Designation: ~~E1563 – 21~~ 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~~ 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**

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.~~

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]

[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.

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 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).~~

3.1.1 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 ~~standards~~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 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

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

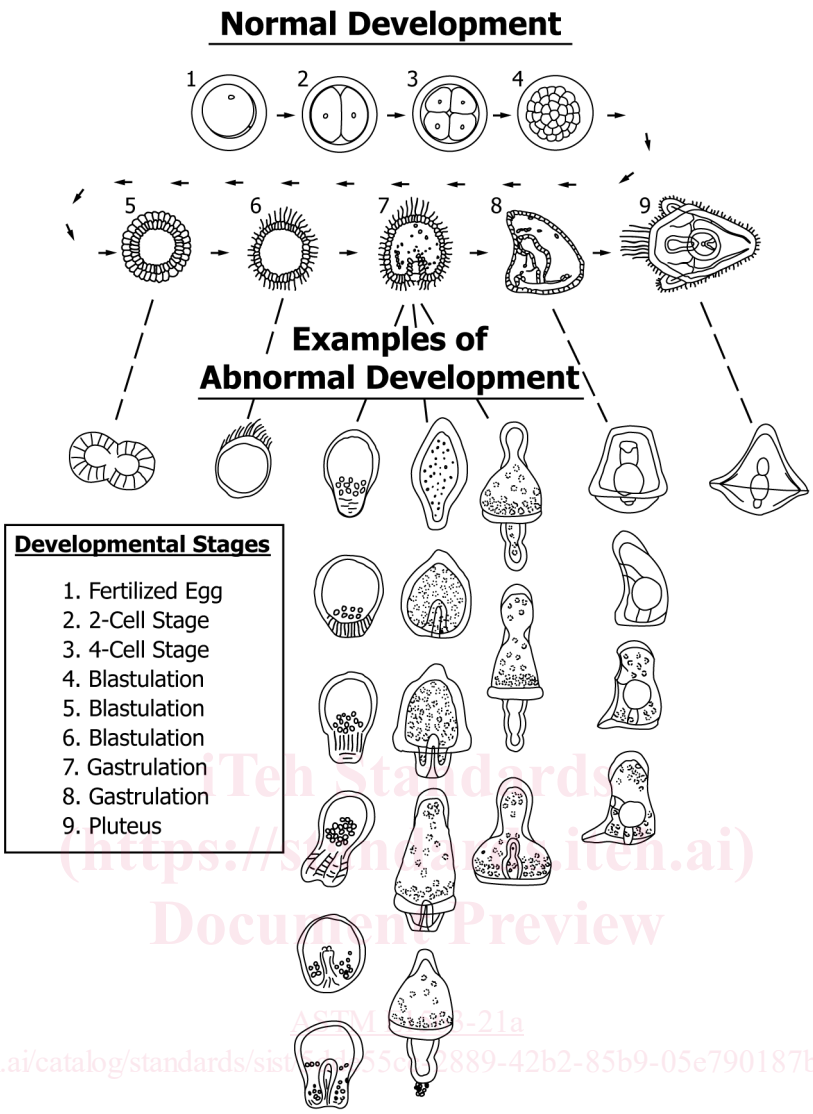

FIG. 1 Drawings Exemplifying Key Developmental Stages of Normal Echinoid Larvae Occurring During the First 48 to 96 h of Development and Examples of Abnormal or Arrested Development (6)

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**).

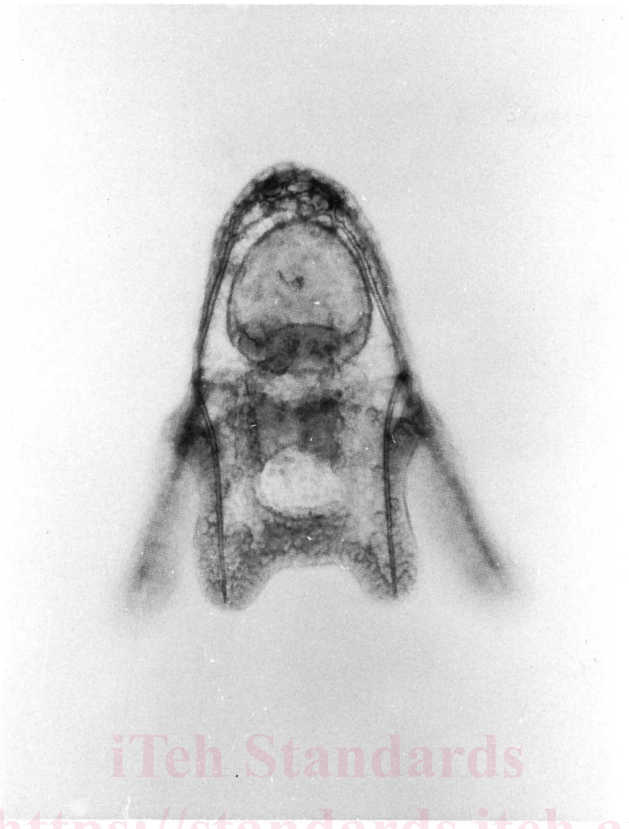

FIG. 2 (a) Examples of Normal and Abnormal Development of Purple Sea Urchin (*Strongylocentrotus purpuratus*) Embryos at the Conclusion of a 72 to 96-h Toxicity Test. Figs. 2a and 2b Show Examples of Normal Echinopluteus' with Four Distinct Arms and Good Symmetrical Development. Fig. 2c Shows a Borderline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

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.

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.

FIG. 2 (b) *(continued)*

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.

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

FIG. 2 (c) *(continued)*

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 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 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

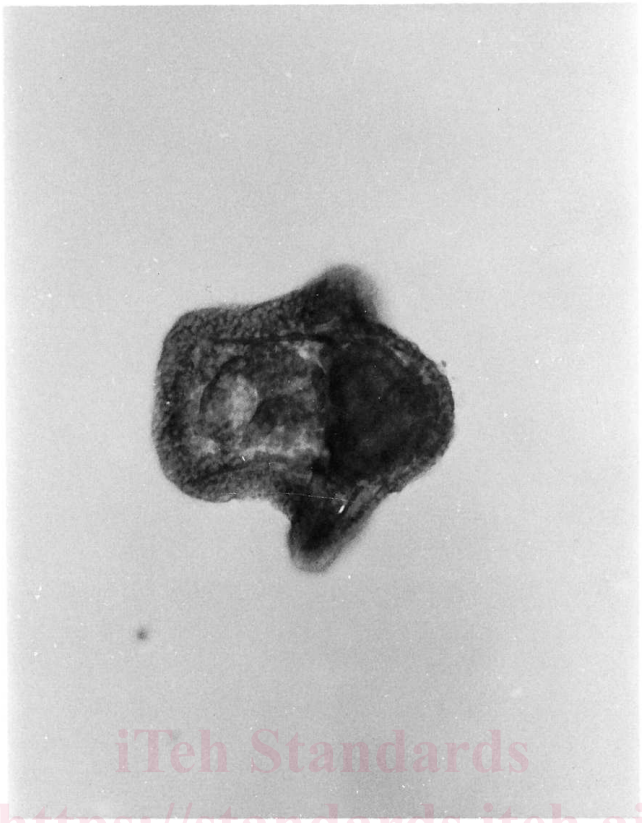

FIG. 2 (d) *(continued)*

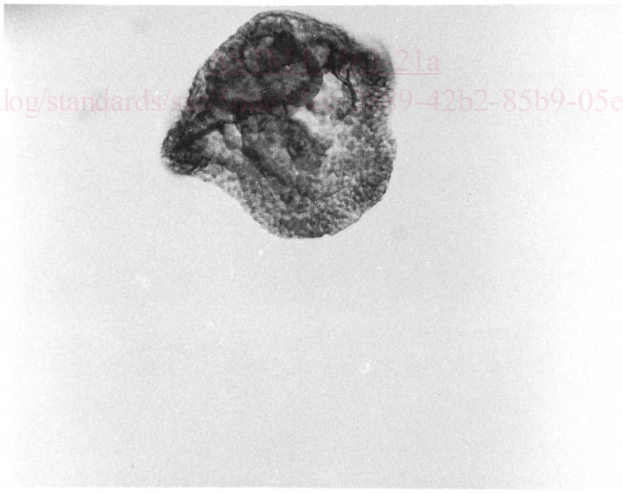

FIG. 2 (e) *(continued)*

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

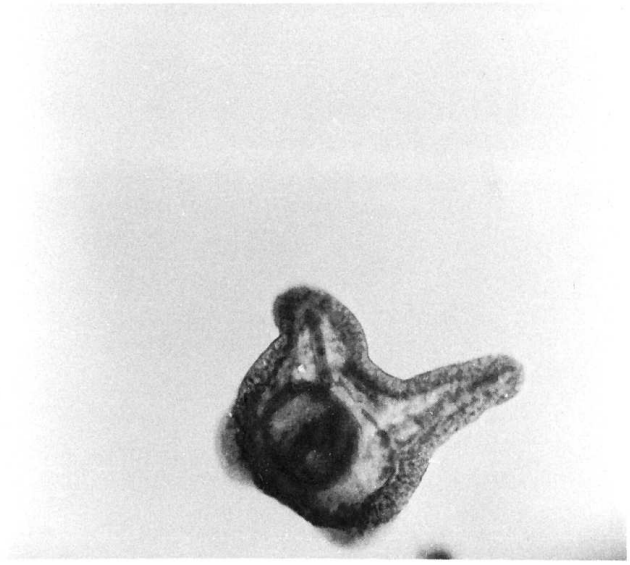

FIG. 2 (f) *(continued)*

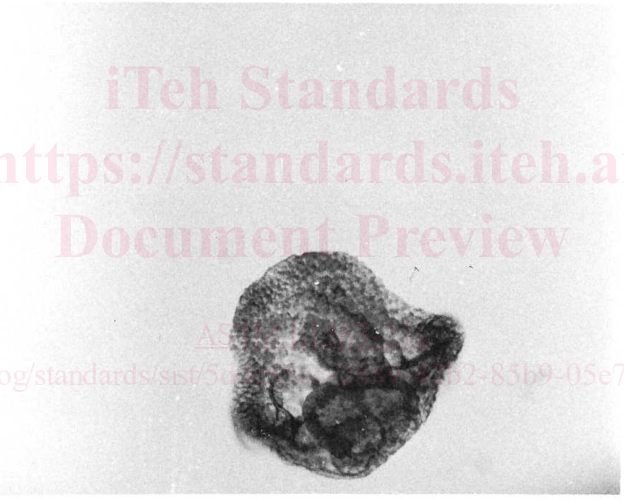

FIG. 2 (g) *(continued)*

embryos that develop 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 (**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.

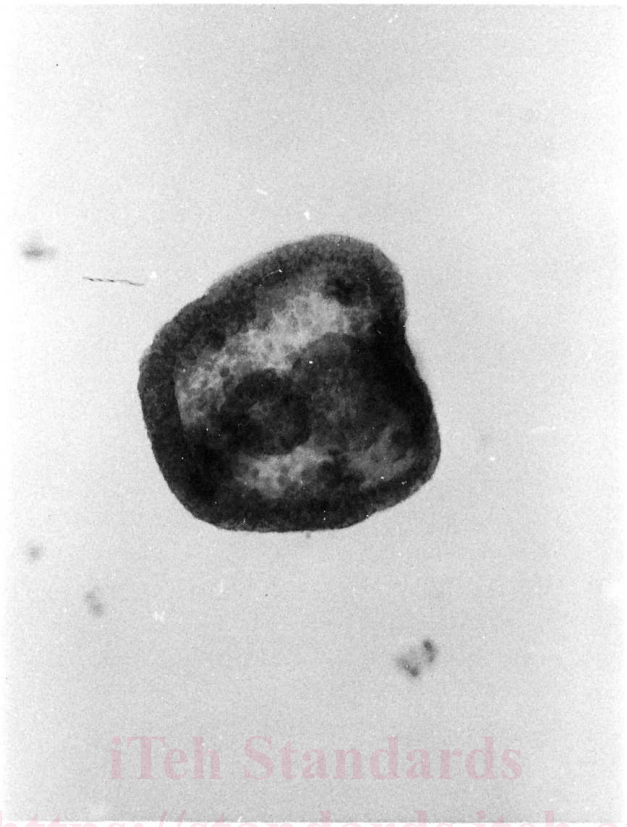

FIG. 2 (h) *(continued)*

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.

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

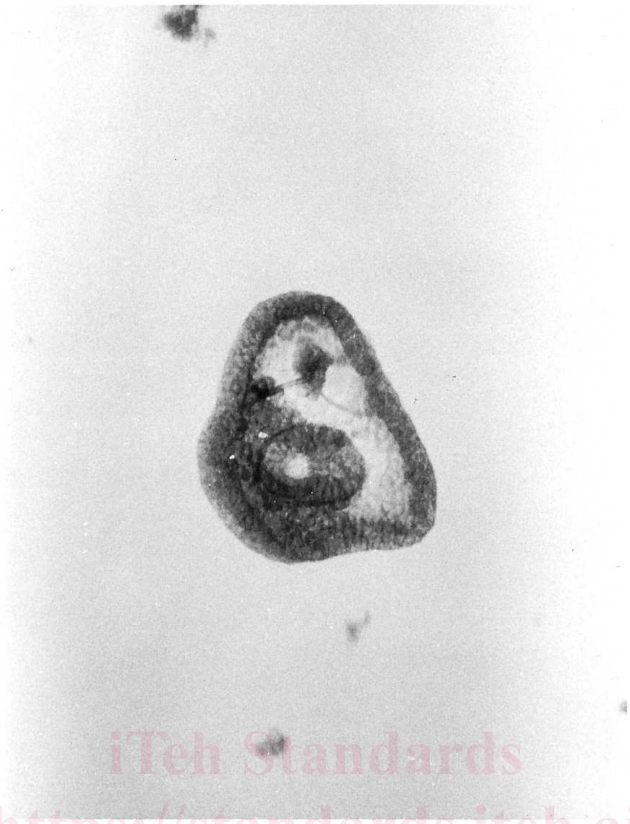

FIG. 2 (i) *(continued)*

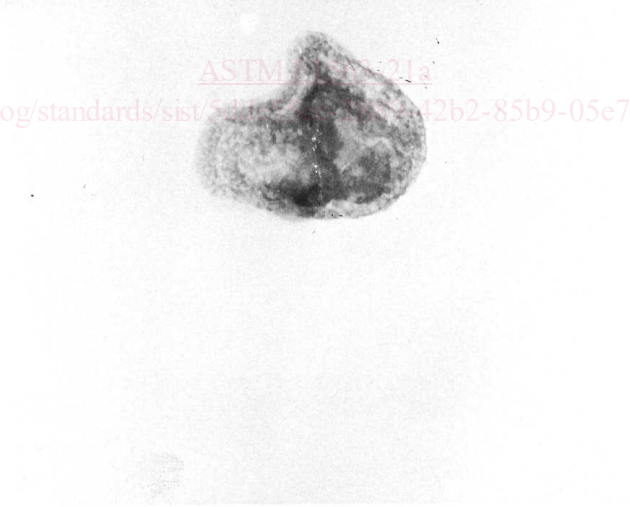

FIG. 2 (j) *(continued)*

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.

8.2.3 *Natural Dilution Water*—If natural salt water is used, it should be obtained from an uncontaminated, uniform quality source.

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.