Designation: E 1367 – 99

Standard Guide for Conducting 10-day Static Sediment Toxicity Tests with Marine and Estuarine Amphipods[1]

This standard is issued under the fixed designation E 1367; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ϵ) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This guide (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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[1] This guide is under the jurisdiction of ASTM Committee E-47 on Biological Effects and Environmental Fate and is the direct responsibility of Subcommittee E47.03 on Sediment Assessment and Toxicology.

Current edition approved April 10, 1999. Published July 1999. Originally published as E 1367 – 90. Last previous edition E 1367 – 92.

[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 Water[4]

D 3976 Practice for Preparation of Sediment Samples for Chemical Analysis[5]

D 4447 Guide for the Disposal of Laboratory Chemicals and Samples[6]

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 Amphibians[6]

E 943 Terminology Relating to Biological Effects and Environmental Fate[6]

E 1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses[6]

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 cause a measured sublethal effect (for example the inability of

[4] *Annual Book of ASTM Standards*, Vol 11.01.

[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 Material Section of all volumes).

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

[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.

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 (HNO_3) 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 encountered during 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 µS/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:*

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 ± 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 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.

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

[A] If the resulting solution is diluted to 1 L, the salinity should be 34 ± 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**).

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 cvollected, bottom temperature and salinity and sediment temperature should be recorded, and a composite sediment sample from all shovelsful, 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 ± 3°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, highdensity 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 ± 3°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 toxicity 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 undesireable 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 waterborne 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 time 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 haustorids 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 *Hyalella 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. A96-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 with a dipnet and transferred to and allowed to bury in sieved sediment from the amphipod collection site. Sieves and containers used to collect and transport amphipods 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 amphipods should be held in sediment during transport to the laboratory, and should be kept at or near collection site temperature or below. During a long transport, it might be necessary to keep containers of sediment and amphipods in coolers and to provide aeration. Collection

site sediment should be saved for control, acclimation, and reburial sediment.

11.4.3 *Holding*—Amphipods should be fully acclimated to the test temperature and salinity by holding them in the laboratory prior to their use in a toxicity test. Amphipods 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.

11.4.3.1 In the laboratory, amphipods can be counted into holding containers with clean sieved sediment to ascertain whether sufficient numbers have been collected. Amphipods should be sieved from transport sediment and gently washed into a clean dish for counting. Active, apparently healthy amphipods can be picked up and removed from detritus with a wide-mouthed bulb pipette and transferred to sieved collection site sediment, into which the amphipods should quickly bury. Enough amphipods should be collected to provide at least one third more individuals than are required for the test. During counting, the temperature of the water containing the amphipods must not exceed the amphipods' tolerance limit, and 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 changes in temperature and salinity are necessary to bring amphipods from the collection site conditions to the test conditions, adjustments should be made gradually to allow amphipods to acclimate. Healthy burrowing amphipods will usually remain in the holding sediment until the initiation of the test, and can be easily retrieved for setup. Supplementary feeding during the acclimation period might or might not be necessary, as some amphipods will find food in the holding sediment (see species specific annexes). Any individuals that fail to bury or make tubes (if they are tube builders) in holding sediment or that appear unhealthy during holding should be discarded. The temperature and salinity of the water in the holding containers should be monitored daily.

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

11.5.1 Amphipods in holding containers should be checked daily before the initiation of a test. Individuals that emerge from the sediment and appear dead or unhealthy should be discarded. If greater than 5 % of the amphipods emerge and appear unhealthy during the 48 h preceding the test, the entire group should be discarded and not used in the test.

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

11.5.3 Survival of amphipods in control sediment during the test is an indication of the health of the population and other factors. If a mean of greater than 10 % mortality occurs in the controls, or if individual replicate control mortality values exceed 20 %, the test must be considered invalid.

11.5.4 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 11.1.4).

11.5.4.1 Reference toxicants can be useful in assessing the sensitivity of different populations or species of amphipods, or seasonal variation in sensitivity of a field-collected population. Such assessment is usually conducted simultaneously with the toxicity test. Many chemicals have been used or evaluated as reference toxicants for use as reference toxicants (**28**). 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 there is not an adequate history of use with each procedure, species and laboratory.

12. Experimental Design

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

12.2 *Controls*—Every test requires a control treatment consisting of sediment from the amphipod collection site or other sediment known to be nontoxic to, and within the geochemical requirements of the test species (see 10.3). The same water, conditions, procedures, and organisms are used as in the other test treatments, except that none of the test material 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 test sediments are replicated. This allows comparisons among experiments and among laboratories of the validity of procedures used in individual tests.

12.2.1 In addition to the standard control, if a field 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 reference sediment controls for these characteristics. The design of field surveys should include an additional field control involving five replicate samples from an area that is free from sediment contamination. This provides a site-specific basis for comparison of potentially toxic and nontoxic conditions, and can account for mortality associated exclusively with subjecting the organisms to nonnative sediments. The concentrations of chemical contaminants 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 If any solvent other than water is present in any of the test chambers, a solvent control is also required. The solvent

control must be identical to the regular control, except that the highest amount of solvent present in any other treatment is added to this treatment. If the test material is a mixture, formulation, or commercial product, none of the ingredients is considered a solvent unless an extra amount is used to prepare the stock solution (see 10.6.5).

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. It is often conducted in areas where little is known about contamination patterns. To allow for maximum spatial coverage, the survey design might include only one sample from each station. The lack of replication precludes statistical comparisons, but samples from sites where 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 effects among negative control or reference sediments and test sediments from several sites. Replicates (that is, separate samples from different grabs taken at the same site) should be taken at each station in the survey. The number of replicates needed per station is a function of the need for sensitivity or power (see 12.3.6). 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 to be true replicates for statistical comparisons among stations (**29, 30**).

12.3.3 Station locations might be distributed among 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. In pre-dredging studies, a sampling design can be prepared to assess the toxicity of samples representative of the project area to be dredged. Such a design must include subsampling cores taken to the project depth.

12.3.4 If no amphipods survive in sediment from a particular field location, it might be useful to conduct toxicity tests with dilutions of the field sediment mixed with control sediment. 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. 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 (**30**). 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.

12.3.6 The power of the toxicity test is a function of the number of replicates and the number of individuals and variability in the response measure. On the basis of historical control data with the species *Rhepoxynius abronius*, with five independent replicates per treatment and 20 amphipods per replicate, there is a 75 % probability of detecting a significant difference ($P < 0.05$) if the difference in mean survival between control and test sediment is 2.8 (see Table 2). For control survival of 18.0 (90 %), this corresponds to a test sediment mean survival of 15.2, about a 15 % reduction. Since the number of survivors in test sediments is often much less than 15, this is a reasonable level of precision for most applications.

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 contaminants in sediment. The test can be used to determine natural limits such as salinity, temperature, etc., to estimate the LC50 of a contaminant in a particular sediment type, to study the interaction among contaminants in sediment, and to assess the effect of complex waste mixtures on the test species in sediment.

12.4.1 An acute test used to calculate an LC50 or an EC50 usually consists of one or more control 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 curve indicates that a different dilution factor is more appropriate. At least one concentration should give a partial response below the LC50 or EC50 and one above the LC50 or EC50. If the estimate of acute toxicity is particularly uncertain, six or more concentrations might be desirable to increase the likelihood of covering the appropriate range.

12.4.2 If it is only necessary to determine (*a*) whether a specific concentration is acutely toxic to the test species or (*b*) whether the LC50 or EC50 is above or below a specific concentration (see 10.7.4), only that concentration and the controls are necessary. Two additional concentrations at about one half and two times the specific concentration of concern are desirable to increase confidence in the results.

12.4.3 An LC or EC near the extremes of toxicity, such as an LC5 or an LC95 should not be calculated unless at least one concentration of test material killed or affected a percentage of

TABLE 2 Precision of the Sediment Toxicity Test Using *Rhepoxynius abronius* in Relation to Sample Size and Replication (3)

Number of Amphipods per Replicate					
10			20		
Number of Replicates	δ[A]	δ/c[B]	Number of Replicates	δ[A]	δ/c[B]
2	6.80	71.6			
4	2.66	28.0	2	8.55	45.0
6	1.94	20.4	3	4.44	23.4
8	1.60	16.8	4	3.35	17.6
10	1.38	14.5	5	2.80	14.7
12	1.25	13.2	6	2.45	12.9
14	1.14	12.0	7	2.20	11.6
16	1.05	11.0	8	2.02	10.6
18	0.98	10.3	9	1.89	10.0
20	0.93	9.8	10	1.76	9.3

[A] δ is the difference between the survival means for which the toxicity test is 75 % certain of detecting statistical significance ($P < 0.05$) (**31**).

[B] δ/c expresses the precision estimate as a percent of the normal control survival in *Rhepoxynius abronius* ($c = 19.0$ for $n = 20$; $c = 9.5$ for $n = 10$).

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test organisms, other than 0 or 100 %, near the percentage for which the LC or EC is to be calculated. This requirement might be met in a test to determine an LC50 or EC50, but special tests with appropriate test concentrations and possibly more replicates per treatment might be necessary. Other ways of providing information concerning the extremes of toxicity are to report the highest concentration of test material that actually killed or affected no greater a percentage of the test organisms than did the control treatment(s), or to report the lowest concentration of test material that actually killed or affected all test organisms exposed to it. These alternatives are normally more reliable than reporting a calculated result such as reporting an LC5 or LC95 unless two or more concentrations resulted in percent killed or affected close to 5 or 95 %.

12.4.4 The primary focus of the physical and experimental design of the test and the statistical analysis of the data is the experimental unit, that is defined as the smallest physical entity to which treatments can be independently assigned (see Guide E 729). Thus, the test chamber is the experimental unit. With respect to factors that might affect results within test chambers and, therefore, the results of the test, all chambers in the test should be treated as similarly as possible. For example, the temperature in all test chambers should be as similar as possible unless the purpose of the test is to study the effect of temperature. Test chambers are usually arranged in one or more rows. Treatments must be randomly assigned to individual test chamber locations. A randomized block design (with each treatment being present in each block, that might be a row or a rectangle) is preferable to a completely randomized design to reduce the probability of chance segregation of treatments (27).

12.4.5 The minimum desirable number of test chambers and organisms per treatment should be calculated from (*a*) the expected variance within test chambers, (*b*) the expected variance between test chambers within a treatment, and (*c*) either the maximum acceptable width of the confidence interval on a point estimate (for example, LC50 or EC50) or the minimum difference that is desired to be detectable using hypothesis testing (32). 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 significance test increases.

12.4.6 Mean survival in control sediment must be 90 % or greater. A difference of about 15 % between mean survival in control and test sediments is usually significant when twenty amphipods are included in each of five replicate test chambers of control and test sediment (see 16.5).

12.4.7 It is desirable to repeat the test at a later time to obtain information concerning the reproducibility of the results.

13. Procedure

13.1 *Dissolved Oxygen*—The concentration of dissolved oxygen (DO) in the water overlying the sediment in the test chambers should be maintained at or near saturation by gently aerating the water (see annexes). Air should be bubbled into the test chambers at a rate that maintains a ≥90 % dissolved oxygen concentration, but does not cause turbulence or disturb the sediment surface. If air flow to the beakers is interrupted for more than an hour, DO should be measured in the beakers to determine whether dissolved oxygen concentrations have dropped to less than 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 amphipods occur in the field. Within an experiment individual temperature readings should not vary by more than 3°C from the selected test temperature, and the time-weighted average measured temperature at the end of the test should be within 1°C of the selected test temperature. When temperature is measured concurrently in more than one test chamber, the highest and lowest temperatures should not differ by more than 2°C.

13.3 *Salinity*—The water overlying the test sediment in sediment 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 experimentally spiked 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 upon 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*—For sediment toxicity tests involving some infaunal amphipod species, lights are usually left on continuously. The constant light increases the tendency of the organisms to remain buried in the sediment, and thus to remain exposed to the test material. For other species a different photoperiod might be desired (see annexes).

13.5 *Feeding*—Infaunal amphipods do not require supplementary feeding during the 10-day toxicity test. Supplemental feeding might be required for longer tests (see Annex A3).

13.6 *Beginning the Test*:

13.6.1 The toxicity test begins when test organisms are first placed in test chambers containing test material.

13.6.2 On the day before the test begins, each test sediment sample should be thoroughly homogenized within its storage container, and an aliquot added 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 randomly assigned to prenumbered test chambers. It is desirable to take subsamples of the test sediment for geochemical analyses as the test chambers are loaded. For some experimental designs it might be desirable to test intact cores.

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). The disk should be removed and rinsed with sea water between replicates of a treatment, and a separate disk should be used for each treatment. The test chambers should then be covered, put in numerical order into a temperature controlled water bath, and aerated 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 amphipods are added.

13.6.4 If the experimental design requires monitoring of sediment chemistry (for example, metals, total volatile solids, pH, Eh, etc.), additional test chambers with sediment and amphipods should be set up for this purpose. Monitoring the quality of the overlying water (for DO, pH, or for certain chemicals) in the test chambers can be accomplished without disturbing the sediment, and may be done in the test chambers containing the test amphipods. Temperature can be measured with a thermometer set in a simulated test beaker containing water and control sediment but no amphipods. If more than one water bath is used to contain the test chambers, a separate temperature beaker should be included in each water bath (see 13.9.3).

13.6.5 The toxicity test is initiated (Day 0) when amphipods are distributed to each test chamber. It is usually not possible to distribute amphipods to all 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. If treatments are replicated, each treatment, including controls, should be represented in each set of test chambers to be processed together. If treatments are not replicated, selection should be random.

13.6.6 A sufficient number of amphipods should be removed from the holding facility at one time to provide about one third more amphipods than are needed for one set of test chambers. This allows selection of active, apparently healthy individuals. Before amphipods are removed, the temperature and salinity of the water in the holding containers should be recorded. Amphipods should be sieved from the holding sediment and transferred to a sorting tray containing water of the holding temperature and salinity. The holding sediment may be saved and returned to the holding containers for use as reburial sediment at the termination of the test. Active, apparently healthy amphipods should be impartially selected from the sorting tray and sequentially distributed among dishes containing approximately 150 mL of prepared toxicity test sea water until each dish contains the required number (usually 20; see annexes) of individuals. The number of amphipods in each dish should be verified by recounting them into a separate dish containing toxicity test water.

13.6.7 Amphipods should be added to test chambers without disruption of the sediment by placing a 6-mil nylon, TFE-fluorocarbon, or polyethylene disk on the water surface, and gently pouring the water and amphipods from the sorting dish over the disk into the test chamber. Any amphipods remaining in the dish should be gently washed into the test chamber. The water level should be brought up to the final test level in the test chamber, the disk removed, and the chamber replaced in the water bath, covered, and aerated. Any amphipods that do not bury within the time specified for the species (see annexes) should be removed and replaced.

13.7 *Duration of Test*—The test begins when amphipods are added to test chambers containing test sediment. Amphipods should be exposed to the test material for ten days. There are no observed substantial effects of starvation or other laboratory artifacts in this amount of time (3). An exposure period of less than ten days is not generally recommended. Experiments with cadmium and field sediments have shown that many amphipods emerge from sediment and are alive but unable to rebury after four days, but most of these amphipods are dead after ten days of exposure (3). For some experimental designs, such as comparison of a 96-h LC50 between species in the presence or absence of sediment, other exposure periods may be used.

13.8 *Biological Data*—Response criteria indicating toxicity of test sediment include mortality and sublethal effects. Sublethal effects include (*a*) emergence from highly toxic sediment during the course of the test, and (*b*) inability of surviving but affected amphipods to rebury in clean, collection site sediment at the termination of the test. Response criteria must be monitored in a "blind" fashion, that is, the observed must have no knowledge of the treatment of the sediment in the test chambers. This is accomplished through randomization of sample numbers.

13.8.1 *Emergence*—Since most infaunal amphipods remain buried during sediment toxicity tests, there is little opportunity to monitor temporal changes in mortality or sublethal effects. An exception is the temporal pattern of emergence from highly toxic sediment. The test should be monitored at least daily (including the day of initiation and the day of termination) for temperature, aeration, lights, and emergence of the amphipods from the test sediment. Each test chamber should be observed by temporarily turning off the air to the test chambers, and gently removing the cover from individual chambers with minimal disturbance of the chamber. The number of amphipods observed completely or partially out of the sediment, either on the sediment surface, swimming in the overlying water, or floating at the water surface, should be recorded. Amphipods that are caught in the surface film should be gently pushed down into the water. Any pertinent observations on the appearance of the sediment (such as color, presence of non-test organisms, growth of mold or algae, or depth of oxidized layer) should be recorded.

13.8.2 *Mortality*—The primary effect of sediment toxicity is mortality of the test amphipods, which is determined at the end of the exposure period. After daily observations have been made and any necessary samples have been taken, the contents of the test chambers should be sieved to remove the test species. Use of a larger screen size sieve for initiation and a smaller screen size sieve for termination reduces the possibility of losing small amphipods through the screen at termination. Screen sizes are specific for various test species (see annexes). Material retained on the screen should be washed into a sorting

tray with clean sea water. The total numbers of live and dead amphipods of the test species should be recorded. The sum of these numbers might be less than the number of amphipods at T_o because of decomposition. If the test species is naturally present in the test sediment, the total number of live and dead amphipods might exceed the number at T_o. Amphipods that are inactive but not obviously dead should be observed under a lowpower microscope and should be counted as alive if there is any sign of movement, such as a neuromuscular pleopod twitch. Gentle prodding may be used in an attempt to elicit movement.

13.8.3 *Reburial*—Data on the ability of the amphipods to rebury in clean sediment at the termination of the sediment toxicity test can be used to detect biologically important sublethal effects. Amphipods that survive the test should be transferred to dishes containing a layer of clean, 0.5 mm sieved control sediment. Sediment saved from the pretest holding containers and kept either in flowing sea water or at 4°C might be appropriate for use as reburial sediment. The numbers of amphipods able to bury within the time period specified for the species should be recorded. These data are used to document sublethal effects on behavior, and can be used to calculate an EC50. Infaunal amphipods unable to rebury are very unlikely to survive in nature. Toxicity data can therefore be analyzed in relation to *effective mortality*, that is, the sum of dead individuals plus those survivors that are not able to rebury. EC50 calculations can be made on the basis of effective mortality. In most cases, amphipods that survive in a ten-day test are able to rebury.

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 properties (see 10.2). A separate sample for faunal analyses 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 at the end of the test. This is especially desirable for field sediments, that 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 at the 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 amphipods. 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 at the termination of the experiment. It is not necessary to add amphipods to chemistry chambers sampled at the initiation of the experiment, but amphipods should be added to those sampled later. Some sediment and water quality characteristics, such as pH, Eh, and dissolved oxygen, can be measured by inserting analytical probes into the test chambers containing amphipods. If radiolabeled test compounds are used, separate chemistry beakers might not be necessary.

13.9.2.1 The concentration of 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 at the 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.

13.9.2.2 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 top, bottom, and sides of the test chamber. Water samples should not contain any surface scum, any material from the sides of the test chamber, or any sediment.

13.9.2.3 Samples for measurement of concentrations of test material 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.

13.9.2.4 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. Care should be taken to ensure that test materials do not undergo transformation, degradation, or volatilization during sample preparation. It should be kept in mind that filtering can remove certain test materials from solution.

13.9.3 *All Tests*—Temperature should be recorded in a separate temperature beaker throughout the test. If test chambers are in more than one temperature controlled water bath, a temperature beaker should be set up in each water bath. Temperature should be monitored at least hourly using a recording thermometer or the daily maximum and minimum temperatures should be monitored (see Guide E 729). Individual temperature measurements should not vary by more than 3°C 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 sediments or overlying water from test chambers, stock solutions, test sediment, or interstitial water are not to be analyzed immediately, they should be handled and stored appropriately (33) to minimize loss of test material or contaminants through such processes as microbial degradation, hydrolysis, oxidation, photolysis, reduction, sorption and volatilization (see Practice D 3976).

14.2 Chemical and physical data should be obtained using appropriate ASTM standards whenever possible. For those measurements for which ASTM standards do not exist or are not sensitive enough, methods should be obtained from other reliable sources (34).

14.3 The analytical method used to measure the concentration of toxicant in test chambers should be validated before beginning the test. The precision of the method should

checked using reference or split samples, interlaboratory comparisons, or alternative (preferably reference or corroborative) methods of analysis.

14.4 Concentrations of test materials in interstitial water should be measured as well as the 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 of the test material is desirable. The free form for organic contaminants 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 must be accounted for.

15. Acceptability of Test

15.1 A 10-day sediment toxicity test is unacceptable if more than a total of 10 % of the control organisms die or show signs of disease or stress, or if mortality in an individual control test chamber exceeds 20 %.

15.2 A 10-day sediment toxicity test should usually be considered unacceptable if one or more of the following occurred:

15.2.1 All test chambers were not identical.

15.2.2 Treatments were not randomly assigned to test chambers.

15.2.3 Test organisms were not randomly or impartially distributed 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, were not all of the same species, or were not of acceptable quality.

15.2.6 Amphipods from a wild population were maintained in the laboratory for more than two weeks, unless the effects of prolonged maintenance in the laboratory has been shown to have no significant effect on sensitivity.

15.2.7 The test organisms were not acclimated at the test temperature and salinity at least 48 h before they were placed in the test chambers.

15.2.8 Temperature, dissolved oxygen, and concentration of test material were not measured, or were not within the range as specified in Section 13.

15.2.9 Aeration to the test chamber was off for an extended time such that dissolved oxygen levels dropped to less than 60 % of saturation.

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

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

15.2.12 Response criteria were not monitored in a blind fashion, that is, observers had knowledge of the treatment of sediments in the test chambers.

16. Interpretation of Results

16.1 The calculating procedure(s) and interpretation of the results should be appropriate to the experimental design. Procedures used to calculate 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 consideration of (a) the advantages and disadvantages of various alternative procedures and (b) appropriate preliminary tests, such as those for outliers and for heterogeneity. Preprocessing of data might be required to meet the assumptions of the analyses.

16.2 LC50 or EC50 and their 95 % confidence limits should be calculated on the basis of (a) the measured initial concentrations of test material, if available, or the calculated initial concentrations, and (b) the mortality or" effective mortality" (see 13.8.3). If other LCs or ECs are calculated, their 95 % confidence limits should also be calculated (see Guide E 729).

16.3 Most acute toxicity tests produce quantal data, that is, counts of the number of organisms in two mutually exclusive categories, such as alive or dead. A variety of methods **(35)** can be used to calculate an LC50 or EC50 and its 95 % confidence limits from a set of quantal data that is binomially distributed and contains two or more concentrations at which the percent dead or affected is between 0 and 100, but the most widely used are the probit, moving average, trimmed Spearman-Kärber and Litchfield-Wilcoxon methods **(35)**. 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 sound information about the LC50 or EC50 even when less than two concentrations kill or affect between 0 and 100 %. The binomial test does not provide a point estimate of the LC50 or EC50, but it does provide a range within which the LC50 or EC50 should lie.

16.4 The results of toxicity tests on field samples without replication may be reported in terms of survival values. A sample should be considered to be toxic if the single sample value lies outside the 95 % tolerance limits of the survival of the controls. Alternately, the field result may be compared with the control survival data using outlier detection methods; the sample may be considered toxic if it would be rejected as an extreme value when considered as part of the control population. Another approach is to use the special case comparison of a single value against a sample, described by Sokal and Rohlf **(36)**. It is strongly recommended that samples be replicated if comparisons among sites are desired (see 12.3.2).

16.5 If samples from field stations are replicated, the mean survival at the stations and the mean control survival should be statistically compared by a one-tailed *t*-test or analysis of variance (ANOVA) followed by a multiple comparison test. 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 of no difference among concentrations (or samples). 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 station samples) were not large enough to be detected

as statistically significant by the experimental design and hypothesis test used.

16.5.1 Following a significant *F*-test result, all exposure concentration effects (or field station samples) can be compared with the control effects by using mean separation techniques such as those explained by Chew (**37**) orthogonal contrasts, Fisher's methods, Dunnett's procedure and William's method. The Dunnett's procedure is a multiple comparison test specifically designed to compare several experimental samples to the concurrent control (**38**). A multiple comparison test is a technique that accounts for the fact that several comparisons are being made simultaneously.

16.6 Daily observations on the numbers of amphipods that have completely or partially emerged from the sediment, either lying on the sediment surface, swimming in the water column, or floating at the water surface, 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 of the sediment during the course of the toxicity test, as amphipods often emerge earlier and in greater numbers from more highly toxic sediment.

17. Report

17.1 The record of the results of an acceptable sediment toxicity test should include the following information either directly or by reference to other available documents:

17.1.1 Names 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, lot number if applicable, composition (identifies and concentrations of major ingredients and impurities if known), 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, and any other pertinent chemical characteristics.

17.1.4 Source of the control, reference and test sediments, dates and methods of collection, method of transport and storage of field sediments, method and dates of treatment of laboratory prepared sediment, and method of distribution to test chambers.

17.1.5 Source and date of collection of the test organisms, scientific name, name of person who identified the organisms and the taxonomic key used, age, life stage, means and ranges of weights and lengths, observed diseases or unusual appearance, treatments, holding and acclimation procedures.

17.1.6 Description of the experimental design, test chambers and covers, the depth, weight, and volume of sediment and water in the chambers, the date, time, and method of beginning the test, numbers of test organisms and chambers, temperature, salinity, and lighting regime.

17.1.7 The average and range of holding and test temperatures, and the method(s) of measuring or monitoring, or both.

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

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

17.1.10 Definition(s) of the effects used to calculate LC50s or EC50s and a summary of general observations of other effects.

17.1.11 A table of the biological data for each test chamber for each treatment (including the control(s)) in sufficient detail to allow independent statistical analyses.

17.1.12 The 10-day LC50s or EC50s and the methods used to calculate them, and their 95 % confidence limits, or the survival or mortality data and their significance relative to the control(s); specify whether results are based on measured or nominal concentrations of the test material.

17.1.13 Results of any other analyses that were made on the same sediment, such as faunal analyses, field notes made while collecting the sediment, chemical measurements made in the chambers, or chemical and geological analyses of subsamples of the sediment.

17.1.14 Anything unusual about the test, any deviation from these procedures, and any other relevant information.

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

18. Keywords

18.1 acute toxicity tests; *ampelisca*; amphipods; benthic amphipods; corophium; EC50 test; eohaustorius; estuarine environments; experimental design; exposure tests; grandidierella; hyalella; LC50 test; marine environments; *pontoporeia*; reference toxicants; *rheoxynius*; saline water; saltwater; sediment; sediment toxicity testing; static test; toxicity; toxicology

ANNEXES

(Mandatory Information)

A1. *RHEPOXYNIUS ABRONIUS*

A1.1 *Ecological Requirements— Rhepoxynius abronius* (**3**) occurs along the West Coast of North America from central California to Puget Sound, Washington (**39**). It is the desired test species where it is available and when the salinity of the interstitial water in the test sediment is 25 g/kg or greater (see Table A1.1). *Rhepoxynius abronius* naturally inhabits clean, fine, sandy sediments. In areas where test sediments are predominantly silts or clays, the experimental design should include a silt-clay control treatment of clean sediment from an uncontaminated reference collection site near that of the test sediments, in addition to the native sediment control.

A1.2 *Collection and Handling Techniques—R. abronius* inhabits clean, fine, sandy sediments from the lower intertidal to a depth of at least 274 m. Amphipods can be collected from a boat using a small biological dredge or a grab sampler. A sieve with a 1.0-mm diameter mesh size can be used to separate adult *R. abronius* from their native sediment. Individual amphipods can be transferred between sorting trays, acclimation dishes, and test chambers by using a bulb pipette of a suitable size (for example, one with a 5-mm diameter opening).

A1.2.1 For acclimation, *R. abronius* can be counted into 10-cm diameter specimen dishes containing a 2-cm deep layer of 0.5-mm sieved collection site sediment, at a density of 20 amphipods per dish. These dishes can be transferred to holding tanks supplied with aerated or flowing sea water at the test temperature and salinity. Two to three days are sufficient for acclimation to the test conditions. A sieve with a 1.0-mm diameter mesh size can be used to separate *R. abronius* from the acclimation sediment immediately prior to the initiation of a toxicity test.

A1.3 *Toxicity Test Specifications*—The toxicity test should be run at 15 ± 3°C using 28 g/kg overlying water in the test chambers. 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 diameter watch glass 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 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 the beakers. Sediment and water should be added to beakers the day before the amphipods are added, to allow suspended sediment particles to settle, and to allow time for equilibration of temperature and the sediment-water interface.

A1.3.1 After the overnight equilibration time, 20 amphipods are distributed to each of the test chambers, with additional toxicity test water to bring the water level up to the 950-mL level. The amphipods should be allowed 5 to 10 min to bury into the test substrate. Any amphipods that have not buried within that time or appear damaged should be replaced, unless the amphipods are repeatedly burrowing into the sediment and immediately emerging in an apparent avoidance response to the test substrate. In that case amphipods are not replaced. Amphipods are not removed from the surface of test sediments during the course of the test even if they appear dead, since some amphipods that seem dead might actually be alive and might later rebury in the test sediments.

A1.3.2 The toxicity test is terminated when amphipods are separated from test substrates using a 0.5-mm mesh-diameter screen. Amphipods are transferred to a sorting tray and numbers of live and dead amphipods are counted. Survivors are transferred to dishes containing a 2-cm deep layer of clean, native sediment and allowed 1 h to rebury. The numbers of survivors unable to rebury in clean sediment can be used to calculate an EC50 for this sublethal effect.

A1.4 *Life Cycle and Age Class— Rhepoxynius abronius* has an annual life cycle (**40**), with recruitment occurring primarily in the late winter through the spring months. Large immature and adult amphipods, 3 to 5 mm total length, should be used in the toxicity test because they are available year round, and their sensitivity to contaminated sediments has been shown to be not greatly different from that of juveniles (**19**). They are also large enough to be easily handled and counted in the toxicity test. Mature males and females, even those carrying eggs, have been found to be equally sensitive to test materials, so it is possible to use a mixed population of both sexes, although very large mature individuals should not be used because they might be senescent. It is necessary to change year classes sometime during the summer, as old amphipods die out and are replaced by the maturing juveniles.

A1.5 *Control Survival*—Control survival using *Rhepoxynius* is generally 95 % or greater, and must be at least 90 % for the toxicity test to be considered valid.

A1.6 *Sensitivity—Rhepoxynius abronius* has been shown to be among the most sensitive of sediment toxicity test organisms to test materials, but is fairly tolerant of handling and to a variety of physical characteristics of sediment (**3**). The genus *Rhepoxynius* is one of the first to disappear from benthic communities impacted by pollution (**3, 39**).

A1.7 *Interpretation and Interferences*—In interpreting the data from 10-day sediment toxicity tests with adult *Rhepoxynius*, it should be kept in mind that the very early life stages, the reproductive ability of amphipods, or their longterm survival might be affected by contaminants at lower concentrations than those that produce a lethal or sublethal effect in mature amphipods in a short-term test. *Rhepoxynius* has been shown to be somewhat adversely affected by very fine-grained sediments (**20**). Despite these limitations, the toxicity test using

TABLE A1.1 Summary of Ecological and Testing Conditions that Should Be Considered When Conducting Ten-Day Sediment Toxicity Tests with Amphipods

NOTE 1— See Annex for further explanation.

NOTE 2—N/A = not applicable; N.D. = no data published at this time.

A1a *Rhepoxynius abronius* (Family Phoxocephalidae)

	Field	Laboratory
Geographic range	Puget Sound to Southern California (23, 39)	N/A
Habitat	Free-burrowing sand dweller, low intertidal to 274 m (21, 23)	clean, fine sand, 2 cm (3)
Life cycle	Annual (40)	N/A—Field collected
Life stage tested	N/A	Mature 3 to 5-mm amphipods, mixed sexes (3)
Temperature	Annual range at collecting site = 8 to 16°C (3)	Standard temperature is 15°C; (3) survives 0 to at least 20°C
Salinity	Annual range at collecting site = near 0 to 35 g/kg (40)	Standard salinity is 28 g/kg, salinity effects noted below 25 g/kg (3)
Sediment type	Well-sorted fine sand to sandy silt (3)	86 % mean survival in sediments with ≥ 80 % silt-clay, up to 100 % in sand sediments (20)
Sediment depth	Usually upper 2 cm, to 6 cm (36, 39)	Test sediment depth 2 cm (3)
Nutrition	Meiofaunal predator, algae, detritus (41)	Amphipods are not fed in the laboratory (3)
Light cycle	Natural light	Continuous light (3)
Control mortality	N/A	≤10 % (3)
Chronic test	N/A	Not developed
96 h LC50, cadmium, water only exposure	N/A	0.92 (0.68–1.25) mg/L (42)

A1b *Eohaustorius estuarius* (Family Haustoriidae)

	Field	Laboratory
Geographic range	Central British Columbia to Central California (24, 27)	N/A
Habitat	Free-burrowing sand dweller, upper to mid-intertidal, shallow subtidal (24, 27)	Clean, fine sand, 2 cm (24)
Life cycle	Probably annual (24)	N/A—Field collected
Life stage tested	N/A	Mature amphipods 3–5 mm, mixed sexes (24)
Temperature	Approximately 0 to 21°C (24, 27)	Standard temperature is 15°C (3, 24); tolerates 5 to at least 21°C (27)
Salinity	Annual range at collecting site = near 0 to 35 g/kg	Standard salinity is 2 to ≤28 g/kg (24, 27)
Sediment type	Clean fine to medium sand (27, 43)	92 % mean survival in sediments with ≥80 % silt-clay, 97 % in sandy sediments (25)
Sediment depth	Approximately top 5 cm	2 cm (3, 24)
Nutrition	Probable deposit feeder (24)	Amphipods are not fed in the laboratory (3, 24)
Light cycle	Natural light	Continuous light (3, 24)
Control mortality	N/A	≤10 % (3, 24)
Chronic test	N/A	Not developed
96 h LC50, cadmium, water only exposure	N/A	9.33 (7.20–12.09) mg/L (24)

A1c *Ampelisca abdita* (Family Ampeliscidae)

	Field	Laboratory
Geographic range	Central Main to Northern Florida, eastern Gulf of Mexico (44), San Francisco Bay (45)	N/A
Habitat	Infaunal tube dweller, low intertidal to 60 m (44, 46)	Collection site sediment, 4 cm (33)
Life cycle	Two to several generations per year, temperature dependent, probably one brood per female (46)	Life cycle approximately 6 weeks at 20°C (48)
Life stage tested	N/A	Immature amphipods, or mature females only
Temperature	Collected in water temperatures from − 2 to 27°C (44)	Standard temperature is 20°C, has been tested in 4 to 10-day tests at 8 to 25°C (48)
Salinity	Fully marine to 10 g/kg (44)	Has been tested from 20 to 35 g/kg (48)
Sediment type	Fine sand and mud to silt (46)	>94 % survival in 90 % silt-clay to 86 % coarse-medium sand, one 10-day test
Sediment depth	Tubes approximately 3.5 cm long (46)	4 cm (49)
Nutrition	Algae, detritus, sediment grains (46, 47)	Diatom culture daily in excess (48)
Light cycle	Natural light	Continuous light
Control mortality	N/A	≤10 %
Chronic test	N/A	Under development
96 h LC50, cadmium water only exposure	N/A	0.33 (0.29–0.38) mg/L total cadmium, one test
96 h LC50, fluoranthen water only	N/A	3.3 to 9.9 μg/L under laboratory lights, one test

Rhepoxynius abronius has been demonstrated to be very useful in detecting sediment toxicity, and can be used in a variety of research and regulatory applications.

TABLE A1.1 *Continued*
A1d *Grandidierella japonica* (Family Corophiidae)

	Field	Laboratory
Geographic range	Japan, San Francisco Bay (50), Southern California bays (51)	N/A
Habitat	Infaunal tube dweller, mid-tidal to shallow subtitial (51)	Collection site sediment, 1 cm (51)
Life cycle	N.D.	4 to 5 life cycles per year at 20°C
Life stage tested	N/A	Immature 3 to 6 mm, no females carrying embryos (51)
Temperature	Collected in California from water temperatures ranging from 9 to 26°C (51)	Standard test temperature is 15 to 19°C; satisfactory survival at 15 to 23°C (51)
Salinity	Full ocean salinity to hyposaline waters of unknown salinity (51)	Standard test salinity is 30 to 35 g/kg; survival at 16 to 34 g/kg, 15 % mortality at 4 g/kg (51)
Sediment type	Mud-sand; occurs in sands, silts, clay (50, 52)	Fine sand to silty clay (51)
Sediment depth	Upper 2–4 cm (51)	2 cm (51)
Nutrition	Algae, detritus, sediment	Suspension of finely ground Tetramin and *Enteromorpha* (51)
Light cycle	Natural light	Continuous light (51)
Control mortality	N/A	≤10 % (51)
Chronic test	N/A	Not developed
96 h LC50, cadmium, water only exposure	N/A	1.17 (0.94–1.46) mg/L (53)

TABLE A1e *Leptocheirus plumulosus* (Family Aoridae)

	Field	Laboratory
Geographic range	Cape Cod, Massachusetts to Northern Florida (54)	N/A
Habitat	U-shaped burrows in fine sand to muddy sediments; shallow subtidal (54, 55)	Collection site or culture sediment, 2 cm (56, 59-62)
Life cycle	Annual; reproduction spring through fall in Chesapeake Bay, at least two broods per female (54, 56)	Multiple broods per year, life span ≥ 7 weeks (62)
Life stage tested	N/A	Immature or mature 3 to 5-mm amphipods; mixed sexes (56, 59-62)
Temperature	Range at collecting site = 0 to 29°C (57)	Routinely tested at 20°C, has been tested at 25°C (56, 59-61)
Salinity	Collected in water ranging from near 0 to 33 g/kg (55, 58)	> 90 % mean survival in salinities 2 to 32 g/kg (56, 59-61)
Sediment type	Fine sand to silty clay (55-58)	Up to 100 % survival with > 90 % silt-clay; 85 % mean survival with > 95 % sand-gravel (56, 59-61)
Sediment depth	Usually in upper 2 cm; rarely deeper than 5 cm	2 cm (56, 59-61)
Nutrition	Surface deposit and suspension feeder (57)	Combination of "amphipod gorp" and micro-algae (62)
Light cycle	Natural light	16h:8h light:dark (56, 59-61)
Control mortality	N/A	≤ 10 %
Chronic test	N/A	Under development
96h LC50, cadmium water only exposure	N/A	1.06 mg/L (0.85–1.33), one test @ 20°C, 20 g/kg

A2. *EOHAUSTORIUS ESTUARIUS*

A2.1 *Ecological Requirements*— *Eohaustorius estuarius* (24) lives in intertidal sands along the North American west coast from British Columbia south to at least central California (26, 43). It is a desirable test species for sediments which have interstitial salinities ranging between 2 and 28 g/kg. Since *E. estuarius* normally inhabits sandy sediments, the experimental design should include a fine-sediment control (in addition to the native sediment control) if test sediments are predominantly silts or clays. This control sediment should consist of clean sediment from an uncontaminated reference collection site near that of the test sediment(s) and have a similar grain size distribution.

A2.2 *Collection and Handling Techniques*—*E. estuarius* can be found in the upper 10 cm of fine, intertidal, estuarine sands +0.5 to +2.0 m above mean low low water (MLLW). The amphipods can be collected by shovel at low tide and sieved from their native sediments with a 1.0–mm mesh-diameter screen. They can be transferred between sorting trays, acclimation dishes, and test chambers with a 5-mm diameter bulb pipette.

A2.2.1 For acclimation, up to 20 *E. estuarius* can be held in 10-cm diameter specimen dishes containing a 2-cm deep layer of native sediment served to ≤0.5 mm. These dishes should be transferred to holding tanks supplied with aerated or flowing sea water at the test temperature and salinity. Two to three days are sufficient for acclimation to test conditions. The amphipods should be separated from the acclimation sediments using a 1.0-mm mesh-diameter sieve immediately prior to initiating the toxicity test.

A2.3 *Toxicity Test Specifications*—The toxicity test should be run at 15 ± 3°C with the overlying water composed of toxicity test seawater diluted to the same salinity as the interstitial water of the test substrate. 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 diameter

watch glass 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 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 the beakers. Sediment and water should be added to beakers the day before the amphipods are added, to allow suspended sediment particles to settle, and to allow time for equilibration of temperature and the sediment-water interface.

A2.3.1 After the overnight equilibration time, 20 amphipods are distributed to each of the test chambers, with additional toxicity test water to bring the water level up to the 950-mL level. The amphipods should be allowed 5 to 10 min to bury into the test substrate. Any amphipods that have not buried within that time should be replaced, unless the amphipods are repeatedly burrowing into the sediment and immediately emerging in an apparent avoidance response to the test substrate. In that case, amphipods are not replaced. Amphipods are not removed from the surface of test sediments during the course of the toxicity test even if they appear dead, since some amphipods that seem dead might actually be alive and might later rebury into the test substrate.

A2.3.2 The toxicity test terminates when the amphipods are sieved from the test substrate using a 0.5-mm mesh-diameter screen and the animals are transferred to a sorting tray. After survivors are counted, the ability of surviving amphipods to rebury into clean native sediments may be used to determine an EC50 for this sublethal effect. Surviving *E. estuarius* should be transferred to specimen dishes containing a 2-cm deep layer of native sediment sieved to ≤0.5 mm, and should be allowed one hour to rebury.

A2.4 *Life Cycle and Age Classes*— *Eohaustorius estuarius* appears to have an annual life cycle, with reproduction occurring from February through July (**27**). Large immature and adult amphipods, 3 to 5 mm total length, should be used in the toxicity test because they are available year round and are easily handled and counted. Larger individuals should not be used as they might be senescent. Age, size, and sex-specific sensitivity of *E. estuarius* to contaminants has not been examined, but mixed-sex populations of animals within their recommended size range show highly replicable responses to laboratory-spiked and field-collected contaminated sediments (**24**). This strongly suggests that both sexes are comparably susceptible to contaminated sediments.

A2.5 *Control Survival*—Control survival using *Eohaustorius* must be at least 90 % for the toxicity test to be considered valid.

A2.6 *Sensitivity*—*Eohaustorius estuarius* is only slightly less sensitive than *Rhepoxynius* to contaminants, and is fairly tolerant of handling. The species is less sensitive than *Rhepoxynius* to a variety of physical characteristics of sediment and is tolerant of salinity levels ranging from about 2 g/kg to at least 35 g/kg.

A2.7 *Interpretation*—When interpreting the results of acute toxicity tests, it should be kept in mind that the early life stages, the reproductive ability, or the long-term survival of *E. estuarius* might be affected by contaminants at concentrations lower than those that produce a lethal or sublethal response. Despite these limitations, the toxicity test using adult *E. estuarius* has been demonstrated to be useful in quickly detecting sediment toxicity in estuarine sediments of widely varying interstitial salinity, and can be used in a variety of research and regulatory applications.

A3. *AMPELISCA ABDITA*

A3.1 *Ecology*—*Ampelisca abdita* is a tube-dwelling amphipod belonging to the family Ampeliscidae, found mainly in protected areas from the low intertidal zone to depths of 60 m. It ranges from central Maine to south-central Florida and the eastern Gulf of Mexico (**44, 63**), and has also been introduced into San Francisco Bay (**45**). It is euryhaline, and has been reported in waters that range from fully marine to 10 parts per thousand salinity (**44**). This species generally inhabits sediments from fine sand to mud and silt without shell, although it can also be found in relatively coarser sediments with a sizable fine component (**46**). *A. abdita* is often abundant in sediments with a high organic content (**47**).

A3.1.1 In the colder waters of its range, *A. abdita*produces two generations per year, an overwintering generation that breeds in the spring and a second that reproduces in mid to late summer (**45, 46**). In New England, breeding of the overwintering generation begins when the water temperature is about 8°C, but in warmer waters south of Cape Hatteras, breeding might be continuous throughout the year. Adults mate in the water column, and intense breeding activity is correlated with the full moon and spring tides. Juveniles are released after approximately two weeks in the brood pouch, at about 1.5 mm in length. It then takes 40 to 80 days for newly released juveniles to become breeding adults (**46**). When *A. abdita* are present, they are often dominant members of the benthic community with densities up to 110 000 m^{-2} (**45, 47, 64**). *Ampelisca abdita* is a particle feeder, feeding both on particles in suspension and on those from the surface of the sediment surrounding its tube. Gut contents of field-collected specimens have been found to include algal material, sediment grains, and organic detritus (**44, 45**).

A3.2 *Collection and Handling Techniques*—*Ampelisca* should be sieved from their native (collection site) sediment as soon as possible after collection. A 2-mm mesh sieve nesting over a 0.5-mm mesh sieve is useful for this procedure. It is desirable for the sediment containing the amphipods to be rinsed first through the upper, 2-mm sieve with a forceful stream of seawater at the collection temperature and salinity. This will break up the sediment material and also force most of the amphipods out of their tubes. The material thus retained on the 0.5-mm sieve should be vigorously shaken and swirled so

the fine sediments pass through and the amphipods are separated from tubes, sediment, and detrital material. If the sieve is then lifted from the water, allowed to drain, and then slowly lowered into a shallow tray of seawater, the *Ampelisca* will be caught on the water's surface tension and can be easily collected with a fine mesh dip net. The amphipods can be held temporarily in large culture dishes in a constant temperature bath, and then separated into two size classes with the use of nested 1.0 and 0.5-mm sieves.

A3.2.1 During acclimation, *Ampelisca* can be held in 1-gal glass jars, each containing approximately a 4-cm deep layer of sieved collection site sediment. If seawater is flowing through the holding containers, a screened overflow must be used to prevent loss of swimming amphipods. Amphipods should have food available on a daily basis during acclimation. Research is currently being conducted to determine optimal food sources for culturing this amphipod. Reasonable growth and reproduction have been obtained when *A. abdita* has been fed the diatom *Phaeodactylum tricornutum* daily in excess (a suggested amount is 0.5 to 1 L of algae per gallon jar, or 3 × 10 4 cells/mL). *Skeletonema costatum* has also been used successfully. Amphipod exposure to the food source will be increased if, during the feeding period (for example, overnight), the holding system is static, with aeration to circulate the algae. Sloping upper sides on the holding containers will aid in movement of algae across the sediment surface. Care should be taken to maintain the temperature with a water bath when seawater is not flowing through the jars. Approximate density in the holding jars should not exceed 300 amphipods. Acclimation to the test temperature should not exceed 3°C per day, and amphipods should be used within 2 weeks after collection.

A3.2.2 *Ampelisca abdita* may be shipped if this is done within one day of collection. Small plastic" sandwich" containers (approximately 500 mL) can be used to hold the amphipods. The containers are filled three quarters full with a minimum depth of 2 cm of sieved collection site sediment and then to the top with well-aerated seawater. No more than 200 amphipods should be added to each container. Amphipods should be allowed to burrow into the sediment and build tubes before the containers are capped. The capping must be done underwater to eliminate any air pockets in the containers. Containers should be shipped by means of overnight delivery in coolers with a few ice packs to prevent extreme temperature changes during transit.

A3.3 *Ten-Day Sediment Toxicity Test*—The variation of *Ampelisca*'s sensitivity to toxic materials under different physical conditions is still being examined. This species is routinely tested at 20°C, but has been tested from 8 to 25°C. In nature, feeding and somatic growth occur at temperatures as low as 3 to 5°C (44). For comparison with other *Ampelisca abdita* test results, 20°C is recommended. Similarly, *A. abdita* is tolerant of a wide salinity range, but most tests have been conducted at salinities of 28 to 35 g/kg. This amphipod inhabits fine-grained sediments, and as with other physical conditions, if it is suspected that a coarse grain size of a test sediment will stress the animals, a grain size control should be included.

A3.3.1 The exposure chamber routinely used to test *A. abdita* is a quart-sized glass canning jar with a narrow mouth. This container was selected because it is inexpensive, easily available, easily drilled if a screened overflow is needed for flow-through tests, and has sloping upper sides to improve circulation of algal material in experiments where growth or reproductive endpoints are measured and feeding is necessary. *Ampelisca abdita* has not been tested in the 1-L beaker exposure chamber used in other amphipod tests, but it is not anticipated that use of beakers would create any problems. With either exposure chamber, the water column should be gently aerated with a glass pipette inserted above the sediment surface. Sediment in the exposure chamber should be 4 cm deep.

A3.3.2 *A. abdita* can be collected throughout the year. However, during certain times of the year, juvenile amphipods might be difficult to obtain. If mature animals are used, adult males must not be tested; they are very active swimmers and they die shortly after mating. *Ampelisca* should be sieved from the holding containers using a 0.5-mm sieve. Twenty to thirty amphipods should be tested per replicate. For each replicate, the contents of a sorting cup can be rinsed into a plastic cup with a 400 or 500-micron screened base and from there into the exposure container. Any animals caught on the water's surface can be gently pushed under using a glass rod. Amphipods should be given 1 h to burrow into the sediment. If the lack of ability to burrow does not show a dose-response, then the animals not burrowed can be replaced with others from the same sieved population.

A3.3.3 The endpoint for the 10-day test is mortality, and dead animals should be counted and removed daily. An amphipod is considered dead if it does not respond to gentle probing. It is also useful to note any animals out of their tubes on the sediment or water surface, amphipods that are nearly dead and only exhibit a muscular pleopod twitch, the presence of molts, and the condition of the tubes built. Emergence from the sediment and the inability to construct a proper tube are sublethal behavioral responses that would ultimately result in death.

A3.3.4 After checking the assay on the last day, the contents of each exposure container should be rinsed through a 0.5-mm sieve. (A smaller mesh sieve can be used for the final sieving if there is concern about losing very small animals, but this will make the sieving process more time-consuming.) If the experiment is small, the material retained on the sieve can be examined that day. If time does not permit same-day examination, the retained material from each jar can be preserved in 5 % buffered formalin with Rose Bengal stain for later examination. Any amphipods that are not accounted for when the sieved material is examined are presumed to have died during the test. Amphipods that have died in their tubes will generally decompose during the test or break apart during sieving. Rarely, an individual that has died during the test will be recovered in the preserved material, and its appearance will be markedly different from those of the amphipods that were alive when preserved. For instance, there might be little tissue left within its exoskeleton, it might be contorted, etc.

A3.4 *Other Testing*—Growth of *Ampelisca abdita* has been measured in 10-day tests. Small juveniles in a narrow size

range should be selected, and when sorting for the initiation of the test at least one additional group of amphipods should be sorted. This extra group represents the initial size and should be preserved in 5 % buffered formalin for later measurement. The amphipods must be fed during the test. Growth is measured by length from the base of the first antennae to the base of the telson. Measurements are done after preservation and counting of test survivors.

A3.4.1 Chronic tests have also been conducted with this species (**48**) and research is underway to determine the optimum conditions for those tests.

A3.5 *Interpretation—Ampelisca abdita* has been shown to be sensitive to a variety of anthropogenic materials in the marine environment. For example, when exposed to dredged material from Black Rock Harbor, Connecticut, in the solid phase, *Ampelisca abdita* was the most sensitive of 11 species of fish and invertebrates tested (**65**). This material was contaminated primarily with polyaromatic hydrocarbons and heavy metals. At a concentration of 5 mg/L suspended Black Rock Harbor sediment, growth, and consequently sexual maturation, were delayed, and effects were seen in the laboratory population structure (**48**). *A. abdita* also showed sensitivity to a series of sediments from New Bedford Harbor, Massachusetts, that were heavily contaminated with polychlorinated biphenyls and heavy metals.

A3.5.1 All routes of exposure have not been fully examined for *A. abdita*. Since it is a particle feeder, it will be exposed to contaminated particles in suspension or on the sediment surface. This amphipod feeds ventral side up in its tube, by using its second antennae to pick up particles or by capturing small particles carried to the mouth in the current created by the action of the pleopods and second antennae (**46**). Therefore *Ampelisca*'s feeding current exposes it to overlying water. Pore water also enters the tube, and research is currently underway to determine the extent to which *A. abdita* is exposed to this interstitial water. In a flow-through system, it is assumed that the sensitivity that this amphipod shows to contaminated sediments is due primarily to exposure to pore water contaminants, since the overlying water contaminants are continually removed. It might be possible to use other species of *Ampelisca* in toxicity tests. For information on congeners, see Bousfield (**44**) and Mills (**46, 63**).

A4. *GRANDIDIERELLA JAPONICA*

A4.1 *Ecological Requirements— Grandidierella japonica* (**52**) was accidently introduced into San Francisco Bay and some other northern California bays by unknown means. It was first collected in 1971 (**50**). Later it was found in southern California where large populations are known from Upper Newport Bay and Shoreline Aquatic Park in Long Beach (**52**). It has proved to be a useful test species for environmental studies in southern California. The toxicity test should be conducted at 15 to 19°C using sea water with salinities between 30 and 35 g/kg. *Grandidierella japonica* lives in a variety of sediment types that makes it possible to conduct tests with a variety of sediment types (sands, silts, or clays).

A4.2 *Collecting and Handling Techniques*—This species can be collected intertidally and subtidally from the localities listed above. The upper 2 to 4 cm of sediment should be collected and placed in a bucket with sea water. The contents should be gently stirred and the supernatant fluid decanted into a 1.0-mm sieve. The material retained on the screen should be transferred to a container for transport to the laboratory. In the laboratory, the material should be placed in a sorting tray (white) containing sea water. Amphipods can be picked up using a bulb pipette with a 5-mm diameter. Females carrying embryos in their marsupium should not be used. This stage in the life cycle can be detected with the naked eye after some experience. These females can be set aside to establish cultures if so desired.

A4.2.1 For acclimation, *G. japonica* can be placed in an aquarium containing a 1-cm deep layer of 0.5-mm sieved sediment from the collection site at a density of about 10 to 15 amphipods per 100 cm^2 of surface area. Two to three days are sufficient for acclimation to the test environment. A sieve with a 1.0-mm diameter mesh size can be used to separate *G. japonica* from the acclimation sediment at the time of the initiation of the sediment toxicity test.

A4.3 *Toxicity Test Specifications*—The toxicity test should be run at 15 to 19 ± 3°C using 30 to 35 g/kg overlying water in the test chambers. 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 diameter watch glass 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 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 chamber should be 2 cm deep, and toxicity test water should be added up to the 700-mL mark on the beakers. Sediment and water should be added to beakers the day before the amphipods are added, to allow suspended sediment particles to settle, and to allow time for equilibration of temperature and the sediment-water interface.

A4.3.1 After the overnight equilibration time, 20 amphipods are distributed to each of the test chambers, with additional toxicity test water to bring the water level up to the 950-mL level. The amphipods should be allowed 5 to 10 min to bury into the test substrate. Any amphipods that have not buried within that time or appear damaged should be replaced, unless the amphipods are repeatedly burrowing into the sediment and immediately emerging in an apparent avoidance response to the test substrate. In that case amphipods are not replaced. Amphipods are not removed from the surface of test sediment during the course of the test even if they appear dead since some amphipods that seem dead are actually alive and might re-bury in the test sediment. At the termination of the test, the

re-burial data can be used to determine an EC50 for a sublethal measurement.

A4.3.2 The toxicity test should be terminated and the amphipods recovered using a 0.5-mm sieve. Surviving amphipods should be allowed 1 h to re-bury in a 2-cm deep layer of clean, collection site sediment.

A4.4 *Life Cycle and Age Class— Grandidierella japonica* has a short life cycle and is capable of completing four or five life cycles a year under laboratory conditions of 20°C. Immature amphipods, 3 to 6 mm in total length, should be used in the toxicity test. No females carrying embryos in their marsupium should be used in these tests. Animals can be cultured in the laboratory on a diet of powdered fish flakes. It is easier to initiate such a culture with females carrying embryos.

A4.5 *Interpretation*—In interpreting the data from acute toxicity tests, it should be kept in mind that the reproductive ability or long-term survival might be affected by contaminants at lower concentrations than those that produce a lethal or sublethal effect in a short-term test. Despite these limitations, the toxicity test using *Grandidierella japonica* has been used in detecting sediment toxicity or toxic elements. Its ability to live in a burrow in a variety of sediment types gives broad application for the use of *G. japonica* in research and regulatory applications.

A5. *LEPTOCHEIRUS PLUMULOSUS*

A5.1 *Ecological Requirements—Leptocheirus plumulosus* (family Aoridae) is an infaunal amphipod distributed subtidally along the east coast of the United States from Cape Cod, Massachusetts to northern Florida (**54**). In Chesapeake Bay, *L. plumulosus* is indigenous to oligohaline and mesohaline regions (**55, 57, 58**), though it can tolerate an even broader salinity range, from near 0 to 33 g/kg (**55, 56, 58**). This species constructs U-shaped burrows in sediments ranging from fine sand to silty clay (**56-58**). Due to its broad salinity and sediment tolerances, it is a desirable test species for east coast estuarine sediments and has been used successfully in the assessment of contaminated sediments in Chesapeake Bay (**59-61**).

A5.2 *Collecting and Handling Techniques—Leptocheirus plumulosus* is most abundant in the upper 2 cm of sediment, rarely penetrating to depths below 5 cm (**66**). Amphipods can be collected with benthic grab samplers (for example, Peterson, Ponar) from various tributaries of Chesapeake Bay. The contents of each grab are sieved through a 0.5-mm mesh screen and the retained material is gently rinsed into polyethylene buckets containing collection site sediment and water. These containers are transported to the laboratory where they are aerated. It is desirable to sort amphipods from collection site debris within 12 hours. A0.5-mm mesh sieve can be used to separate amphipods from transport sediment. The material retained on the screen can be rinsed into sorting trays containing collection site water. Healthy, active amphipods can be removed from detritus by using a bulb pipette of a suitable size (for example, one with a 5-mm diameter bulb).

A5.2.1 For acclimation, *L. plumulosus* can be placed in an aquarium (for example, 40-L) containing a 1–2 cm deep layer of 0.5-mm sieved collection site sediment at a density of approximately 200 to 300 per aquarium. Aeration should be vigorous. Two to three days are sufficient for acclimation to the test environment. A gradual change from collection site water to test water is desirable. This can be accomplished by gradually increasing the proportion of test water in the tanks over 2 to 3 days.

A5.2.2 Culture techniques are being refined. Presently, laboratory populations can be maintained through several generations in shallow plastic tubs or glass aquaria containing a 1–2 cm layer of fine grained sediment from the amphipod collection site or a texturally similar sediment (**62**). Water exchange is static-renewal, with 30 to 50 percent of water volume in each container replaced 2 to 4 times per week. Culture containers are aerated, maintained at a temperature of approximately 20°C, a salinity of 20 g/kg and a photoperiod of 16h light:8h dark. Cultures receive a mixture of micro-algae (for example, *Pseudoisochrysis paradoxa, Phaeodactylum tricornutum, Tetraselmis suecica*) and approximately 0.1 g of amphipod "gorp" (a mixture of fish food flakes, yeast, alfalfa powder, ground cereal leaves and shrimp maturation feed) 2–3 times per week (**62**). Amphipods can be separated from acclimation or culture sediments using a 0.5 mm sieve immediately prior to initiating the toxicity test.

A5.3 *Toxicity Test Specifications*—The effects of different physical conditions on the sensitivity of *L. plumulosus* to toxic materials are currently under investigation. This species is routinely tested at 20°C, but has been tested at 25°C. Salinity of overlying water will depend on the objectives of the study. Toxicity test seawater can be diluted to the same salinity as the interstitial water of the test sediment, the ambient bottom salinity at the test site or a selected test salinity in the range of 2 to 32 g/kg. Laboratory investigations indicate *Leptocheirus* is tolerant of a range of sediment types (**56**); however, a grain size reference should be included for coarse sediments since these may be somewhat stressful. Fine grained sediments from the amphipod collection site or laboratory cultures are desirable as the negative control. The exposure chamber routinely used to test *L. plumulosus* is a 1-L glass container with an internal diameter of 10.0-cm (for example, standard 1-L beaker). The exposure chamber should be covered with a watch glass to reduce contamination of the contents and evaporation of the water and test materials. Aeration can be provided to each test chamber through a 1-mL glass pipette positioned not closer than 2 cm from the sediment surface. Each test chamber should contain a 2-cm deep layer of sediment and enough overlying water to create approximately a 4:1 (v/v) water to sediment ratio. Sediment and water should be added to the test chambers the day before the amphipods are added to allow suspended sediment particles to settle, and to allow time for equilibration of temperature and the sediment-water interface.

A5.3.1 After overnight equilibration of the test chambers, amphipods can be randomly distributed to each of the containers. It is desirable to sacrifice a random sample of at least 20 animals from those being sorted on day 0 to provide an initial size range estimate of test animals. Twenty amphipods should be tested per replicate. Animals caught on the water's surface can be gently pushed under using a glass rod. Amphipods should be allowed 5 to 10 min to burrow into the test sediments. Amphipods that have not burrowed within that time should be replaced with healthy animals, unless the amphipods are repeatedly burrowing into the sediment and immediately emerging in an apparent avoidance response. In that case, the amphipods are not replaced. Amphipods are not removed from the surface of test sediments during the course of the toxicity test even if they appear dead, since some amphipods that seem dead might actually be alive and might later rebury into test substrate.

A5.3.2 The toxicity test can be terminated after 10 days by sieving amphipods from test sediments using a 0.5-mm mesh screen. Mortality is the endpoint for this short-term test. Burrows generally disintegrate during sieving and animals can be transferred to a sorting tray for enumeration. The ability of surviving amphipods to rebury into clean sediments can be used as a sublethal test endpoint.

A5.3.3 *Other Testing*— Partial life cycle tests (28 to 30 days) initiated with juveniles are being conducted with this species, with amphipod length and survivorship as viable endpoints. Research is currently underway to determine the optimum conditions for these tests.

A5.4 *Life Cycle and Age Classes—Leptocheirus plumulosus* is an annual species capable of producing a least two broods, with peak periods of reproduction in early to mid spring and in the fall **(56, 67)**. Gravid females have been observed in Chesapeake Bay as late as December and as early as February, indicating that timing of reproduction varies yearly depending on climatic conditions. In cultured populations, females produce multiple broods and gravid females are available year round **(62)**. Size range of field-collected test organisms might depend on the size structure of the field population, as the mean size of amphipods collected in early spring is generally greater than those collected in the summer or fall. Size range of cultured amphipods is less variable seasonally. Immature and adult amphipods, approximately 3 to 5 mm as measured from the base of the first antenna to the end of the third pleon segment along the dorsal surface, should be used in toxicity tests because they are easy to handle and count. The potential effects of age, size, sex, and seasonal variation of field-collected organisms on the sensitivity of *L. plumulosus* to contaminants is currently being examined. Evidence to date indicates mixed-sex populations within the recommended size range show consistent responses to field-collected contaminated sediments and 96 h water only exposures to cadmium **(56, 59-61)**.

A5.5 *Control Survival*—Mean control survival using *Leptocheirus* must be at least 90 % for the toxicity test to be considered valid.

A5.6 *Sensitivity—Leptocheirus plumulosus* is tolerant of handling and a range of sediment types and salinities. The sensitivity of this species is comparable to *Hyalella azteca* in 96 h water only exposures to cadmium **(56, 61)**. A review of benthic surveys and sediment contamination in Chesapeake Bay indicates a negative correlation between the presence of *L. plumulosus*and the degree of contamination **(66, 68)**.

A5.7 *Interpretation*—When interpreting the results of acute toxicity tests, it should be kept in mind that the early life stage, the reproductive ability, or the long-term survival of *L. plumulosus* might be affected by contaminants at concentrations lower than those that produce a lethal response. Partial life cycle sediment toxicity test procedures are under development for *L. plumulosus* and should resolve these questions.

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