. At that point, any other animals in

the same spawning chamber should be moved to other spawning chambers lest they be induced to spawn, thus producing a mixture of gametes. The spawner should be left in the chamber until release of gametes ceases, at which time it should be returned to a holding tank. 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. 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. 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.

10.6.5 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.

10.6.6 Eggs should be passed through a 75-µm 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 length. Several plungers should be prepared of various diameters suitable for the various culture and other vessels in which they will be used. The diameter of the discs should be just small enough so that the plunger moves freely up and down in the vessel. When used, the plunger should be moved gently the full length of the water column several times to ensure adequate mixing of the suspension.) Agitation by aeration or stirring does not uniformly distribute the eggs or embryos. A 1-mL sample should be immediately removed using an automatic pipet, placed on a Sedgwick-Rafter cell and counted at 400×. Unrounded eggs should not be counted because they probably cannot be fertilized. The total number of eggs should be calculated to determine whether it is sufficient to perform the test. The egg density should be adjusted a range of 20 to 50 eggs/mL before adding sperm.

10.6.7 After sperm have been verified by microscopic examination, the sperm suspension should be passed through a 37-µm screen to remove feces and other extraneous material. Sperm counts can be made on the sperm suspension by using a hemocytometer or other suitable counting cell. Precise sperm counts are unnecessary after one gains a little experience.

10.6.8 To fertilize the eggs, sufficient sperm suspension should be added to the egg suspension to yield 10^5 to 10^7 sperm/mL in the final mixture (**28**). Fertilization should be accomplished at the spawning temperature (or slightly below this temperature if spawning occurred at a lethal temperature for embryos), and the suspension held at that temperature at least until it is determined that fertilization has been accomplished.

10.6.9 After the eggs have been fertilized, the embryo suspension should be poured through a 54-µm screen to remove debris. Excess sperm, small protozoa, and bacteria should be removed by pouring the embryos onto a 22-µm screen, washing with dilution water, and backwashing into a suitable container with dilution water. The resulting embryo suspension should be maintained at 20°C for *C. gigas,* 20 to 25°C for *C. virginica* and *M. mercenaria,* and 16°C for *M. edulis.* The test must be begun within 4 h after fertilization and the embryos should be kept suspended during this period by frequent agitation with a perforated plastic plunger. Agitation helps maintain a homogeneous distribution of sperm, eggs, and embryos, preventing polyspermy that can arise if the sperm are allowed to concentrate around eggs.

10.7 *Quality:*

10.7.1 The adults from which the eggs and sperm were obtained should be analyzed for the test material, if it might be present in the environment, and other chemicals to which they were probably exposed.

10.7.2 The chances of the embryos being of good quality are increased if (*a*) they are obtained from adults with high condition factors (see 10.5.1), (*b*) the adults spawned after the first stimulation (see 10.6.4), and (*c*) other adults from the same batch produced high quality embryos.

10.7.3 Reference toxicants might be useful for assessing the quality of embryos and larvae, but such assessment can only be conducted simultaneously with the toxicity test; it cannot be completed before the toxicity test is begun. Many chemicals have been used or evaluated as reference toxicants (**36**), but none has been proven to be a reliable indicator of the overall quality of any species or test results. Antimycin has been found useful for detecting stressed freshwater fish in some situations (**37**) and might be useful for other species. A reference toxicant is likely to be more useful in tests on materials that have the same mode of action as the reference toxicant than in other tests.

11. Procedure

11.1 *Experimental Design:*

11.1.1 Decisions concerning such aspects of experimental design as the dilution factor, number of treatments, and number of test chambers per treatment, should be based on the purpose of the test and the type of procedure that is to be used to calculate results (see Section 14). A unique feature of an acute test starting with embryos of bivalve molluscs is that it is not too difficult to isolate and obtain large numbers of embryos from individual male-female pairs so that the between-spawn variance can be determined (see 10.6.1) and compared with the between-chamber and between-count variances. One of the following two types of experimental design will probably be appropriate in most cases.

11.1.1.1 An acute test intended to allow calculation of an EC50 usually consists of three replicates of each treatment

including the control treatment(s). In dilution-water and solvent control(s) (see 9.2.5), embryos are exposed to dilution water to which no test material has been added. Except for the control(s) and the highest concentration of the test material, each concentration should be at least 60 % of the next higher one, unless information concerning the concentration-effect curve indicates that a different dilution factor is more appropriate. At a dilution factor of 0.6, five properly chosen concentrations are a reasonable compromise between cost and the risk of all concentrations being either too high or too low. If the estimate of acute toxicity is particularly uncertain (see 9.3.1), six or seven concentrations might be desirable to increase the chances of covering the appropriate range.

11.1.1.2 If it is only necessary to determine whether (*a*) a specific concentration is acutely toxic to embryos of the test species or (*b*) the EC50 is above or below a specific concentration (see 9.3.1.2), three replicates of that single concentration and of the control(s) are necessary. Two additional concentrations at about one-half and two times the specific concentration of concern are desirable to increase confidence in the results.

11.1.1.3 If an endpoint near the extremes of toxicity, such as an EC5 or EC95, is to be calculated, at least one concentration of the test material should have affected a percentage of test organisms, other than 0 or 100 %, near the percentage for which the EC is to be calculated. This requirement might be met in a test designed to determine an EC50, but a special test with appropriate concentrations of test material usually will be necessary.

11.1.2 The primary focus of the physical and experimental design of the test and the statistical analysis of the data is the experimental unit, that is defined as the smallest physical entity to which treatments can be independently assigned (**38**). Thus the test chamber is the experimental unit. As the number of test chambers (that is, experimental units) per treatment increases, the number of degrees of freedom increases, the width of the confidence interval on a point estimate decreases, and the power of an hypothesis test increases. With respect to factors that might affect results within test chambers and, therefore, the results of the test, all chambers in the test should be treated as similarly as possible. For example, the temperature in all test chambers should be as similar as possible unless the purpose of the test is to study the effect of temperature. Treatments must be randomly assigned to individual test chamber locations and may be randomly reassigned during the test. A randomized block design (with each treatment being present in each block, which may be a row or a rectangular matrix) is preferable to a completely randomized design.

11.1.3 The minimum desirable number of individual spawns used per test and the number of test chambers per spawn per treatment should be calculated from (*a*) the expected variance of counts within test chambers, (*b*) the expected variance in the percent live larvae with completely developed shells between test chambers within a spawn, (*c*) the expected variance between spawns within a treatment, and (*d*) the maximum acceptable confidence interval of the EC50 (**39**). If such calculations are not made, spawns from at least three male-female pairs should be exposed to each treatment that contains test material. Replicate test chambers (that is, experimental units) for at least some spawns in at least some treatments are necessary in order to allow estimation of experimental error (**38**). Because up to 30 % of the oyster embryos and 40 % of the hard clam embryos might not result in live larvae with completely developed shells, and because of the importance of the mean and variance of this percentage, there should be more test chambers for each spawn for each control treatment than for any of the concentrations of the test material.

11.1.4 It is desirable to repeat the test at a later time to obtain information concerning the reproducibility of the results.

11.2 *Dissolved Oxygen:*

11.2.1 The dissolved oxygen concentration in each test chamber must be between 60 and 100 % of saturation (**19**) at all times during the test. Embryos of the hard clam can tolerate dissolved oxygen concentrations as low as 0.5 mg/L (7 % of saturation) but growth of larvae is reduced at oxygen concentrations below 4.2 mg/L (58.8 % of saturation) (**40**). There is no data, however, indicating whether reduced oxygen concentrations affect EC50s.

11.2.2 Test solutions should not be aerated during the test because bubbles can collect within the mantle cavity of the larvae.

11.3 *Temperature:*

11.3.1 Tests with *C. gigas* should be conducted at 20°C, with *C. virginica* and *M. mercenaria* at 25°C, and with *M. edulis* at 16°C (see Table 2).

11.3.2 For each individual test chamber in which temperature is measured, the time-weighted average measured temperature at the end of the test should be within 1°C of the selected test temperature. The difference between the highest and lowest time-weighted averages for the individual test chambers must not be greater than 1°C. Each measured temperature must be within 3°C of the mean of the time-weighted averages. Whenever temperature is measured concurrently in more than one test chamber, the highest and lowest temperatures must not differ by more than 2°C.

11.4 *Beginning the Test:*

11.4.1 A large enough batch of the dilution water should be prepared or obtained so that (*a*) the desired volume can be placed in each control test chamber, (*b*) the necessary volume of each test solution containing the test material can be prepared, and (*c*) the desired analyses can be performed on the dilution water (see 11.8). Enough test solution should be prepared for each treatment containing test material so that (*a*) the desired volume can be placed in each test chamber and (*b*) any desired analyses of water quality, test material, and so forth (see 11.8) can be performed.

11.4.2 About 1 h after adding sperm suspension to the egg suspension, the concentration of embryos in the embryo suspension should be determined by mixing the solution with a perforated plunger, withdrawing a 1-mL sample, placing it in a Sedgwick-Rafter cell, and counting the number of embryos that have developed to a 2-cell stage or beyond. An electronic particle counter should not be used because it cannot differentiate between embryos (fertilized eggs) and unfertilized eggs.

The accuracy of this count is not crucial because it is merely used to plan the preparation of the test solutions.

11.4.3 The concentration of embryos in the test solutions should be between 15 and 30 embryos per mL. Although concentrations up to 100 embryos/mL do not impair normal development of Pacific oyster embryos (**41**), the other three species develop abnormally at concentrations above 30 embryos/mL.

11.4.4 Within 4 h after fertilization, equal volumes of the homogeneously mixed embryo suspension should be placed in each test container, which already contains test solution, in a random order by using an automatic pipet.

11.4.4.1 The variation in the concentration of embryos in the various test solutions should be minimized by (*a*) keeping the embryo suspension well mixed by using a perforated plunger, and (*b*) carefully using a high precision automatic pipet. This is especially important if the concentration of embryos in each individual test solution at the beginning of the test will not be determined directly. The pipet should be calibrated before the test by weighing at least eight individual water-delivery volumes to determine the coefficient of variation. A pipet whose coefficient of variation at the desired volume is greater than 5 % should not be used. The volume to be delivered by the automatic pipet and the concentration of embryos in the embryo suspension should be adjusted to result in the desired concentration of embryos in the test solutions, with the embryo suspension volume representing less than 5 % of the total volume, and an acceptable coefficient of variation.

11.4.5 The test begins when embryos are first placed in solutions containing test material.

11.4.6 Three methods may be used to determine the initial number of embryos added to the test chambers: direct counting of samples from each test chamber; direct counting of samples from three or more chambers prepared as surrogates for the sole purpose of determining the initial number; estimation by calculation from a precise count of the number of embryos in the stock suspension and the dilution rate.

11.4.6.1 *Method 1*—After the embryos have been added to the test containers, the suspension in each test chamber should be mixed and a sample taken immediately in which to count the embryos. Each sample should contain 100 to 300 embryos and should be preserved with 5 % buffered formalin in a labeled and closed vial. The test solutions should be mixed and sampled carefully to minimize the sampling error. This test method provides a direct measure of the initial embryo density (*N*) for each test chamber. This is the preferred test method, especially if the initial stock volume is so small that homogeneous mixing of the stock larval suspension cannot be ensured.

11.4.6.2 *Method 2*—Three containers should be prepared in addition to the number needed for the basic test. One should be prepared as the first container in the otherwise random series to be prepared, one should be prepared last, and the third should be prepared in the middle of the preparation of test containers. After all containers have been prepared, these three extra containers should be sampled as described in 11.4.6.1. The initial embryo density (*N*) is then defined as the average of the concentrations in these three extra containers and the variance of the average can be calculated.

11.4.6.3 *Method 3*—In the case when the stock suspension is of much larger volume than the total volume needed to prepare all test containers, and direct counts will not be made on the initial number in each test chamber, the concentration of embryos in the stock suspension should first be precisely determined. From this number, calculate the embryo density in the test containers as follows:

$$N = S(V_s/V_t)$$

where:

S = the mean embryo density in the stock suspension,
V_s = the volume of embryo stock suspension added to the test container, and
V_t = the total volume of test solution.

11.5 *Feeding*—The organisms should not be fed during the test because uneaten food might decrease the concentration of dissolved oxygen and the biological activity of some test materials, and because the embryos and resulting larvae can survive for more than 72 h without being fed.

11.6 *Duration of Test:*

11.6.1 The embryos and resulting larvae must be exposed to the test material for a total of 48 h because embryos in the control treatment(s) will usually develop into straight hinge larvae with completely developed shells in 20 to 30 h, but will be stressed, and possibly die, if they are deprived of food for much longer than 72 h.

11.7 *Biological Data:*

11.7.1 Forty-eight h after the beginning of the test, the solution in each test chamber should be carefully mixed and a sample immediately removed and preserved using exactly the same sampling and preservation procedures and sample volume as was used to sample the test chambers at the beginning of the test in accordance with 11.4.6.

11.7.2 The embryos and larvae in the samples obtained in accordance with 11.4.6 and 11.7.1 should be placed in a Sedgwick-Rafter cell for counting. Because the volume of the cell is 1 mL, it might be necessary to prepare and count several slides to enumerate all embryos and larvae in each sample. Embryos and larvae usually sink after preservation, and it is frequently possible to discard most of the liquid before transferring the residual volume containing the organisms to the cell. Some larvae might not settle when preserved; therefore it should be determined periodically that embryos or larvae are not being discarded inadvertently.

11.7.3 In samples obtained in accordance with 11.4.6, all embryos exhibiting cell division must be counted.

11.7.4 In samples obtained in accordance with 11.7.1, all larvae with completely developed shells containing meat must be counted. Empty shells, even if they are completely developed, must not be counted because the larvae were not alive at the end of the test. Live larvae possessing misshapen or otherwise malformed shells must be included in the count of live larvae if the shell is completely developed, because it is considered unlikely that a malformed shell will reduce survival in the natural environment. Larvae with incompletely developed shells after 48 h might be morphologically normal, but

the retarded development is considered likely to reduce survival in the natural environment.

11.7.5 A total count of all live larvae including those with incomplete or no shell growth might be desirable. If substantial numbers of larvae with malformed shells are present, it might also be desirable to ascertain the proportion of live larvae with malformed shells.

11.7.6 Data concerning the effect of the test material on the time(s) to reach trochophore or straight hinge stages may be obtained, if desired, by collecting additional samples for counts at various times during the test.

11.7.7 All organisms used in the test should be destroyed at the end of the test.

11.8 *Other Measurements:*

11.8.1 The salinity and pH of the dilution water should be measured prior to each test, and measurement of particulate matter, total dissolved gases, and TOC is desirable. The concentration of dissolved oxygen must be measured at the beginning and end of the test in the required control(s) and the high, medium, and low concentrations of test material. The pH should be measured at the beginning and end of the test in the required control(s) and the high, medium, and low concentrations of test material.

11.8.2 *Temperature:*

11.8.2.1 Throughout conditioning, either temperature should be measured at least hourly or the maximum and minimum temperatures should be measured daily with a minimum/maximum thermometer.

11.8.2.2 Throughout the test, in at least one test chamber, either the temperature must be measured at least hourly or the maximum and minimum temperatures must be measured daily with a minimum/maximum thermometer. Near the beginning and the end of the test, temperature must be measured concurrently in all test chambers. If the test chambers are in a water bath, measurement of temperature at least hourly, or daily measurement of the maximum and minimum temperatures may be made instead of any measurements in test chambers. If the test chambers are in a constant temperature room or incubator, measurement of the air temperature at least hourly, or daily measurement of the maximum and minimum air temperatures, may be made instead of any measurements in test chambers because the temperature of the air will probably fluctuate more than that of the test solutions.

11.8.3 *Test Material:*

11.8.3.1 The concentration of test material should be measured, if possible, at the beginning and end of the test in all treatments. Measurements of the concentration of dissolved test material in each treatment might be desirable. If the test organisms are probably exposed to substantial concentrations of one or more impurities or degradation or reaction products, measurement of the impurities and products is desirable.

11.8.3.2 If the test material is uniformly dispersed throughout the test chamber, water samples should be taken by pipetting or siphoning through glass or fluorocarbon plastic tubing from a point midway between the top, bottom, and sides of the test chamber and should not include any surface scum or material stirred up from the bottom or sides. The submersed end of the siphon or pipet should be covered with a piece of 26-μm mesh nylon screen to prevent removal of embryos or larvae. If test material might be lost due to sorption onto the walls of the sample container, the container and siphon or pipet should be rinsed with test solution before collecting the sample. Water samples should be collected directly into appropriate-sized containers from which the test material can be extracted or analyzed directly. If the test material is not uniformly dispersed in the test chamber, the whole volume of solution in the test chamber should be (*a*) used as the sample or (*b*) treated appropriately (for example by adding acid, base, or surfactant and mixing thoroughly), to uniformly distribute the test material before a sample is taken.

12. Analytical Methods

12.1 If samples of dilution water, stock solutions, or test solutions cannot be analyzed immediately, they should be handled and stored appropriately **(42)** to minimize loss of test material by such things as microbial degradation, hydrolysis, oxidation, photolysis, reduction, sorption, and volatilization.

12.2 Chemical and physical data should be obtained by using appropriate ASTM standards whenever possible. For those measurements for which ASTM standards do not exist or are not sensitive enough, methods should be obtained from other reliable sources **(43)**.

12.3 Methods used to analyze tissues of brood stock should be obtained from reliable sources **(44)**.

12.4 The precision and bias of each analytical method used should be determined in an appropriate matrix, for example, in water samples from control test chambers or in tissue from brood stock. When appropriate, reagent blanks, recoveries, and standards should be included whenever samples are analyzed.

13. Acceptability of Test

13.1 An acute toxicity test started with embryos of bivalve molluscs should usually be considered unacceptable if one or more of the following occurred, except that if temperature was measured numerous times, a deviation of more than 3°C (see 13.1.10) in any one measurement might be inconsequential. However, if temperature was measured only a minimal number of times, one deviation of more than 3°C might indicate that more such deviations would have been found if temperature had been measured more often.

13.1.1 All test chambers were not identical.

13.1.2 Treatments were not randomly assigned to individual test chamber locations.

13.1.3 A required dilution-water or solvent control was not included in the test.

13.1.4 All animals in the brood stock were not obtained from the same location.

13.1.5 The test was begun with embryos more than 4 h after they were fertilized.

13.1.6 Less than 70 % of oyster embryos or 60 % of hard clam embryos introduced into a required control treatment resulted in live larvae with completely developed shells at the end of the test (see 9.2.4 and 14.4).

13.1.7 Dissolved oxygen and temperature were not measured as specified in 11.8.

13.1.8 Any measured dissolved oxygen concentration was not between 60 and 100 % of saturation.

13.1.9 The difference between the time-weighted average measured temperatures for any two test chambers from the beginning to the end of the test was greater than 1°C.

13.1.10 Any single measured temperature in any test chamber was more than 3°C different from the mean of the time-weighted average measured temperatures for the individual test chambers.

13.1.11 At any one time the difference between the measured temperatures in any two test chambers was more than 2°C.

13.1.12 Ammonia concentrations in the overlying water were not measured at test initiation and test end when it was known or suspected that the sediments were high in organics or ammonia, or both.

13.2 Calculation of an EC50 should usually be considered unacceptable if one or both of the following occurred:

13.2.1 No treatment other than a control treatment resulted in an average *E* (see 14.6) less than 37 %.

13.2.2 No treatment resulted in an average *E* (see 14.6) greater than 63 %.

14. Calculation

14.1 For each set of data, the EC50 should be calculated on the basis of (*a*) the measured initial concentrations of test material, if available, or the calculated initial concentrations, and (*b*) the number of embryos that resulted in live larvae with completely developed shells after 48 h. The 95 % confidence limits on the EC50 or any other ECs should be calculated if possible. In addition, the LC50 may be calculated on the basis of (*a*) the measured initial concentrations of test material, if available, or the calculated initial concentrations, and (*b*) the number of embryos that resulted in live larvae after 48 h.

14.2 Most acute toxicity tests produce quantal data, that is, counts of the number of organisms in two mutually exclusive categories, such as alive or dead. A variety of methods (see Guide E729) can be used to calculate an EC50 and its 95 % confidence limits from a set of quantal data that is binomially distributed and contains two or more concentrations at which the percent affected lies between 0 and 100 % (**45**). Even though acute tests started with embryos of bivalve molluscs produce quantal data, the data are not likely to be binomially distributed because (*a*) there is between-chamber variance within a treatment in the percentage of the embryos that result in live larvae with completely developed shells, and (*b*) there is within-chamber error in the sampling and counting of the number of live larvae that have completely developed shells at the end of the test.

14.3 For each test chamber in each treatment, including the control treatment(s), the calculation should be as follows:

$$A = 100(N - B)/N$$

where:

A = the percentage of the embryos that did not result in live larvae with completely developed shells,

B = the number of live larvae with completely developed shells in the sample taken from that test chamber at the end of the test, and

N = the number of embryos in the 2-cell stage or beyond, in the sample taken from that test chamber at the beginning of the test.

14.4 *M* should be calculated as the average of *A*'s (see 14.3), where *M* = the average percentage of the embryos that did not result in live larvae with completely developed shells for the test chambers in the control treatment(s) (see 9.2.3). The test is unacceptable if *M* is >30 % for oysters or >40 % for hard clams (see 13.1.6).

14.5 For each test chamber in each treatment other than the control treatment(s), *E* should be calculated by using Abbott's formula (**45**), as follows:

$$E = 100(A - M)/(100 - M)$$

where:

E = the percentage of introduced live embryos that did not result in live larvae with completely developed shells adjusted for the controls,

A = (see 14.3), and

M = (see 14.4).

14.6 The EC50, but not its 95 % confidence limits, can be determined graphically by plotting *E* for each test chamber against the corresponding measured or initial nominal concentration of test material (see 14.1) after transformation of *E* or concentration or both, if appropriate. The EC50 can then be obtained from a visually fitted line of best fit by determining the concentration corresponding to *E* = 50 %. If *E* is between 0 and 100 % for fewer than two test chambers, only an approximate EC50 can be determined. Alternatively, if two or more test chambers gave *E* between 0 and 100 %, an appropriate linear or nonlinear inverse regression technique (**46**) can be used to calculate the EC50 and its 95 % confidence limits. If *E* covers an appropriate range (see 13.2), a variety of regression models will usually give nearly the same EC50 from a set of data. Only the correct model, which is not known to be available at this time, will appropriately take into account the number of test chambers per treatment, the number of test organisms exposed in each chamber, the range of concentrations tested, and the variance within each treatment, especially within the control treatment(s), and give the correct confidence limits.

14.7 For each data set, the LC50 may be calculated by substituting the number of live larvae in the equation in 14.3 and proceeding through 14.6. The same procedure may be used to calculate an EC50 or LC50 for any other time interval for which appropriate data were collected.

14.8 An endpoint near an extreme of toxicity, such as an EC5 or EC95, should not be calculated unless at least one concentration of test material killed or affected a percentage of test organisms, other than 0 and 100 %, near the percentage for which the EC (or LC) is to be calculated. Other ways of providing information concerning the extremes of toxicity are to report the highest concentration of test material that actually

killed or affected no greater a percentage of the test organisms than did the control treatment(s), or to report the lowest concentration of test material that actually killed or affected all test organisms exposed to it. These alternatives are normally more reliable than reporting a calculated result such as an EC5 or EC95 unless several percent killed or affected were obtained close to 5 or 95 %.

14.9 It might be desirable to perform a hypothesis test to determine which of the tested concentrations of test material killed or affected a statistically significant number of the exposed organisms. If a hypothesis test is to be performed, the data should first be examined using appropriate outlier detection procedures and tests of heterogeneity. Then a pairwise comparison technique, contingency table test, analysis of variance, or multiple comparison procedure appropriate to the experimental design should be used. Presentation of results of each hypothesis test should include the test statistic and its corresponding significance level, the minimum detectable difference, and the power of the test.

15. Report

15.1 The record of the results of an acceptable acute toxicity test started with embryos of bivalve molluscs should include the following information either directly or by reference to available documents:

15.1.1 Names of test and investigator(s), name and location of laboratory, and dates of initiation and termination of test.

15.1.2 Source of test material, its lot number, composition (identities and concentrations of major ingredients and major impurities), known chemical and physical properties, and the identity and concentration(s) of any solvent used.

15.1.3 Source of the dilution water, its chemical characteristics, a description of any pretreatment, and results of any demonstration of the acceptability of the water to an aquatic species.

15.1.4 Location from which the brood stock was obtained, date of collection, scientific name, name of person who identified the organisms and the taxonomic reference used, duration and temperature of conditioning, food for brood stock, method used to induce spawning, and the time (in hours) from fertilization to the beginning of the test.

15.1.5 Description of the experimental design, test chambers and covers, the depth and volume of solution in the chambers, number of female/male pairs used, the number of test chambers per female/male pair per treatment, and the lighting.

15.1.6 The average and range of the measured dissolved oxygen concentration (as % of saturation) for each treatment.

15.1.7 The averages and ranges of the conditioning and test temperatures and the method(s) of measuring or monitoring or both.

15.1.8 Schedule for obtaining samples of test solutions, and methods used to obtain, prepare, and store them.

15.1.9 Methods used for, and results (with standard deviations or confidence limits) of, chemical analyses of water quality and concentration(s) of test material, impurities, degradation and reaction products, including validation studies and reagent blanks.

15.1.10 A table of data on the number of live embryos at the beginning of the test and the number of live larvae with completely developed shells at the end of the test in each test chamber in each treatment, including the control(s), in sufficient detail to allow independent statistical analyses.

15.1.11 The 48-h EC50 (and its 95 % confidence limits if possible) and the method used to calculate them; the highest concentration of test material that did not reduce the number of live larvae with completely developed shells. Specify whether results are based on measured or unmeasured concentrations of the test material, and for formulations and commercial products, specify whether results are based on whole mixture or active ingredient.

15.1.12 Anything unusual about the test, any deviation from these procedures, and any other relevant information.

15.2 Published reports should contain enough information to clearly identify the procedures used and the quality of the results.

16. Keywords

16.1 acute toxicity test; bivalve mollusc larvae; bivalve molluscs embryos; blue mussel; *Crassostrea gigas*; *Crassostrea virginica*; hard clam; *Mercenaria mercenaria*; *Mytilus edulis*; oyster

ANNEX

(Mandatory Information)

A1. SEDIMENT TESTS

A1.1 *Purpose*—This Annex describes techniques that have been adapted for testing the toxicity of marine and estuarine sediments using embryos and larvae of bivalve molluscs (mussels, clams, and oysters). Methods for testing sediments with embryos and larvae of molluscs are well-developed on the U.S. and Canadian West Coast (**47-49**). It is probable that embryos of many bivalve mollusc species can be used with this test. This sediment test can be used to assess the toxicity of field-collected sediments or to determine toxic effects associated with manipulation of sediment or other environmental conditions in the laboratory. This Annex describes the exposure of developing mollusc embryos and larvae to aliquots of sediments that have been mixed in a specified amount of seawater (to release toxicants into the aqueous phase), and the sediments allowed to settle prior to inoculation with fertilized eggs. The embryos and larvae develop for 48 hours in the

TABLE A1.1 Summary of Conditions for Conducting Sediment Toxicity Tests with Embryos of Molluscan Bivalves

Parameter	Conditions
1. Test Type:	Sediment static toxicity test
2. Test Species:	Bivalve mollusc embryos (see Standard Guide E724)
3. Test Chamber:	One-litre beaker or ~950-mL Canning jar
4. Amount of Sediment:	Eighteen grams, wet weight
5. Amount of Filtered Seawater:	Fill chambers to 900 mL final volume
6. Sediment Preparation:	Shake or stir for 10 seconds with the final volume of seawater and let settle for 4 hours prior to embryo additions
7. Treatments:	1. Test sediments 2. Control and/or reference sediments 3. Seawater control (no sediment) 4. Five extra seawater control chambers for collecting subsamples for T_0 embryo counts and monitoring embryo development progress
8. Number of Test Chambers Per Treatment:	Five, plus one extra for taking water quality measurements
9. Embryo Inoculation Density:	20,000 to 40,000 embryos/chamber
10. Randomization:	Randomize all chambers, including extra water quality monitoring chambers
11. Exposure Time:	48 hours (development time may vary by species)
12. Test Salinity and Temperature:	Varies by species; see Sections 8 and 10.5.2 of Standard Guide E724
13. Test pH:	Measure only in overlying water—do not adjust
14. Aeration:	Not required. However, aeration is advised if highly organic sediments are being tested or if dissolved oxygen in any treatment drops below 60 % saturation. If aeration is used, all chambers must be aerated by using a pipette inserted mid-depth in the water column with an air flow of about 100 bubbles/minute
15. Test Termination:	Ensure that larval development in the extra seawater control chambers is satisfactory. Slowly decant chambers with sediment into empty 1-L containers (beakers or canning jars), leaving behind the sediments. Mix containers with a perforated plunger (do not stir) and remove the desired number of 10-mL subsamples. Preserve with 5 % buffered formalin
16. Water Quality Measurements:	Required at 24-h intervals: temperature, salinity, pH, DO Recommended at T_0 and T_{end}: Ammonia and sulfide. Ammonia and sulfide must be measured when sediments are known to be high in organic content
17. Test Acceptability:	Seawater controls must produce at least 70 % (oysters) or 60 % (clams) normally developed prodissoconch I larvae at T_{end} relative to those introduced at T_0
18. Ammonia Toxicity Threshold:	Unionized ammonia >0.13 mg/L in the overlying water at pH 7.8 to 8.1
19. Range of Sulfide Toxicity Threshold:	Total sulfide >0.5 mg/L in the overlying water

presence of the sediments, which have formed layers on the bottom of the test chambers. As such, this test combines elements of solid phase, pore water, and elutriate testing. Other methods for testing pore water or elutriates prepared from sediments are not addressed in this annex.

A1.2 *Test Material*—Sediment toxicity tests may be performed using field-collected sediments or laboratory-prepared sediments that have been spiked with one or more chemical compounds or otherwise manipulated. Techniques describing collection, handling, and preparation of these sediments are described in detail in Guides E1367 and E1391. If field-collected sediments are being tested, reference sediments should be tested in addition to control sediments, or reference sediments can be considered the control sediments. As defined in Guide E943, control sediments are sediments that are essentially free of contaminants and are used routinely to assess the acceptability of a test. Reference sediments are whole sediments collected near an area of concern and used to assess sediment conditions exclusive of material(s) of interest. Additional information about control and reference sediments may be found in Section 3 of Guide E1706.

A1.2.1 *Sediment Storage*—Sediments for toxicity tests should be stored in the dark at 4 ± 2°C (never frozen) prior to testing. Since the effects of storage time on sediment toxicity are poorly defined and unpredictable (**50-52**), it is recommended that sediments be tested as soon as possible after collection. Presently accepted holding times for sediments range from 2 to 8 weeks (**49, 53-55**; Guides E1391 and E1706). It might sometimes be preferable to store sediments in a nitrogen environment by replacing the head-space air in storage containers with nitrogen gas (**49**).

A1.3 *Test Methods*—The following sediment procedures are derived primarily from Puget Sound Estuary Program (PSEP) protocols (**53**) , where these procedures have been used since the 1980's. A summary of the basic test conditions appears in Table A1.1.

A1.3.1 Test chambers should be prepared either before or while the test organisms are spawning. Test chambers may be either 1-L glass beakers or (approximately) 950-mL glass canning jars, which are loosely covered by watchglasses or non-toxic plastic. If canning jar lids are used, they should be covered with a Teflon liner.[6] Six replicates are typically tested for each treatment, five chambers for assessing toxicity and one chamber for monitoring water quality (temperature, pH, salinity, dissolved oxygen, and so forth) without disturbing the actual test chambers. Eighteen grams of sediment (wet weight) are weighed into each replicate chamber and covered with filtered seawater to a final volume of 900 mL. Stir the contents of each chamber vigorously for 10 seconds. Mixing for 10 seconds facilitates release of toxicants in the pore water into the overlying water, which is the phase that developing embryos are primarily exposed to. After mixing, the contents

[6] Most, if not all, tests using the methods presented in this Annex have used 1-L or 950-mL chambers. However, use of smaller chambers (and equivalent sediment weights/water volumes) may be satisfactory, although comparative data have not yet been developed.

are allowed to settle for 4 h prior to adding embryos. No additional agitation is performed during the test.

A1.3.2 Aeration of the test chambers is optional; however, experiences in the Puget Sound region have shown that many organic-rich sediments require aeration to maintain satisfactory dissolved oxygen (DO) concentrations (**1**). Thus, DO concentrations should be monitored at frequent intervals when testing organic-rich sediments. If the DO concentration in any chamber falls below 60 % saturation, then all chambers should be aerated by approximately 100 bubbles/minute of oil-free air injected by pipette at mid-depth in the water column.

A1.3.3 For sediment tests, two negative (non-toxic) controls are used: a seawater-only control and a sediment control. The seawater control contains only filtered seawater. This control is used to judge the quality of embryo development without the influences of sediment, and the larvae at T_{end} must meet basic performance criteria for the test to be valid (Table 1). The seawater control typically consists of six chambers (five test and one monitoring), each containing only 900 mL of filtered seawater. A duplicate set of five seawater-only control chambers should be prepared to provide T_0 embryo density counts. Embryos in these extra chambers can then be monitored for embryo development progress as the test proceeds. The second negative control consists of 18 g of clean control (or reference) sediment with filtered seawater added to a final volume of 900 mL.

A1.3.4 If the seawater-only control passes test validation criteria (see item 17, Table A1.1), then the control (or reference) sediment is used as the basis for judging the toxicity of the test sediments. Pass-fail criteria may also be established for the performance of the control (reference) sediment, but universal criteria are not presently available. One example of a reference sediment performance standard is the Puget Sound Dredge Disposal Analysis (PSDDA) Program criterion that reference sediment combined mortality and abnormality must not be greater than 35 %, normalized to the seawater-only control (**57**).

A1.3.5 Water quality parameters (temperature, salinity, pH, DO) should be measured in each treatment (in the sixth replicate) just prior to embryo inoculation. If other chemical variables (that is, ammonia, sulfide) are of interest, they should also be measured at this time.

A1.3.6 After the sediments have settled for 4 hours, each test chamber (including the water quality chambers) should be inoculated with 20 000 to 40 000 fertilized embryos within 2 h of egg fertilization. To confirm the embryo inoculation density, the contents in each of the five extra seawater control replicates are gently mixed with a perforated plunger (not stirred) (see Section 10.6.6 for a description of the plunger design), and one (or more as desired) 10-mL aliquot is removed by precision pipette from each chamber. Each aliquot should be transferred to a screw-top vial and preserved in 5 % buffered formalin for subsequent enumeration. Because the presence of sediment in the test chambers does not allow for collection of a homogeneous subsample of embryos from every test chamber at T_0, this is the only technique that can be used to confirm inoculation density. Note that T_0 counts from the concentrated stock embryo solution are **not** satisfactory for this purpose. Once inoculation is complete, the test chambers should be covered loosely with watch glasses or another non-toxic lid type and incubated under conditions described in Sections 11.2-11.6.

A1.4 *Test Monitoring*—Test chambers should be left undisturbed throughout the exposure period. Water quality parameters should be measured every 24 h in the extra designated (sixth replicate) chambers. If aeration was not provided at T_0, and the dissolved oxygen concentration drops below 60 % saturation in any of the chambers, gentle aeration should be provided to all treatments for the remainder of the test. Embryo development progress may be monitored by examining subsamples removed from the extra set of seawater control chambers.

A1.5 *Test Termination*—At the end of the exposure period, the water and larvae in each chamber are carefully decanted into clean 1-L containers (that is, beakers or canning jars). This process must be done slowly so that the sediments are not disturbed, although a small amount of sediment transfer will not interfere with subsequent embryo counting. The decanted water should then be gently mixed with a perforated plunger to resuspend the larvae. One or more 10-mL aliquots of test solution should be immediately removed by precision pipette, transferred to screw-top glass vials, and preserved with 5 % buffered formalin. The larvae are later evaluated by counting and assessing the normality of **all** embryos in each subsample (for examples of normal and abnormal larvae, see Figs. 1 and 2). Normal or inverted compound microscopes can be used for this step. Although most embryos will be swimming in the water column at T_{end} . Some normal and potentially abnormal larvae may be mixed with the bottom sediments and left behind during the decanting step. “Missing” larvae in the test treatments that exceed the “missing” counts from the control (or reference) sediment are considered to have died or been so abnormal as to have been trapped in the sediments. Thus, “missing larvae” are considered to be non-survivors for the purposes of this test.

A1.5.1 Water quality parameters should be measured in each extra “water quality” replicate at T_{end} and samples for analysis of other variables (that is, ammonia, sulfide) should also be collected at this time.

A1.6 *Data Analysis*—Calculations of test results for mollusc embryo sediment tests are the same as for tests without sediments. See Section 14 for the details of data analysis.

A1.7 *Evaluating Toxicity Associated with Ammonia and Sulfide*—Ammonia and sulfide are two naturally-occurring compounds that are known to affect development and survival of bivalve mollusc embryos during sediment testing. The interpretation of ammonia or sulfide as toxicants versus interferences depends on the design and objective of the study. Beginning and final ammonia and sulfide concentrations should be measured for all sediment tests using bivalve mollusc embryos and must be measured when sediments are known to be high in organic content.

A1.7.1 A recent study of the effects of ammonia on Pacific oyster (*Crassostrea gigas*) larvae found a NOEC of 4.68 mg/L total ammonia (0.08 mg/litre unionized ammonia; test pH range 7.8 to 8.1 and salinity range 27 to 28 ppt), and found that concentrations of total ammonia of 9.79 mg/L or greater caused high mortality and abnormal development **(57)**. Results of this study led to a recommended interim effect threshold value for unionized ammonia of 0.13 mg/L (for pH range of 7.8 to 8.1) for the oyster larval test **(49, 57)**. Values of unionized ammonia measured in water from the larval test chambers in excess of 0.13 mg/L are considered to have the potential for producing toxicity in the test. For sediment tests at pHs outside the range of 7.8 to 8.1, this toxicity threshold value will need to be adjusted, since toxicity of ammonia is dependent on pH.

A1.7.2 Few experimental data exist on the effects of sulfide concentrations on developing bivalve larvae. One study on the effects of natural sediment properties on Pacific oyster larvae **(59)** calculated possible elutriate sulfide concentration effects levels of 0.34 mg/L (EC50) and 0.10 mg/L (NOEC). However, no actual measurements were made in this study, so these values should be used with caution. Presently, the Puget Sound PSDDA program has specified interim limits of 0.50 mg/L total sulfide as a sulfide toxicity threshold in both echinoid and bivalve mollusc embryo tests of sediments **(60)**. This value may change as more data on sulfide effects on bivalve larvae become available.

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