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

Designation: E1563 – 21a

Standard Guide for Conducting Short-Term Chronic Toxicity Tests with Echinoid Embryos[1,2]

This standard is issued under the fixed designation E1563; 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 short-term chronic effects of a test material on echinoderm embryos and the resulting larvae (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 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 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 tests on temperature, dissolved oxygen, and pH and on such materials as aqueous effluents (see also Guide E1192), leachates, oils, particulate matter, surface waters, effluents, 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 short-term chronic 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 toxic to embryos or whether the EC50 is above or below a specific concentration.

1.5 This guide is arranged as follows:

[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 E50 on Environmental Assessment, Risk Management and Corrective Action and is the direct responsibility of Subcommittee E50.47 on Biological Effects and Environmental Fate.

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

***A Summary of Changes section appears 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, health, and environmental practices and determine the applicability of regulatory limitations prior to use. Specific precautionary statements are given in Section 7.*

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

2. Referenced Documents

2.1 *ASTM Standards:*[4]

E380 Practice for Use of the International System of Units (SI) (the Modernized Metric System) (Withdrawn 1997)[5]

E724 Guide for Conducting Static Short-Term Chronic Toxicity Tests Starting with Embryos of Four Species of Saltwater Bivalve Molluscs

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

E943 Terminology Relating to Biological Effects and Environmental Fate

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

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

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

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

E1525 Guide for Designing Biological Tests with Sediments

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

E1733 Guide for Use of Lighting in Laboratory Testing

3. Terminology

3.1 *Definitions:*

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

3.1.2 For definitions of other terms used in this guide, refer to Guide E729 and Terminology E943. For sediment tests (Annex A1), refer to Guides E1391 and E1525. For an explanation of units and symbols, refer to Practice E380.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *embryo, n*—the stages of a multicellular organism's development that occur between the fertilization of the egg and the pluteus larva.

3.2.2 *pluteus larva, n*—a juvenile lifecycle stage characteristic of all echinoids 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).

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, heat or 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

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

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

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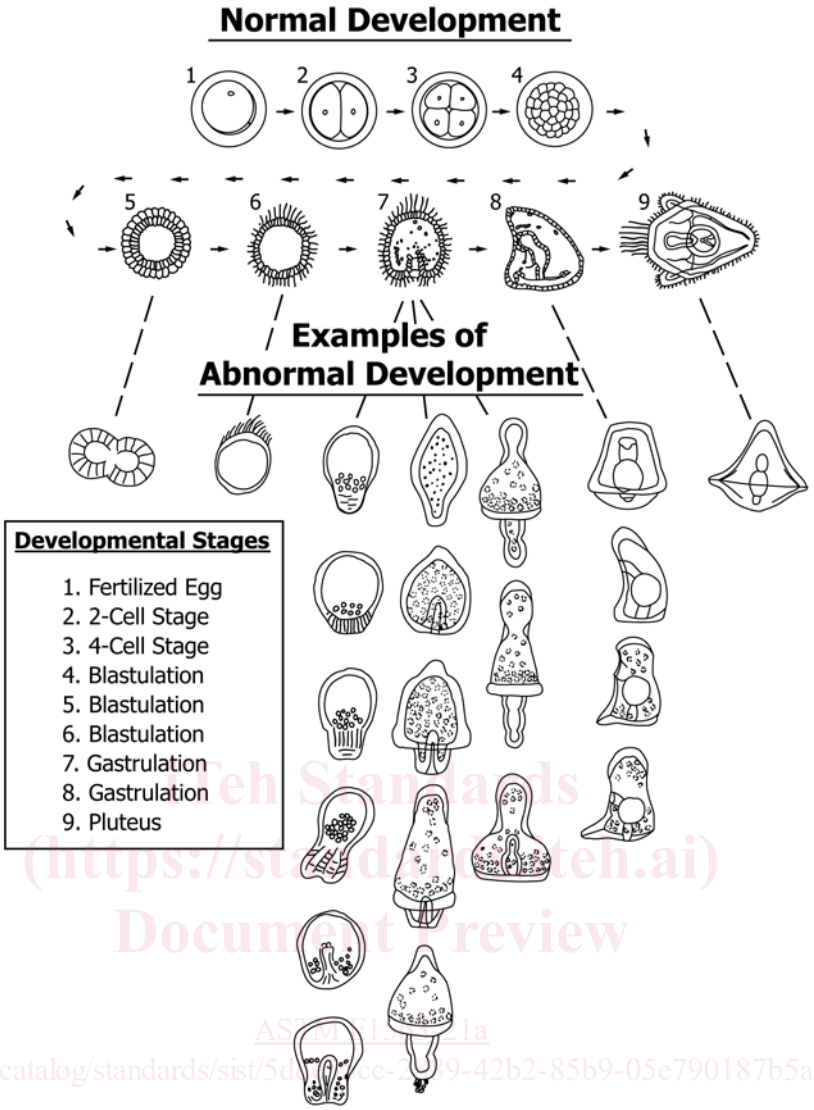

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FIG. 1 Drawings Exemplifying Key Developmental Stages of Normal Echinoid Larvae Occurring During the First 48 to 96 h of Development and Examples of Abnormal or Arrested Development (6)

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 assess effects of a short-term exposure of organisms to a test material under specific experimental conditions. An acute toxicity test does not provide information concerning whether delayed effects will occur and typically evaluates effects on survival. A chronic test is typically longer in duration and includes a sublethal endpoint to assess effects on a population that might occur beyond the exposure period. Because the echinoderm embryo development test includes a sublethal endpoint, but is also short in duration, these tests are considered to be short-term chronic tests, consistent with EPA guidance.

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 tests are often considered to be a good indication of the acceptability of pollutant concentrations to saltwater species in general. The results of these toxicity tests are often assumed to be an important consideration when assessing the hazard of materials to other saltwater organisms (see Guides E724 and E1023) or when deriving water quality criteria for saltwater organisms (**7**).

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

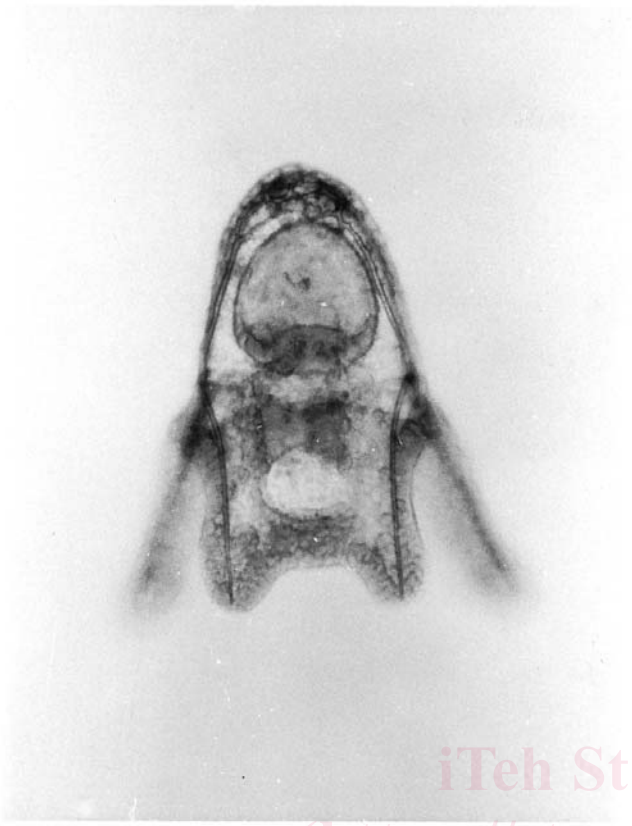

FIG. 2 (a) Examples of Normal and Abnormal Development of Purple Sea Urchin (*Strongylocentrotus purpuratus*) Embryos at the Conclusion of a 72 to 96-h Toxicity Test. Figs. 2a and 2b Show Examples of Normal Echinopluteus' with Four Distinct Arms and Good Symmetrical Development. Fig. 2c Shows a Borderline 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)*

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5.4 The results of short-term chronic tests might be used to compare the 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 short-term chronic toxicity tests might be useful for studying the biological availability of, and structure-activity relationships between, test materials.

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

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

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

6. Apparatus

6.1 *Facilities:*

6.1.1 Flow-through troughs with appropriate trays should be available for holding and conditioning test animals (**8**). 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 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.

FIG. 2 (c) *(continued)*

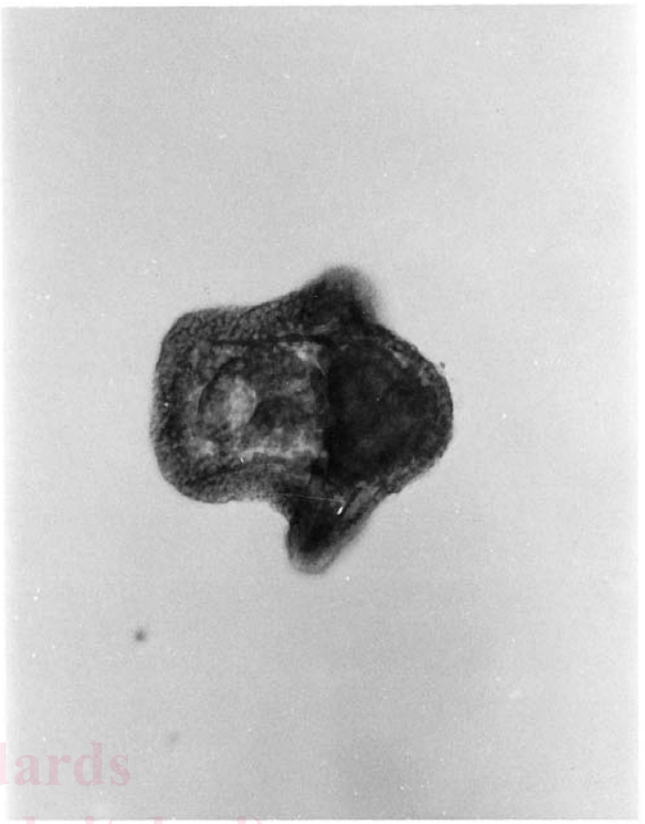

FIG. 2 (d) *(continued)*

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6.1.2 It is desirable, if feasible, 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 (**9**). 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

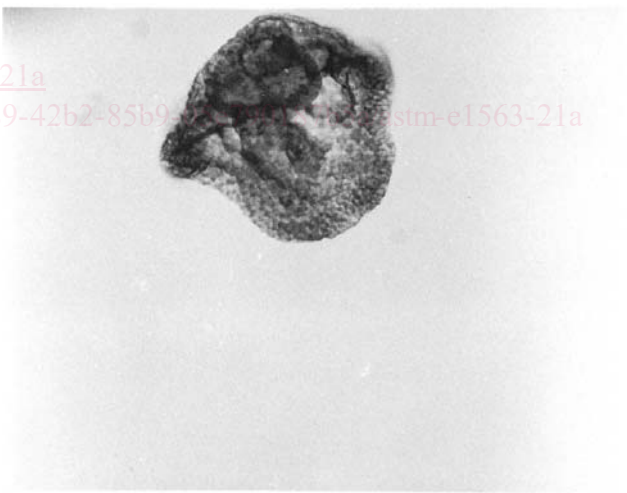

FIG. 2 (e) *(continued)*

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:*

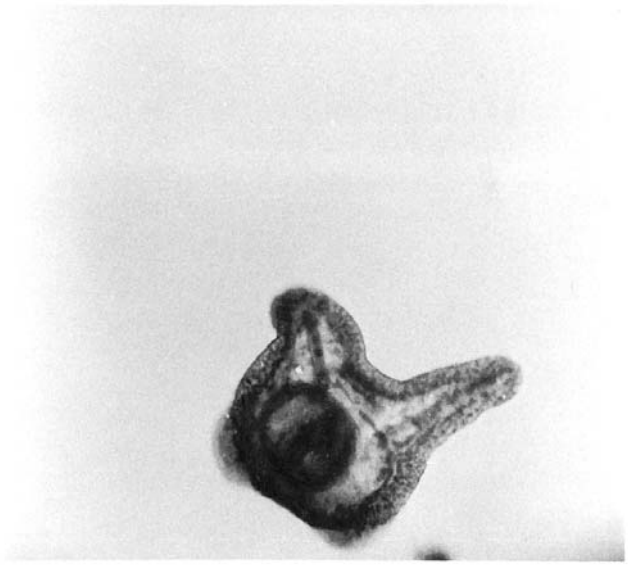

FIG. 2 (f) *(continued)*

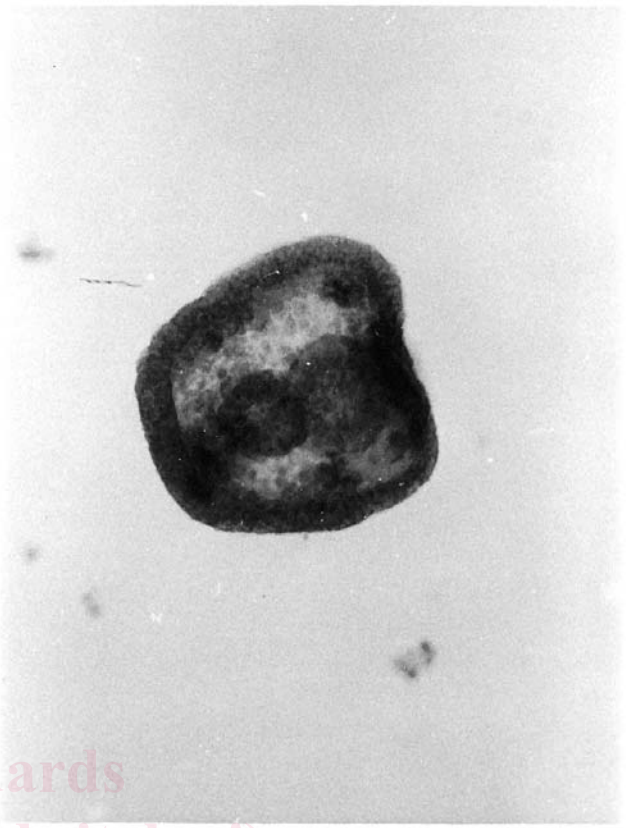

FIG. 2 (h) *(continued)*

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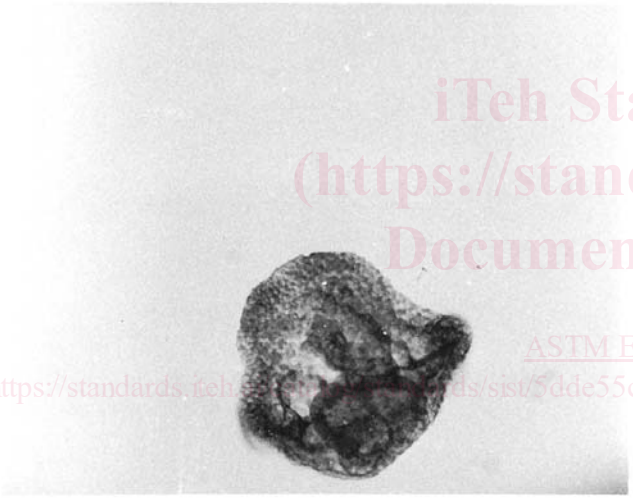

FIG. 2 (g) *(continued)*

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 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 (**10**), 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 (**11**). The test chambers should be rinsed with dilution water just before use.

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 into normal larvae. It is also highly recommended that each

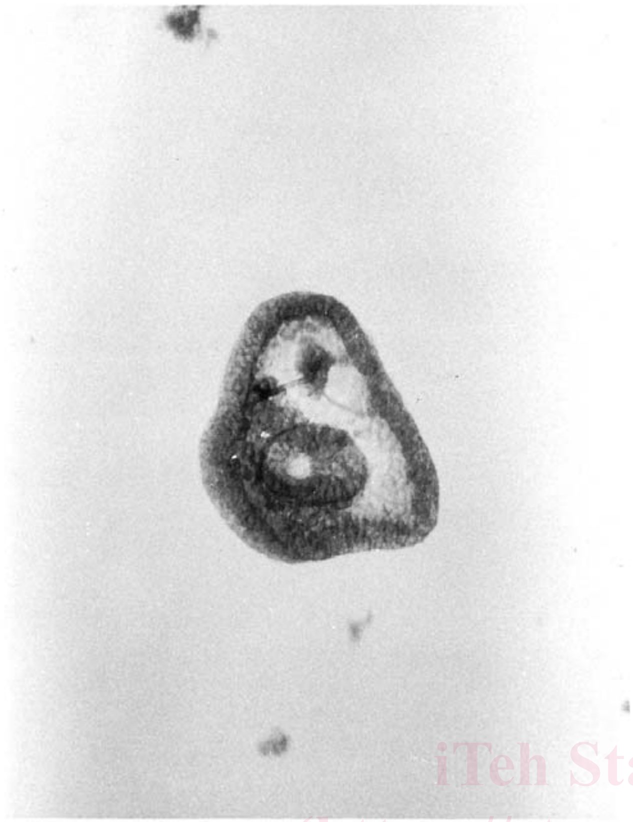

FIG. 2 (i) *(continued)*

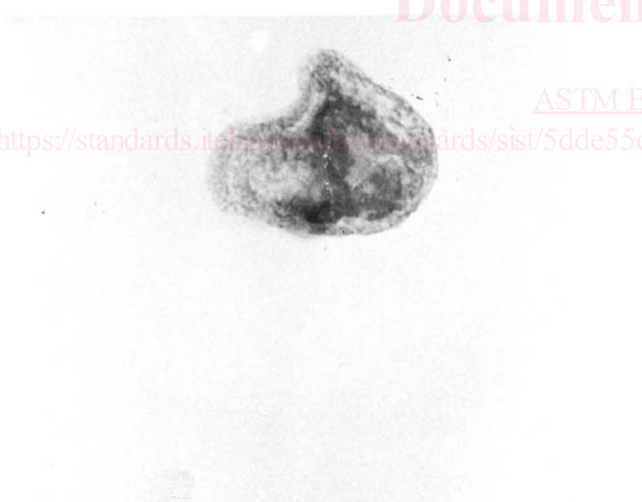

FIG. 2 (j) *(continued)*

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 (**12**), recommended handling procedures (**13**), 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 (**14**) and with materials that are, or are suspected of being, carcinogenic (**15**).

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 **Warning—**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.

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7.6 *Precaution*—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.

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

8. Dilution Water

8.1 *Requirements:*

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 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, certain toxic algae species are present such as some dinoflagellates (**16**).

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 or parts per thousand (ppt) (17) and within a test should not vary by more than 1 ppt among treatments or any renewals during a test. When a test is conducted on an effluent, brine, drilling mud, or other material that affects salinity when mixed with dilution water, it might be desirable to adjust salinity by adding artificial sea salts or natural saline brines to raise the salinity or by adding distilled or deionized water to lower the salinity. If salts are added, the adjusted material should be well mixed and allowed to equilibrate for minimum of 2 h with gentle aeration (24 h preferred) and salinity confirmed as salts dissolve. The addition of artificial salts can produce artifactual toxicity that is ameliorated with aging.

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

8.2.2 Reconstituted water is prepared by adding a commercially available sea salt or specified amounts of reagent-grade chemicals (18) to high-quality water with (*1*) conductivity less than 1 µS/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 1—Add the following reagent-grade chemicals (18)[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	700 mg
$CaCl_2 \cdot 2H_2O$	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 E729). 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 (15). 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 (19). Some organic chloramines, however, react slowly with sodium bisulfite (20). 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 (21), 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

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stones, surface aerators, or column aerators (22) 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 (23) 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 (22, 24).

8.3.2 The salinity and pH of dilution water may be adjusted by the addition of appropriate reagent-grade chemicals (18),[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 (25) 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 (26).

9. Test Material

9.1 *General*—Test materials may include a range of sample types such as effluents, materials or products, specific chemicals, solvents, oils, surface waters, drilling fluids, stormwater, and sediments. For chemical or product testing studies the test material should be reagent-grade (18)[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 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 (27). 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 (28). Other water-miscible organic solvents

[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.

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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 **(18)** 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 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

Test Specimen
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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. 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. The echinoid species, like bivalve mollusks, are seasonally gravid, but con often be maintained in a gravid condition with 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 When gravid, 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.

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)	15 (29)
Strongylocentrotus droebachiensis	8 to 12	12

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 (**30**) and kill the adults. Following proper acclimation to the laboratory holding conditions, some test animals can be test-spawned to check gamete quality if enough animals 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 also recommended that sand dollars be provided a bed of 1-2 in. of sand from their collection location or similar during holding periods lasting more than a few days. 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 tanks 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 holding tanks should be drained, all animals removed to other tanks, and the tanks 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