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Standard Guide for Conducting 10-day Static Sediment Toxicity Tests with Marine and Estuarine Amphipods¹

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1. Scope

1.1 This guide (3)^{2,3} describes procedures for obtaining laboratory data concerning the short-term adverse effects of potentially contaminated sediment, or of a test material experimentally added to contaminated or uncontaminated sediment, on marine or estuarine infaunal amphipods during static 10-day exposures. These procedures are useful for testing the effects of various geochemical characteristics of sediments on marine and estuarine amphipods, 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 10-day static sediment toxicity tests are described for the following species: *Rhepoxynius abronius*, *Eohaustorius estuarius*, *Ampelisca abdita*, *Grandidierella japonica*, and *Leptocheirus plumulosus*.

1.2 Two documents (USEPA 1994 (1), USEPA-USACE 1999 (2)) provide additional guidance on methods for conducting sediment toxicity tests with estuarine and marine amphipods. This additional guidance includes supplemental information on: 1. sediment collection and storage (Section 10.4), 2. sediment spiking (Section 10.6), 3. collection, handling, and culturing of amphipods (Section 11.4), and 4. statistical analyses (Section 16). USEPA-USACE (1999) also provides guidance on a method for conduction 28-d sediment toxicity tests with the amphipod *Leptocheirus plumulosus*. Endpoints measured in this 28-d test include survival, growth, and reproduction.

1.3 Modifications of these procedures might be appropriate for other sediment toxicity test procedures such as flow-through or partial life-cycle tests. 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 amphipods, other crustaceans, polychaetes, and bivalves.

1.4 Other modifications of these procedures might be justified by special needs or circumstances. Although using appropriate procedures is more important than following prescribed procedures, results of tests conducted using unusual procedures are not likely to be comparable to results of many other tests. Comparisons of 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.

1.5 These procedures are applicable to sediments containing most chemicals, either individually or in formulations, commercial products, and known or unknown mixtures. With appropriate modifications these procedures can be used to conduct sediment toxicity tests on factors such as temperature, salinity, dissolved oxygen, and natural sediment characteristics (for example, particle size distribution, organic carbon content, total solids). These methods can also be used to conduct bioconcentration tests and in situ tests, and to assess the toxicity of potentially contaminated field sediments, or of such materials 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 either adhering to sediment particles or dissolved in interstitial water can be tested.

1.6 Results of short-term toxicity tests with test materials experimentally added to sediments may be reported in terms of an LC50, and sometimes an EC50 where “concentration” refers to dry or wet weight concentration in sediment. Results of a field survey with single samples to determine a spatial or temporal distribution of sediment toxicity may be reported in terms of percent mortality (see Section 16). 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.

1.7 This guide is arranged as follows:

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² Boldface numbers in parentheses refer to the list of references at the end of this guide.

³ This guide is based largely on Guide E 729 and Ref (3).

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

1.9 *This standard does not purport to address all of the safety problems, 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.* While some safety considerations are presented in this guide, it is beyond the scope of this guide to encompass all safety requirements necessary to conduct sediment toxicity tests. Specific hazard statements are given in Section 8.

2. Referenced Documents

2.1 ASTM Standards:

D 1129 Terminology Relating to Water⁴

D 3976 Practice for Preparation of Sediment Samples for Chemical Analysis⁵

D 4447 Guide for the Disposal of Laboratory Chemicals and Samples⁶

E 380 Practice for Use of the International System of Units (SI) (the Modernized Metric System)⁷

E 729 Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians⁶

E 943 Terminology Relating to Biological Effects and Environmental Fate⁶

E 1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses⁶

3. Terminology

3.1 Definitions of Terms Specific to This Standard:

3.1.1 The term “*sediment*” is used here to denote a naturally occurring particulate material that has been transported and deposited at the bottom of a body of water. The procedures described can also be applied using an experimentally prepared substrate within which the amphipods can burrow.

3.1.1.1 *clean sediment*—denotes sediment that does not contain concentrations of toxicants that cause apparent stress to the test organisms or reduce their survival.

3.1.1.2 *solid-phase sediment*—distinguished from elutriates and resuspended sediments in that the whole, intact sediment is used to expose the organisms, not a form or derivative of the sediment.

3.1.2 *toxicity*—the property of a material or combination of materials, to adversely affect organisms (see Terminology E 943).

3.1.3 *exposure*—contact with a chemical or physical agent (see Terminology E 943).

3.1.4 *interstitial water*—the water within a wet sediment that surrounds the sediment particles. The amount of interstitial water in sediment is expressed as the percent ratio of the weight of the water in the sediment to that of the wet sediment.

3.1.5 *overlying water*—the water that is added to the test chamber over the solid phase of the sediment in a toxicity test.

3.1.6 *spiking of sediment*, refers to the experimental addition of a test material such as a chemical or mixture of chemicals, sewage sludge, oil, particulate matter, or highly contaminated sediment to a clean negative control or reference sediment to determine the toxicity of the material added. After the test material is added, sometimes with a solvent carrier, the sediment is thoroughly mixed to evenly distribute the test material throughout the sediment.

3.1.7 The LC50 is the statistically or graphically derived best estimate of the concentration of test material added to or contained in sediment that is expected to be lethal to 50 % of the test organisms under specified conditions within the test period (see Terminology E 943).

3.1.8 The EC50 is the statistically or graphically estimated concentration of test material in sediment that is expected to

⁵ Annual Book of ASTM Standards, Vol 11.02.

⁶ Annual Book of ASTM Standards, Vol 11.05.

⁷ Annual Book of ASTM Standards, Vol 14.02 (excerpts in Related Materials Section of all volumes).

⁴ Annual Book of ASTM Standards, Vol 11.01.

cause a measured sublethal effect (for example the inability of amphipods to rebury in clean sediment at the end of the test period), in 50 % of the test organisms under specified conditions (see Terminology E 943).

3.1.9 The words “must,” “should,” “may,” “can,” and “might” have very specific meanings in this guide.

3.1.9.1 “Must” is used to express an absolute requirement, that is, to state that the test ought to be designed to satisfy the specified condition, unless the purpose of the test requires a different design. “Must” is only used in connection with factors that directly relate to the acceptability of the test (see Section 15).

3.1.9.2 “Should” is used to state that the specified condition is recommended and ought to be met if possible. Although violation of one “should” is rarely a serious matter, violation of several will often render the results questionable. Terms such as “is desirable,” “is often desirable,” and “might be desirable” are used in connection with less important factors.

3.1.9.3 “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.”

3.2 For definitions of other terms used in this guide, refer to Terminology D 1129, Guide E 729, Terminology E 943, and Guide E 1023. For an explanation of units and symbols, refer to Practice E 380.

4. Summary of Guide

4.1 The relative toxicity of marine or estuarine sediments can be determined through a 10-day static test with solid phase sediment and overlying water in aerated 1-L glass test chambers. Mortality and sublethal effects such as emergence from sediment and inability to bury in clean sediment are determined after exposure of a specific number (usually 20) of amphipods to a quantity of test sediment. Response of the amphipods to the test sediment is compared with response in control sediment. A negative control or reference sediment is used to provide (a) a measure of the acceptability of the test by providing evidence of the health and relative quality of the test organisms, and the suitability of the overlying water, test conditions and handling procedures, etc., and (b) the basis for interpreting data obtained from the test sediments.

4.1.1 The toxicity of field collected sediment is indicated by the percent mortality of amphipods exposed to that sediment compared to those exposed to control sediment. The toxicity of field sediments may also be assessed by testing dilutions of a highly toxic test sediment with clean sediment to obtain information on the toxicity of proportions of that sediment.

4.1.2 The toxicity of a toxicant experimentally added to sediments can be expressed by analyzing the mortality and reburial data to determine an LC50 and an EC50 for the toxicant for the duration of exposure.

5. Significance and Use

5.1 The test procedure in this guide is not intended to exactly simulate the exposure of benthic amphipods to contaminants 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 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. Protection of the most sensitive species within a community will theoretically protect the community as a whole.

5.3 Amphipods are an abundant component of the soft bottom marine and estuarine benthic community. They are a principal prey of many fish, birds, and larger invertebrate species. Some species are predators of smaller benthic invertebrates. Others ingest sediment particles and thus are directly exposed to contaminants. Amphipods are among the first taxa to disappear from benthic communities impacted by pollution, and have been shown to be more sensitive to contaminated sediments than several other major taxa (4). The ecological importance of amphipods, their wide geographical distribution, ease of handling in the laboratory, and their sensitivity to contaminated sediments make them appropriate species for sediment toxicity testing.

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

5.5 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 (a) motile organisms might avoid exposure when possible and (b) toxicity to benthic organisms can be dependent on sediment characteristics, dynamics of equilibrium partitioning, and the route of exposure to the benthic organisms.

5.6 The amphipod 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 in toxicity. Mortality data can be used to indicate the relative toxicity of field collected sediments.

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

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

5.9 Results of acute sediment toxicity tests might be an important consideration when assessing the hazards of materials to aquatic organisms (see Guide E 1023) or when deriving sediment quality criteria for aquatic organisms (5). Sediment toxicity tests might be useful in making decisions regarding the extent of remedial action needed for contaminated sites.

6. Interferences

6.1 Due to the limited time sediment toxicity tests have been practiced, the methodology continues to develop and evolve with time and research needs. Because of the developmental nature of sediment toxicity testing, there are limitations to the methods described in this guide.

6.2 Results of acute sediment toxicity tests will depend, in part, 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 results from static sediment toxicity tests might include:

6.2.1 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 is extremely difficult. The sediment environment is composed of a myriad of microenvironments, redox gradients, and other interacting physiochemical and biological processes. Many of these characteristics influence sediment toxicity and bioavailability to benthic and planktonic organisms, microbial degradation, and chemical sorption. Any disruption of this environment complicates 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 contaminant solubility, partitioning coefficients, and other physical and chemical characteristics.

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

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

6.2.4 Realism of using spiked sediment (that is, whether the spiked sediment is at equilibrium and evenly mixed).

6.2.5 Photolysis and other processes degrading test chemicals.

6.2.6 Maintaining acceptable quality of overlying water.

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

6.2.8 Resuspension of sediment during the toxicity test.

6.2.9 Limited opportunity for biological observations during the test because organisms bury in test sediment.

6.2.10 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 Recovery of test organisms from the test system.

6.2.12 Endemic organisms which might be present in field collected sediments including (a) predators, (b) species that might be the same as or closely related to the test species, (c) microorganisms (for example, bacteria, molds), and algae colonizing sediment and test chamber surfaces.

6.3 Static tests might not be applicable to materials that are highly volatile or are rapidly biologically or chemically transformed. Furthermore, the overlying water quality might change considerably from the initial overlying water. Because the experimental chambers are aerated, the procedures can usually be applied to materials that have a high oxygen demand.

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 contaminant 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*—Flow-through troughs or aquaria containing either clean (uncontaminated) natural sea water or reconstituted sea water should be used for holding amphipods after field collection and prior to a test. The holding tanks and any areas used for manipulating live amphipods should be located in a room or space separate from that in which toxicity tests are to be conducted, stock solutions or test materials are prepared, or equipment is cleaned. The water supply system should be equipped with salinity and temperature control and aeration.

7.1.1 Test chambers containing sediment should be held in a well-lighted (at least 100 lx at the test sediment surface), constant temperature room, incubator, or recirculating water bath to maintain the experimental temperature. Air used for aeration should be free of fumes, oil, and water; filters to remove oil and water are desirable. The area containing the test chambers must be well ventilated and free of fumes, both to prevent contamination of test materials and to protect researchers from exposure to toxic volatile materials that might be released from the test sediments. Enclosures may be needed to ventilate the area surrounding test chambers.

7.1.2 The exposure room should be equipped with a timing device for photoperiod control. If a photoperiod other than continuous light is used, it might be desirable to incorporate a 15 to 30-min transition period when lights go on or off to reduce stress to the organisms from sudden large changes in light intensity (10). It is also desirable to have the room temperature and light controls and the aeration on emergency power to protect the experiment in case of a power failure.

7.2 *Construction Materials*—Equipment and facilities that contact stock solutions, test solutions, or any water or sediment into which test organisms will be placed should not contain substances that can be leached or dissolved by aqueous solutions in amounts that adversely affect test organisms. In addition, equipment and facilities that contact stock or test solutions or sediment should be chosen to minimize sorption of test materials from water. Glass, Type 316 stainless steel, nylon, high-density polyethylene, polycarbonate and fluorocarbon plastics should be used whenever possible to minimize dissolution, 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 sea water, for a week or more before use (11). Brass, copper, lead, cast iron pipe, galvanized metal, and natural rubber should not contact test sea water, stock solutions, or test sediment before or during the test. Tubing used in making up test sea water and in aerating the test chambers should be

nontoxic vinyl.⁸ New tubing should be aged at least one week prior to use. Separate sieves, dishes, containers, and other equipment should be used to handle test sediment or other toxic materials and these should be kept and stored separately from those used to handle live animals prior to testing.

7.3 Test Chambers—Species specific information on test chambers is given in Annex A2–Annex A5. The test chambers should be placed in water bath to minimize temperature fluctuations, and should be aerated. Aeration can be provided as in 13.1.

7.4 Cleaning—Test chambers and other glassware, and equipment used to store and prepare test sea water, stock solutions, and test sediment should be cleaned before use. All glassware should be cleaned before each use by washing with laboratory detergent, followed by three distilled water rinses, 10 % nitric (HNO₃) or hydrochloric (HCl) acid rinse, and at least two distilled water rinses. Metals, sulfides, and carbonate deposits are removed by the acid rinse. Organic chemicals should be removed by a water-miscible organic solvent rinse followed by a distilled water rinse, or by baking for 8 h at 300 to 400°C. The use of hypochlorite solution is not recommended, because it is highly toxic to the test organisms (12) and difficult to remove from some materials. At the end of each test, all items that are to be used again should be immediately (a) emptied, (b) rinsed with water, (c) cleaned by a procedure appropriate for removing the test material, and (d) rinsed at least twice with deionized, distilled, or clean sea water. Large plastic containers used only for non-toxic sediments and water may be rinsed after use with clean sea water. They should be used only for toxicity tests and stored in a room that is free from toxic fumes. Glassware used only for live animals, not exposed to toxicants, may be cleaned using only clean distilled or sea 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 “nontoxicant” test in which all test chambers contain control sediment and clean sea water. Survival of the test species will demonstrate whether facilities, water, control sediment, and handling techniques are adequate to result in acceptable (≥90 %) control survival in the absence of toxicants.

8. Hazards

8.1 Many materials can affect humans adversely if precautions are inadequate. Therefore, skin contact with all toxicants, overlying water, and sediments should be minimized by such means as wearing appropriate protective gloves (especially when washing equipment or putting hands into test sediments or solutions), laboratory coats, aprons, and glasses. Special precautions, such as covering test chambers and ventilating the area surrounding the chambers, should be taken when conducting tests on volatile materials. Information on toxicity to humans (6), recommended handling procedures (7), 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 (8) and with materials that are, or are suspected of being, carcinogenic (9).

8.2 Field sediments to be tested, especially those from effluent areas, might contain organisms that can be pathogenic to humans. Special precautions when dealing with these sediments might include immunization prior to sampling and 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 exposure of researchers to toxic compounds. Mixing of toxic sediments in open containers, spiking of laboratory prepared sediments, and loading of toxic sediments into test chambers should be done in a well-ventilated area, preferably a chemical fume hood. Face shields or protective goggles should be worn during any operations that might involve accidental splashing of sediments, such as sieving, mixing and loading into test chambers.

8.4 Health and safety precautions and applicable regulations for disposal of stock solutions, overlying water from test chambers, test organisms, and sediments should be considered before beginning a test (see Guide D 4447). Consideration of cost as well as detailed regulatory requirements might be necessary. For tests involving spiked sediments with known toxicants, removal or degradation of toxicants before disposal of stock solutions, test sediments, and water is sometimes desirable.

8.5 Cleaning of equipment with a volatile solvent such as acetone, should be performed only in a well-ventilated area in which no smoking is allowed and no open flame, such as a pilot light, is present. Cleaning equipment with acids should be done only in a well-ventilated area, and protective gloves and safety goggles should be worn.

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

8.7 Use of ground fault systems and leak detectors is strongly recommended to help prevent electrical shocks because salt water is a good conductor of electricity.

9. Toxicity Test Water

9.1 General Requirements—Besides being available in adequate supply, water used in toxicity tests should be acceptable to test organisms and the purpose of the test. The minimum requirement for acceptable water for use in acute toxicity tests is that healthy test organisms survive in the water, and 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 contaminants and the range of temperature and salinity are controlled.

⁸ Tygon R-3603, a registered trademark of Norton Co., Performance Plastics, 335 N Diamond St., Ravenna, OH 44266, or equivalent, has been found suitable for this purpose.

the holding period do not adversely affect the survival of the test organisms in the holding tanks or in the control treatments during the test.

9.2 Source:

9.2.1 Natural Salt Water—If natural salt water is used, it should be obtained from an uncontaminated area known to support a healthy, reproducing population of the test species or a comparable sensitive species. The water intake should be positioned to minimize fluctuations in quality and the possibility of contamination, and to maximize the concentration of dissolved oxygen to help ensure low concentrations of sulfide and iron. A specially designed system might be necessary to obtain salt water from a natural water source. To ensure uniform quality, water should be monitored as in 9.4. These precautions are intended to ensure that test organisms are not apparently stressed by water quality during holding, acclimation, and testing and that water quality does not unnecessarily affect test results.

9.2.2 Reconstituted Salt Water—Reconstituted salt water can be prepared by adding a commercially available sea salt or specified amounts (see Guide E 729 and Table 1) of reagent-grade chemicals (13) to high-quality water with (a) conductivity less than 1 $\mu\text{S}/\text{cm}$ and (b) either total organic carbon (TOC) less than 2 mg/L or chemical oxygen demand (COD) less than 5 mg/L. Acceptable water can usually be prepared using properly operated deionization or distillation units. Reconstituted salt water should be intensively aerated before use, and aging for one to two weeks might be desirable. If a residue or precipitate is present, the solution should be filtered before use. The water should meet the criteria given in 9.1.

9.2.3 Chlorinated water must never be used in the preparation of water for toxicity tests, because residual chlorine and chlorine-produced oxidants are highly toxic to many aquatic animals (12). Dechlorinated water should be used only as a last resort because dechlorination is often incomplete. Municipal drinking water is not recommended for use because in addition to residual chlorine, it often contains unacceptably high concentrations of metals, and quality is often highly variable (see Guide E 729).

9.3 Preparation:

TABLE 1 Reconstituted Salt Water (14) for Marine and Estuarine Crustaceans

Add the following reagent-grade (13) 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
$\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$	20 mg
H_3BO_3	30 mg
KBr	100 mg
KCl	700 mg
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	1.47 g
Na_2SO_4	4.00 g
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	10.78 g
NaCl	23.50 g
$\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$	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 time of use. The reconstituted salt water should be stripped of trace metals (15).

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 (16) or a filter effective to 0.45 μm or less.

9.3.1.1 If necessary, the salinity should be reduced by diluting the sea water with high-quality deionized or distilled water (see 9.2.2). Salinity can be raised by addition of clean filtered oceanic water or prepared brine. Common practice is to use a 60 to 90-g/kg saltwater brine. Such brines have been successfully prepared using slow, heat-concentration of natural salt water, or by the addition of artificial sea salts or reagent grade (13) salts to a natural salt water (see 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 $4 \pm 3^\circ\text{C}$ 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 test chambers. Additional water might be required for sieving control sediment to adjust salinity or for holding the test amphipods prior to the test.

9.3.3 For certain applications the experimental design might require use of sea water from the test sediment collection site. In other instances, experimental treatments might involve manipulation of the test sea water conditions.

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 or chlorinity, pH, particulate matter, total organic carbon (TOC), organophosphorus pesticides, organic chlorine (or organic chlorine pesticides plus polychlorinated biphenyl (PCBs)), chlorinated phenoxy herbicides, ammonia, cyanide, sulfide, bromide, fluoride, iodide, nitrate, phosphate, sulfate, calcium, magnesium, potassium, aluminum, arsenic, beryllium, boron, cadmium, chromium, cobalt, copper, iron, lead, manganese, mercury, molybdenum, nickel, selenium, silver, and zinc.

9.4.2 In estuarine areas, where large diurnal, tidal, and seasonal variations in concentrations of organics, heavy metals and water quality might occur, more frequent monitoring might be necessary. 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 (a) be accurate and precise enough to adequately characterize the toxicity test water or (b) have detection limits below concentrations that have been shown to adversely affect the test species (17).

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 contaminants (see Section 8).

10.2 Characterization—Sediments chosen for use should be characterized and at least the following should be determined: pH, organic carbon content (total organic carbon 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 in 14.4. 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 amphipod collection site or from another area that is within the geochemical requirements of the test species and that can provide a nontoxic reference sediment for evaluation of the condition of the test population subject to laboratory procedures, and for statistical comparison with test sediment. Control sediment should be brought to the sieving area in a clean collecting basin. Any water overlying the sediment or used to rinse the sediment into the collecting basin should be saved so that fine particles contained in the water can be recombined into the sediment. Any sediment that shows evidence of contamination (for example, oil sheen) should be discarded. As the sediment is collected, bottom temperature and 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 water content, particle size distribution, and organic content.

10.3.1.1 At least annually, control sediment should be empirically characterized as in 10.2.

10.3.2 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 as in 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, sediment should be left undisturbed for a sufficient time to allow settling of fine particles (usually at least overnight). 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 sediment should be allowed to settle, overlying water should be decanted, and the sediment should be thoroughly mixed to evenly distribute fine particles that settle on the surface.

10.3.3 Storage—The control sediment should be stored in glass or rigid plastic containers at $4 \pm 3^{\circ}\text{C}$ until the test chambers are prepared. The sediment should be stored in the dark and must not be frozen or allowed to dry during storage.

10.4 Field-Collected Test Sediment:

10.4.1 Collection—The spatial or temporal distribution of sediment toxicity can be determined by collecting potentially contaminated sediment from field sites. A benthic grab or core should be used rather than a dredge to minimize disruption of the sample. If the sediment is collected with a grab, glass cores

should be used to collect a sample from the upper 2 cm, or desired layer, of the test sediment. This operation is facilitated if the grab can be opened from the top so that the undisturbed sediment surface is exposed. The sample should be transferred to a clean (see 7.4) glass, high-density polyethylene or fluorocarbon plastic sample container. It is desirable as much as possible to avoid contact of the sample with metals, including stainless steel, and plastics including polypropylene and low-density polyethylene as contaminant interactions might occur. The sample must be cooled in the field to about 4°C , and stored at $4 \pm 3^{\circ}\text{C}$ until the sediment is distributed to the test chambers. Test sediment should be stored in the dark, no longer than two weeks before the initiation of the test, and must not be frozen or allowed to dry. Freezing and longer storage might change sediment properties and have been shown to change the toxicity of stored sediment (18, 19). Field collected test sediments should not be wet sieved, but if obvious large predators or other large organisms are present, they should be removed by forceps. For some applications, it might be desirable to remove small objects by press sieving the sediment through a 2-mm sieve. If sediment is stored longer than two weeks, it should be retested to confirm that toxicity has not changed.

10.4.1.1 If sediment is collected from multiple field samples and pooled to meet technical objectives, the sediment should be thoroughly homogenized by stirring or mixing by hand, or with the aid of a rolling mill as in 10.8.

10.4.2 Additional samples may be taken from the same grab for other kinds of sediment analyses (see 10.2). Sediment temperature, interstitial water salinity, pH, and Eh can be recorded in the field. Qualitative description of the sediment might include color, texture, depth of oxidized layer, and presence of plants, animals, tracks, or burrows. Monitoring the odor of sediment samples should be avoided, especially if the odor is associated with potentially hazardous chemical contaminants. A core or the remainder of the sediment in the grab can be sieved to provide a macrobenthos sample.

10.4.3 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. Controls for such factors as particle size, organic content, salinity, etc. should be run if the limits are exceeded in the test sediments (20).

10.5 Reference Sediment—A reference sediment is a clean sediment collected from the field that represents the test sediments in sedimental characteristics (for example, TOC; particle size, pH, Eh, salinity). This provides a site-specific basis for comparison of potentially toxic and non-toxic conditions. It should be handled in the same manner as field collected test sediment (see 12.2.1).

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 (21). The resulting

of substances either dissolved in the interstitial water or adsorbed to sediment particles can be determined experimentally.

10.6.2 Test Chemicals—Chemicals experimentally added to sediment should be reagent-grade (**13**) or better, unless a test on a formulation, commercial product, or technical-grade or use-grade material is specifically needed. Before a test is begun, the following should be known about the chemical used: identities of major ingredients and impurities, solubility and stability in test water, estimated toxicity to the test species and to humans, and recommended handling and disposal procedures.

10.6.3 Stock Solution—Toxic chemicals to be tested in sediment are usually dissolved in a solvent to form a stock solution that is then added to the sediment. The concentration and stability of the chemical in the stock solution should be determined before the beginning of the test. If the chemical is subject to photolysis, the stock solution should be shielded from the light both before and during the process of mixing into the sediment.

10.6.4 The preferred solvent is prepared toxicity test sea water at the test salinity. If a substance is insoluble in salt water, deionized water may be used, if salinity is adjusted accordingly if necessary. Several techniques have been specifically developed for preparing aqueous stock solutions of slightly soluble materials (**22**). The minimum necessary amount of a strong acid or base may be used in the preparation of an aqueous stock solution, but such reagents might affect the pH of stock solutions appreciably. Use of a more soluble form of the test material, such as chloride or sulfate salts of organic amines, sodium or potassium salts of phenols and organic acids, and chloride or nitrate salts of metals, might affect the pH more than the use of the necessary minimum amount of a strong acid or base.

10.6.5 If a solvent other than water is used, it should be of reagent grade. Its concentration in the sediment should be kept to a minimum, and should be low enough that it does not affect the test species. Triethylene glycol is often a good organic solvent for preparing stock solutions because of its low toxicity to aquatic animals, low volatility, and high ability to dissolve many organic chemicals. Other water-miscible organic solvents such as methanol, ethanol, or acetone may be used, but they might affect total organic carbon levels, introduce toxicity, alter the geochemical properties of the sediment, or stimulate undesirable growths of microorganisms. Acetone is highly volatile and might leave the system more readily than methanol or ethanol. A surfactant should not be used in the preparation of a stock solution because it might affect the bioavailability, form, and toxicity of the test material.

10.6.6 If a solvent other than water is used, both a solvent control with control sediment and a clean sediment control must be included in the test. The solvent control must contain the highest concentration of solvent present in sediment in any other treatment and must use solvent from the same batch used to make the stock solution. The percentage of organisms that show signs of stress, such as inability to rebury at the end of the test, or death, must be 10 % or less in both controls. Greater

than 10 % mortality in the controls or obvious sublethal stress in 10 % or more of the control animals invalidates the test (see 12.2.2).

10.6.7 If the test contains both a clean sediment (negative) control and a solvent control, the survival, reburial, or other endpoint determined in the two controls should be compared. If a statistically significant difference in any endpoint is detected between the two controls, only the solvent control may be used for meeting the acceptability of the test and 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 may be pooled for meeting the acceptability of the test and as the basis for calculation of results.

10.7 Test Concentration(s):

10.7.1 If the test is designed to calculate an LC50 or other effect level, the test concentrations should bracket the predicted effect level. The prediction might be based on the results of a test on the same or a 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 ten.

10.7.2 If necessary, concentrations above aqueous solubility can be used because in the real world organisms are sometimes exposed to concentrations above solubility and because solubility is often not well known. The toxicity of the test material in sediments might be quite different from the toxicity 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 organic compounds might be normalized to organic carbon content.

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

10.8 Addition of Test Material to Sediment:

10.8.1 Test material such as an effluent, a toxic sediment, or a solution of a chemical can be added to sediment and evenly distributed by thorough hand mixing, by use of a rolling mill, or by adding the test material to a slurry of the test sediment that is allowed to settle. The test material might also be added to water flowing over or through the sediments, and allowed to partition onto the sediment. Other methods of mixing might also be appropriate provided the test material is shown to be evenly distributed in the sediment.

10.8.2 Modifications of the mixing technique might be necessary to allow time for a test material to equilibrate with sediment. If tests are repeated, mixing conditions such as duration and temperature of mixing, and time of mixing before the initiation of the test should be kept constant, unless it is

after spiking is an experimental variable. Care should be taken to ensure that a test material added to sediment is thoroughly and evenly distributed within the sediment. If necessary, sub-samples of the sediment within a mixing container can be analyzed to determine degree of mixing and homogeneity.

11. Test Organisms

11.1 *Species*—The species of infaunal amphipod to be used in the sediment toxicity test should be selected based on availability, sensitivity to test materials, tolerance to 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 selection of a particular species. Ideally, species or genera with wide geographical distributions should be selected, so that test results can be compared among laboratories with similar species. Species used should be identified with an appropriate taxonomic key, and identifications should be verified by a taxonomic authority. The annexes to this guide give guidance as to requirements and methods of handling for various species of amphipods. Use of the species listed in the annexes is encouraged to increase comparability of results.

11.1.1 *Rhepoxynius abronius* is a free-burrowing amphipod that has been successfully used in sediment toxicity testing since the late 1970's (3). The sensitivity of this species to salinities less than 25 g/kg limits its use to testing sediments from marine areas, but the large data base that has been developed for the response of *R. abronius* to a variety of sediments and chemicals establishes its usefulness as a test species as well as a reference species for comparing the sensitivity of other species. Species of the genus *Rhepoxynius* are widely distributed on the West Coast of North America and are present on the East Coast (23).

11.1.2 *Eohaustorius estuarius* (see Annex A2), *E. sencillus*, and *E. washingtonianus* have been successfully used in sediment toxicity tests (24, 25) and haustoriids in general are more abundant than phoxocephalids on the East and Gulf Coasts (26). Their sand-burrowing habits, availability, ease of handling, tolerance to a wide range of salinity and temperature, and ecological importance as probable prey of shorebirds and fishes (27) make them good candidates for test species, especially for estuarine areas.

11.1.3 A variety of other benthic amphipod species have been used successfully to test the toxicity of marine and estuarine sediments using similar methods or the same method described here. These species include: *Corophium salmonis*, *C. spinicorne*, and the freshwater amphipods *Hyaella azteca* and *Pontoporeia hoyi*.

11.1.4 The environmental requirements and sensitivity of a prospective test species of amphipod to test materials and to various sediment characteristics should be established before it is widely used 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 contaminant effects. Choice of the scale of the test chamber, density of test organisms, temperature, salinity, and control sediment might have to be modified to accommodate the

requirements of the test species. Required modifications should be based on conditions at the natural habitat of the species.

11.1.5 The sensitivity of a prospective new test species of amphipod should be compared with a reference species such as *R. abronius* before the new species is used in routine toxicity testing. A 96-h reference toxicity test using water only could eliminate the relative effects of sediment particle size and other sediment characteristics (see 11.5.4). The test should be set up as in Section 13, 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 (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 amphipods are used in sediment toxicity testing, it should be kept in mind that the amphipods 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 utilizing interstitial water. They might feed on particulate materials that either are suspended in the water column or 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 routes of exposure to adsorbed or dissolved sediment contaminants. Amphipods that emerge from the sediment and either swim in overlying water or crawl on the sediment surface might not be continually exposed 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 sensitivity to test materials is not affected by state of maturity, reproduction, seasonality, etc. (see Annexes for species, specific requirements).

11.3 *Source*—All individuals in a test should be from the same source, because different populations of the same species might have different acute sensitivities to contaminants. Marine amphipods are usually obtained directly from a wild population in a clean area, although attempts have been made to culture some species. Collecting permits for field collected amphipods might be required by some local and state agencies.

11.3.1 If test organisms are cultured or held for an extended period of time in the laboratory, the response of laboratory-held organisms to test materials should be compared to that of animals freshly collected from the field to assure that laboratory stresses do not affect their sensitivity to test materials (19).

11.4 Collection and Handling:

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

11.4.2 *Collection*—Amphipods can be collected intertidally with a shovel or subtidally with a small biological dredge or a grab. Sediment containing amphipods can be gently sieved to separate the amphipods. The amphipods can then be collected