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

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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 *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:

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

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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 and health practices and determine the applicability of regulatory limitations prior to use.* Specific hazards statements are given in Section 8.

2. Referenced Documents

2.1 *ASTM Standards:*[2]

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

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

3.2.3 *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.

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

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

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

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

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

[2] 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.

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

3.2.10 *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). 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.

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

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

3.2.13 *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 to 28-day test, which determines the effect of contaminated sediment on survival and growth. If smaller worms are used, such as *N. arenaceodentata*, five worms are placed in a 1-L glass test chamber with a minimum sediment depth of 2 to 3 cm and the overlying water aerated. Either young adult or recently emerged juveniles (2 to 3 weeks post-emergence) worms are used in the 10-day test; only recently emerged (2 to 3 weeks) juveniles are used in the 20 to 28-day test. 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 to 28-day test, except for the test duration (see Annex A1). If larger worms are used, such as *N. virens*, ten worms are placed in a glass aquaria (4 to 37 L) with a minimum sediment depth of 10 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 *N. 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**).[3] 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

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

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 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 Due to the limited time that sediment toxicity tests have been practiced, 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 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*—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 fluorocarbon plastics, 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. 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. Five worms 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 ± 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 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 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. 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 twice 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

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
$SrCl_2·6H_2O$	20 mg
H_3BO_3	30 mg
KBr	100 mg
KCl	700 mg
$CaCl_2·2H_2O$	1.47 g
Na_2SO_4	4.00 g
$MgCl_2·6H_2O$	10.78 g
$NaCl$	23.50 g
$Na_2SiO_3·9H_2O$	20 mg
$NaHCO_3$	200 mg

[A] If the resulting solution is diluted to 1 L, the salinity should be 34 ± 0.5 g/kg 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.

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 at 4 ± 3°C 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 twice 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. 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 ± 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. 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.

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 **(30)**. Moreover, results from sediment-spiking studies should be compared with the response of test organisms to chemical concentrations in natural sediments **(31)**.

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 **(30)**; or (3) coated onto silica sand (e.g., 5 % w/w of sediment) which is added to the sediment **(32)**. 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; **(32)**). 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 (Guide E1241). 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 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 (**33**). The sensitivity of this species to salinities below 28 parts per thousand (ppt) limits its use to testing sediment from areas of low salinities. 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, 34**).

11.1.2 *N. 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, Nepthys incisa, 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 *N. virens* before the new species is used in routine toxicity testing. A96-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 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 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 species-specific annexes). Any individuals that appear unhealthy during holding should be discarded. The temperature and salinity of the water in the holding containers should be monitored daily.

11.6 *Quality*—All polychaetes used in a test must be of acceptable quality. A qualified polychaete taxonomist must be consulted to ensure that the animals in the test population are all of the same species.

11.6.1 Polychaetes in holding containers should be checked daily before the initiation of a test. Individuals that emerge from the sediment and appear to be dead or unhealthy should be discarded. If greater than 5 % of the polychaetes appear to be unhealthy during the 48 h preceding the test, the entire group should be discarded and not used in the test. It may be difficult to obtain good field-collected polychaetes during the winter months. Individuals collected during this time should be examined carefully for scars and missing parapodia or posterior ends; those appearing limp should be discarded.

11.6.2 If it might be present in the environment, analysis of the test organisms for the test material and other chemicals to which exposure might have occurred is desirable. Polychaetes may be used without analysis of chemical concentration if the polychaetes are obtained from an area monitored for chemical contamination (see 10.2) and known to be free of toxicants and they are held in clean, uncontaminated water and facilities. Polychaetes from contaminated areas should not be used in sediment toxicity tests unless the experimental design specifically requires the use of that population.

11.6.3 The survival of polychaetes in control or reference sediment during the test is an indication of the health of the population and other factors. Mean (survival in controls must be 90 % or greater and 80 % or greater in an individual replicate control for the test to be considered valid.

11.6.4 Reference toxicants might be useful for assessing the quality and sensitivity of test organisms and can be employed using 96-h toxicity tests without sediment to generate LC50 values (see 11.1.3). Data from these reference toxicity tests could be used to assess genetic strain or life-stage sensitivity to select chemicals. Physiological measurements such as lipid

content might also provide useful information regarding the health of the test organisms. Periodic reference toxicity testing should be conducted as an indication of overall comparability of results among laboratories (at a minimum, 6 tests over a 3 year period should be conducted to evaluate potential differences in life stage or genetic strain of test organisms). In particular, reference-toxicity tests should be performed more frequently when organisms are obtained from outside sources. The supplier should also certify the species identification of the test organisms, and provide the taxonomic references, or name(s) of the taxonomic expert(s) consulted. See Test Method E1706 for additional detail.

11.6.4.1 Reference toxicants can be useful for assessing the sensitivity of different populations or species of polychaetes, or seasonal variation in sensitivity of a field-collected population. Such assessment should be conducted simultaneously with the toxicity test. Many chemicals have been used or evaluated for use as reference toxicants (**35**). None has been proven to be a reliable indicator of the overall quality of any species or test results. A reference toxicant is likely to be more useful when used in conjunction with tests on materials that have the same mode of action as the reference toxicant. However, frequent changing among reference toxicants can reduce the value of reference toxicant data if an adequate history of use with each procedure, species, and laboratory does not exist.

12. Experimental Design

12.1 Decisions concerning such aspects of experimental design as concentrations of materials added to sediment, number of treatments, and numbers of test chambers per treatment should be based on the purposes of the test and the time of procedure to be used to calculate the results (see Section 16). The polychaete sediment toxicity test can be used to test the toxicity of sediment in the field (12.3) or to address a great variety of sediment and water quality manipulations in the laboratory (12.4). Every test requires one or more control or reference sediment treatments (12.2).

12.2 *Controls*—Every test requires a control sediment treatment consisting of sediment known to be nontoxic to, and within the geochemical requirements of, the test species (10.3). The same water, conditions, procedures, and organisms as used in the other treatments, except that none of the test material being tested is added to the control sediment or water. At least five laboratory replicates of the control sediment should be included in all tests, regardless of whether the test sediments are replicated. This allows comparisons among experiments and laboratories of the validity of the procedures used in individual tests.

12.2.1 In addition to the standard control sediment, if a reference sediment has properties such as grain size or organic content that might exceed the tolerance range of the test species, it is desirable to include nontoxic sediment such as silica sand for these parameters. The design of field surveys should include an additional field control involving five replicate samples from an area free of sediment contamination. This provides a site-specific basis for the comparison of potentially toxic and nontoxic conditions and can account for the mortality associated exclusively with subjecting the organisms to non-native sediments. The concentrations of chemicals should be measured in these field control sediments in order to justify the assumption that they are contaminant-free (see 10.3).

12.2.2 Both a sediment solvent control and a sediment negative control or reference sediment must be included in the test if a solvent other than water is used. The solvent control must contain the highest concentration of solvent present and must use solvent from the same batch used to make the stock solution (see Guide E729). The same concentration of solvent should be used in all treatments. (See Section 10.6.5 for additional detail).

12.3 *Field Survey Design*—Field surveys can be designed to provide either a qualitative reconnaissance of the distribution of sediment toxicity or a quantitative statistical comparison of toxicity among stations.

12.3.1 The object of a qualitative reconnaissance survey is to identify sites of potential toxic conditions that warrant further study. A quantitative survey is often conducted in areas in which little is known concerning contamination patterns. The survey design might include only one sample from each station to allow for maximum spatial coverage. The lack of replication precludes statistical comparisons, but samples for which mortality exceeds the control range can be identified for further study.

12.3.2 The object of a quantitative statistical comparison is to test for statistically significant differences in survival and growth among control sediments and test sediments from several stations. Replicates should be taken at each station in the survey. Depending on the design of the survey, the replicates can represent separate samples from different grabs taken at the same station, separate samples from the same grab, or split samples of composited sediment from one or more grabs. The number of replicates necessary per station is a function of the need for resolution. Separate subsamples from the same grab may be used to test for within-grab variability, or split samples of composited sediment from one or more grabs may be used for comparisons of test procedures (such as comparative sensitivity among test species), but these subsamples should not be considered true replicates for statistical comparisons among stations (**36, 37**).

12.3.3 Station locations might be distributed along a known pollution gradient, in relation to the boundary of a disposal site, or at sites identified as being potentially toxic in a reconnaissance survey. Comparisons can be made in both space and time. A sampling design can be prepared to assess the toxicity of samples representative of the project area to be dredged in predredging studies. Such a design needs to include subsampling cores taken to the project depth.

12.3.4 It might be useful to conduct toxicity tests with dilutions of the field sediment mixed with control sediment if no polychaetes survive in sediment from a particular field location. Concentrations should be expressed as percent dilutions on a wet weight basis, that is, wet weight of field sediment/total wet weight of field and control sediment mixture (**33**). Experimental designs for sediment dilution experiments are the same as those described in 12.4 for other laboratory experiments.

12.3.5 Sediment toxicity surveys are usually part of more comprehensive analyses of biological, chemical, geological, and hydrographic conditions. A useful summary of field sampling design is presented by Green (**37**). Statistical correlation can be increased and costs reduced if subsamples for sediment toxicity tests, geochemical analyses, and benthic community structure are taken simultaneously from the same grab or at the same station.

12.4 *Laboratory Experiments*—Sediment toxicity tests can be applied in the laboratory to provide information on a variety of problems related to the action of chemicals in sediment. The test can be used to determine natural limits such as salinity or temperature to estimate the LC50 or growth of a chemical in a particular sediment type, to study the interaction among chemicals in sediment, and to assess the effect of complex waste mixtures on the test species in sediment.

12.4.1 A test used to calculate an LC50 usually consists of one or more control sediment treatments and a geometric series of at least five concentrations of test material. Except for the control(s) and the highest concentration, each concentration should be at least 60 % of the next higher one, unless information concerning the concentration-effect survey indicates that a different dilution factor is more appropriate. At least one concentration should give a partial response below the LC50 and one above the LC50. Six or seven concentrations might be desirable to increase the likelihood of covering the appropriate range if the estimate of toxicity is particularly uncertain of covering the appropriate range.

12.4.2 A test is used to calculate a statistically significant difference between one or more control sediment treatments and one or more treatments of test material. Five replicates should typically be included for each concentration of sediment tested plus controls. However, the number of replicates should be based on the need to meet statistical testing requirements, as in the case of a reconnaissance survey, to identify potentially contaminated sediments.

12.4.3 The primary focus of the physical and experimental design of the test and the statistical analysis of the data is the experimental unit, which is defined as the smallest physical entity to which treatments can be assigned independently (see Guide E729). Thus the test chamber is the experimental unit. All chambers in the test should be treated in a similar fashion with respect to factors that might affect results within test chambers and therefore the results of the test. Treatments need to be assigned impartially to individual test chamber locations. A randomized block design (with each treatment being present in each block, which might be a row or a rectangle) is preferable to a completely randomized design (**38**).

12.4.4 The minimum desirable number of test chambers and organisms per treatment should be calculated from (*1*) the expected variance within test chambers, (*2*) the expected variance between test chambers within a treatment, and (*3*) either the maximum acceptable width of the confidence interval on a point estimate (for example, LC50) or minimum difference that is desired using hypothesis testing (**39**). As the number of test chambers (that is, experimental units) per treatment increases, the number of degrees of freedom increases, and therefore the width of the confidence interval on a point estimate decreases, and the power of a significant test increases.

12.4.5 Mean survival in control or reference sediment must be 90 % or greater and 80 % or greater in an individual replicate control. A difference of approximately 15 % between mean survival in control and test sediments is usually significant when five polychaetes are included in each of five replicate test chambers of control or reference test sediment (see 16.4).

12.4.6 It is desirable to repeat the test at a later time to obtain information concerning the reproducibility of the results. However, the toxicity of stored sediment may change over time.

13. Procedure

13.1 *Dissolved Oxygen (DO)*—Maintain the concentration of DO in the water overlying the sediment in each test chamber at or near the saturation level by aerating the water gently. Aeration can be provided to each test chamber through a 1-mL glass pipette that extends to a depth not closer than 2 cm from the surface of the sediment. Air should be bubbled into the test chamber at a rate that maintains a desirable DO concentration of ≥90 % but does not cause turbulence or disturb the sediment surface. Aeration should be the same in all test chambers, including the control(s), throughout the test. If air flow to the beakers is interrupted for more than an hour, measure the DO in the beakers to determine whether DO concentrations have dropped to below 60 % of saturation (see 15.2.7).

13.2 *Temperature*—The temperature selected should be within the natural range of temperatures in the area from which the polychaetes occur in the field. The temperature at which the tests are conducted should be within the range of the temperatures in the laboratory if the polychaetes are taken from laboratory cultures. Individual temperature readings should not vary by more than 3°C from the selected test temperature within an experiment, and the time-weighted average measured temperature at the end of the test should be within 1°C of the selected test temperature. The highest and lowest temperatures should not differ by more than 2°C when temperature is measured concurrently in more than one test chamber.

13.3 *Salinity*—The salinity of the water overlying the test sediment in the toxicity tests must be within the tolerance range of the selected test species (see annexes). The salinity of the interstitial water of test sediments from the field should not be adjusted because such an operation might change the toxicological properties of the sediment. The salinity of the interstitial water of sediments spiked experimentally in the laboratory with contaminants may be adjusted prior to spiking.

13.3.1 If test sediments are collected from low-salinity areas, the salinity of the overlying water in the test chambers should be approximately the same as the interstitial water or as the water above the sediment at the collection site. Depending on the experimental design, it might be desirable to use water from the sediment collection site or to adjust the salinity of prepared salt water to the collection site salinity (see 7.3).

13.4 *Light*—Polychaetes do not generally require a definite light regime.

13.5 *Feeding*—Infaunal polychaetes do not require feeding during the 10-day test. See the annexes for feeding requirements for 20 to 28-day tests.

13.6 *Beginning the Test:*

13.6.1 The toxicity test begins when the test organisms are first placed in test chambers containing test sediment and overlying sea water.

13.6.2 On the day before the toxicity test is initiated, thoroughly homogenize each test sediment sample within its storage container, and add an aliquot to a test chamber to a depth specific for the test species (see annexes). In the case of replicate sediment samples, it might be desirable to calculate the net weight of sediment necessary to make a layer of the desired depth in the first chamber, and then add the same net weight of sediment to the other replicates within a treatment. The same procedure might be applied to control sediments, measuring the required weight for replicates of each treatment separately, because different sediments might have different densities. Treatments should be assigned randomly to prenumbered test chambers. The same procedure should be applied to control sediments. It is desirable to take subsamples of the test sediment for geochemical analyses as the test chambers are loaded. It might be desirable to test intact cores for some experimental designs.

13.6.3 The sediment within the test chamber should be settled by tapping the test chamber against the side of the hand, or by smoothing the sediment surface with a nylon, fluorocarbon, or polyethylene spatula. A disk cut from 6-mil nylon, TFE-fluorocarbon, or polyethylene sheeting to fit the inside diameter of the test chamber, and attached to a length of nylon monofilament for removal, can be placed on the sediment surface to minimize sediment disruption as prepared toxicity test sea water is added up to the desired level in the test chambers (see annexes). Remove the disk and rinse it with sea water between replicates of a treatment, and use a separate disk for each treatment. Then cover the test chambers, and put it in numerical order into a temperature-controlled water bath or in a temperature-controlled room, and aerate overnight. The system should be left overnight to allow suspended particles to settle and an equilibrium to be established between sediment and overlying water before the polychaetes are added.

13.6.4 If the experimental design requires monitoring of the sediment chemistry (for example, metals, total volatile solids, pH, Eh, etc.), additional test chambers with sediment and polychaetes should be set up for this purpose. Monitoring the quality of the overlying water (for DO, pH, or certain chemicals) in the test chambers can be accomplished without disturbing the sediment, and it may be conducted in the test chambers containing the test polychaetes. A separate temperature beaker should be included in each water bath if more than one water bath is used to contain the test chambers (see 13.9.3).

13.6.5 The initiation of the toxicity test is identical for both the 10-day and 20 to 28-day tests. The test begins when worms are distributed to each test chamber. It is usually not possible to distribute worms to the test chambers at the same time, so it is necessary to select a set of test chambers (usually 10 to 15) to be processed together. Each treatment, including controls, should be represented in each set of test chambers to be processed together if treatments are replicated. The selection should be random if treatments are not replicated.

13.6.6 A sufficient number of polychaetes should be removed from the holding facility at one time to provide approximately one third more specimens than is necessary for the test. This allows the selection of active, apparently healthy polychaetes. The temperature and salinity of the water in the holding containers should be recorded before the polychaetes are removed. The polychaetes should be transferred from the holding sediment to a sorting tray containing water of the holding temperature and salinity. Active, apparently healthy amphipods should be selected impartially from the sorting tray and distributed sequentially among dishes containing approximately 50 mL of prepared toxicity test sea water until each dish contains the required number (usually 5 for smaller worms and 10 for larger worms; see annexes) of individuals. The number of polychaetes in each dish should be verified by recounting them into a separate dish containing toxicity test water. The estimated individual dry weight for the 20 to 28-day growth test should be determined on five subsamples (five specimens per subsample) selected randomly from the same pool of polychaetes used to initiate the test.

13.6.7 Worms should be added to the test chambers by pouring the water and worms gently from the sorting dish into the test chamber. Any worms remaining in the dish should be washed gently into the test chamber. The water level should be raised to 750 mL in the test chamber for tests with smaller worms. The water level should be brought to approximately 5 cm from the top of the aquarium in tests with larger worms.

13.7 *Duration of the Test*—The test begins when the worms are added to the test chambers containing test sediment. The test period is 10 days for the short-term test and 20 to 28 days for the long-term test.

13.8 *Biological Data*—Survival of the test organisms is used as a criterion for both tests. As measured by dry body weight, growth is a sublethal criterion used in conjunction with death in the 20 to 28-day test.

13.8.1 *Mortality*—The primary criterion of the 10-day sediment toxicity test is death of the test species, which is determined at the end of the exposure period. However, if the sediment is very toxic, worms may crawl to the surface of the sediment and die before the end of the experimental period. Death is determined by the lack of movement when touched gently with a glass probe. Dead worms are usually white and their proboscis everted (see Annex A1). The contents of the test chambers in both tests should be sieved to remove the test species for counting after the experimental period and any necessary samples have been taken. Use a 0.5-mm sieve for separating the worms from the sediment. Material and worms retained on the sieve should be washed into a sorting tray with clean sea water. Record the total number of live and dead worms for each replicate. The sum of living and dead worms might be less than 5 in a replicate because of decomposition.

13.8.2 *Growth*—Survival and growth are used as criteria for the 20 to 28-day test. Measure growth by the dry weight of the surviving test worms within a replicate. Compare the results with the weight of the worms at the beginning of the test (see 13.6.5) and with the control(s) and the test concentrations of

sediment. At the conclusion of the 20 to 28-day test, sieve the contents of each replicate chamber through a 0.5-mm screen, and count the number of living worms retained on the screen. Then place the surviving worms within a replicate in a vial containing clean seawater. Rinse the contents of each vial with distilled water, place on a preweighed aluminum pan, and dry at 50°C to a constant weight. Process a randomly selected subsample of worms in the same way at the initiation of the experiment. Record weight, as dry weight, by specimen to the nearest 0.1 mg to account for a possible difference in the number of surviving worms present at the termination of the test. It would be advantageous to allow the worms to depurate before weighing; however, this is not generally done. If depuration is conducted, it should occur over a 24-h period, and if so, it should be stated in the results of the growth experiment. It should be noted that worms frequently feed on their fecal material.

13.8.3 Record the 20 to 28-day average individual dry weight in each exposure chamber (see 17.1.11), and calculate the mean and standard deviation for each treatment.

13.9 *Other Measurements:*

13.9.1 *Field Sediment*—If the sediment to be tested is collected from a potentially contaminated site in the field, sediment samples should be collected from the same grab for analysis of various geochemical parameters (see 10.2). A separate sample for faunal analysis is also desirable. These samples may be stored under appropriate conditions for possible future analysis, after the results of the sediment toxicity test are known. Sediment Eh and pH should be measured both in the field and in the test chambers at the beginning and end of the test. This is especially desirable for field sediments, which might contain high concentrations of organic materials. All measurements should also be taken in control samples.

13.9.2 *Laboratory-Spiked Sediments*—In experiments in which a known test material is added to sediment, the concentration of the test material should be determined in stock solutions or mixtures added to sediment, and in test chambers at the beginning and end of the test. Sea water and sediment samples can be taken as test chambers are loaded, and small water samples can be taken from the test chambers containing polychaetes. To monitor changes in sediment or interstitial water chemistry during the course of the experiment, separate sediment chemistry beakers should be set up and sampled at the initiation and termination of the experiment. It is not necessary to add polychaetes to chemistry chambers sampled at the initiation of the experiment, but polychaetes should be added to those samples later. Some sediment and water quality characteristics, such as pH, Eh, and DO, can be measured by inserting analytical probes into the test chambers containing polychaetes. Separate chemistry beakers might not be necessary if radiolabeled test compounds are used.

13.9.3 *Water Quality*—Salinity should be measured daily during the test. The pH and ammonia should be measured at the beginning and end of the experiment for each test concentration plus control(s). The DO concentration must be measured in at least one test chamber in each concentration at the beginning and end of the experiment for each test concentration plus control(s).

13.9.4 *Temperature*—The temperature of the overlying water should be measured in a test chamber for each concentration each day for the duration of the experiment. The temperature should not vary by more than 1°C from the test temperature.

13.9.5 The concentration of the test material in water and sediment should be measured at several concentrations and as often as practicable during the test. At a minimum, the concentration of the test material should be measured at the beginning and end of the test in the control and at low, medium, and high concentrations. Measurement of degradation products of the test material might also be desirable.

13.9.5.1 Measurement of test material concentrations in water can be accomplished by pipetting water samples through glass or fluorocarbon plastic tubing from a point midway between the top, bottom, and sides of the test chamber. Water samples should not contain any surface scum, material from the sides of the test chamber, or sediment.

13.9.5.2 Measurements of test material concentrations in sediment can be taken by siphoning off the overlying water without disturbing the surface of the sediment and then taking appropriate aliquots of the sediment for chemical analysis.

13.9.5.3 Interstitial water can be sampled by using the water that comes to the surface in a rolling mill jar or in a sample container as the sediment settles, by centrifuging a sediment sample to separate the sediment particles from the interstitial water, or by using a filter apparatus to extract interstitial water from a sediment sample (see Guide E1391 and Test Method E1706 for additional detail on sampling of interstitial water). Care should be taken to ensure that chemicals do not undergo transformation, degradation, or volatilization during sample preparation. It should be kept in mind that filtering can remove certain chemicals from solution.

13.9.6 *All Tests*—Temperature should be recorded in a separate temperature beaker throughout the test. A temperature beaker should be set up in each water bath if the test chambers are in more than one temperature-controlled water bath. Temperatures should be monitored hourly using a recording thermometer, or the daily maximum and minimum temperature should be monitored (see Guide E729). The temperature should not vary by more than 3°C from the designated test temperature, and the time-weighted average should not differ by more than 1°C from the designated test temperature (see 13.2).

14. Analytical Methodology

14.1 If samples of the dilution water stock solution or test solutions cannot be analyzed immediately, they should be handled and stored appropriately **(40)** to minimize the loss of test material or chemicals through such processes as microbial degradation, hydrolysis, oxidation, photolysis, reduction, sorption, and volatilization (see Practice D3976).

14.2 Chemical and physical data should be obtained using appropriate ASTM standards whenever possible. Methods should be obtained from other reliable sources **(41)** for those measurements for which ASTM standards do not exist or are not sufficiently sensitive. The concentration of nonionized ammonia may be calculated from pH, temperature, and total ammonia concentration **(42)**.

14.3 Methods used to analyze food or test organisms (see section 10.9.1) should be obtained from appropriate sources (**42**).

14.4 The analytical method used to measure the concentration of toxicant in test chambers needs to be validated before beginning the test. The precision and bias of the test method in an appropriate matrix should be determined whenever samples are analyzed using reference samples, reagent blanks, split samples, spike recoveries, interlaboratory comparisons, or alternative methods of analysis when appropriate.

14.5 Test material concentrations in interstitial water should be measured as well as bulk sediment concentrations. In addition to measuring the total concentration of test material in interstitial water or in the overlying water from test chambers, measurement of the apparent dissolved or free form for organic compounds is that which is not bound to either particulates or to dissolved organic carbon, and for metals it is the ionic form of the element. The apparent dissolved fraction is usually defined and determined as that which passes through a 0.45-µm membrane filter. However, passing solutions through membrane filters can result in significant sorptive losses that need to be accounted for.

15. Acceptability of Test

15.1 A toxicity test is unacceptable if mortality is greater than 5 % of the animals during the holding period before the start of the test, if survival is below an average of 90 % of the control animals, or if survival is below 80 % in an individual control test chamber.

15.2 A sediment toxicity test should usually be considered unacceptable if one or more of the following occurred:

15.2.1 All test chambers and compartments were not identical.

15.2.2 Treatments were not assigned randomly to test chambers.

15.2.3 Test organisms were not distributed randomly or impartially to test chambers.

15.2.4 Required negative, reference sediment, positive, or solvent controls were not included in the test.

15.2.5 All test animals were not from the same population or culture, not of the same species, or not of acceptable quality.

15.2.6 Test specimens of *N. arenaceodentata* used in the 20 to 28-day growth test were older than 2 to 3 weeks post-emergence.

15.2.7 Individual test organisms were not assigned impartially or randomly to test chambers or compartments.

15.2.8 Dissolved Oxygen and temperature were not measured as specified in Section 13.

15.2.9 Polychaetes from a wild population were maintained in the laboratory for more than two weeks, unless the effects of prolonged maintenance in the laboratory have been shown to have no significant impact on sensitivity. If juvenile polychaetes were collected from wild populations for use in the 20 to 28-day growth test, they either were not maintained under laboratory conditions for a sufficient length of time or they were too large for the test (see 15.2.5).

15.2.10 Aeration to a test chamber(s) was off for an extended time, and the DO levels dropped to below 60 % of saturation.

15.2.11 The concentration of solvent, if required, in the range used affected survival, growth, or reproduction of the test species (see species-specific annexes).

15.2.12 The analytical method used to measure the concentration of test material in the test chamber was not validated before beginning the test.

15.3 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.

16. Interpretation of Results

16.1 The calculating procedure(s) and interpretation of results should be appropriate to the experimental design. Procedures used to calculate the results of toxicity tests can be divided into two categories: those that test hypotheses and those that provide point estimates. No procedure should be used without careful considerations of (*1*) the advantages and disadvantages of various alternative procedures and (*2*) appropriate preliminary tests, such as those for outliers and heterogeneity. Preprocessing of data might be required to meet the assumptions of the analyses. See Test Method E1706 for additional detail on statistical analyses of data.

16.2 The LC50 and their 95 % confidence limits for 10-day and 20 to 28-day tests should be calculated on the basis of the measured initial concentrations of the test material, if available, or the calculated initial concentrations. If other LC50s are calculated, their 95 % confidence limits should also be calculated (see Guide E729).

16.3 Most acute toxicity tests produce quantal data, that is, counts of the number of alive. A variety of methods (**43**) can be used to calculate an LC50 and its 95 % confidence limits from a set of quantal data that is distributed binomially and contains two or more concentrations at which the percent dead is between 0 and 100, but the most widely used are the probit, moving average, Spearman-Karber, and Litchfield-Wilcoxon methods. The method used should appropriately take into account the number of test chambers per treatment and the number of test organisms per chamber. The binomial test can usually be used to obtain statistically reliable information concerning the LC50, even when less than two concentrations kill or between 0 and 100 %. The binomial method does not provide a point estimate of the LC50 or EC50, but it does provide a range within which the LC50 should lie.

16.4 The results of a field survey without replication may be reported in terms of survival values for the 10-day test or survival and growth as measured by body weight for the 20 to 28-day test. A sample should be considered to be toxic if survival lies outside the 95 % confidence limits of the survival of the control or reference sediments for both tests or if the body weight values lie outside the 95 % confidence limits compared to the control. If samples from field stations are replicated, the mean survival for both tests and growth for the 20 to 28-day test at the station can be compared statistically with mean control survival by t-test or analysis of variance (ANOVA).

16.5 The precision of the toxicity test is dependent on the number of replicates, number of individuals, variability of the response among replicates, and how the test is conducted by the laboratory personnel. Acute and chronic toxicity tests with polychaetes usually use five test concentrations plus control(s) with five replicates of five worms per replicate. Increasing the number of replicates per test concentration may improve the precision of the acute toxicity test.

16.6 If samples from field sites are replicated, the site effects can be compared statistically by t-tests and ANOVA. Analysis of variance is used to determine whether any of the observed differences among the concentrations (or samples) are statistically significant. This is a test of the null hypothesis that no differences exist in the effects at all of the concentrations (or samples) and the control. If the F-test is not statistically significant ($P > 0.05$), it can be concluded that the effects observed in the toxicant treatments (or field sites) were not large enough to be detected as statistically significant by the experimental design and hypothesis test used.

16.6.1 Following a significant F-test result ($P = \leq 0.05$), all exposure concentration responses (or field sites) can be compared with the control response by using mean separation techniques. A multiple-comparison test is a technique that accounts for the fact that several comparisons are being made simultaneously. Some techniques are orthogonal contrasts, Duncan's methods, Fisher's methods, Dunnett's procedure, and Williams' method (**44**). Gelber, et al (**45**) state that the Dunnett's procedure is a multiple-comparison test designed specifically to compare several experiment samples to the concurrent control.

16.7 Daily observations on the numbers of polychaetes that have emerged completely or partially from the sediment, either lying on the sediment surface or having crawled to the water-air interface, can be used to document an apparent avoidance response to the sediment. Emergence data plotted against time can give the observer an impression of the degree of toxicity test, as polychaetes often emerge earlier and in greater numbers from more highly toxic sediment. These data can be analyzed using reliability techniques to detect significant differences in emergence time from controls.

17. Report

17.1 Include the following information, either directly or by reference to other available documents, in the record of the results of an acceptable acute toxicity test:

17.1.1 Name of test and investigator(s), name and location of laboratory, and dates of initiation and termination of the test.

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

17.1.3 Source and method of preparation of water used, its salinity, any other notable chemical characteristics: and the results of any demonstration of the acceptability of the water to the test species.

17.1.4 Source of the polychaetes, place and date of collection (if from a wild population) of the test organisms, scientific name, name of the person who identified the organisms and the taxonomic key used, observed diseases or unusual appearance, treatments, holding and acclimation procedures, and age, means, and ranges of dry weight of the worms at the beginning of the test.

17.1.5 Description of the experimental design, test chambers and covers, and depth and volume of the sediment and water in the chambers. The date, temperature, salinity, lighting, time, and method of beginning the test, and the number of worms and chambers used per treatment.

17.1.6 Average and range of the measured concentration of DO (as % of saturation) for each treatment and a description of any aeration performed on the test solutions before or during the test.

17.1.7 Averages and ranges of the acclimation and test temperatures, and the method(s) of measuring or monitoring, or both.

17.1.8 Schedule for obtaining sediment samples and water for geochemical analyses, and the methods used to obtain, prepare, and store them.

17.1.9 Methods used for, and results (with standard deviations or confidence limits) of, chemical analysis of the water quality and concentration(s) of test material, sediment geochemical analyses, and concentrations of test materials in sediment, including validation studies and reagent blanks.

17.1.10 Definition of the effect(s) used for calculating LC50s, and a summary of general observations on other effects.

17.1.11 A table of data on the number of test organisms surviving each test chamber in each test concentration as well as their total biomass and individual dry weight (determined by dividing the total body weight by the number of surviving worms in a test chamber), recorded in tabular form. Include the dry weight of a reference group at the initiation of the experiment in the table. The estimated growth rate of the individual worm during the experimental period is calculated by the following formula:

$$G = \frac{DW_t - DW_i}{T} \tag{1}$$

where:

G = estimated individual growth rate, mg dry weight day^{-1},
DW_t = mean estimated individual dry weight at the termination of the experiment, mg,
DW_i = mean estimated individual dry weight at the initiation of the experiment, mg, and
T = exposure time, days.

Expressing growth as a rate function is useful for comparing the results with the other test concentrations and other experiments. Tests are not initiated in practice with the same size (weight) polychaetes. Expressing growth as a rate rather than an absolute mass per worm normalizes the results for these differences. Furthermore, the use of growth allows for flexibility when comparing from tests in which the exposure period varies between 20 and 28 days.

17.1.12 The LC50 and 95 % confidence limits, and the methods used to calculate it, should be included with the data table (17.1.11).

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

17.2 Reports should contain enough information to identify clearly the procedures used and the quality of the results.

18. Keywords

18.1 acute toxicity tests; chronic toxicity tests; estuarine environments; experimental design; exposure tests; growth; LC50 test; marine environments; *Neanthes*; polychaetes; reference toxicants; saline water; saltwater; sediment; sediment toxicity testing; static test; terminology; toxicity; toxicology; 10-day test; 20 to 28-day test

ANNEXES

(Mandatory Information)

A1. *NEANTHES ARENACEODENTATA*

A1.1 *Ecological Requirements—Neanthes arenaceodentata* is distributed widely throughout the world; it has been collected in New England, Florida, California, Baja California, Hawaii, Central Pacific, and Europe primarily from estuarine intertidal sand or muddy sand beaches **(1)**. Population densities as high as 1000/m^2 have been observed. The polychaete lives in a variety of sediment types, where it constructs nonpermanent mucoid tubes that consist of sediment, food, and fecal material. Specimens will measure up to 10 cm in length and are tan to yellow in color.

A1.1.1 *Identification—* Since many members of the family Nereidae are similar in size, color, and appearance, *N. arenaceodentata* can be distinguished from the other species by the possession of a continuous ring of paragnaths around Areas V–VIII of the everted proboscis and by a hooked tip present on the blade of the neuropodial heterogomph seta (Fig. A1.1 and Fig. A1.2). It is necessary to kill the animal in order to see these distinguishing characters.

FIG. A1.2 *Neanthes Arenaceodentata* , Neuropodial Heterogomph Falcigerous Seta With a Hook at the Tip

A1.2 *Collection—Neanthes arenaceodentata* inhabits silts and sandy sediments from intertidal to subtidal depths in bays, harbors, and estuaries. It is more convenient to collect them with a shovel from the intertidal shores during a low tide. A sieve with a 1.0-mm diameter mesh size can be used to separate specimens from the sediment. The material retained on the sieve is transferred to a sorting tray, where individual worms can be removed by using either a small brush or a bulb pipette of a suitable size (for example, one with a 5-mm diameter opening). A laboratory population of this species has been maintained by the Department of Biology, California State University, Long Beach, Long Beach, CA, since 1964. Cultures of this species are also maintained by the Environmental Division, U.S. Army Corps of Engineers, Vicksburg, MI. It may be more convenient to obtain laboratory-reared specimens from one of these laboratories rather than to obtain them from field collections. Pesch, et al and Weinberg, et al **(46)** reported a difference in the chromosome numbers between the laboratory population originally collected from Southern California and specimens collected from the field in Connecticut and Hawaii. Thus far, no one has used either the Connecticut or Hawaiian populations as a test organism for toxicological studies.

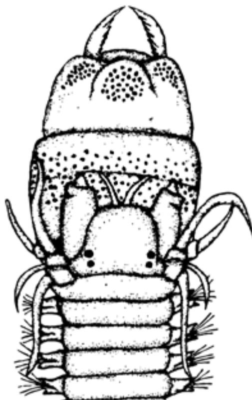

FIG. A1.1 *Neanthes Arenaceodentata* , Anterior End With Everted Proboscis Covered With Paragnaths

A1.3 *Feeding Requirements—Neanthes arenaceodentata* has been cultured through its complete life cycle using a variety of foods. A comparison of the different foods used to culture this polychaete was made by Pesch and Schauer **(47)**. They found that a diet consisting of flake food, such as Tetramarin, in combination with a dried algae, such as *Enteromorpha* spp. to supply tube-building material and provided the best growth and survival in cultures. The water can be fouled easily with Tetramarin if the culture is overfed. Other foods that have been used to culture *N. arenaceodentata* include powered alfalfa flour, powered green algae (*Enteromorpha* spp.

or *Ulva* spp.), or commercial rabbit food. Strands of *Enteromorpha* spp. are good for feeding and tube building (see A1.4.2).

A1.4 *Culture Techniques for N. arenaceodentata:*

A1.4.1 *Juvenile and Adult Stages*—Stock colonies can be maintained at 18 to 24°C in aquaria of approximately 37 to 57-L size with a maximum population of 75 to 100 worms. Sediment is not required to culture this species. Two air stones at opposite sides of the aquarium provide an adequate DO supply and water circulation. Approximately 1.5 to 2.5 g of dried food should be provided once per week, depending on the size and number of the worms. The food must be soaked in sea water prior to feeding. The amount of food provided will vary according to the size of the worms, and it should be determined whether the worms are obtaining too much or too little food prior to feeding. Overfeeding usually leads to the growth of mold or fungus on the bottom of the aquarium. The water level of the aquarium should be checked periodically and distilled water added to replace that lost by evaporation. Nearly all of the water in the aquarium should be changed once per month. To remove worms from the aquarium for an experiment or other purposes, gather the clumps with a fine net from the bottom and place them in a white pan with approximately 1 cm of sea water. The worms will free themselves from the tube masses, and they can be picked up with a pipette or a fine art brush (**1**,**2**, **48**).

A1.4.2 *Reproductive Stage*—It is impossible to distinguish immature females from males on the basis of morphology. However, a behavioral difference occurs that can be used to distinguish the sexes (**48**). Males will fight males and females will fight females. Fighting consists of everting their proboscis and extending their two jaws to grasp the other worm (Fig. A1.3). They can be cannibalistic. A male and female placed together will come along side each other and lie side by side. A maturing female can be determined by observing yellow-orange ova maturing in her coelom. Place a female with developing ova in a petri dish with a sexually unknown worm and observe their behavior when they come into contact with one another. The two worms are both females if they fight; the unknown worm is a male if they lie side by side, and the pair can be used for reproduction. Place the pair in a 3.78-L jar containing 2.5 L of sea water, an air supply, and some soaked *Enteromorpha* spp., which will provide food and material for tube construction.

A1.4.2.1 The mature ova of *N. arenaceodentata* measure 0.5 to 0.6 mm in diameter. The eggs are probably released through breaks in the body wall between successive parapodia. The female dies within two to three days or can be eaten by the male after laying her eggs. Fertilization has never been observed, but it is probably external. The fertilized eggs are clumped in the central part of the tube around the mid-body region of the male (**48**).

A1.4.3 *Embryo and Larval Stages*—The early stages of development through the 18 to 21-segmented stage proceeds within the tube of the male. The male circulates water through the tube with his body undulations. With its lecithotrophic egg, *N. arenaceodentata* lacks a free-swimming trochophore larval stage characteristic of most polychaetes. The larvae do not feed, but rather use the yolk as a source of energy. The larval body shape is distorted as a result of the yolk reserves (Fig. A1.4 and Fig. A1.5). When the yolk supply is depleted at approximately the 18 to 21-segmented stage, the young worm leaves the tube and commences feeding (Fig. A1.6, Figs. A1.7-A1.11). The early development within the male's tube generally takes three weeks under laboratory conditions (**48**). As soon as the young are observed leaving the tube, the juvenile worms should be placed in an aquarium and handled as indicated in A1.3.2. The male should be fed while the larvae develop since he may otherwise eat the larvae (**48**).

A1.5 *Toxicity Test Specifications*—The toxicity test procedures are the same for both the 10-day and the 20 to 28-day test period. The toxicity test should be run at 20 ± 1°C. The test chamber is usually a standard 1-L glass beaker with a 10-cm internal diameter. Beakers should be covered with an 11.4-cm watchglass to reduce contamination of the contents and evaporation of the water and test material. Aeration can be provided to each test chamber through a 1-mL opening glass pipette that extends between the beaker spout and the watchglass cover to a depth not closer than 2 cm from the sediment surface. Sediment in the test chambers should be 2 cm deep, and toxicity test water should be added up to the 700-mL mark on

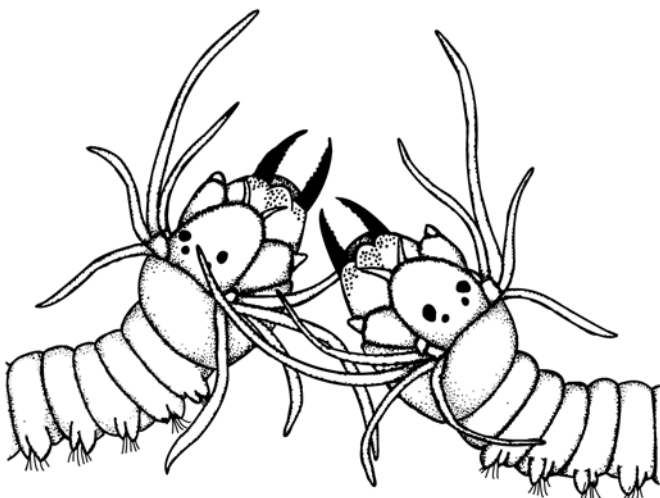

FIG. A1.3 *Neanthes Arenaceodentata* of the Same Sex in Fighting Position

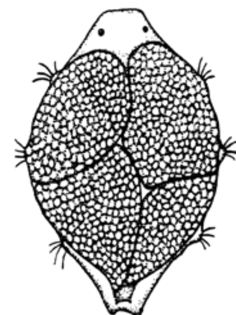

FIG. A1.4 *Neanthes Arenaceodentata* , Three-Segmented Larva

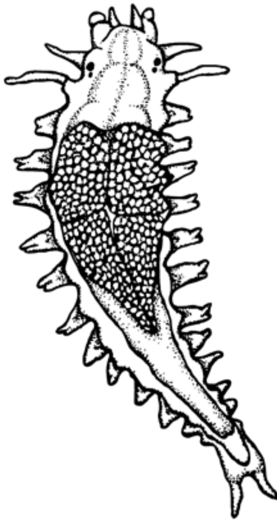

FIG. A1.5 *Neanthes Arenaceodentata* , 12-Segmented Stage

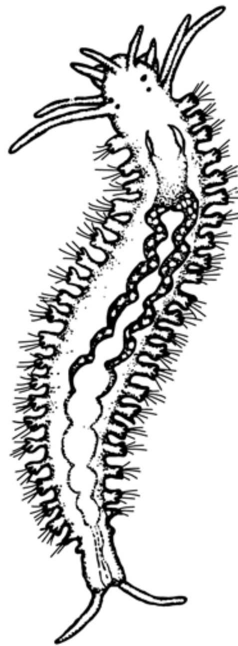

FIG. A1.6 *Neanthes Arenaceodentata* , Juvenile, with 21 Segments and Having Just Left the Parents' Tube and Commenced Feeding

the beakers. Sediment and water should be added to beakers the day before the worms are added, to allow suspended sediment particles to settle, and to allow time for equilibration of the temperature and sediment-water interface (33).

A1.5.1 After the overnight equilibration time, five worms are distributed to each test chamber, with additional toxicity test water to raise the water level to the 750-mL level. Initial (that is, 1 h) observations of burrowing should be made following placement of the worms in the test chambers. If a worm, or group of worms, do not appear to be burrowing and the observer believes that the nonburrowing behavior results from factors other than sediment toxicity (for example, reduced viability or damage to test organisms), those worms should be replaced.

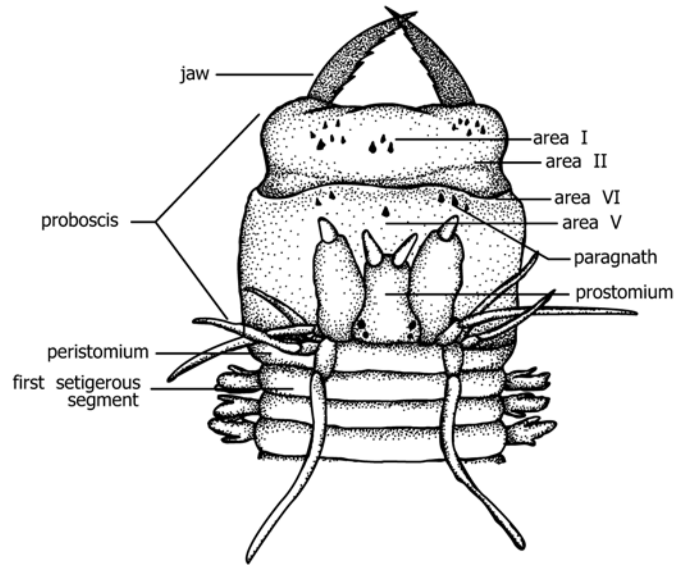

FIG. A1.7 *Neanthes Virens* , Anterior End, Dorsal View, With Everted Proboscis

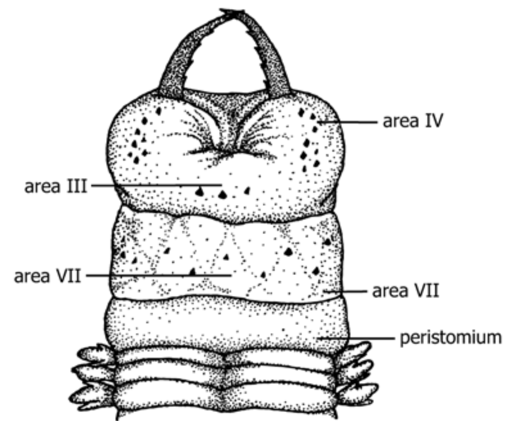

FIG. A1.8 *Neanthes Virens* , Anterior End, Ventral View, with Everted Proboscis

A1.5.2 The toxicity test is terminated when worms are separated from the test sediment at either 10 days or 20 to 28 days. The contents of the test chamber are emptied into a white sorting tray and surviving worms are separated at the end of the test period. Some of the worms may remain in their mucoid tube; if this is the case, a worm can be removed from the tube by probing gently one end of the tube, which causes the worm to leave its tube. Count and record the number of survivors and dead worms. The 10-day experiment is terminated at this time.

A1.5.3 After the number of survivors and dead worms has been determined in the 20 to 28-day test, the worms from each replicate are placed in a clean petri dish and washed in distilled water. The worms from each replicate are placed in a pre-weighed aluminum pan, dried at 50°C to a constant weight, and weighed to the nearest 0.1 mg.

A1.6 *Control Survival*—Mean control survival in both the 10-day and the 20 to 28-day tests using *N. arenaceodentata* is generally 95 % or greater, and must be at least 90 % and 80 %

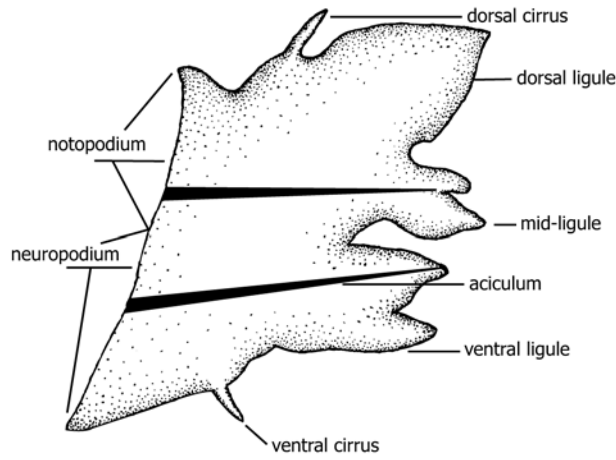

FIG. A1.9 *Neanthes Virens* , Parapodium in Side View

FIG. A1.10 *Neanthes Virens* , Spiniger Seta from the Notopodial Region (Dorsal) of Parapodium with Equal Sides to the Shaft

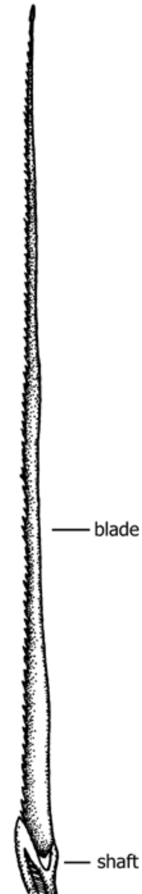

FIG. A1.11 *Neanthes Virens* , Spiniger Seta from the Neuropodial Region (Ventral) of Parapodium with Unequal Sides to the Shaft

or greater in an individual replicate control for the toxicity test to be considered valid.

A1.7 *Sensitivity—Neanthes arenaceodentata* is a sensitive animal in both aquatic and sediment toxicity tests and has been found to be more sensitive than the amphipod *Rhepoxynius abronius* in one test (**49**). Table A1.1 summarizes the ecological and test conditions for this species.

TABLE A1.1 Summary of the Ecological and Test Conditions that Should Be Considered When Conducting Sediment Toxicity Tests with *N. Arenaceodentata*

Geographical	California Baja, California, Hawaii, Central Pacific, New England, Florida, Europe (**1**, **49**, **50**)
Habitat	burrows into intertidal to subtidal silts and sands (**2**)
Life cycle	3 to 4 months under laboratory conditions (**1**)
Temperature	optimum between 15 and 25°C (**1**)
Salinity	optimum between 28 and 36 ppt (**1**)
Sediment type	silts and sands (**2**)
Sediment depth	usually upper 5 cm (**1**)
Nutrition	detrital (field), variety of foods (laboratory) (**1**)
Light cycle	no special requirement
Control mortality	not to exceed 10 %
Chronic test	20 to 28-day sediment test developed (**23**)
Life cycle test	sediment (**51**); water (**1**)
Test results, water[A]	
Aluminum	>2.0 96-h LC50 (**50**)
Arsenic	7.4 96-h LC50 (**2**)
Cadmium	5.6 (at 15°C); 11.8 (at 20°C) 96-h LC50 (**50**)
Chromium VI	3.9 96-h LC50 (**2**)
Copper	0.13 96-h LC50 (**2**)
Lead	>10 (at 15°C); 7.7 (at 20°C) 96-h LC50 (**50**)
Mercury	0.02 96-h LC50 (**50**)
Nickel	49 96-h LC50 (**50**)
Silver	0.2 96-h LC50 (**50**)
Zinc	1.5 96-h LC50 (**50**)
Life cycle[B]	
Cadmium	1.0 (**50**)
Lead	3.1 (**50**)
Zinc	0.32 (**50**)

[A] Data as milligrams per litre.
[B] Concentration in milligrams per litre at which there was significant suppression of reproduction.

A2. *NEANTHES VIRENS*

A2.1 *Introduction—Neanthes virens* is distributed widely in the North Atlantic Ocean from the Arctic to Virginia, Iceland, and in Europe from the British Isles, North Sea, and France (**49**). It is usually found in intertidal waters and from shallow subtidal depths, but specimens have been collected from 150-m depth. It is found in all types of sediments, including coarse to fine sands, clay, peat, and water-soaked wood. It is common on mud flats bordering the mouths of rivers. Also known as *Nereis virens* and *Nereis (Neanthes) virens, N. virens* is referred to commonly as the "sandworm" or "clamworm." It is a commercially important worm for fish bait and as a biological species for education and research. Specimens measure up to 90 cm in length and 4.3 cm in width and possess more than 200 segments. It is necessary to kill the animal in order to make positive identification. The everted proboscis (Fig. A1.7 and Fig. A1.8) are characterized as follows: Area I, 0 to 7 paragnaths; Area II, 1 to 8 paragnaths in an arch on each side; Area III, 1 to 3 irregular rows of 4 to 14 paragnaths; Area IV, 5 to 27 paragnaths in an arch on each side; Area V, 0 to 4 paragnaths; Area VI, 0 to 5 paragnaths on each side; and Areas VII–VIII, 2 to 3 irregular rows of paragnaths. Parapodia (Fig. A1.9) are similar from anterior to posterior end with a well-developed dorsal ligule. Setae (Fig. A1.10 and Fig. A1.11) are similar in appearance with a long blade; however, the sides of the shaft may be equal from the notopodial region (Fig. A1.10) or unequal in the neuropodial region (Fig. A1.11) of the parapodium.

A2.2 *Source of Test Animals—Neanthes virens* can be collected from intertidal sand-mud flats during low tides. Specimens may be injured during collection; they should be discarded. Bait shops should not be used as a source of test animals because of the potential variability of the condition of the worm.

A2.3 *Feeding Requirements—Neanthes virens* feeds on a variety of food in its natural environment, including algae and living and dead animals. It is not usually necessary to feed this species if it is being kept in the laboratory for only 2 to 3 weeks. Provide the animals with green or brown algae or

TABLE A2.1 Summary of the Ecological and Test Conditions that Should Be Considered When Conducting Sediment Toxicity Tests with *N. virens*

Geographical	atlantic coast from the Artic region south to Virginia, Iceland, Norway, British Isles, North Sea, France
Habitat	intertidal to subtidal in coarse and fine muddy sand, mussel beds, roots of decaying marsh and eelgrass (**52**)
Life cycle	1 to 2 years from egg to sexual maturity (**49**, **53**)
Temperature	optimum 10 ± 5°C (**49**, **54**)
Salinity	optimum 30 to 35 ppt (**49**, **54**)
Sediment types	common in fine muddy sand but may occur in all types from coarse sand to clay and peat (**52**)
Sediment depth	burrows not uncommon to depths of 20+ cm
Nutrition	omnivorous; can be held for extended periods without food (**54**)
Light cycle	no special requirements
Control mortality	should not exceed 10 %
Chronic test	10 to 28-day bioaccumulation test method (**44**)
Test results (**37**)	
Cadmium	>40.0 mg/L 96-h LC50 (water)
Chlordane	0.22 mg/L 288-h LC50 (water)
	<–5.8 mg/kg 288-h LC50 (sediment)
DDT	>0.3 mg/L 288-h LC50 (water)
	>16.5 mg/kg 288-h LC50 (sediment)
Diedrin	>0.17 mg/L 288-h LC50 (water)
	>13.0 mg/kg 288-h LC50 (sediment)
Endosulfan	0.1 mg/L 288-h LC50 (water)
	0.34 mg/kg 288-h LC50 (sediment)
Endrin	>11.0 mg/L 288-h LC50 (water)
	>28.0 mg/kg 288-h LC50 (sediment)
Pentachlorophenol	>0.12 mg/L 7-week LC50 (water)

freshly chopped clams if feeding is necessary. If, however, animal food is provided, such as freshly chopped clams, do not overfeed since excess food can foul the water readily (**53**).

A2.4 *Holding Requirements*—Many animals, approximately 10/600 cm^2, can be placed in an aquarium provided with seawater and aeration. *Neanthes virens* is capable of living in a wide salinity range (from 15 to 35 ppt) but prefers the higher salinities within this range.

A2.5 *Life History*—*Neanthes virens* leaves its burrow and swims to the surface when it is sexually mature. Large numbers of swarmers have been observed, during both the day and night (**49, 53**). Both sexes release their gametes into the water and die following spawning. Laboratory colonies of this species have not been established (**53**) because they are sexually mature for only a short period of time (hours) and the life cycle is long (1 to 2 years).

A2.6 *Toxicity Test Specifications*—Toxicity tests with *N. virens* are generally conducted in small glass aquaria (4 to 38 L). A minimum sediment depth of 10 cm is recommended for adult animals. The loading of each test chamber should not exceed 0.8 g/L in static and renewal exposures. In renewal exposure tests, one third of the exposure water is exchanged with fresh sea water every third day during the course of the experiment. Gentle aeration should be provided to ensure that the DO concentration remains above ≥90 % saturation. Sediment should be layered in the exposure chamber before the gentle addition of overlying water to minimize sediment resuspension. Sediment and water should be added to the test chambers at least one day prior to the start of the test to allow suspended sediment particles to settle and to allow time for temperature equilibration. The surviving animals can be removed from the sediment with a medium-sized mesh aquarium net or sieve at the termination of the test (**54**).

A2.7 *Control Survival*—Mean control survival in the 10-day test with *N. virens* is generally greater than 95 % and must be at least 90 % and 80 % or greater in an individual replicate control for the toxicity test to be considered valid.

A2.8 *Sensitivity*—The mortality end point is relatively insensitive to contaminants. Numerous biochemical and physiological measurements of sublethal responses have been investigated with this species due to its relative large size, which also makes *N. virens* desirable for bioaccumulation studies.

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SUMMARY OF CHANGES

The primary changes from the previous version of this guide are summarized in this Section.

E1611 – 99 and E1611 – 00:
The following sections were revised in 1999 and 2000:
(*1*) Sediment Collection (10.1);
(*2*) Sediment Storage (10.4);
(*3*) Sediment Spiking (10.6);
(*4*) Reference Toxicity: USEPA 2000; Test Method E1706.

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