Designation: E1611 – 21

Standard Guide for Conducting Sediment Toxicity Tests with Polychaetous Annelids[1]

This standard is issued under the fixed designation E1611; 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 adverse effects of potentially contaminated sediment, or of a test material added experimentally to contaminated or uncontaminated sediment, on marine or estuarine infaunal polychaetes during 10-day or 20 to 28-day exposures. These procedures are useful for testing the effects of various geochemical characteristics of sediments on marine and estuarine polychaetes and could be used to assess sediment toxicity to other infaunal taxa, although modifications of the procedures appropriate to the test species might be necessary. Procedures for the 10-day static test are described for *Neanthes arenaceodentata* and *Alitta virens* [2] (formerly *Nereis virens* and *Neanthes virens*) and for the 20 to 28-day static-renewal sediment toxicity for *N. arenaceodentata*.

1.2 Modifications of these procedures might be appropriate for other sediment toxicity test procedures, such as flow-through or partial life-cycle tests. The methods outlined in this guide should also be useful for conducting sediment toxicity tests with other aquatic taxa, although modifications might be necessary. Other test organisms might include other species of polychaetes, crustaceans, and bivalves.

1.3 Other modifications of these procedures might be appropriate for special needs or circumstances. Although using appropriate procedures is more important than following prescribed procedures, the results of tests conducted using unusual procedures are not likely to be comparable to those of many other tests. Comparisons of the results obtained using modified and unmodified versions of these procedures might provide useful information concerning new concepts and procedures for conducting sediment tests with infaunal organisms.

1.4 These procedures are applicable to sediments contaminated with most chemicals, either individually or in formulations, commercial products, and known or unknown mixtures. These procedures can be used with appropriate modifications to conduct sediment toxicity tests on factors such as temperature, salinity, dissolved oxygen (DO), and natural sediment characteristics (for example, particle size distribution, organic carbon content, and total solids). These procedures can also be used to conduct bioconcentration tests and in situ tests, and to assess the toxicity of potentially contaminated field sediments, or of materials such as sewage sludge, oils, particulate matter, and solutions of toxicants added to sediments. A median lethal concentration (LC50) or median sublethal effect concentration (EC50) of toxicants or of highly contaminated sediment mixed into uncontaminated sediment can be determined. Materials adhering to sediment particles or dissolved in interstitial water can be tested.

1.5 The results of 10-day toxicity tests with contaminated sediments can be reported as a LC50 if a series of concentrations is tested or as a percent mortality relative to a control or reference sediment. The results of 20 to 28-day toxicity tests with contaminated sediments can be reported as a LC50 if a series of concentrations is tested or as a percent mortality or growth relative to a control or reference sediment.

1.6 This guide is arranged as follows:

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

Current edition approved Nov. 1, 2021. Published December 2021. Originally approved in 1994. Last previous edition approved in 2013 as E1611 - 00(2013). DOI: 10.1520/E1611-21.

[2] World Register of Marine Species (WoRMS) at https://www.marinespecies.org/aphia.php?p=taxdetails&id=234851

*A Summary of Changes section appears at the end of this standard

1.7 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.8 *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 hazards statements are given in Section 8.

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

2. Referenced Documents

2.1 *ASTM Standards:*[3]

D1129 Terminology Relating to Water

D3976 Practice for Preparation of Sediment Samples for Chemical Analysis

D4447 Guide for Disposal of Laboratory Chemicals and Samples

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

E943 Terminology Relating to Biological Effects and Environmental Fate

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

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

E1241 Guide for Conducting Early Life-Stage Toxicity Tests with Fishes (Withdrawn 2022)[4]

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

E1847 Practice for Statistical Analysis of Toxicity Tests Conducted Under ASTM Guidelines (Withdrawn 2022)[4]

SI10-02 IEEE/ASTM SI 10 American National Standard for Use of the International System of Units (SI): The Modern Metric System

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 the strongest possible recommendation, just short of an absolute requirement, that is, to state that this test ought to be designed to satisfy the specific condition, unless the purpose of the test requires a different design. "Must" is used only in connection with factors that relate directly to the acceptability of the test (see Section 14). "Should" is used to state that the specific condition is recommended and ought to be met if possible. Although the violation of one "should" is rarely a serious matter, the 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 Terminologies D1129 and E943, Guides E729, E1023, E1192, E1367, and E1525. For an explanation of units and symbols, refer to SI10-02 IEEE/ASTM SI 10.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *ash-free dry weight*—Organism weight determined by subtracting the standard dry weight from the ashed (550 °C dried) weight to remove the inorganic contribution.

3.2.1.1 *Discussion*—AFDW is therefore the weight of the organic content of the organism.

3.2.2 *clean sediment, n*—sediment that does not contain concentrations of toxicants that cause apparent stress to the test organism or reduce their survival.

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

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

3.2.3 *control sediment, n*—a sediment that is essentially free of contaminants and is used routinely to assess the acceptability of a test.

3.2.4 *estimated individual dry weight, n*—a value that is calculated by dividing the total dry weight by the number of surviving worms within a replicate.

3.2.5 *exposure, n*—contact with a chemical or physical agent (see Terminology E943).

3.2.6 *interstitial water, n*—water occupying the space between sediment or soil particles; a synonym for *pore water*.

3.2.7 *overlying water, n*—the water added to the test chamber over the solid phase of the sediment in a toxicity test.

3.2.8 *pore water, n*—water occupying the space between sediment particles; a synonym for *interstitial water*.

3.2.9 *reference sediment, n*—a whole sediment near the area of concern used to assess sediment conditions exclusive of material(s) of interest.

3.2.10 *sediment, n*—particulate material that usually lies below water. Formulated particulate material that is intended to lie below water in a test.

3.2.11 *short-term toxicity tests, n*—generally used to determine the concentration of test material that produces a specific adverse effect on a specific percentage of test organisms during a short exposure (for example, 10 days).

3.2.11.1 *Discussion*—Because death is obviously an important adverse effect and is detected easily for many species, the most common end point is survival. Both survival and growth are used as end points in the 20 to 28-day test. Effect on 50 % of a group of test organisms is the most experimentally reproducible and easily determined measure of toxicity, and 96 h is often a convenient, useful exposure duration. The measure used most often in acute tests is therefore the 96-h LC50 value. In tests with contaminated sediment, however, the exposure period is generally 10 days or 20 to 28 days. Death is used as the measure of toxicity in the 10-day test; the results are reported as a 10-day LC50 or response relative to a control or reference sediment. Dry body weight is used as the measure of effect in the 20 to 28-day test or the 20 to 28-day LC50 if dilutions are tested. Ash-free dry weight may also be measured to differentiate the influence of the gut contents from final tissue mass.

3.2.12 *spiked sediments, n*—a sediment to which a material has been added for experimental purposes.

3.2.13 *toxicity, n*—the property of a material or combination of materials that affects organisms adversely (see Terminology E943).

3.2.14 *whole sediment, n*—sediment that has not had material extracted or removed.

4. Summary of Guide

4.1 Two procedures are used to measure the relative toxicity of marine or estuarine sediments to polychaetes: (*1*) the 10-day test, which measures the effect of contaminated sediment on survival; and (*2*) the 20-day to 28-day test, which determines the effect of contaminated sediment on survival and growth. If smaller worms are used, such as *N. arenaceodentata*, a minimum of five worms are placed in a 1-L glass test chamber with a minimum sediment depth of 2 cm to 3 cm and the overlying water aerated. The survival of the worm exposed to the test sediment is compared with the survival in a negative control or reference sediment in the 10-day test. The same procedure is used in the 20-day to 28-day test, except for the test duration (see Annex A1). If larger worms are used, such as *A. virens*, a minimum of ten worms are placed in beakers or glass aquaria (4 L to 37 L) with a minimum sediment depth of 5 cm and the overlying water aerated. A negative control or reference sediment is used to give a measure of the acceptability of the test by (*1*) providing evidence of the health and relative quality of the test organisms, suitability of the overlying water, test conditions, and handling procedures, etc.; and (*2*) providing a basis for interpreting data obtained from the test sediments.

4.1.1 The percent survival of polychaetes exposed to field-collected sediment is compared to those exposed to a negative control or reference sediment in 10-day tests. The survival and body weight of the animals surviving in field-collected sediment is compared to those exposed to negative control or reference sediment in 20 to 28-day tests. The toxicity of field sediments may also be assessed by testing dilutions of highly toxic test sediments with clean sediments to obtain information on the toxicity of proportions of that sediment.

4.1.2 The toxicity of a material added experimentally to sediments can be expressed by analyzing the survival and growth data to determine a LC50 for the material for the duration of exposure.

4.2 The annexes at the end of this guide outline the techniques for collecting, identifying, holding, and testing *N. arenaceodentata* and *A. virens* and culturing *N. arenaceodentata*.

5. Significance and Use

5.1 The test procedure covered in this guide is not intended to simulate exactly the exposure of benthic polychaetes to chemicals under natural conditions, but rather to provide a conveniently rapid, standard toxicity test procedure yielding a reasonably sensitive indication of the toxicity of materials in marine and estuarine sediments.

5.2 The protection of a community of organisms requires averting detrimental contaminant-related effects on the number and health of individuals and species within that population. Sediment toxicity tests provide information on the toxicity of test materials in sediments. Theoretically, projection of the most sensitive species within a community will protect the community as a whole.

5.3 Polychaetes are an important component of the benthic community. They are preyed upon by many species of fish, birds, and larger invertebrate species, and they are predators of smaller invertebrates, larval stages of invertebrates, and, in

some cases, algae, as well as organic material associated with sediment. Polychaetes are sensitive to both organic and inorganic chemicals (**1, 2**).[5] The ecological importance of polychaetes, their wide geographical distribution and ability to be cultured in the laboratory, and sensitivity to chemicals, make them appropriate toxicity test organisms.

5.4 An acute or 10-day toxicity test is conducted to obtain information concerning the immediate effects to a test material on a test organism under specified experimental conditions for a short period of time. An acute toxicity test does not necessarily provide information concerning whether delayed effects will occur, although a post-exposure observation period, with appropriate feeding, if necessary, could provide such information.

5.5 The results of acute sediment toxicity tests can be used to predict acute effects likely to occur on aquatic organisms in field situations as a result of exposure under comparable conditions, except that (*1*) motile organisms might avoid exposure when possible and (*2*) toxicity to benthic organisms can be dependent on sediment characteristics, the dynamics of equilibrium partitioning, and the route of exposure to the benthic organisms.

5.6 The polychaete sediment toxicity test might be used to determine the temporal or spatial distribution of sediment toxicity. Test methods can be used to detect horizontal and vertical gradients to toxicity. Mortality data can be used to indicate the relative toxicity of field-collected sediments.

5.7 The results of acute tests with toxicants added experimentally to sediments can be used to compare the acute sensitivities of different species and acute toxicities of different test materials, and to define the effects of various environmental factors on the results of such tests.

5.8 The results of acute sediment toxicity tests are useful for studying the biological availability of, and structure-activity relationships between, test materials in sediment.

5.9 The results of acute sediment toxicity tests might be an important consideration when assessing the hazards of materials to aquatic organisms (see Guide E1023) or when deriving the sediment quality for aquatic organisms (**3**). Sediment toxicity tests might be useful for making decisions regarding the extent of remedial action necessary for contaminated sites.

5.10 A 10-day test provides data on the short-term effects that are useful for comparisons to other species but does not provide information on delayed effects. Results of the 20-day to 28-day sediment toxicity test, which measures growth in addition to survival, can be useful indicators of the effects of contaminated sediments over a longer time period.

6. Interferences

6.1 The methodology continues to develop and evolve with time and research needs. There are limitations to the methods described in this guide because of the developmental nature of sediment toxicity testing.

6.2 The results of sediment toxicity tests will depend partly on the temperature, water quality, physical and chemical properties of the test sediment, condition of the test organisms, exposure technique, and other factors. Factors potentially affecting the results from static sediment toxicity tests might include the following:

6.2.1 The alteration of field sediments in preparation for laboratory testing.

6.2.1.1 Maintaining the integrity of the sediment environment during its removal, transport, and testing in the laboratory, which is extremely difficult (Guide E1391). The sediment environment is composed of a myriad of microenvironments, redox gradients, and other interacting physiochemical and biological processes. Many of these characteristics influence the sediment toxicity and bioavailability to benthic and planktonic organisms, microbial degradation, and chemical sorption. Any disruption of this environment complicates the interpretations of treatment effects, causative factors, and in situ comparisons.

6.2.1.2 Testing of sediments at temperatures or salinities other than those at which they were collected might affect chemical solubility, partitioning coefficients, and other physical and chemical characteristics.

6.2.2 Interactions among the sediment particles, overlying water, interstitial water, humic substances, and the sediment to overlying water ratio.

6.2.3 Interactions among chemicals that might be present in the test sediment.

6.2.4 The realism of using spiked sediment (that is, whether the spiked sediment is at equilibrium and mixed evenly or represents the bioavailability of naturally occurring chemicals).

6.2.5 Photolysis and other processes degrading the test chemicals.

6.2.6 Maintaining an acceptable quality of the overlying water.

6.2.7 Excess food might change the sediment partitioning and water quality parameters.

6.2.8 Resuspension of sediment during the toxicity test.

6.2.9 A limited opportunity for biological observation during the test because organisms bury in the sediment.

6.2.10 The natural geochemical properties of test sediment collected from the field that might not be within the tolerance limits of the test organisms.

6.2.11 It may be difficult to recover the worms from sediment if growth is stunted.

6.2.12 Endemic organisms that might be present in field-collected sediments, including (*1*) predators; (*2*) species that might be the same as or closely related to the test species; (*3*) microorganisms (for example, bacteria and molds); and (*4*) algae colonizing sediment and test chamber surfaces.

6.3 Static tests might not be applicable to materials that are highly volatile or are rapidly transformed biologically or chemically. Furthermore, the overlying water quality might change considerably from the initial overlying water. The procedures can usually be applied to materials that have a high oxygen demand because the experimental chambers are aerated. Materials dissolved in interstitial waters might be removed from solution in substantial quantities by adsorption to

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

sediment particles and to the test chamber during the test. The dynamics of chemical partitioning between solid and dissolved phases at the initiation of the test should therefore be considered, especially in relation to assumptions of chemical equilibrium.

7. Apparatus

7.1 *Facilities*—Beakers, aquaria, or tanks containing either clean (uncontaminated), natural sea water, or reconstituted sea water should be used for holding the polychaetes before a test. The holding tanks and any area used for manipulating live polychaetes should be located in a room or space separated from that in which the toxicity tests are to be conducted, stock solutions or test materials are to be prepared, or equipment is to be cleaned. The sea water should be monitored periodically to ensure a constant salinity. The holding tanks, water supply, or room in which they are kept should be equipped with temperature control. Aeration can be provided to ensure that the DO is greater than 60 % saturation and that there is adequate water circulation in the tanks.

7.2 *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 stock or test solutions should be chosen to minimize the sorption of test materials from water. Glass, Type 316 stainless steel, nylon, and polypropylene, or polyethylene should be used whenever possible to minimize dissolutions, leaching, and sorption, except that stainless steel should not be used in tests on metals in salt water. Fluorocarbon plastics should be avoided especially in studies evaluating these types of chemicals with increasing environmental concern (Per- and polyfluoroalkyl substances (PFAS)). Concrete and rigid plastics may be used for holding tanks and in the water-supply system, but they should be soaked, preferably in flowing dilution water, for a week or more before use (**4**). Brass, copper, lead, cast iron pipe, galvanized metal, and natural rubber must not contact the test sea water, stock solutions, or test sediment before or during the test. A specially designed system is usually necessary to obtain salt water from a natural water source (**5**). Tubing used in making up test sea water and in aerating the test chambers should be nontoxic vinyl. New tubing should be aged in sea water at least one week prior to use. Separate sieves, dishes, containers, and other equipment should be used to handle the test sediment or other toxic materials, and these should be kept and stored separately from those used to handle the live animals prior to testing. Items made from other materials not mentioned previously should not be used unless it has been shown that their use will not affect either the survival, growth, or reproduction of the polychaetes adversely.

7.3 *Test Chambers*—Species-specific information on test chambers is given in the annexes. The test chambers should be placed in either a temperature-controlled room or a water bath to minimize temperature fluctuations, and they should be aerated. Aeration can be provided as described in 13.1.

7.3.1 Test chambers are defined as the smallest physical unit between which there are no water connections in a toxicity test with aquatic organisms. The test chambers for both the 10-day and 20 to 28-day sediment toxicity tests are 1-L glass containers with an inside diameter of approximately 10 cm. An appropriate number of worms, as specified by the scope of the study (13.6.7), are placed within each test chamber, which gives each juvenile worm approximately 15 cm^2 of surface area. The chambers are covered with glass lids to reduce contamination of the contents and minimize evaporation of the water or test material. The test chambers are maintained at 20 °C ± 1 °C in either a shallow water bath or a constant temperature room. The test chambers should be aerated with air free of fumes, oil, and water. The air is delivered to the test chamber by non-toxic tubing connected to a 1 mL glass pipette that is suspended 3 to 4 cm below the water surface. Aeration should be bubbled into the test chambers at <100 bubbles/min or at a rate that maintains a ≥90 % dissolved oxygen (DO) concentration (see 13.1). Larger-sized specimens are used in some sediment toxicity tests, which requires a larger-sized test chamber. A surface area of approximately 30 cm^2 to 40 cm^2 per specimen is necessary when larger-sized worms are used.

7.4 *Cleaning*—The test chambers and other glassware and equipment used to store and prepare test sea water, stock solutions, and test sediments should be cleaned before use. New items should be cleaned before each use by washing with laboratory detergent; rinsing with water, a weak-miscible organic solvent, water, and acid (10 % nitric or hydrochloric acid); and rinsing twice with distilled, deionized, or dilution water. Metals, sulfides, and carbonate deposits are removed by the acid rinse, and organics are removed by the organic solvent rinse. A dichromate-sulfuric acid cleaning solution may be used in place of both the organic solvent and acid rinses, but it might attack silicone adhesives. At the end of each test, all items to be used again should immediately be (*1*) emptied; (*2*) rinsed with water; (*3*) cleaned by a procedure appropriate for removing the test material (for example, acid to remove metals and solvents to remove organics); and (*4*) rinsed at least twice with deionized, distilled, or dilution water. Acid is often used to remove mineral deposits, and 200 mg of hypochlorite (ClO^-) per litre is often used to remove organic matter and for disinfection. (A solution containing approximately 200 mg of ClO^-/L may be prepared by adding 6 mL of liquid household chlorine bleach to 1 L of water. However, ClO^- is quite toxic to many aquatic animals (**6**) and is difficult to remove from some construction materials. It is often removed by soaking in a sodium thiosulfate, sodium sulfite, or sodium bisulfite solution, by autoclaving in distilled water for 20 min, or by drying the item and letting it sit for at least 24 h before use. An item cleaned or disinfected with hypochlorite should not be used unless it has been demonstrated at least once that the test polychaete species do not show signs of apparent stress, such as discoloration, unusual behavior, or death, when held for at least 48 h in static dilution water in which the item is soaking than when held in static dilution water containing a similar item that was not treated with ClO^-.) Glassware used only for live animals, not exposed to test material, may be cleaned using

only clean distilled or dilution water, since the use of detergents is sometimes detrimental to live organisms.

7.5 *Acceptability*—The acceptability of new holding or testing facilities should be demonstrated by conducting a non-toxicant test in which all test chambers contain sediment and clean sea water. The survival of the test species will demonstrate whether the facilities, water, control sediment, and handling techniques are adequate to result in acceptable (≥90 %) control level survival in the absence of toxicants.

8. Hazards

8.1 Many materials can affect humans adversely if precautions are inadequate. Skin contact with all test materials and their solutions should therefore be minimized by such means as wearing appropriate protective gloves (especially when washing equipment or putting hands into the 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 on toxicity to humans (**7**), recommended handling procedures (**8**), and chemical and physical properties of the test material should be studied before a test is begun. Special precautions might be necessary with radiolabeled test materials (**9**) and test materials that are, or are suspected of being, carcinogenic.

8.2 Field sediments to be tested, especially those from effluent areas, might contain organisms that can be pathogenic to humans. When dealing with these sediments, special precautions might include immunizations prior to sampling and the use of bactericidal soaps after working with the sediments.

8.3 Sediments collected from the field might be contaminated with unknown concentrations of many potentially toxic materials, and laboratory-prepared sediments might be spiked with high concentrations of toxicants. Any potentially contaminated sediments should be handled in a manner to minimize the exposure of researchers to toxic compounds. Mixing of toxic sediments in open containers, spiking of laboratory-prepared sediments, and loading of toxic sediments into the test chambers should be performed in a well-ventilated area, preferably a chemical fume hood. Face shields or protective goggles should be worn during any operations that might involve the accidental splashing of sediments, such as sieving, mixing, and loading into test chambers.

8.4 Health and safety precautions and applicable regulations for the disposal of stock solutions, overlying water from test chambers, test organisms, and sediments should be considered before beginning a test (see Guide D4447). Consideration of cost as well as detailed regulatory requirements might be necessary. Removal or degradation of the toxicants before disposal of the stock solutions, test sediments, and water is sometimes desirable for tests involving spiked sediments, with known toxicants.

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

8.6 Cleaning of the 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. Cleaning of the equipment with acids should be performed only in a well-ventilated area, and protective gloves and safety goggles should be worn. Hexane might also be used as a solvent for removing non-ionic organic compounds. However, acetone is preferable if only one organic solvent is used to clean equipment.

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

8.8 Concentrated acid should be added to water, not vice versa, to prepare dilute acid solutions. Opening a bottle of concentrated acid and adding concentrated acid to water should be performed only in a well-ventilated room or chemical fume hood.

9. Test Water

9.1 *General Requirements*—In addition to being available in adequate supply, water used in sediment toxicity tests should be acceptable to the test organisms and purpose of the test. The minimum requirement for acceptable water for use in sediment toxicity tests is that healthy test organisms survive (≥90 %) in the water with sediment for the duration of holding and testing without showing signs of disease or apparent stress such as unusual behavior, changes in appearance, or death. The water in which the test organisms are held prior to the test should be uniform in quality in that the concentration of chemicals and the range of temperature and salinity encountered during the holding period do not adversely affect the survival of the test organisms in the holding tanks or control treatments during the test. A better criterion for an acceptable sea water is that in which the test species will survive and grow.

9.2 *Source:*

9.2.1 *Natural Sea Water*—If natural salt water is used, it should be obtained from an uncontaminated area known to support a healthy, naturally reproducing population of the test organism or a comparably sensitive species. The water intake should be positioned to minimize fluctuations in quality and the possibility of contamination, and to maximize the concentration of DO to help ensure low sulfide and iron concentrations. A specially designed system might be necessary to obtain salt water from a natural water source (see Guide E729). The water should be monitored in accordance with 9.4 to ensure uniform quality. These precautions ensure that the test organisms are not stressed by the water quality during holding, acclimation, and testing and that the water quality does not affect the test results unnecessarily.

9.2.2 *Reconstituted Salt Water*—Reconstituted salt water can be prepared by adding a commercially available sea salt or specified amounts (see Guide E729 and Table 1) of reagent grade chemicals (**10-12**) to high-quality water with (*1*) a conductivity below 1 µS/cm and (*2*) either a total organic carbon (TOC) below 2 mg/L or a chemical oxygen demand (COD) below 5 mg/L. Commercial sea salt mixes should be "bioassay grade" without the addition of the metal chelating agent ethylenediaminetetraacetic acid (EDTA) or dechlorination chemicals such as sodium thioosulfate. These compounds are present in hobbyist salts and may mask the toxicity of

TABLE 1 Reconstituted Salt Water (from Guide E729)

NOTE 1—Add the following reagent grade (**10**) chemicals in the amounts and order listed to 890 mL of water. Each chemical must be dissolved before the next is added.[A]

Chemical	Amount
NaF	3 mg
$SrCl_2 \cdot 6H_2O$	20 mg
H_3BO_3	30 mg
KBr	100 mg
KCl	700 mg
$CaCl_2 \cdot 2H_2O$	1.47 g
Na_2SO_4	4.00 g
$MgCl_2 \cdot 6H_2O$	10.78 g
NaCl	23.50 g
$Na_2SiO_3 \cdot 9H_2O$	20 mg
$NaHCO_3$	200 mg

[A]If the resulting solution is diluted to 1 L, the salinity should be 34 g/kg ± 0.5 g/kg and the pH 8.0 ± 0.2. The desired test salinity is attained by dilution at the time of use. The reconstituted salt water should be stripped of trace metals.

environmental or spiked sediment samples. Acceptable water can usually be prepared using properly operated deionization or distillation units. Conductivity should be measured on each batch, and TOC or COD should be measured at least once per year and whenever significant changes might be expected. The TOC or COD should be measured on each batch if the water is prepared from a surface water. The reconstituted water should be aerated intensively before use. The solution should be filtered if a residue or precipitate is present. Problems have been encountered with some species in some salt reconstituted waters, but these problems have sometimes been overcome by aging the reconstituted water for one or more weeks. The salt water should meet the criteria given in 9.1.

9.2.3 Chlorinated water must never be used in the preparation of salt water for toxicity tests because residual chlorine and chlorine-produced oxidants are highly toxic to many aquatic animals (**6**). The use of dechlorinated water should be avoided because dechlorination is often incomplete. Municipal drinking water is not recommended for use because it often contains unacceptable high concentrations of metals in addition to residual chlorine, and the quality is often highly variable (see Guide E729).

9.3 *Preparation:*

9.3.1 Sea water used in the sediment toxicity test should be passed through a filter effective to 5 µm or less to remove suspended particles and organisms from the water. Water that might be contaminated with facultative pathogens should be passed through a properly maintained ultraviolet sterilizer (**13**) or filter with a pore size of 0.45 µm or less.

9.3.1.1 The salinity should be reduced by diluting the sea water with a high-quality deionized distilled water (see 9.2.2) if necessary. Salinity can be raised by the addition of clean filtered oceanic water, brine, or reagent grade chemicals in accordance with 9.2.2.

9.3.2 Fresh sea water used in the test should be prepared within two days of the test and stored in clean, covered containers until sediment and water are added to the test chambers. It might be necessary to age reconstituted sea water for one to two weeks before use. Sufficient water should be prepared at one time for all of the test chambers. Additional water might be required for sieving control sediment to adjust the salinity or for holding the test worms prior to the test.

9.3.3 The experimental design might require the use of sea water from the test sediment collection site for certain applications. Experimental treatments might involve manipulation of the test water conditions in other instances.

9.4 *Characterization*—The following items should be measured at least once each year, and more often if such measurements have not been made semiannually for at least two years:

9.4.1 Salinity, pH, particulate matter, TOC, organophosphorus pesticides, organic chlorine (or organochlorine pesticides and polychlorinated biphenyls (PCBs)), chlorinated phenoxy herbicides, ammonia, cyanide, sulfide, fluoride, iodide, nitrate, phosphate, sulfate, calcium, chromium, cobalt, copper, iron, lead, manganese, mercury, molybdenum, nickel, selenium, silver, tributyltin, and zinc.

9.4.2 More frequent monitoring might be necessary in estuarine areas, in which large diurnal, tidal, and seasonal variations in the concentrations of organics, heavy metals, and water quality might occur. In particular, daily measurements of salinity, temperature, and pH, and quarterly monitoring of other parameters over a tidal cycle, might be desirable.

9.4.3 The methods used (see 14.2) should either (*1*) be accurate and precise enough to characterize the toxicity test water adequately or (*2*) have detection limits below concentrations that have been shown to affect the test species adversely (**14**).

10. Test and Control Sediments

10.1 *General*—Before the preparation or collection of test sediment, an approved written procedure should be prepared for the handling of sediment that might contain unknown quantities of many potentially toxic chemicals (see Section 8). Sediments are spatially and temporally variable. Replicate samples should be collected to determine variance in sediment characteristics. Sediment should be collected with as little disruption as possible; however, subsampling, compositing, or homogenization of sediment samples may be necessary for some experimental designs. Sampling may cause loss of sediment integrity, change in chemical speciation, or disruption of chemical equilibrium (Guide E1391). A bethic grab or core should be used rather than a dredge to minimize disruption of the sediment sample. Sediment should be collected from a depth that will represent expected exposure.

10.2 *Characterization*—Sediments chosen for use should be characterized, and at least the following should be determined: salinity, pH, ammonia, hydrogen sulfide, organic carbon content (TOC or total volatile solids), particle size distribution (percent sand, silt, and clay), and percent water content. Other analyses on sediments might include biological oxygen demand, chemical oxygen demand, Eh or pE, total inorganic carbon, metals, synthetic organic compounds, oil and grease, organosilicones, and petroleum hydrocarbons. Interstitial water might also be analyzed as described in 14.4 and in Test Method E1706. Toxicological results can identify samples that should be subjected to more intensive physical, chemical, or biological testing.

10.3 *Control Sediment:*

10.3.1 *Collection*—Control sediment should be collected from the polychaete collection site or from another area that is within the geochemical requirements of the test species and can provide a nontoxic reference sediment for evaluation of the condition of the test population subject to laboratory procedures, and for statistical comparison with the test sediment (see Guide E1847). Control sediment should be brought to the sieving area in a clean container. Any water overlying the sediment or used to wash the sediment into the container should be saved so that any fine particles contained in the water can be recombined into the sediment. Any sediment showing evidence of contamination (for example, oil sheen) should be discarded. As the sediment is collected, the bottom temperatures, salinity, and sediment temperature should be recorded, and a composite sediment sample from all shovelful, dredge hauls, or grabs should be collected for analysis of the water content, particle size distribution, and organic content.

10.3.2 Control or reference sediment should be characterized empirically as described in 10.2 at least annually.

10.3.3 *Sieving*—A separate clean container should be set up to sieve and contain the control sediment. Control sediment should be sieved twice: first to remove individuals of the test species and other macrobenthos, and second to adjust interstitial water to the test salinity if necessary. Water for sieving should be clean sea water prepared in accordance with Section 9. The entire contents of the collecting basin, including water and suspended particles, should be sieved (for example, through a 0.5 mm screen) without allowing overflow from the sieving container. After the first sieving, the sediment should be left undisturbed for a sufficient time to allow the settling of fine particles (usually overnight). The overlying water should then be decanted and the sediment resieved (for example, through a 0.5 mm screen) into water of a salinity calculated to bring the interstitial water salinity to the test level, taking into account the estimated quantity and salinity of the interstitial water. Again, the overlying water should be decanted, and the sediment should be mixed thoroughly to distribute evenly the fine particles that settle on the surface.

10.3.4 *Storage*—The control sediment should be stored covered, in clean glass or rigid plastic containers, at 4 °C ± 3 °C until the test chambers are prepared. The sediment should be stored covered in the dark and must not be frozen or allowed to dry during storage (Guide E1391).

10.4 *Field-Collected Test Sediment:*

10.4.1 *Collection*—The sediment for metals should be stored in the absence of air to minimize the oxidation of reduced forms. Nitrogen can be used to fill the headspace in the container. Glass containers are recommended for sediments polluted with either metals or organic compounds, although high-density polyethylene, polypropylene, or PTFE containers are also acceptable. PTFE containers should be avoided if evaluating these types of chemicals or chemical byproducts (Per- and polyfluoroalkyl substances (PFAS)). Remove large organisms and extraneous material, such as bivalves or twigs, from the sediment before storing.

10.4.2 Since the chemicals of concern and influencing sediment characteristics are not always known, it is desirable to hold the sediments after collection in the dark at 4 °C. Traditional convention has held that sediment tests should be started as soon as possible following collection from the field, although actual recommended storage times range from two weeks (Guide E1391) to less than eight weeks (USEPA-USACE, 1998) (**15**). Discrepancies in recommended storage times reflected a lack of data concerning the effects of long-term storage on the physical, chemical, and toxicological characteristics of the sediment. However, numerous studies have recently been conducted to address issues related to sediment storage (see Refs (**16-22**). The conclusions and recommendations offered by these studies vary substantially and appear to depend primarily upon the type or class of chemical(s) present. Considered collectively, these studies suggest that the recommended guidance that sediments be tested sometime between the time of collection and 8 weeks storage is appropriate. Additional guidance is provided below and in Guide E1391 and Test Method E1706.

10.4.3 Extended storage of sediments that contain high concentrations of labile chemicals (for example, ammonia, volatile organic compounds) may lead to a loss of these chemicals and a corresponding reduction in toxicity or bioavailability. Under these circumstances, the sediment should be tested as soon as possible after collection, but not later than within 2 weeks (**20**). Sediments that exhibit low-level to moderate toxicity or contamination can exhibit considerable temporal variability in toxicity or contamination, although the direction of change is often unpredictable (**18,19,22**). For these types of sediments, the recommended storage time of <8 weeks may be most appropriate. In some situations, a minimum storage period for low-to-moderately contaminated sediments may help reduce variability. For example, DeFoe and Ankley (1998) (**22**) observed high variability in survival during early testing periods (for example, <2 weeks) in sediments with low toxicity. DeFoe and Ankley (1998) (**22**) hypothesized that this variability partially reflected the presence of indigenous predators that remained alive during this relatively short storage period. Thus, if predatory species are known to exist, and the sediment does not contain labile chemicals, it may be desirable to store the sediment for a short period before testing (for example, 2 weeks) to reduce potential for interferences from indigenous organisms. Sediments that contain comparatively stable compounds (for example, high molecular weight compounds such as PCBs) or which exhibit a moderate-to-high level of toxicity, typically do not vary appreciably in toxicity in relation to storage duration (**19,22**). For these sediments, long-term storage (for example, >8 weeks) can be undertaken.

10.4.4 If sediment is collected from multiple field samples and pooled to meet technical objectives, the sediment should be homogenized thoroughly by stirring or mixing by hand, or with the aid of a rolling mill as described in 10.7.1.1 (see Guide E1391).

10.4.5 Additional samples may be taken from the same grab sample for other kinds of sediment analyses (see 10.2). The sediment temperature, interstitial water salinity, pH, and Eh can be recorded in the field. Qualitative description of the sediment might include the color, texture, presence of macroscopic plants and animals, and tracks or burrows. The odor of

the sediment can be recorded if any is noted at the time of sampling or subsampling, but monitoring the odor specifically should be avoided, especially if the odor is associated with potentially hazardous chemical chemicals. A core or the remainder of the sediments in the grab can be sieved to provide a macrobenthic sample.

10.4.6 The natural geochemical properties of test sediment collected from the field must be within the tolerance limits of the test species. The limits for the test species should be determined experimentally in advance. However, many polychaetes can live in a wide range of sediment particle sizes **(23)**.

10.5 *Reference Sediment*—A whole sediment near the area of interest used to assess sediment conditions exclusive of the material(s) of concern. See Test Method E1706 for additional detail on reference sediment.

10.6 *Laboratory-Spiked Test Sediment:*

10.6.1 Test sediment can also be prepared in the laboratory by manipulating the properties of control sediment. This can include adding various concentrations of toxic chemicals, highly toxic sediment, or complex waste mixtures (for example, sewage sludge) to the clean sediment **(24)**. The toxicity of substances either dissolved in the interstitial water or adsorbed to sediment particles can be determined experimentally. Limited studies have been conducted comparing appropriate methods for spiking chemicals in sediment. Additional research is needed before more definitive recommendations for spiking of sediment can be outlined in this guide. The guidance provided in the following sections has been developed from a variety of sources. Spiking procedures that have been developed using one sediment or test organism may not be applicable to other sediments or test organisms. See USEPA (1997) and Guide E1391 for additional detail regarding sediment spiking techniques.

10.6.2 *Test Chemicals*—Chemicals added to sediment experimentally should be reagent grade **(10)** or better, unless a test on a formulation, commercial product, or technical-grade or use-grade material is required specifically. The following should be known concerning the chemical used before a test is begun: identities of major ingredients and impurities, solubility in test water, estimated toxicity to the test species and to humans, and recommended handling and disposal procedures.

10.6.3 Test sediment can be prepared by manipulating the properties of a control sediment. Additional research is needed before formulated sediments are used routinely for sediment spiking procedures (for example, identifying standardized and representative sources of organic carbon; see Test Method E1706). Mixing time **(25)** and aging **(26-28)** of spiked sediment can affect bioavailability of chemicals in sediment. Many studies with spiked sediment are often started only a few days after the chemical has been added to the sediment. This short time period may not be long enough for sediments to equilibrate with the spiked chemicals (Section 10.6.4.3). Consistent spiking procedures should be followed in order to make interlaboratory comparisons. It is recommended that spiked sediment be aged at least one month before starting a test; however equilibration for some chemicals may not be achieved for long periods of time. See USEPA (1997)**(29)**, Guide E1391 and Test Method E1706 for additional detail regarding sediment spiking. **(30)**

10.6.4 The test material(s) should be at least reagent grade, unless a test using a formulated commercial product, technical-grade, or use-grade material is specifically needed. Before a test is started, the following should be known about the test material: (1) the identity and concentration of major ingredients and impurities, (2) water solubility in test water, (3) log Kow, BCF (from other test species), persistence, hydrolysis, and photolysis rates of the test substance, (4) estimated toxicity to the test organism and to humans, (5) if the test concentration(s) are to be measured, the precision and bias of the analytical method at the planned concentration(s) of the test material, and (6) recommended handling and disposal procedures. Addition of test material(s) to sediment may be accomplished using various methods, such as a: (1) rolling mill, (2) feed mixer, or (3) hand mixing (Guide E1391; USEPA 1997) **(29)**. Modifications of the mixing techniques might be necessary to allow time for a test material to equilibrate with the sediment. Mixing time of spiked sediment should be limited from minutes to a few hours and temperature should be kept low to minimize potential changes in the physico-chemical and microbial characteristics of the sediment (Guide E1391). Duration of contact between the chemical and sediment can affect partitioning and bioavailability **(26)**. Care should be taken to ensure that the chemical is thoroughly and evenly distributed in the sediment. Analyses of sediment subsamples is advisable to determine the degree of mixing homogeneity **(31)**. Moreover, results from sediment-spiking studies should be compared with the response of test organisms to chemical concentrations in natural sediments **(32)**.

10.6.4.1 Organic compounds have been added: (1) directly in a dry (crystalline) form; (2) coated on the inside walls of the container **(31)**; or (3) coated onto silica sand (e.g., 5 % w/w of sediment) which is added to the sediment **(33)**. In techniques 2 and 3, the chemical is dissolved in solvent, placed in a glass spiking container (with or without sand), then the solvent is slowly evaporated. The advantage of these three approaches is that no solvent is introduced to the sediment, only the chemical being spiked. When testing spiked sediments, procedural blanks (sediments that have been handled in the same way, including solvent addition and evaporation, but contain no added chemical) should be tested in addition to regular negative controls.

10.6.4.2 Organic solvents such as triethylene glycol, methanol, ethanol, or acetone may be used, but they might affect TOC levels, introduce toxicity, alter the geochemical properties of the sediment, or stimulate undesirable growths of microorganisms (Guide E1391). Acetone is highly volatile and might leave the system more readily than triethylene glycol, methanol, or ethanol. A surfactant should not be used in the preparation of a stock solution because it might affect the bioavailability, form, or toxicity of the test material.

10.6.4.3 Sufficient time should be allowed after spiking for the spiked chemical to equilibrate with sediment components. For organic compounds, it is recommended that the sediment be aged at least one month before starting a test. Two months

or more may be necessary for chemicals with a high log Kow (for example, >6; (33)). For metals, shorter aging times (1 to 2 weeks) may be sufficient. Periodic monitoring of chemical concentrations in pore water during sediment aging is highly recommended as a means to assess the equilibration of the spiked sediments. Monitoring of pore water during spiked sediment testing is also recommended.

10.6.5 Direct addition of a solvent (other than water) to the sediment should be avoided if possible. Addition of organic solvents may dramatically influence the concentration of dissolved organic carbon in pore water. If an organic solvent is to be used, the solvent should be at a concentration that does not affect the test organism. The solvent control must contain the highest concentration of solvent present and must be from the same batch used to make the stock solution (Guide E729).

10.6.6 If the test contains both a negative control and a solvent control, the survival, growth, or reproduction of the organisms tested should be compared in the two controls. If a statistically significant difference is detected between the two controls, only the solvent control may be used for meeting the acceptability of the test and as the basis for calculation of results. The negative control might provide additional information on the general health of the organisms tested. If no statistically significant difference is detected, the data from both controls should be used for meeting the acceptability of the test and as the basis for calculation of results (see Guides E1241 and E1847). If performance in the solvent control is markedly different from that in the negative control, it is possible that the data are comprised by experimental artifacts and may not accurately reflect the toxicity of the chemical in natural sediments.

10.7 *Test Concentration(s):*

10.7.1 If the test is designed to calculate an LC50, the test concentrations should bracket the predicted effect level LC50. The prediction might be based on the results of a test on the same or similar test material on 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 the organisms are exposed to a control and three or more concentrations of the test material that differ by a factor of 10.

10.7.2 Concentrations above aqueous solubility can be used if necessary because organisms are sometimes exposed to concentrations above solubility in the real world and because solubility is often not well known. The toxicity of the test material in sediments might be quite different from that in water-borne exposures.

10.7.3 Bulk sediment chemical concentrations might be normalized to factors other than dry weight. For example, concentrations of non-ionic compounds might be normalized to organic carbon content.

10.7.4 In some (usually regulatory) situations, it is necessary to determine only (*1*) whether a specific concentration of test material is acutely toxic to the test species or (*2*) whether the LC50 is above or below a specific concentration. For example, the specific concentration might be that occurring in a particular sediment or that in a dredge material to be deposited at a disposal site. When there is only interest in a particular concentration, it might be necessary to test only that concentration and the negative and solvent controls.

11. Test Organisms

11.1 *Species*—The species of benthic polychaete to be used in sediment toxicity tests should be selected based on availability, sensitivity to the test materials, tolerance to the ecological conditions (for example, temperature, salinity, and grain size), ecological importance, and ease of handling in the laboratory. The source and type of sediment being tested or the type of test to be implemented might dictate the selection of a particular species. Species or genera with wide geographical distributions should ideally be selected, so that the test results can be compared among laboratories with similar species. The species used should be identified with an appropriate taxonomic key, and identifications should be verified by a taxonomic authority. The annexes of this guide provide information on the identification of test species as well as guidance concerning the requirements and methods of handling these species. Use of the species listed in the annexes is encouraged in order to increase the comparability of results.

11.1.1 *N. arenaceodentata* (see Annex A1) is a free-burrowing polychaete that constructs non-permanent mucoid tubes that has been used successfully in acute, chronic, and life cycle sediment and aquatic testing since the late 1960s (**1**) and was the subject of a sediment test protocol (**34**). The sensitivity of this species to salinities below 25 parts per thousand (ppt) limits its use to testing sediment from areas of low salinities, except when sediments have been acclimated to test conditions. This species is distributed widely in salt waters of the United States and elsewhere (**1**). It is available from laboratory cultures. The annex of this guide details the procedures used in culturing this species. A large database has been developed for the response of *N. arenaceodentata* to a variety of chemicals and sediments that establishes its usefulness as a test species as well as a reference species for comparing the sensitivity of other test species (**1, 2, 35**).

11.1.2 *A. virens* (see Annex A2) is a free-burrowing species that has been used successfully in sediment and aquatic testing, especially on the east coast of the United States (**1**). It is used as fish bait throughout the United States, but bait shops should not be used as a source because of the potential variability of condition of the animal.

11.1.3 Other species of polychaetes have been used successfully for testing the toxicity of marine and estuarine sediments using the same or similar methods described herein. However, since these species vary in size, it may be necessary to modify the size of the container and amount of sediment used in the test to accommodate the species. These species include the following: *Capitella capitata, Ophryotrocha diadema, Glycera dibranchiata, Nephtys incisa, Nephtys caecoides, Abarenicola pacifica, Ctenodrilus serratus*, and *Dinophilus gyrociliatus*.

11.1.4 The environmental requirements and sensitivity of a prospective test species of polychaete to test materials and to various sediment characteristics should be established before it is used widely in toxicity tests. The tolerance of a test species to variations in sediment characteristics such as particle size distribution, organic enrichment, and interstitial water salinity

should be established before responses can be ascribed to chemical effects. Choice of the scale of the test chamber, density of test organisms, temperature, salinity, and control sediment might require modification in order to accommodate the requirements of the test species. The required modifications should be based on conditions at the natural habitat of the species (see Annexes).

11.1.5 The sensitivity of a prospective new test species of polychaete should be compared with a reference species such as *N. arenaceodentata* or *A. virens* before the new species is used in routine toxicity testing. A 96-h reference toxicity test using water only could eliminate the relative effects of sediment particle size and other sediment characteristics (see section 11.5.4). The test should be set up as in Section 3, but without the addition of sediment. A non-ionic organic compound whose binding properties are not affected by salinity could be used to compare species at different salinity levels (for example, polynuclear aromatic hydrocarbons such as fluoranthene). It might be desirable to also test a metal such as cadmium. Any factor (such as salinity, pH, redox state, carbonates, or sulfides) that might affect the toxicity or bioavailability of the reference toxicant should be held constant.

11.1.6 If tube-building polychaetes are used in sediment toxicity testing, it should be kept in mind that the polychaetes might not be directly in contact with test sediment after their tubes are built, and they might pump overlying water through their tubes rather than using interstitial water. They might feed on particulate materials that either have settled on the sediment surface, while burrowing species might feed on particles or meiofauna found within the sediment. Thus, tube builders and burrowing species might have different exposure routes to adsorbed or dissolved sediment chemicals. Polychaetes that emerge from the sediment and crawl on the sediment surface might not be exposed continually to the test sediment.

11.2 *Age*—All organisms should be as uniform as possible in age and size. The age or size class for a particular species should be chosen so that the sensitivity to test materials is not affected by the state of maturity, reproduction, seasonality, etc. See the Annexes for the age requirements of the test species.

11.3 *Feeding*—It is generally not necessary to feed polychaetes during a 10-day sediment test; however, it may be necessary to feed the test species for longer than the 10-day test period. See the Annexes for the food requirements of the test species.

11.4 *Source*—All individuals in a test should be from the same source because different populations of the same species might have different sensitivities to chemicals. Marine and estuarine polychaetes are obtained either from laboratory cultures or by collecting specimens from a field population in a clean area. Permits for collecting specimens from the field might be required by some local or state agencies. See the Annexes for further information on the source of test species.

11.4.1 If test organisms are cultured or held for an extended period of time in the laboratory, the responses of laboratory-held organisms to chemicals should be compared to that of animals collected freshly from the field to determine whether laboratory stresses do not change sensitivities to test materials (**23**).

11.5 *Collection and Handling:*

11.5.1 Polychaetes should be handled as little as possible. When handling is necessary, it should be performed carefully, gently, and quickly so that organisms are not stressed unnecessarily. Polychaetes that touch dry absorbent surfaces or are injured during handling should be discarded.

11.5.2 *Collection*—Polychaetes can be collected intertidally with a shovel or subtidally with a small biological dredge or a grab. Sediment-containing polychaetes can be sieved gently to separate the polychaetes. The polychaetes can then be transferred to and allowed to bury in sieved sediment from the polychaete collecting site. Sieves and containers used to collect and transport polychaetes should be marked “live only” and should never be used for working with formalin or any other toxic materials. Water used for sieving should be at the same temperature and salinity as bottom water at the collection site. Infaunal polychaetes should be held in sediment or algae during transport to the laboratory and should be kept at or near the collection site temperature or below. It might be necessary to keep containers of sediment and polychaetes in coolers and to provide aeration during a long transport. Collection-site sediments should be saved for control and acclimation.

11.5.3 *Holding*—Polychaetes should be acclimated to the test temperature and salinity by holding them in the laboratory prior to their use in a toxicity test. Polychaetes should be collected from the field three or four days before use, but field-collection animals should not be held in the laboratory for more than two weeks before the initiation of a test. The same procedures should be followed if the animals are obtained from a supplier.

11.5.3.1 Polychaetes can be counted into holding containers with clean sieved sediment in order to ascertain whether sufficient numbers have been obtained in the laboratory. Polychaetes should be washed gently into a clean dish for counting. Active, apparently healthy animals can be picked up and removed from detritus with a wide-mouthed bulb pipette and transferred to a sieved collection-site sediment. Enough polychaetes should be collected to provide at least one third more individuals than are required for the test. The temperature of the water containing the animals must not exceed the polychaetes’ tolerance limit during counting, and it should remain close to the holding temperature. The holding containers should be provided with flowing or aerated sea water at or near the test temperature and salinity. If temperature and salinity changes are necessary to bring polychaetes from the collection site conditions to the test conditions, adjustment should be made gradually in order to allow polychaetes to acclimate over a 5–day to 7-day period. Healthy polychaetes will remain on either the surface or the sediment, or burrow into it during the holding period, until the initiation of the test and can be retrieved easily for setup. Supplementary feeding during the acclimation period is not necessary, as some polychaetes will find food in the holding sediment (see