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Standard Guide for Conducting Static Acute Toxicity Tests with Echinoid Embryos[1,2]

This standard is issued under the fixed designation E 1563; 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 covers procedures for obtaining laboratory data concerning the acute effects of a test material on embryos and the resulting larvae of echinoid embryos (sea urchins and sand dollars) during static 48- to 96-h exposures. These procedures have generally been used with U.S. East Coast (*Arbacia punctulata* and *Strongylocentrotus droebachiensis*) **(1)**[3] and West Coast species (*Strongylocentrotus purpuratus, S. droebachiensis,* and *Dendraster excentricus*) **(2)**. The basic procedures described in this guide first originated in Japan and Scandanavia **(3)**, and parallel procedures have been used with foreign species, especially in Japan and the Mediterranean **(4)**. These procedures will probably be useful for conducting static acute toxicity tests with embryos of other echinoid species, 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, the results of tests conducted by using unusual procedures are not likely to be comparable with those of many other tests. The comparison of results obtained by using modified and unmodified versions of these procedures might provide useful information concerning new concepts and procedures for conducting acute tests starting with embryos of echinoids.

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 E 1192), leachates, oils, particulate matter, surface waters and sediments (Annex A1). Renewal tests might be preferable to static tests for materials that have a high oxygen demand, are highly volatile, are rapidly transformed biologically or chemically 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 echinoid embryos should usually be reported as the 50 % effect concentration (EC50) based on the total abnormally developed embryos and larvae. 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:

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[1] A Standard Guide is a document, developed using the consensus mechanisms of ASTM that provides guidance for the selection of procedures to accomplish a specific test, but which does not stipulate specific procedures.

[2] This guide is under the jurisdiction of ASTM Committee E47 on Biological Effects and Environmental Fateand is the direct responsibility of Subcommittee E47.01 on Aquatic Assessment and Toxicology.

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

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1.6 The values stated in SI units are to be regarded as the standard.

1.7 *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.* Specific precautionary statements are given in Section 7.

2. Referenced Documents

2.1 *ASTM Standards:*

E 380 Practice for Use of the International System of Units (SI)[4]

E 724 Guide for Conducting Static Acute Toxicity Tests Starting with Embryos of Four Species of Saltwater Bivalve Molluscs[5]

E 729 Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians[5]

E 943 Terminology Relating to Biological Effects and Environmental Fate[5]

E 1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses[5]

E 1192 Guide for Conducting Acute Toxicity Tests on Aqueous Effluents with Fishes, Macroinvertebrates, and Amphibians[5]

E 1367 Guide for Conducting 10-day Static Sediment Toxicity Tests with Marine and Estuarine Amphipods

E 1391 Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing

E 1525 Guide for Designing Biological Tests with Sediments

E 1706 Method for Measuring the Toxicity of Sediment-Associated Contaminants with Fresh Water Invertebrates

3. Terminology

3.1 *Definitions:*

3.1.1 The term "embryo" is used herein to denote the stages between the fertilization of the egg and the pluteus larva. The term "larva" is used herein to refer to the pluteus larva characteristic of all echinoids **(5)** (Fig. 1 and Fig. 2).

3.1.2 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."

3.1.3 For definitions of other terms used in this guide, refer to Guide E 729 and Terminology E 943. For sediment tests (Annex A1), refer to standards E 1391 and E 1525. For an explanation of units and symbols, refer to Practice E 380.

4. Summary of Guide

4.1 Adult sea urchins and sand dollars are brought into the laboratory and identified to species. If the gonads are not ripe, the sea urchins or sand dollars should be held and fed until the gonads are brought into a suitable reproductive state. Echinoids 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, electrical current) or chemical (for example, potassium chloride).

4.2 In each of two or more treatments, embryos and the resulting larvae of one species are maintained for 48 to 96 h, depending on the species and test temperature. 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 (*1*) 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, etc.; and (*2*) 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 EC50 is calculated based on the proportion of larvae that develop into normal pluteus larvae in chambers containing the test material relative to normal larvae in the controls at the termination of the 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 echinoid species, and because some of these species are commercially and recreationally important, the results of these acute tests are often considered to be a good indication of the acceptability of pollutant concentrations to saltwater species in general. The results of these acute toxicity tests are often assumed to be an

[4] *Annual Book of ASTM Standards,* Vol 14.02.

[5] *Annual Book of ASTM Standards,* Vol 11.05.

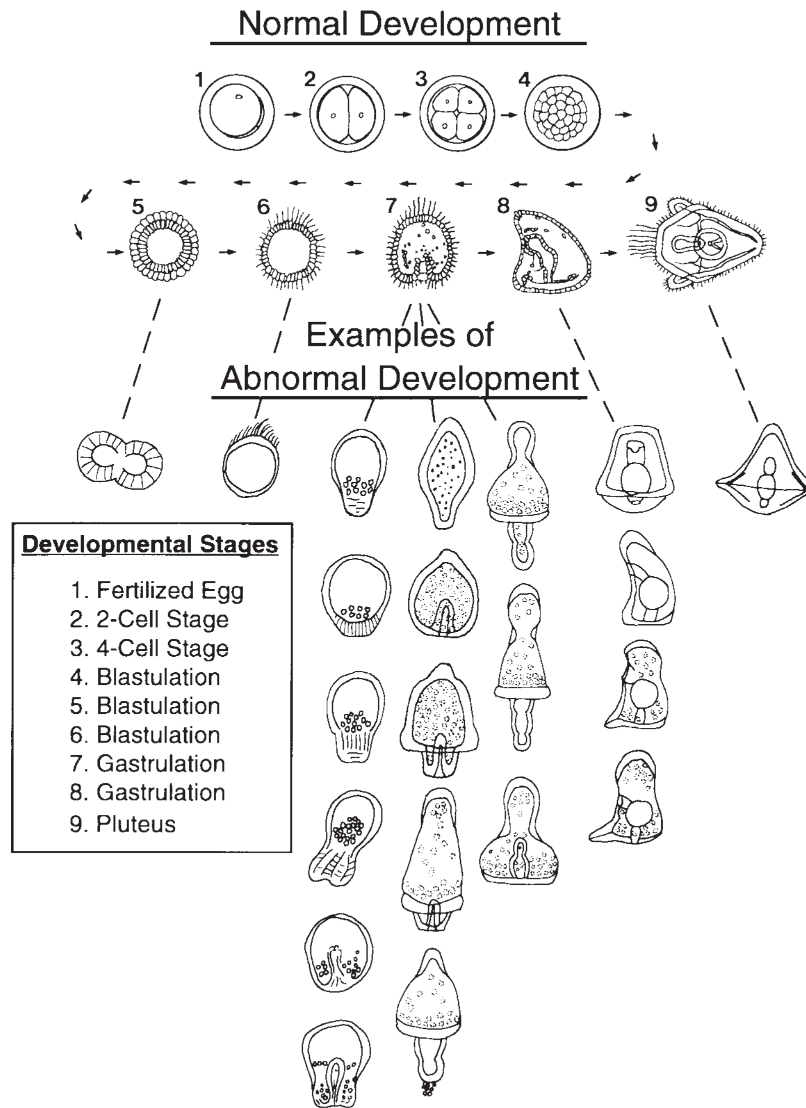

FIG. 1 Drawings Exemplifying Key Developmental Stages of Normal Echinoid Larvae Occurring During the First 48 to 96 h of Development and Examples of Abnormal or Arrested Development (38)

important consideration when assessing the hazard of materials to other saltwater organisms (see Guides E 724 and E 1023) or when deriving water quality criteria for saltwater organisms **(6)**.

5.3 The 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 The 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 the results of such tests.

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

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

6. Apparatus

6.1 *Facilities*:

6.1.1 Flow-through troughs with appropriate trays should be available for holding and conditioning test animals **(7)**. 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 may 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 the test organisms by test materials and other substances, especially volatile ones, holding and conditioning troughs should not be in a room in which the toxicity tests are conducted, stock solutions or test solutions

FIG. 2 (a) Examples of Normal and Abnormal Development of Purple Sea Urchin (*Strongylocentrotus purpuratus*) Embryos at the Conclusion of a 72 to 96-h Toxicity Test. Figs. 2a and 2b Show Examples of Normal Echinopluteus' with Four Distinct Arms and Good Symmetrical Development. Fig. 2c Shows a Borderline Abnormal Pluteus with Poor Symmetrical Form and One Missing Arm. Figs. 2d through 2j Show Examples of Increasing Abnormal and/or Retarded (Considered Abnormal) Development. Other Species of Sea Urchins and Sand Dollars Will Have the Same General Larval Form, But Will Vary in Size, Conformation, and Number of Larval Arms. Each Investigator Using a Different Species Should Carefully Compare Well-Developed Embryos from Controls with Gradations of Abnormal Development in a Toxicant to Identify Distinctions between Normal and Abnormal for Their Given Species

FIG. 2 (b) *(continued)*

are prepared, or equipment is cleaned. Organisms should be shielded from disturbances, with curtains or partitions, to prevent unnecessary stress during holding, conditioning, and testing.

6.1.2 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 autodialing alarms if the water flow or temperature deviates from preset limits. If the 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.

6.2 *Construction Materials*—Equipment and facilities that contact stock solutions, test solutions, or any water into which the test organisms will be placed should not contain substances that can be leached or dissolved by aqueous solutions in amounts that affect the test organisms adversely. In addition, equipment and facilities that contact the stock solutions or test solutions should be chosen to minimize the 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 (**8**). Brass, copper, lead, galvanized metal, cast-iron pipe, and natural rubber should not contact the dilution water, stock solutions, or test solutions before or during the test. Items made of neoprene rubber and other materials not mentioned above should not be used unless it has been shown that the embryos and resulting larvae of the test species do not show more signs of stress, such as discoloration, abnormal development, or death, when held for 48 to 96 h in the static dilution water in which the item is soaking than when held in static dilution water that does not contain the item.

6.3 *Test Chambers*:

6.3.1 In a toxicity test with aquatic organisms, test chambers are defined as the smallest physical units between which there are no water connections. The chambers should be covered to keep out extraneous contaminants and bacteria and to minimize the evaporation of test solution and material. Substantial concentrations of bacteria in the test solutions might reduce the

FIG. 2 (c) *(continued)*

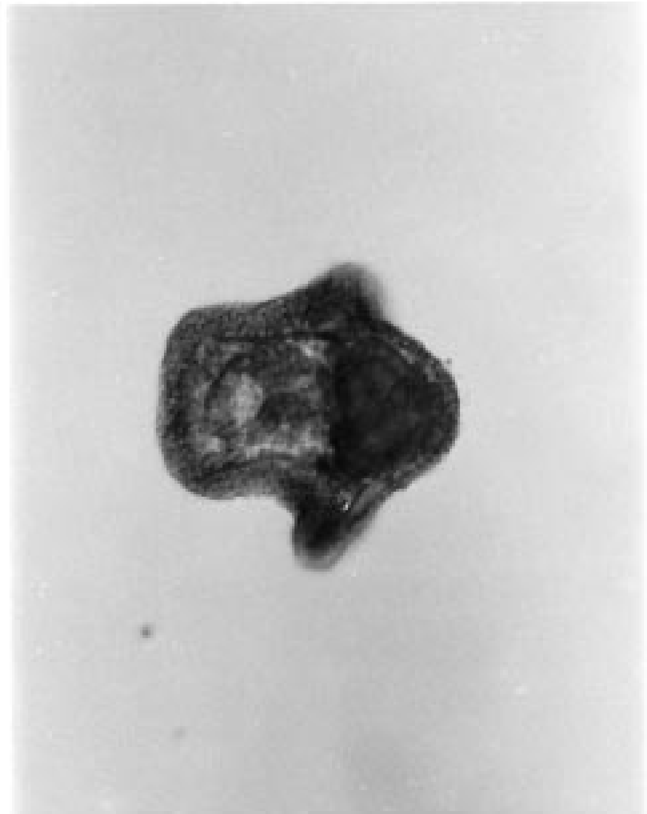

FIG. 2 (d) *(continued)*

survival of the embryos and resulting larvae severely, whereas differences in the amount of evaporation among test chambers will contribute directly to between-chamber variation in survival. All chambers in a test must be identical.

6.3.2 Tests are usually conducted in glass chambers that are 250 mL to 1 L in capacity. Very small test chambers, containing as little as 10 to 30 mL **(9)**, and sealed test chambers may be used if the survival and development of the embryos and resulting larvae in the control(s) are acceptable (see 11.8).

6.4 *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 immediately be (*1*) emptied; (*2*) rinsed with water; (*3*) cleaned by a procedure appropriate for removing the test material from the item (for example, acid for removing metals and bases and detergent or organic solvent for removing organic chemicals); and (*4*) 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 echinoid larvae **(10)**. The test chambers should be rinsed with dilution water just before use.

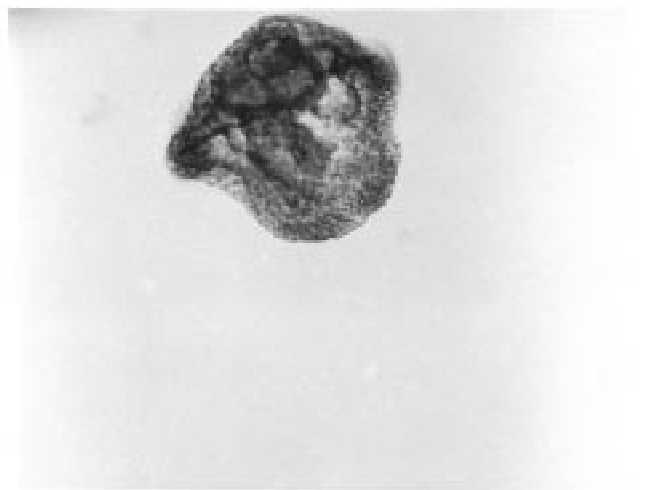

FIG. 2 (e) *(continued)*

6.5 *Acceptability*— Before a test is begun with echinoid embryos in new test facilities, it is desirable to conduct a "non-toxicant" test in which all test chambers contain dilution water with no added test material to determine (*1*) whether embryos will survive and develop acceptably (see 11.8); (*2*) whether the dilution water, handling procedures, etc., are acceptable; (*3*) whether there are any location effects on either survival or development; and (*4*) the magnitude of between-chamber variance in the percentage of embryos that develop

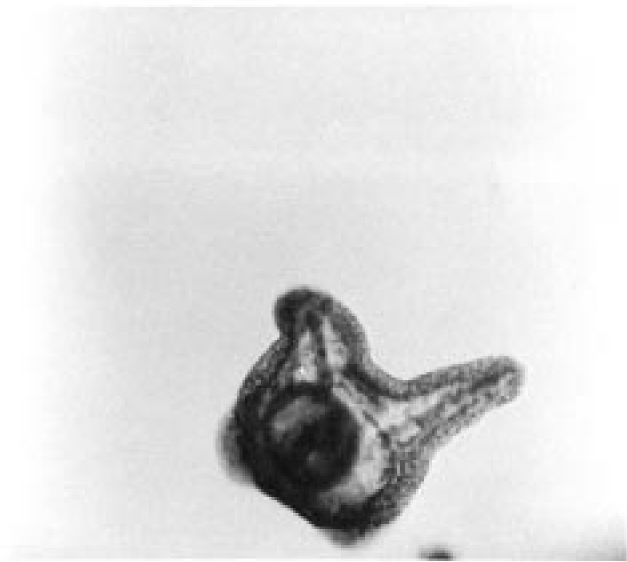

FIG. 2 (f) *(continued)*

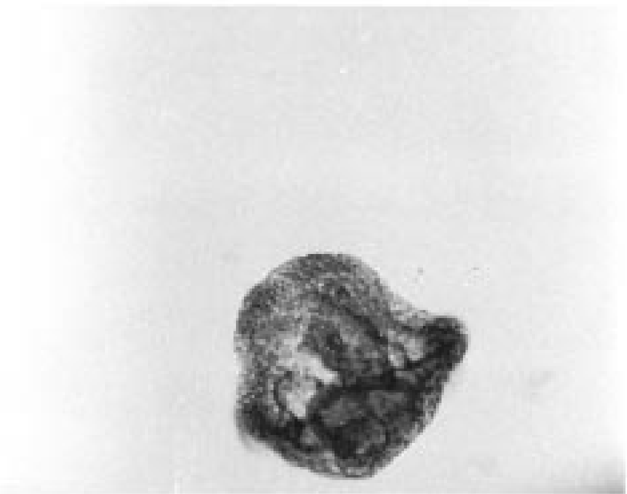

FIG. 2 (g) *(continued)*

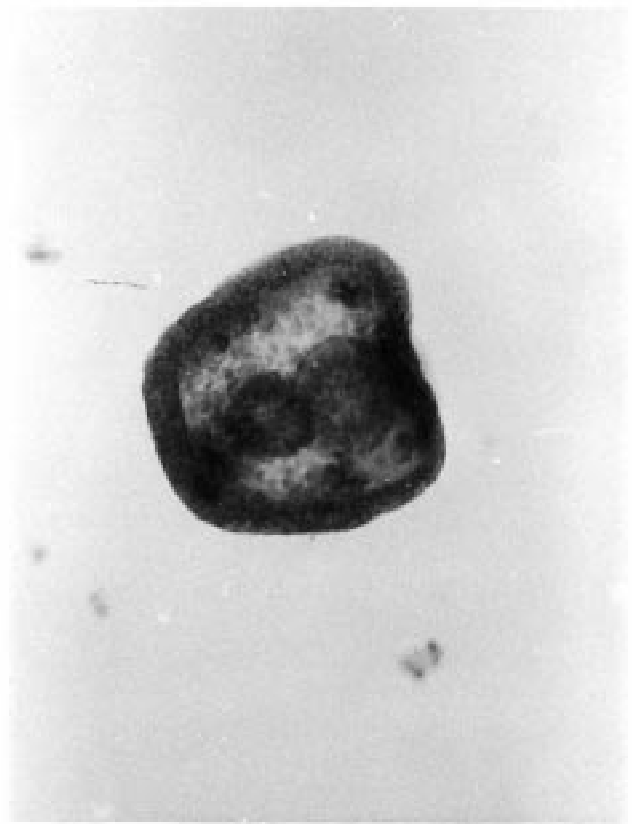

FIG. 2 (h) *(continued)*

into normal larvae. It is also highly recommended that each laboratory develop and maintain a "control chart" of the results of routine reference toxicant testing and control responses.

7. Safety Precautions

7.1 Many materials can affect humans adversely if precautions are inadequate. Therefore, skin contact with all test materials and their solutions 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 the test chambers and ventilating the area surrounding the chambers, should be taken when conducting tests on volatile materials. Information concerning toxicity to humans **(11)**, recommended handling procedures **(12)**, 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 **(13)** and with materials that are, or are suspected of being, carcinogenic **(14)**.

7.2 Although in most cases the 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 the stock and test solutions.

7.3 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.

7.4 An acidic solution should not be mixed with a hypochlorite solution because hazardous fumes might be produced.

7.5 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.

7.6 The use of ground-fault systems and leak detectors is recommended strongly to help prevent electrical shocks because salt water is a good conductor of electricity.

7.7 Care should be exercised when collecting and handling sea urchins to avoid puncture wounds from spines. Where possible, species with blunt spines should be selected over those with long, sharp spines.

8. Dilution Water

8.1 *Requirements*:

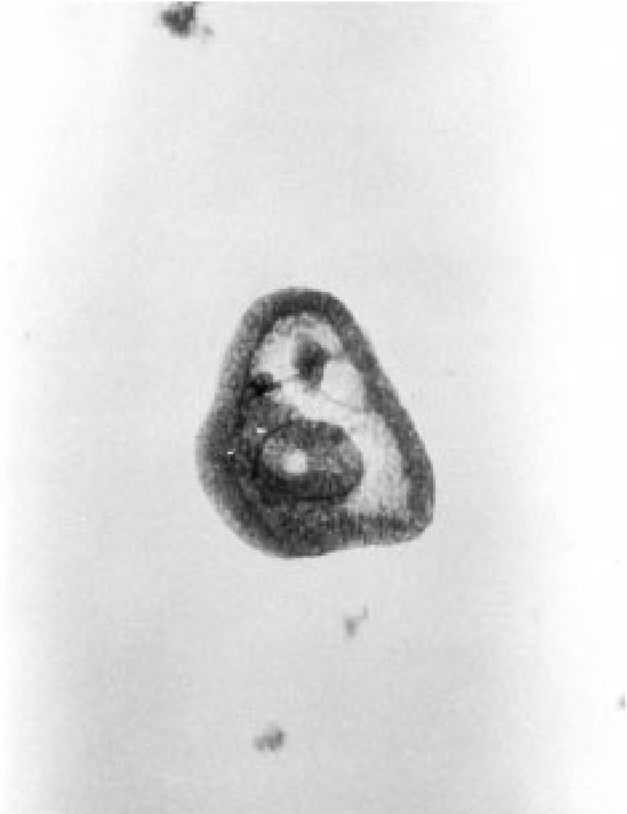

FIG. 2 (i) *(continued)*

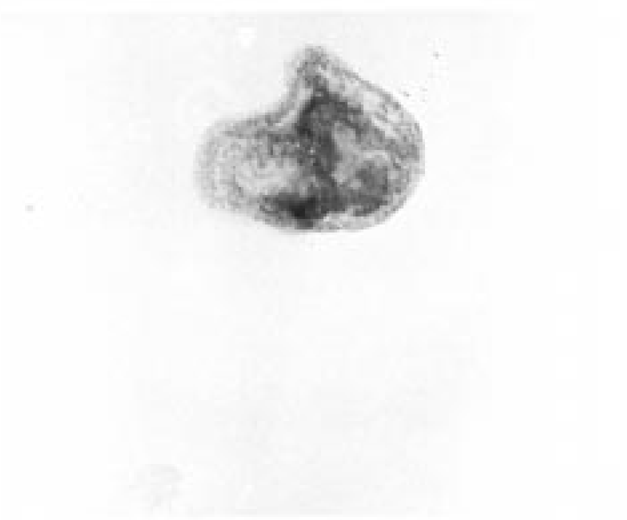

FIG. 2 (j) *(continued)*

8.1.1 In addition to being available in adequate supply, the dilution water should (*1*) be acceptable to adult echinoids and their embryos and larvae, (*2*) be of uniform quality, and (*3*) not affect the test results unnecessarily.

8.1.2 The minimal requirement for an acceptable dilution water for acute toxicity tests starting with embryos of sea urchins or sand dollars is that at least 70 % of the embryos resulting from eggs and sperm produced by appropriately conditioned adults result in normal larvae while being maintained in the dilution water for 48 to 96 h. Natural salt water varies in quality enough that even though it is usually acceptable, it might occasionally be toxic to embryos or larvae if, for example, dinoflagellates are present **(15)**.

8.1.3 The quality of the dilution water should be sufficiently uniform that the test animals are held and conditioned and that the test is conducted in water of the same quality. In particular, the salinity should always be between 27 and 36 g/kg **(16)** 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 artificial sea salts or natural saline brines to raise the salinity or by adding distilled or deionized water to lower the salinity.

8.1.4 The dilution water should not affect the test unnecessarily because of such things as sorption or complexation of test material. Therefore, 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 can be greater than 5 mg/L in the water in which the test animals are held and conditioned, since food will normally be present in the holding tanks.

8.1.5 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 water that is naturally or artificially high in TOC or particulate matter or low in dissolved oxygen. If such water is used, it is important that adequate analyses be performed to characterize the water and that a comparable test be available or be conducted in a more usual dilution water to facilitate interpretation of the results obtained in the special water.

8.2 *Source*:

8.2.1 *Reconstituted Water*—Use of reconstituted water is often not worth the effort for tests starting with echinoid embryos because of (*1*) the large volume needed for holding and conditioning the test animals, (*2*) the necessity of providing adequate food for the test animals (see 10.5.5), and (*3*) frequently poor survival and development of the embryos and resulting larvae.

8.2.2 Reconstituted water is prepared by adding a sea salt or specified amounts of reagent-grade chemicals **(17)** to high-quality water with (*1*) conductivity less than 1 micromho/cm and (*2*) either TOC less than 2 mg/L or chemical oxygen demand (COD) less than 5 mg/L. A formula for reconstituted water that may be acceptable for use with echinoids is given in Table 1. Acceptable water for the 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 per 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.

TABLE 1 Reconstituted Salt Water

NOTE—Add the following reagent-grade chemicals **(17)**[6] in the amounts and order listed to 890 mL of fresh 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	
$CaCl_2 \cdot 2H_2O$	1470 mg
Na_2SO_4	4000 mg
$MgCl_2 \cdot 6H_2O$	10 780 mg
NaCl	23 500 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 g/kg ± 0.2. The reconstituted water should be stripped of trace metals (33). If necessary, the water should be diluted to the desired salinity at the time of use.

[B] It is presently unknown if NaF affects the development of echinoid embryos and larvae. Initial tests should be conducted with and without NaF if reconstituted water is used for test animal holding or test water.

8.2.3 *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 saline surface water, but acceptability based on embryo and larval survival and normal development should be assessed. If surface water is used, it should be obtained from an area known to support a healthy, naturally reproducing population of echinoids. The water intake should be positioned (for example, approximately 5 to 10 m below the surface) to minimize fluctuations in quality and the possibility of contamination and to maximize the concentration of dissolved oxygen and healthy phytoplankton (see 10.5.5). A specially designed system is usually necessary to obtain salt water from a natural source (see Guide E 729). Chlorinated water should not be used as, or in the preparation of, saline dilution water because chlorine-produced oxidants are quite toxic to the embryos and larvae of sea urchins and sand dollars **(14)**. 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 **(18)**. Some organic chloramines, however, react slowly with sodium bisulfite **(19)**. In addition to residual chlorine, municipal drinking water often contains high concentrations of copper, lead, zinc, and fluoride, and the quality is often rather variable. The concentrations of most metals can usually be reduced by using a chelating resin **(20)**, but the use of different-dilution water might be preferable.

8.3 *Treatment*:

8.3.1 Unless natural seawater is used, dilution water should be aerated intensively for 24 to 48 h by such means as air stones, surface aerators, or column aerators **(21)** before addition of the 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 the pH and concentrations of dissolved oxygen and other gases into equilibrium with air and minimize the oxygen demand and concentrations of volatiles. The concentration of dissolved oxygen in dilution water should be between 90 and 100 % saturation **(22)** to help ensure that dissolved oxygen concentrations are acceptable in the test chambers. Supersaturation by dissolved gases, which can be caused by heating the dilution water, should be avoided to prevent possible symptoms similar to gas-bubble disease in fish **(21, 23)**.

8.3.2 The salinity and pH of dilution water may be adjusted by the addition of appropriate reagent-grade chemicals **(17)**,[6] sea salts or brine (especially to prevent excessive decreases in salinity, see 8.2), 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 echinoids (see 10.5.5), filtration through bag, sand, sock, or depth-type (honeycomb) cartridge filters may be used to keep the concentration of particulate matter acceptably low (see 8.1.4) 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 **(24)** 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, with the finest effective to 1.0 µm or less to remove the embryos and larvae of marine animals, parasites, and predators. If bacteria are to be removed by filtration, a filter effective to 0.45 µm or less must be used. 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 per year and more often if such measurements have not been made semiannually for at least two years or if 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 Section 12), the detection limit should be below either (*1*) the concentration in the dilution water or (*2*) the lowest concentration that has been shown to affect embryos, larvae, or adults of sea urchins and sand dollars unacceptably **(25)**.

[6] *Reagent Chemicals, American Chemical Society Specifications,* American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals,* BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary,* U.S. Pharmaceutical Convention, Inc. (USPC), Rockville, MD.

9. Test Material

9.1 *General*—The test material should be reagent-grade **(17)**[6] or better, unless a test on a formulation, commercial product, or technical-grade or use-grade material is specifically needed. Before a test is begun, the following should be known about the test material:

9.1.1 Identities and concentrations of major ingredients and major impurities, that is, impurities that constitute more than approximately 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 larval stage of another echinoid or marine invertebrate.

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.

9.1.6 Recommended handling procedures (see 7.1).

9.2 *Stock Solution*:

9.2.1 In some cases, the test material can be added directly to the dilution water, but often it is dissolved in a solvent to form a stock solution that is then added to the dilution water. If a stock solution is used, the concentration and stability of the test material in it should be determined before beginning 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 test treatments will not be affected, deionized or distilled water may be used. Several techniques have been developed specifically for preparing aqueous stock solutions of slightly soluble materials **(26)**. 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, low volatility, and high ability to dissolve many organic chemicals, triethylene glycol is often a good organic solvent for preparing stock solutions **(27)**. 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 **(17)** or better,[6] 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 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 echinoids, at least 70 % of the embryos introduced into the control treatment must result in normal larvae at the end of the test. These stipulations may be species-specific and may be too high or too low for some lesser tested echinoid species.

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 normal larvae at the end of the test must be at least 70 % of the initial number in the solvent control. If a dilution-water control is included in the test, the number of embryos that result in normal larvae at the end of the test should be at least 70 % of the initial number in the dilution-water control.

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 normal larvae 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.

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 the calculation of an EC50, the test concentrations 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 to 96 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.

9.3.2 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.

9.3.3 In some situations, usually related to regulatory activities, it is necessary to determine only whether (*1*) a specific concentration of test material is acutely toxic to the embryos or larvae of the test species or (*2*) 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 only necessary to test only that concentration plus a control (see 11.1), and it is not necessary to determine an EC50.

10. Test Organisms

10.1 *Species*—Whenever possible, either Atlantic sea urchins (*Arbacia punctulata*), green sea urchins (*Strongylocentrotus droebachiensis,* available on the northern Atlantic and Pacific coasts), Pacific purple sea urchins (*S. purpuratus*), or Pacific eccentric sand dollars (*Dendraster excentricus*) should be used as the test species. These species were selected on the basis of availability, commercial importance of several of the species, past successful use, and ease of handling in the laboratory. Their use is encouraged to increase the comparability of results and availability of much information on a few species rather than little information on many species. The species used should be identified by using an appropriate taxonomic key. Successful toxicity tests can be conducted with embryos of other echinoid species, but the comparability of the results will be less.

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

10.3 *Source of Embryos*—Embryos used to begin a test can be obtained from females and males that have been collected freshly from the field or that had been maintained in the dilution water in the laboratory before they were stimulated to spawn.

10.4 *Handling*—The organisms should be handled as little as possible. When handling is necessary, it should be done carefully, gently, and quickly, so that the organisms are not stressed unnecessarily. Adults that are injured during handling should be discarded. Equipment used to transfer embryos (but not exposed to toxicants) should be cleaned between uses by washing with clean sea water or distilled or deionized water. Hands should be washed before and after handling brood stock.

10.5 *Test Animal Source and Condition*:

10.5.1 For any one test or a series of related tests, all test animals should be collected from the same location, which should be known precisely. The test animals may be obtained from a commercial source only if the original specific collection location of the sea urchins or sand dollars can be identified. To minimize the possibility of genetic or physiological adaptation to chemicals or aberrant water quality, the organisms should be collected from a location that is not subject to obvious point or non-point source pollution and has water that is comparable in quality to that which will be used for the holding and testing. Large mature individuals should be obtained. Sex ratios are generally 1:1, although sea urchins may sometimes aggregate by sex in the field. Hermaphroditism is rare but can occur. Unlike bivalve mollusks, it is difficult to condition sea urchins and sand dollars to spawn out of season. However, proper laboratory manipulation of the holding conditions (that is, temperature, light, and salinity) and food supply (both quantity and quality) hasten the conditioning or urchins and sand dollars undergoing active gametogenesis and can prolong their period of ripeness (for example, defer spawn-out time).

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 echinoids is to hold a population with mature gonads at an appropriate holding temperature (see Table 2). If done correctly, this will prevent both undesired spontaneous spawning and resorption of gametes. Some species of sea urchins and sand dollars can yield viable gametes for up to four months past their normal spawn-out time under suitable conditions.

10.5.3 During certain periods of the year, ripe adult echinoids can be induced easily to spawn with 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 and placed in sea 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. To avoid spawning during transport, it is usually best to ship the animals “dry,” packed in moist native algae (or a similar moist packing material) in an ice chest (for cold water species) supplied with packaged ice in the bottom. Upon arrival in the laboratory, it should be ensured that spawning is not induced in the test animals when put into the laboratory holding tanks. Any urchins that start to spawn should not be put into community holding tanks. If possible, it is best to separate males and females, since sperm from one spawning male can trigger other sea urchins to spawn out. (Brief electrical stimulation (see 10.6.3) or needle biopsy have been used by some laboratories to sex sea urchins). The limiting factor in most bioassays is the availability of good quality eggs; sperm for egg fertilization are almost always available.

10.5.4 When the test animals are first brought into the laboratory, they should be acclimated to dilution water over a period of two or more days to prevent stress due to abrupt changes in water quality. The temperature may generally be changed at a rate not to exceed 2°C/day, and the salinity at a rate not to exceed 1 g/kg/day. An abrupt increase in temperature or salinity might not only induce spawning, especially of males, but also harm the gametes seriously **(28)** and kill the

TABLE 2 Recommended Holding and Test Temperatures (°C)

Species	Holding	Test
Arbacia punctulata	18 to 22	20
Dendraster excentricus	12 to 16	15
Strongylocentrotus purpuratus	8 to 12 (WA, OR, AK)	12
Strongylocentrotus purpuratus	12 to 16 (CA)	14
Strongylocentrotus droebachiensis	8 to 12	12

adults. Following proper acclimation to the laboratory holding conditions, some test animals can be test-spawned to check gamete quality if enough are available. The animals can be spawned by the method of choice (see 10.6.2) and the amount of spawn and quality of gametes noted. Spawning should begin within minutes of stimulation and should be vigorous. Sperm should be highly motile and eggs free of germinal vesicles or signs of deterioration; egg fertilization should be >90 %.

10.5.5 If the test animals do not contain ripe gonads, they should be conditioned prior to any attempt to induce spawning. To condition sea urchins, food in the form of a preferred species of macroalgae should be supplied in excess, and the troughs cleaned and resupplied every day. Sand dollars should be supplied with an adequate source of natural plankton or perhaps pureed artificial food (for example, various types of dried fish food). It is important to condition the adult animals under proper conditions for an appropriate duration to promote gametogenesis and the production of mature gametes. Spawning of adults before or after optimum maturation will usually result in unsatisfactory gametes.

10.5.6 The brood stock should be carefully observed daily during holding and conditioning for signs of stress and mortality. Animals showing signs of spine loss should be discarded. Holding and conditioning trays should not be drained (changes in hydrostatic pressure can induce spawning in ripe test animals), but debris and fecal material should be siphoned out of the tanks every day to prevent the accumulation of organic matter and bacteria. Dead or stressed animals should be removed daily. If animals have begun to decompose, the troughs should be drained, all animals removed to other tanks, and the troughs cleaned with detergent and rinsed with fresh water. More frequent cleaning might be appropriate with enriched waters and elevated conditioning temperatures. Apparently healthy animals removed from tanks with dead or diseased individuals should be isolated from other brood stock and not used for testing until their health and gamete quality are verified.

10.6 *Spawning and Fertilization*:

10.6.1 Toxicity tests can be designed to assess differences in sensitivity resulting from parentage by subjecting the 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 for more accurate calculation of means 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 (and more common for past echinoid testing), 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 most echinoid species can usually be induced to spawn by one of two commonly used methods. First, the animals may be injected with 0.5 molar potassium chloride (KCl). For most sea urchins, approximately 1.0 mL of KCl is injected into the coelom with a small syringe by inserting the needle through the peristomal membrane surrounding the mouth on the oral side. The urchins are then inverted (oral side up) so that the gametes can be collected by extrusion into a beaker of sea water ("wet" spawning) or into a watchglass or similar container without saltwater ("dry" spawning). "Wet" spawning immediately introduces the gametes into seawater and activates the sperm cells (the intensity of activation is proportional to the amount of dilution in sea water). "Dry" spawning keeps the sperm in an inactive state, as they were in the gonad; thus, activation can be deferred until a later time by storing the sperm on ice. Eggs should not be dry-spawned but rather collected within an hour or two of use and washed very gently with one or two changes of sea water, decanting after the eggs settle to the bottom. Sand dollars are spawned in the same manner except that less KCl is used (~0.5 mL), and it is injected at an angle through the mouth.

10.6.3 The second common spawning method is by electrical stimulation with a 12-V system. The electrodes are placed on either side of the urchin, and spawning is induced until the electrodes are removed. Although this method requires more equipment and is generally confined to the laboratory, one advantage is that the test animals can be sexed (and gamete quality checked) by brief application of the electrodes **(29)**. Neither the KCl or the electrical spawning methods kill healthy test animals, which may often be respawned in 30 to 60 days if held under the proper physical and feeding conditions.

10.6.4 Fertilization is more likely to be successful if sperm is obtained from test animals that spawn readily by chemical or electrical stimulation. If sperm cannot be obtained by these methods, sperm may be stripped from males following excision of the gonads. While the gonads are held over a beaker containing suitable seawater, several incisions should be made through each gonad. The gametes should be allowed to exude from the gonads and should be rinsed into a beaker with a gentle stream of dilution water from a squirt bottle. The use of eggs stripped from female urchins is not recommended because it often results in an excess of poorly developed and malformed embryos.

10.6.5 Eggs can be passed through a fine-mesh screen to remove debris and fecal pellets that may be present. 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 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 distribute the eggs or embryos uniformly. A small subsample (0.1 to 1.0 mL) should be removed immediately using an automatic pipet, placed on a Sedgwick-Rafter cell, and counted at 40 to 100×. Eggs with obvious germinal

vesicles (clear spots) are immature and will not fertilize or develop correctly, or both. Eggs from riper females should be used if there are more than a few percent of immature eggs. The total number of eggs should be calculated to determine whether it is sufficient to perform the test. The egg density should be adjusted to the range of 20 to 50 eggs/mL before adding sperm.

10.6.6 After sperm motility has been verified by microscopic examination, the sperm suspension may 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. For embryo assays, precise sperm counts are unnecessary after one gains a little experience.

10.6.7 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. Fertilization should be accomplished at the spawning (acclimation) temperature and the suspension held at that temperature at least until it is determined that fertilization has been accomplished (10 to 20 min). Fertilized eggs will have an obvious raised fertilization (vitelline) membrane surrounding the egg. Embryo tests must begin within 4 h after fertilization.

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 (*1*) they are obtained from adults in prime spawning condition, (*2*) the adults spawned readily after the first stimulation, and (*3*) 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 **(30)**, but none has been proven to be a reliable indicator of the overall quality of any species or test results. A reference toxicant is likely to be most useful in tests on materials that have the same mode of action as the reference toxicant.

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 the results (see Section 14). A unique feature of an acute test starting with embryos of echinoids 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 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. An acute test intended to allow the calculation of an EC50 usually consists of three replicates of each treatment including the control treatment(s). In dilution-water and solvent control(s), 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, six or seven concentrations might be desirable to increase the chances of covering the appropriate range.

11.1.2 If it is necessary to determine only whether (*1*) a specific concentration is acutely toxic to embryos of the test species or (*2*) the EC50 is above or below a specific concentration, three replicates of that single concentration and of the control(s) are necessary. Two additional concentrations at approximately one-half and two times the specific concentration of concern are desirable to increase confidence in the results.

11.1.3 If an EC 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 will usually be necessary.

11.1.4 The primary focus of the physical and experimental design of the test and the statistical analysis of the data is the experimental unit, which is defined as the smallest physical entity to which treatments can be assigned independently **(31)**. 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 assigned randomly to individual test chamber locations and may be reassigned randomly 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.

11.1.5 The minimum desirable number of individual spawns used per test and the number of test chambers per spawn per treatment should be calculated from the (*1*) expected variance of counts within test chambers, (*2*) expected variance in the percent of normally developed larvae between test chambers within a spawn, (*3*) expected variance between spawns within a treatment, and (*4*) maximum acceptable confidence interval of the EC50 **(32)**. 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 the estimation of experimental error **(31)**. Because up to 30 % of echinoid embryos might not result in normal pluteus larvae 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. It is desirable to repeat the test at a later time to obtain information concerning the reproducibility of the results.

11.2 *Dissolved Oxygen*— The dissolved oxygen concentration in each test chamber must be between 60 and 100 % of saturation **(22)** at all times during the test. There are no data, however, indicating whether reduced oxygen concentrations effect EC50s. Ideally, test solutions should not be aerated during the test because aeration can increase the loss of toxicants from solution and possibly disrupt embryo development.

11.3 *Temperature*— Test temperatures should be selected to accommodate the test species (see Table 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 individual temperature measurement 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 (*1*) the desired volume can be placed in each control test chamber, (*2*) the necessary volume of each test solution containing the test material can be prepared, and (*3*) the desired analyses can be performed on the dilution water. Enough test solution should be prepared for each treatment containing test material so that (*1*) the desired volume can be placed in each test chamber and (*2*) any desired analyses of water quality, test material, etc. can be performed.

11.4.2 Approximately 15 to 30 min after adding sperm suspension to the egg suspension, the concentration of embryos in the stock embryo suspension should be determined by mixing the solution with a perforated plunger, withdrawing a 0.1 to 1.0 mL sample, placing it in a Sedgwick-Rafter cell, and counting the number of embryos. The accuracy of this count is not crucial because it is used merely 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/mL, although concentrations up to 50 embryos/mL probably do not impair the normal development of echinoid embryos.

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, by using an automatic pipet.

11.4.5 The variation in the concentration of embryos in the various test solutions should be minimized by (*1*) keeping the embryo suspension well mixed by using a perforated plunger and (*2*) 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 (15 to 30 embryos/mL), with the embryo suspension volume representing less than 1 % of the total volume, and an acceptable coefficient of variation.

11.4.6 The test begins when embryos are first placed in solutions containing the test material. Three methods may be used to determine the initial number of embryos added to the test chambers: (*1*) direct counting of samples from each test chamber, (*2*) direct counting of samples from three or more chambers prepared as surrogates for the sole purpose of determining the initial number, or (*3*) by 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 one or more 10 mL samples taken immediately in which to count the embryos. Each sample should contain 150 to 300 embryos and should be preserved with 5 % buffered formalin (or similar) in a labeled and closed vial. (Smaller samples can be taken, or see Method 2 when test volumes are very small.) The test solutions should be mixed and sampled carefully to minimize the sampling error. This method provides a direct measure of the initial embryo density (*N*) for each test chamber. This is the preferred method 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 (or more) containers should be prepared in addition to the number needed for the basic test. At least one should be prepared as the first container in the otherwise random series to be prepared, one should be prepared last, and a third should be prepared in the middle of the preparation of test containers. After all of the containers have been prepared, these extra containers should be sampled as described for Method 1. The initial embryo density (*N*) is then defined as the average of the concentrations in these extra containers, and the variance of the average can be calculated. This method is generally preferred to Method 1 since it minimizes the total number of samples to be counted, produces a measure of variance, and does not disrupt the experimental chambers.

11.4.6.3 *Method 3*—In the case in which the stock suspension is of much larger volume than the total volume needed to prepare all of the 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 determined precisely from several samples. From this mean number, calculate the embryo density in the test containers as follows:

$$N = S(V_s/V_t) \quad (1)$$

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 embryo/larvae must 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 96 h without being fed.

11.6 *Duration of Test*— The embryos and resulting larvae must be exposed to the test material for a total of 48 to 96 h. The duration of each test will depend on the species used and the test temperature. Experience with local test species will dictate the length of the test, which should be either 48, 72, or 96 h. In some cases, durations longer than 96 h have been reported. In all cases, the duration of a test will be based on the time to development to the pluteus stage of at least 70 % of the embryos in the control solutions. If a test requires additional time beyond the usual for a given species and temperature, continue the test as necessary for ≥70 % control development to normal pluteus larvae, but record this time extension as a test deviation.

11.7 *Biological Data*:

11.7.1 At the test conclusion, the solution in each test chamber must be mixed carefully and a sample removed immediately and preserved using the same sampling and preservation procedures and sample volume as was used to sample the test chambers (or surrogate chambers) at the beginning of the test in accordance with 11.4.6. Ensure that the bore diameter of the pipet is large enough to accommodate the relatively large pluteus larvae.

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; it should therefore be determined periodically that embryos or larvae are not being discarded inadvertently.

11.7.3 All embryos must be counted in samples obtained in accordance with 11.4.6. In samples obtained in accordance with 11.7.1, all larvae that have developed to a reasonably identifiable pluteus larva must be counted as normal, even if there are signs that the larva died after developing to the pluteus stage. Grossly deformed pluteus larvae or embryos that failed to develop beyond the prism stage must be counted as abnormal. Analyst training and experience are key factors in arriving at successful determinations of the difference between normal and abnormal pluteus larvae.

11.7.4 Data concerning the effect of the test material on the time(s) to reach the pluteus stage may be obtained, if desired, by collecting additional samples for counts at various times during the test.

11.8 *Control Performance*—For a toxicity test with echinoid embryos to be acceptable, an average of ≥70 % of the embryos maintained in the dilution water control chambers must be judged to be normally developed pluteus larvae (see Fig. 1 and Fig. 2).

11.9 *Other Measurements*:

11.9.1 The salinity and pH of the dilution water must be measured prior to each test, and the measurement of particulate matter, total dissolved gases, and TOC is desirable. The concentration of dissolved oxygen and pH 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.

11.9.2 *Temperature*— While holding animals in the laboratory, temperatures should be measured daily with a min-max thermometer. Throughout the test, in at least one test chamber, either the temperature must be measured several times per day, or the maximum and minimum temperatures must be measured daily with a min-max thermometer. Near the beginning and the end of the test, the temperature must be measured concurrently in all test chambers. If the test chambers are in a water bath, measurement of the temperature of the water bath 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 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.

11.9.3 *Test Material*— If possible, the concentration of test material should be measured 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.

11.9.4 If the test material is dispersed uniformly 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 particulates stirred up from the bottom or sides. The submersed end of the siphon or pipette should be covered with a piece of 26-μm mesh plastic screen to prevent the 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 pipette should be rinsed with test solution before collecting the sample. Water samples should be collected directly into appropriately sized containers from which the test material can be extracted or analyzed directly. If the test material is not dispersed uniformly in the test chamber, the whole volume of solution in the test chamber should be (*1*) used as the sample or (*2*) treated appropriately (for example, by adding acid, base, or surfactant and mixing thoroughly) to distribute the test material uniformly before a sample is taken. However, these steps can be executed only at the end of the test, after all embryo samples have been collected.

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 **(33)** to minimize the 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 sufficiently sensitive, methods should be obtained from other reliable sources **(34)**.

12.3 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 begun with echinoid embryos should usually be considered unacceptable if one or more of the following occurred, except that if the temperature was measured numerous times, a deviation of more than 3°C in any one measurement might be inconsequential. However, if the 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 the temperature had been measured more often.

13.1.1 All test chambers were not identical.

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

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

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

13.1.5 Less than 70 % of the echinoid embryos introduced into a required control treatment resulted in normal pluteus larvae at the end of the test.

13.1.6 Dissolved oxygen, salinity, pH, and temperature were not measured as specified in 11.9.

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

13.1.8 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.

13.1.9 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.

13.1.10 The difference between the measured temperatures in any two test chambers was more than 2°C at any one time.

13.2 The 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 effect less than 37 %.

13.2.2 No treatment resulted in an average effect greater than 63 %.

14. Calculation of Results

14.1 For each set of data, the EC50 should be calculated on the basis of (*1*) the measured initial concentrations of test material, if available, or the calculated initial concentrations and (*2*) the number of embryos that resulted in normal pluteus larvae after the test duration. The 95 % confidence limits on the EC50 or any other ECs should be calculated, if possible.

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 E 729) can be used to calculate an EC50 and its 95 % confidence limits from a set of quantal data that is distributed binomially and contains two or more concentrations at which the percent affected lies between 0 and 100 % **(35)**. Even though acute tests started with echinoid embryos produce quantal data, the data are not likely to be binomially distributed because (*1*) there is between-chamber variance within a treatment in the percentage of the embryos that result in normal pluteus larvae, and (*2*) there is within-chamber error in the sampling and counting of the number of normal pluteus larvae at the end of the test.

14.3 For each test chamber in each treatment, including the control treatment(s), *A* (the percentage of the embryos that did not result in normal pluteus larvae) should be calculated as follows:

$$A = 100(N - B)/N \quad (2)$$

where:

N = the number of eggs in the sample taken from that test chamber at the beginning of the test, and

B = the number of normal pluteus larvae in the sample taken from that test chamber at the end of the test

M (the average percentage of the embryos that did not result in normal pluteus larvae in the control treatment(s)) should be calculated as the average of the *A*s for the test chambers in the control treatment(s). The test is unacceptable if *M* is greater than 30 %.

14.4 For each test chamber in each treatment other than the control treatment(s), *E* (the percentage of introduced embryos that did not result in normal pluteus larvae adjusted for the controls) should be calculated, by using Abbott's formula **(35)**, follows:

$$E = 100(A - M)/(100 - M) \quad (3)$$

14.5 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 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 regression technique **(36, 37)** can be used to calculate the EC50 and 95 % confidence limits. If *E* covers an appropriate range, 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, number of test organisms exposed in each chamber, range of concentrations tested, and variance within each treatment, especially within the control treatment(s), and give the correct confidence limits.

14.6 An EC 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 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 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 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 affected were obtained close to 5 or 95 %.

14.7 It might be desirable to perform a hypothesis test to determine which of the tested concentrations of test material 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 heterogeneity tests. Then, a pairwise comparison technique, contingency table test, analysis of variance, or multiple comparison procedure appropriate to the experimental design should be used. The presentation of results of each hypothesis test should include the test statistic and its corresponding significance level, minimum detectable difference, and power of the test.

14.8 For each data set, the EC50 may be calculated by substituting the number of normal pluteus larvae in the equation and proceeding as above. The same procedure may be used to calculate an EC50 for any other time interval for which appropriate data were collected.

15. Report

15.1 Include the following information, either directly or by reference to available documents, in the record of results of an acceptable acute toxicity test with echinoid embryos:

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 test animals were obtained, date of collection, scientific name, name of person who identified the organisms and the taxonomic reference used, duration and temperature of holding, food, 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, depth and volume of solution in the chambers, number of female-male pairs used, number of test chambers per female-male pair per treatment, and lighting.

15.1.6 Average and range of the measured dissolved oxygen concentration (as percent of saturation) for each treatment.

15.1.7 Averages and ranges of the holding and test temperature and 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, and degradation and reaction products, including validation studies and reagent blanks.

15.1.10 A table of data on the number of fertilized eggs at the beginning of the test and the number of normal pluteus larvae 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 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 normal pluteus larvae; specification of whether the results are based on measured or unmeasured concentrations of the test material; for formulations and commercial products, specification of whether the results are based on whole mixture or active ingredient.

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

15.2 Identify the procedures used and quality of the results clearly. Include enough information in published reports.

16. Keywords

16.1 acute toxicity test; *Arbacia punctulata*; Dendraster excentricus; echinoid; EC50 test; embryo development; marine water quality; pluteus larva; sand dollar; sea urchin; sediment test; static toxicity test; *Strongylocentrotus droebachiensis*; Strongylocentrotus purpuratus; toxicity; toxicology

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 echinoids (sea urchins and sand dollars). Methods for testing sediments with embryos and larvae of echinoids are well-developed on the U.S. and Canadian West Coast **(39)**. It is probable that embryos of many echinoid 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 echinoid 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 to 96 hours (development times are species-dependent) in the 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 E 1367 and E 1391. 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.

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 **(40)**, 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 (**39**, **41**; Guides E 1391 and E 1706). It might sometimes be preferable to store sediments in a nitrogen environment by replacing the head-space air in storage containers with nitrogen gas **(1)**.

A1.3 *Test Methods*—The following sediment procedures are derived primarily from Puget Sound Estuary Program (PSEP) protocols **(1)**, 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

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

Parameter	Conditions
1. Test Type:	Sediment static toxicity test
2. Test Species:	Echinoid embryos
3. Test Chamber:	One-L 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 h 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 to 96 hours (development time varies by species)
12. Test Salinity and Temperature:	Varies by species; see Sections 8 and 10.5.4 of this Standard Guide
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-hour 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 % normal pluteus larvae at T_{end} relative to those introduced at T_0
18. Possible Interferences:	Unionized ammonia >0.04 mg/L or total sulfide >0.5 mg/L in the overlying water

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.[7] 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 on.) 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 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. The first negative control is a seawater control, containing 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 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 **(42)**.

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 (e.g., ammonia, sulfide) are of interest, they should also be measured at this time.

A1.3.6 After the sediments have settled for 4 h, 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 10.6.5 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 loosely covered 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-liter 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 Fig. 1 and Fig. 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.

[7] 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.

A1.6 *Data Analysis*—Calculations of test results for echinoid 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 echinoid embryos during sediment testing. For many marine dredging projects, ammonia and sulfide are considered to be natural compounds of relatively little concern. However, ammonia and sulfide can reach toxic concentrations in the test chambers when sediments contain high amounts of organic materials, and thereby mask the effects of "toxicants of concern." Beginning and final ammonia and sulfide concentrations should be measured for all sediment tests using echinoid embryos and must be measured when sediments are known to be high in organic content.

A1.7.1 Ammonia toxicity to echinoid larvae was first investigated by Kobayashi in 1980 **(43)**. He found that three echinoid species (*Peronella japonica, Heliocidaris erythrogramma* and *Anthocidaris crassispina*) all showed ammonia toxicity thresholds in the range of 0.2 to 1.0 mg/L total ammonia. Parallel work with echinoid larvae (sand dollar, *Dendraster excentricus*) found a NOEC of 1.24 mg/L total ammonia (0.014 mg/L unionized ammonia) **(44)**. Results of this study **(44)** led to recommended interim effect threshold value for unionized ammonia of 0.04 mg/L for echinoid larval tests **(39, 45)**. Values of unionized ammonia measured in water from the larval test chambers in excess of 0.04 mg/L are considered to have the potential for producing false positives in the test.

A1.7.2 Relatively little experimental data exist for sulfide effects on echinoid embryo/larval assays. One study by Thompson et al. **(46)** exposed adult Southern California sea urchins (*Lytechinus pictus*) to hydrogen sulfide in sediments for 49 days. Chronic effects to gonad maturation were observed at hydrogen sulfide concentrations as low as 0.033 mg/L. Presently, the Puget Sound PSDDA program has specified interim limits of 0.50 mg/L total sulfide to protect from possible false positives in the echinoid embryo test of sediments **(45)**. This value may change as more data on sulfide effects become available.

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