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Standard Guide for Conducting Sediment Toxicity Tests with Freshwater Invertebrates[1]

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1. Scope

1.1 This guide covers procedures for obtaining laboratory data to evaluate adverse effects of contaminants associated with whole sediment on freshwater organisms. The methods are designed to assess the toxic effects on invertebrate survival, growth, or reproduction, from short (for example, 10 days) or long-term tests, in static or flow-through water systems. Sediments to be tested may be collected from field sites or spiked with known compounds in the laboratory. Test procedures are described for (*1*) *Hyalella azteca*, (*2*) *Chironomus tentans*, (*3*) *Chironomus riparius*, and (*4*) *Daphnia sp. and Ceriodaphnia sp.* Methods described in this document should also be useful for conducting sediment toxicity tests with other aquatic species, although modifications may be necessary.

1.2 Modification of these procedures might be justified by special needs. Results of tests conducted using unusual procedures are not likely to be comparable to results using this guide. Comparison of results obtained using modified and unmodified versions of these procedures might provide useful information concerning new concepts and procedures for conducting sediment toxicity tests with freshwater organisms.

1.3 The results from field collected sediments used in toxicity tests to determine a spatial or temporal distribution of sediment toxicity may be reported in terms of the biological effects on survival, growth, or reproduction (see Section 16, Calculation). In addition, these procedures are applicable to most sediments or chemicals added to sediment. Materials either adhering to sediment particles or dissolved in interstitial water can be tested. With appropriate modifications these procedures can be used to conduct sediment toxicity tests when factors such as temperature, dissolved oxygen, pH, and sediment characteristics (for example, particle size, organic carbon content, total solids) are of interest, or when there is a need to test such materials such as sewage sludge, oils and particulate matter. These methods might also be useful for conducting bioaccumulation tests.

1.4 Results of toxicity tests with test materials experimentally added to sediments may be reported in terms of an LC50 (median lethal concentration), and sometimes an EC50 (median effect concentration). Results of tests may be reported in terms of an NOEC (no observed effect concentration) and LOEC (lowest observed effect concentration).

1.5 This guide is arranged as follows:

1.6 *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.* Specific hazard statements are given in Section 7.

2. Referenced Documents

2.1 *ASTM Standards:*

D 1129 Terminology Relating to Water[2]
D 1193 Specification for Reagent Water[2]
D 4387 Guide for Selecting Grab Sampling Devices for Collecting Benthic Macroinvertebrates[3]
D 4447 Guide for the Disposal of Laboratory Chemicals and Samples[3]
D 4823 Guide for Core-Sampling Submerged, Unconsolidated Sediments[4]
E 380 Practice for Use of the International System of Units (SI) (the Modernized Metric System)[5]
E 729 Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians[3]
E 943 Terminology Relating to Biological Effects and Environmental Fate[3]
E 1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses[3]

[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 Toxicology.

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[2] *Annual Book of ASTM Standards*, Vol 11.01.
[3] *Annual Book of ASTM Standards*, Vol 11.04.
[4] *Annual Book of ASTM Standards*, Vol 11.02.
[5] *Annual Book of ASTM Standards*, Vol 14.02.

E 1241 Guide for Conducting Early Life-Stage Toxicity Tests with Fishes[3]
E 1295 Guide for Conducting Three Brood, Renewal Toxicity Tests with Ceriodaphnia Dubia[3]
E 1297 Test Method for Measuring Fast Neutron Reaction Rates by Radioactivation of Niobium[6]
E 1367 Guide for Conducting 10-day Static Sediment Toxicity Tests With Marine and Estuarine Amphipods[3]
E 1391 Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing[3]

3. Terminology

3.1 The words "must", "should", "may", "can", and "might" have very specific meanings in this guide. "Must" is used to express an absolute requirement, that is, to state that the test ought to be designed to satisfy the specified conditions, unless the purpose of the test requires a different design. "Must" is only used in connection with the factors that directly relate to the acceptability of the test (see Section 15). "Should" is used to state that the specified condition is recommended and ought to be met if possible. Although a 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. "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 *Descriptions of Terms Specific to this Standard:*

3.2.1 *clean*—denotes a sediment or water that does not contain concentrations of test materials which cause apparent stress to the test organisms or reduce their survival.

3.2.2 *concentration*—the ratio of weight or volume of test material(s) to the weight or volume of sediment.

3.2.3 *interstitial water*—the water within a wet sediment that surrounds the sediment particles, expressed as the percent ratio of the weight of the water in the sediment to the weight of the wet sediment.

3.2.4 *overlying water*—the water placed over the whole sediment in the test chamber for the conduct of the toxicity test, and may also include the water used to manipulate the sediments.

3.2.5 *sediment*—a naturally occurring particulate material which has been transported and deposited at the bottom of a body of water, or an experimentally prepared substrate within which the test organisms can interact.

3.2.6 *spiking*—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, such that the toxicity of the material added can be determined. After the test material is added, which may involve a solvent carrier, the sediment is thoroughly mixed to evenly distribute the test material throughout the sediment.

3.2.7 *whole 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.

3.3 *Definitions*—For definitions of other terms used in this guide, refer to Guides E 729, E 1023, E 1241, Terminology E 943 and D 1129. For an explanation of units and symbols, refer to Practice E 380.

4. Summary of Guide

4.1 The toxicity of contaminated whole sediments is assessed during continuous exposure of aquatic organisms, using either static or flow-through exposure systems. Sediments tested may either be collected from field sites or spiked with a known compound(s). A negative control sediment or a reference sediment is used to (*a*) give a measure of the acceptability of the test; (*b*) provide evidence of the health and relative quality of the test organisms; (*c*) determine the suitability of the overlying water, test conditions, food, handling procedures; and (*d*) provide a basis for interpreting data obtained from the test sediments. A reference sediment is collected from the field in a clean area and represents the test sediments in sediment characteristics (for example, TOC, particles size, pH). Specified data are obtained to determine the toxic effects on survival, growth, or reproduction, from short (for example, 10 days), or long-term exposures to aquatic invertebrates.

5. Significance and Use

5.1 Protection of a species requires averting detrimental contaminant related effects on the survival, growth, reproduction, health, and uses of the individuals of that species (**1**).[7] Sediment toxicity tests provide information concerning the bioavailability of contaminants associated with sediments to aquatic organisms. Invertebrates occupy an essential niche in aquatic ecosystems and are an important food source for fish, wildlife, and larger invertebrates. A major change in the availability of invertebrates as either a food source, or as organisms functioning properly in trophic energy transfer and nutrient cycling, could have serious adverse ecological effects on the entire aquatic system.

5.2 Results from sediment toxicity tests might be an important consideration when assessing the hazards of materials on aquatic organisms (see Guide E 1023) or when deriving sediment quality concentrations for aquatic organisms (**2**).

5.3 Information might also be obtained on accumulation of contaminants associated with sediments by analysis of animal tissues for the contaminant(s) being monitored.

5.4 The 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.

5.5 Results of sediment toxicity tests with test materials experimentally added to sediments could be used to compare the sensitivities of different species, the toxicity of different test materials, and to study the effects of various environmental factors or results of such tests. Results of sediment

[6] *Annual Book of ASTM Standards*, Vol 12.02.

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

toxicity tests are useful for studying biological availability of test materials, and structure-activity relationships.

5.6 Results of sediment toxicity tests can be used to predict effects likely to occur with aquatic organisms in field situations as a result of exposure under comparable conditions, except that (*a*) motile organisms might avoid exposure and (*b*) toxicity to benthic organisms can be dependent on sediment physical characteristics, dynamics of equilibrium partitioning, and the route of exposure.

5.6.1 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 sites.

5.6.2 Sediment toxicity surveys are usually part of more comprehensive analyses of biological, chemical, geological, and hydrographic conditions. Statistical correlation can be improved and costs reduced if subsamples for sediment toxicity tests, geochemical analyses, and benthic community structure are taken simultaneously from the same grab of the same site.

5.7 Sediment toxicity tests can be an important tool for making decisions regarding the extent of remedial action needed for contaminated aquatic sites.

6. Interferences

6.1 Limitations to the methods described in this guide might arise and thereby influence sediment toxicity test results and complicate data interpretation. The following factors should be considered when testing whole sediments:

6.1.1 Alteration of field samples in preparation for laboratory testing (for example, sieving),

6.1.1.1 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.

6.1.1.2 Sediments tested at temperatures other than that at which they are collected might affect contaminant solubility, partitioning coefficients, and other physical and chemical characteristics.

6.1.2 Interaction between sediment and overlying water and the influences of the ratio of sediment to overlying water,

6.1.3 Interaction among chemicals present in the sediment,

6.1.4 Use of laboratory spiked sediment that might not be representative of contaminants associated with sediments in the field,

6.1.5 Maintenance of acceptable quality of overlying water,

6.1.6 Addition of food (3) or solvents to the test chambers that might obscure the adverse influence of contaminants associated with sediment, provide an organic substrate for bacterial or fungal growth, and might affect water quality characteristics (4),

6.1.7 Resuspension of sediment during the toxicity test,

6.1.8 Natural geochemical properties of test sediment collected from the field that might not be within the tolerance limits of the test species,

6.1.9 Recovery of test organisms from the sediment,

6.1.10 Field collected sediments that may contain indigenous organisms including predators, the same or closely related species to that being tested, and microorganisms (for example, bacteria and molds) and algae species that might grow in or on the sediment and test chamber surfaces, and

6.1.11 Test material concentrations that might be reduced in the overlying water in flow-through testing, and compounds such as ammonia that might increase during testing.

6.2 Static tests might not be applicable with materials that are highly volatile or rapidly transform biologically or chemically. The dynamics of test material partitioning between solid and dissolved phases at the start of the test should therefore be considered, especially in relation to assumptions of chemical equilibria.

7. Hazards

7.1 Many substances pose health risks to humans if adequate precautions are not taken. Information on toxicity to humans, recommended handling procedures, and chemical and physical properties of the test material should be studied before a test is begun and made aware to all personnel involved (5, 6, 7, 8). Contact with test materials, overlying water and sediments should be minimized.

7.1.1 Many materials can adversely affect humans if precautions are inadequate. Skin contact with test materials and solutions should be minimized by such means as wearing appropriate protective gloves, laboratory coats, aprons, and safety glasses, and by using dip nets, sieves or tubes to remove test organisms from overlying water. When handling hazardous sediments the proper handling procedures might include sieving and distributing sediments under a ventilated hood or in an enclosed glove box, enclosing and ventilating the toxicity testing water bath, and use of respirators, aprons, safety glasses, and gloves. Field collected sediments might contain toxic materials and should be treated with caution to minimize occupational exposure to workers. Worker safety should also be considered when working with spiked sediments containing organics or inorganic contaminants, those that are radio-labeled, and with materials that are, or are suspected of being, carcinogenic (7).

7.2 Careful considerations should be given to those chemicals which might biodegrade, transform to more toxic components, volatilize, oxidize, or photolyze during the test period.

7.3 For tests involving spiked sediments with known test materials, removal or degradation of test material before disposal of stock solutions, overlying water, and sediments is sometimes desirable.

7.4 Health and safety precautions and applicable regulations for disposal of stock solutions, test organisms, sediments, and overlying water should be considered before beginning a test (see Guide D 4447).

7.5 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.

7.6 An acidic solution should not be mixed with a

hypochlorite solution because hazardous fumes might be produced.

7.7 To prepare dilute acid solutions, concentrated acid should be added to water, not vice versa. Opening a bottle of concentrated acid and adding concentrated acid to water should be performed only in a fume hood.

7.8 Use of ground fault systems and leak detectors is strongly recommended to help prevent electrical shocks.

8. Apparatus

8.1 *Facilities*—The facility should include constant temperature areas for culturing and testing to reduce the possibility of contamination by test materials and other substances, especially volatile compounds. Holding, acclimation, and culture tanks should not be in a room in which toxicity tests are conducted, stock solutions or test solutions are prepared, or equipment is cleaned. Test chambers may be placed in a temperature controlled recirculating water bath or a constant-temperature area. Air used for aeration should be free of fumes, oil, and water. Filters to remove oil, water, and bacteria are desirable. Air filtration through a 0.22 μm bacterial filter or other suitable system may be used. The test facility should be well ventilated and free of fumes. Enclosures might be desirable to ventilate test chambers.

8.1.1 If a photoperiod other than continuous light is used, a timing device should be used to provide a light:darkness cycle. A 15- to 30-min transition period (9) when lights go on and off might be desirable to reduce the possibility of test organisms being stressed by instantaneous illumination; a transition period when lights go off might also be desirable.

8.2 *Construction Materials*—Equipment and facilities that contact stock solutions, test solutions, sediment and overlying water, into which test organisms will be placed, should not contain substances that can be leached or dissolved in amounts that adversely affect the test organisms. In addition, equipment and facilities that contact sediment or water 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 leaching, dissolution, and sorption. Concrete and rigid (unplasticized) plastics may be used for holding, acclimation, and culture tanks, and in the water-supply system, but these materials should be soaked, preferably in flowing water, for a week or more before use (10). Cast-iron pipe should probably not be used in freshwater-supply systems because colloidal iron will be added to the overlying water and strainers will be needed to remove rust particles. Copper, brass, lead, galvanized metal, and natural rubber should not contact overlying water or stock solutions before or during the test. Items made of neoprene rubber and other materials not mentioned above should not be used unless it has been shown that their use will not adversely affect survival, growth, or reproduction of the test organisms.

8.3 *Water Delivery System*—The water delivery system used in flow-through testing can be one of several designs. The system should be capable of delivering water to each replicate test chamber. Several designs of diluter systems are currently in use; Mount and Brungs (11) diluters have been successfully modified for sediment testing and other diluter systems have also been useful according to Ingersoll and Nelson (4) and Maki (12). Various metering systems, using different combinations of siphons, pumps, solenoids, valves, etc., have been used successfully to control the flow rates of overlying water.

8.3.1 The metering system should be calibrated before the test by determining the flow rate of the overlying water through each test chamber. The general operation of the metering system should be visually checked daily throughout the conduct of the test. If necessary, the water delivery system should be adjusted during the test. At any particular time during the test, flow rates through any two test chambers should not differ by more than 10 %.

8.4 *Test Chambers:*

8.4.1 In a toxicity test with aquatic organisms, test chambers are defined as the smallest physical units between which there are no water connections. However, screens, cups, etc., may be used to create two or more compartments within each chamber. Therefore, the overlying water can flow from one compartment to another within a test chamber but, by definition, cannot flow from one chamber to another. All test chambers and compartments if used, in a sediment toxicity test, must be identical. For the static tests, cover watch glasses may be used to fit over the top of the test chambers such that an aeration tip is accommodated.

8.4.2 Test chambers may be constructed in several ways of various materials, depending on the experimental design and the contaminants of interest. Clear silicone adhesives, suitable for aquaria, sorb some organic compounds which might be difficult to remove. Therefore, as little adhesive as possible should be in contact with test solution. If extra beads of adhesive are needed, they should be on the outside of the test chambers rather than on the inside. To leach potentially toxic compounds from the adhesive, all new test chambers constructed using silicone adhesives should be acclimated at least 48 h in overlying water used in the sediment toxicity test.

8.4.3 Species-specific information on test chambers is given in Annexes A1 through A4.

8.5 *Cleaning:*

8.5.1 Test chambers, water delivery systems, equipment used to prepare and store overlying water, and stock solutions should be cleaned before use. New items should be washed in the following manner: (*a*) detergent wash, (*b*) water rinse, (*c*) water-miscible organic solvent wash, (*d*) water rinse, (*e*) acid wash (such as 10 % concentrated hydrochloric acid), and (*f*) rinsed at least twice with distilled, deionized, or overlying water. Test chambers should be rinsed with overlying water just before use.

8.5.2 Many organic solvents leave a film that is insoluble in water. A dichromate-sulfuric acid cleaning solution can generally be used in place of both the organic solvent and the acid (see Guide E 729), but the solution might attack silicone adhesive and leave chromium residues on glass.

8.5.3 Upon completion of a test, all items to be used again should be immediately emptied of sediment and overlying water (and properly disposed), rinsed with water, cleaned by a procedure appropriate for removing the test material (for example, acid to remove metals and bases; detergent, organic solvent, or activated carbon to remove organic chemicals), and rinsed at least twice with distilled, deionized, or overlying water, in that order.

8.6 *Acceptability*—Before a toxicity test is conducted in new test facilities, it is desirable to conduct a "non-toxicant" test, in which all test chambers contain a negative control or reference sediment, and overlying water with no added test material. Survival, growth, or reproduction of the test species will demonstrate whether facilities, water, control sediment, and handling techniques are adequate to result in acceptable species-specific control numbers. The magnitude of the within-chamber and between-chamber variance should also be determined.

9. Overlying Water

9.1 *Requirements*—Besides being available in adequate supply, overlying water used in toxicity tests, and water used to hold organisms before testing, should be acceptable to test species and uniform in quality. To be acceptable to the test species, the water must allow satisfactory survival and growth, without showing signs of disease or apparent stress, such as discoloration, or unusual behavior.

9.2 *Source:*

9.2.1 Natural overlying water should be uncontaminated and of constant quality and should meet the following specifications as established in Guide E 729. The values stated help to ensure that test organisms are not apparently stressed during holding, acclimation, and testing, and that test results are not unnecessarily affected by water quality characteristics:

Particulate matter	<5 mg/L
TOC	<5 mg/L
COD	<5 mg/L
Residual chlorine	<11 µg/L

9.2.1.1 A natural overlying water is considered to be of uniform quality if the monthly ranges of the hardness, alkalinity, and specific conductance are less than 10 % of their respective averages and if the monthly range of pH is less than 0.4 unit. Natural overlying waters should be obtained from an uncontaminated well or spring, if possible, or from a surface water source. If surface water is used, the intake should be positioned to minimize fluctuations in quality and the possibility of contamination and maximize the concentration of dissolved oxygen and to help ensure low concentrations of sulfide and iron. Municipal water supplies often contain unacceptably high concentrations of copper, lead, zinc, fluoride, chlorine or chloramines, and quality is often variable (13). Chlorinated water should not be used for, or in the preparation of, overlying water because residual chlorine and chlorine-produced oxidants are toxic to many aquatic animals (14). Dechlorinated water should only be used as a last resort, because dechlorination is often incomplete.

9.2.2 For certain applications the experimental design might require use of water from the test sediment collection site.

9.2.3 Reconstituted water is prepared by adding specified amounts of reagent grade[8] chemicals to high quality distilled or deionized water (see Guide E 729). Acceptable water can be prepared using deionization, distillation, or reverse osmosis units. Conductivity, pH, hardness and alkalinity should be measured on each batch of reconstituted water. If the water is prepared from a surface water, total organic carbon or chemical oxygen demand should be measured on each batch. Filtration through sand, rock, bag, or depth-type cartridge filters may be used to keep the concentration of particulate matter acceptably low. The reconstituted water should be intensively aerated before use, except that buffered soft fresh waters should be aerated before, but not after, addition of buffers. Problems have been encountered with some species in some fresh reconstituted waters, but these problems can be overcome by aging the reconstituted water for one or more weeks.

9.3 *Characterization:*

9.3.1 The following items should be measured at least twice each year, and more often if such measurements have not been determined semiannually for at least two years, or if surface water is used: pH, particulate matter, TOC, organophosphorus pesticides, organic chlorine (or organochlorine pesticides plus PCBs), chlorinated phenoxy herbicides, ammonia, cyanide, sulfide, bromide, chloride, fluoride, iodide, nitrate, phosphate, sulfate, calcium, magnesium, sodium, potassium, aluminum, arsenic, beryllium, boron, cadmium, chromium, cobalt, copper, iron, lead, manganese, mercury, molybdenum, nickel, selenium, silver, and zinc, hardness, alkalinity, and conductivity (see Guide E 729).

9.3.2 For each method used the detection limit should be below the concentration in the overlying water, or below the lowest concentration that has been shown to adversely affect the test species (14).

9.3.3 Water that might be contaminated with facultative pathogens may be passed through a properly maintained ultraviolet sterilizer (15) equipped with an intensity meter and flow controls or passed through a filter with a pore size of 0.45 µm or less.

9.3.4 Water might need intense aeration using air stones, surface aerators, or column aerators (16, 17, 18). Adequate aeration will stabilize pH, bring concentrations of dissolved oxygen and other gases into equilibrium with air, and minimize oxygen demand and concentrations of volatiles. The concentration of dissolved oxygen in water should be between 90 % and 100 % saturation (19) to help ensure that dissolved oxygen concentrations are acceptable in test chambers.

10. Sediment Characterization

10.1 *General*—Before the preparation or collection of sediment an approved written procedure should be prepared for the handling of sediments which might contain unknown quantities of toxic contaminants (see Section 7, Hazards). All sediments should be characterized and at least the following determined: pH, organic carbon content (total organic carbon TOC) or total volatile sulfides, particle size distribution (percent sand, silt, clay), and percent water content (20, 21). Other analyses on sediments might include biological oxygen demand, chemical oxygen demand, cation exchange capacity, Eh, pE, total inorganic carbon, total volatile solids, acid volatile sulfides, total ammonia, metals, organosilicones, synthetic organic compounds, oil and grease, petroleum hydrocarbons, and interstitial water analysis. Macrobenthos

[8] "Reagent Chemicals, American Chemical Society Specifications," Am. Chemical Soc., Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see "Reagent Chemicals and Standards," by Joseph Rosin, D. Van Nostrand Co., Inc., New York, NY, and the "United States Pharmacopeia."

may be determined by a subsample of the field collected sediment. Toxicological results might provide information directing a more intensive analysis. Sediment toxicity testing procedures are detailed in Section 13, Procedures.

10.2 *Negative Control and Reference Sediment*—A negative control sediment or a reference sediment is used to (*a*) give a measure of the acceptability of the test, (*b*) provide evidence of the health and relative quality of the test organisms, (*c*) determine the suitability of the overlying water, test conditions, food, handling procedures, and (*d*) provide a basis for interpreting data obtained from the test sediments. Every test requires a negative sediment control (sediment known to be nontoxic to, and within the geochemical requirements of the test species) or a reference sediment. A reference sediment should be collected from the field in a clean area and represent the test sediment in sediment characteristics (for example, TOC, particles size, pH). This provides a site-specific basis for comparison of toxic and non-toxic conditions. The same overlying water, conditions, procedures, and organisms should be used as in the other treatments, except that none of the test material(s) being tested, or contaminated field collected sediments, is added to the negative control or reference sediment test chambers.

10.2.1 If a field sediment has properties such as grain size and organic content that might exceed the tolerance range of the test species, it is desirable to include a reference sediment for these characteristics.

10.3 *Field Collected Test Sediment:*

10.3.1 *Collection* (see Section 7, Hazards)—A benthic grab or core should be used rather than a dredge to minimize disruption of the sample (see Guides D 4387 and E 1391). If the sediment is obtained with a grab, it is preferable to collect a sediment sample from the upper 2 cm. 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 8.5) sample container. If the contaminants associated with sediments include compounds that readily photolyze, minimize direct sunlight during collection. All sediment samples should be cooled to 4 ± 2°C in the field.

10.3.2 *Storage*—Sediment samples should be stored at 4 ± 2°C and for no longer than two weeks before the start of the test. Freezing and longer storage might change sediment properties and should be avoided (see Guide E 1391). Sediment may be stored in containers constructed of suitable quality as outlined in 8.2. It is desirable to avoid contact with metals, including stainless steel and brass sieving screens, and some plastics. The samples should be thoroughly mixed and may be wet-press sieved through a suitably sized sieve to remove large particles and indigenous organisms, especially predators. Sediment may be diluted and mixed in a 1 to 1 ratio with overlying water to facilitate sieving (22) (see Section 6, Interferences).

10.3.3 If the experimental design prescribes not sieving a field collected sediment, obvious large predators or other large organisms should be removed by using forceps. If sediment is to be collected from multiple field samples and pooled to meet technical objectives, the sediment should be thoroughly homogenized by stirring, or with the aid of a rolling mill, feed mixer, or other suitable apparatus (see Guide E 1391).

10.3.4 Additional samples may be taken from the same grab for other kinds of sediment analyses (see 10.1). Qualitative descriptions of the sediment may include color, texture, presence of macrophytes, animals, tracks, and burrows. Monitoring the odor of sediment samples should be avoided because of hazardous volatile contaminants (see Section 7, Hazards).

10.3.5 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 (see 10.2). Controls for such factors as particle size distribution, organic carbon content, pH, etc., should be run if the limits are exceeded in the test sediments (23).

10.4 *Laboratory Spiked Sediment*—Test sediment can also be prepared in the laboratory by manipulating the properties of the negative control or the reference sediment. This can include adding chemicals or complex waste mixtures (see 1.4) (24). The toxicity of substances either dissolved in the interstitial water or adsorbed to sediment particles can be determined experimentally.

10.4.1 The test material(s) should be reagent grade[7] or better, unless a test on formulation commercial product (25), or technical-grade or use-grade material is specifically needed. Before a test is started, the following should be known about the test material: (*a*) the identity and concentration of major ingredients and impurities, (*b*) water solubility in test water, (*c*) estimated toxicity to the test species and to humans, (*d*) precision and bias of the analytical method at the planned concentration(s) of the test material, if the test concentration(s) are to be measured, and (*e*) recommended handling and disposal procedures. The toxicity of the test material in sediments may be quite different from the toxicity in water borne exposures.

10.4.2 *Stock Solution(s)*—Test material(s) to be tested in sediment should be dissolved in a solvent to form a stock solution that is then added to the sediment. The maximum concentration of the solvent in the sediment should be at a concentration that does not affect the test species. The concentration and stability of the chemical in the stock solution should be determined before beginnning the test. If the chemical(s) is subject to photolysis, the stock solution should be shielded from the light both before and during the process of mixing into the sediment. If a solvent other than water is necessary (the preferred solvent is water), it should be one which can be driven off (for example, evaporated) leaving only the test chemical on the sediments. Concentrations of the chemical in the water and sediment should be monitored before the test begins.

10.4.3 If a solvent other than water is used, both a sediment solvent control and a sediment negative control or reference sediment must be included in the test. The solvent control must contain the highest concentration of solvent present and must use solvent from the same batch used to make the stock solution (see Guide E 729). The same concentration of solvent should be used in all treatments.

10.4.3.1 Triethylene glycol is often a good organic solvent for preparing stock solutions because of its low toxicity to aquatic animals, low volatility, and ability to dissolve many

organic chemicals. Other water-miscible organic solvents, such as methanol, ethanol or acetone may be used, but they might affect total organic carbon levels, introduce toxicity, alter the geochemical properties of the sediment, or stimulate undesirable growths of microorganisms (see Section 6, Interferences). 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.

10.4.4 If the concentration of solvent is not the same in all test solutions that contain test material, either a solvent test should be conducted to determine whether survival, growth, or reproduction of the test organisms is related to the concentration of the solvent over the range used in the toxicity test, or such a solvent test already conducted using the same overlying water and test species. If survival, growth, or reproduction is found to be related to the concentration of solvent, a sediment toxicity test with that species in that amount of solvent is unacceptable if any treatment contained a concentration of solvent in that range.

10.4.4.1 If the test contains both a negative control and a solvent control, the survival, growth, or reproduction of the organisms tested in the two controls should be compared (see Guide E 1241). If a statistically significant difference in either survival, growth, or reproduction is detected between the two controls, only the solvent control may be used for meeting the acceptability of the test and as the basis for calculation of results. The negative control might provide additional information on the general health of the organisms tested. If no statistically significant difference is detected, the data from both controls should be used for meeting the acceptability of the test and as the basis for calculation of results (see 9.2.4.3 of Guide E 1241).

10.4.5 *Test Concentration(s) for Laboratory Spiked Sediments:*

10.4.5.1 If the test is intended to allow calculation of an LC50, the test concentrations should bracket the predicted LC50. 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. The LC50 of a particular compound may vary depending on physical and chemical sediment characteristics. If a useful prediction is not available, it is 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.

10.4.5.2 If necessary, concentrations above aqueous solubility can be used, as indigenous organisms are at times exposed to concentrations above solubility in the real world (see Guide E 729).

10.4.5.3 Bulk sediment chemical concentrations might be normalized to factors other than dry weight. For example, concentrations of non-polar organic compounds might be normalized to sediment organic carbon content, and metals normalized to acid volatile sulfides.

10.4.5.4 In some situations (for example, regulatory) it might be necessary to only determine whether a specific concentration of test material is toxic to the test species, or whether the LC50 is above or below a specific concentration. When there is interest in a particular concentration, it might only be necessary to test that concentration and not to determine the LC50.

10.4.6 Addition of test material(s) to sediment may be accomplished using various methods, such as a (*a*) rolling mill, (*b*) feed mixer, or (*c*) hand mixing (see Guide E 1391).

10.4.6.1 Modifications of the mixing techniques might be necessary to allow time for a test material to equilibrate with the sediment. If tests are repeated, mixing conditions such as duration and temperature of mixing, and time of mixing before the test starts, should be kept constant. Care should be taken to ensure that a test material added to sediment is thoroughly and evenly distributed within the sediment. If necessary, subsamples of the sediment within a mixing container can be analyzed to determine degree of mixing and homogeneity.

11. Test Organisms

11.1 *Species*—Whenever possible and appropriate, tests should be conducted with species listed in the Appendices. Use of these species is encouraged to increase comparability of results. The source and type of sediment being tested or the type of test to be implemented might dictate selection of a particular species. The species used should be selected based on availability, sensitivity to a test material(s), and tolerance to ecological conditions such as temperature, grain size, and ease of handling in the laboratory. The species used should be identified using an appropriate taxonomic key.

11.2 *Age*—All organisms should be as uniform as possible in age and size class. The age or size class for a particular test species should be chosen so that sensitivity to test materials is not affected by state of maturity, reproduction, or other intrinsic life-cycle factors (see Annexes A1 through A4).

11.3 *Source*—All organisms in a test must be from the same source. Organisms may be obtained from laboratory cultures, commercial, state or federal institutions, or natural populations from clean areas. Laboratory cultures of test species can provide organisms whose history, age, and quality are known. Local and state agencies might require collecting permits.

11.4 *Quality*—Analysis of the test organisms for the test material(s) is desirable, as it might be present in the environment, and other chemicals to which major exposure might have occurred.

11.5 *Brood Stock*—Brood stock should be cared for properly so as not to be unnecessarily stressed (see Annexes A1 through A4). To maintain organisms in good condition and avoid unnecessary stress, they should not be crowded and should not be subjected to rapid changes in temperature or water quality characteristics.

11.6 *Handling*—Test organisms should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and as quickly as possible. Organisms should be introduced into solutions beneath the air-water interface. Any organisms that touch dry surfaces, are dropped, or injured during handling should be discarded.

12. Experimental Design

12.1 Decisions concerning the various aspects of experimental design, such as the number of treatments, number of test chambers and test organisms per treatment, and water quality characteristics, should be based on the purpose of the

test and the type of procedure that is to be used to calculate results (see Section 16, Calculation). A test intended to allow calculation of a specific endpoint such as an LC50 should consist of a negative control sediment, a solvent control(s), a reference sediment, and several test sediments (see Section 10, Sediment Characterization).

12.2 The object of a qualitative reconnaissance survey is to identify sites of 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 site. The lack of replication usually precludes statistical comparisons, but identification of samples for further study is possible, where survival, growth, or reproduction differ from the negative control or reference sediment. A useful summary of field sampling design is presented by Green (26).

12.2.1 The object of a quantitative statistical comparison is to test for statistically significant differences in effects (see 13.12) among negative control or reference sediments and test sediments from several sites. The number of replicates needed per site is a function of the need for sensitivity or power. Replicates (for example, separate samples from different grabs taken at the same site) should be taken at each site in the survey. Separate subsamples from the same grab might be used to test for within-grab variability, or split samples of composited sediment from one or more grabs might 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 sites.

12.2.2 Site locations might be distributed along a known pollution gradient, in relation to the boundary of a disposal site, or at sites identified as being toxic in a reconnaissance survey. Comparisons can be made in both space and time (see Section 16, Calculation). 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 should include subsampling cores taken to the project depth.

12.3 *Laboratory Experiments*—The primary focus of the physical and experimental test design, and statistical analysis of the data, is the experimental unit, which is defined as the smallest physical entity to which treatments can be independently assigned (27). Because overlying water or air cannot flow from one test chamber to another the test chamber is the experimental unit (see 8.4). As the number of test chambers per treatment increases, the number of degrees of freedom increases, and, therefore, the width of the confidence interval on a point estimate, such as an LC50, decreases, and the power of a significance test increases (see Section 16, Calculation). Because of factors that might affect results within test chambers and results of the test: (*a*) all test chambers should be treated as similarly as possible, such as temperature and lighting (unless these are the variables tested), and (*b*) each test chamber, including replicate test chambers, must be physically treated as a separate entity. Treatments must be randomly assigned to individual test chamber locations. Assignment of test organisms to test chambers must be randomized.

13. Procedure

13.1 *Sediment into Test Chambers*—The day before the toxicity test is started (Day −1) each test sediment, reference sediment, and negative control sediment should be mixed and a sample added to the test chambers (4, 24, 28). Sediment depth in the test chamber is dependent on the experimental design and the test species (see Annexes A1 through A4 and 6.1.2). Each test chamber and replicates must contain the same amount of sediment, determined either by volume or weight.

13.1.1 The sediment aliquot in each test chamber should be settled by smoothing with a utensil constructed of a suitable material (see 8.2). If beakers are used, bubbles can be removed by either tapping the test chamber against the palm of the hand or by displacement of bubbles with the utensil. After the sediment is placed in the test chambers, overlying water should be added. The overlying water should be gently poured along the side of the test chamber to prevent resuspension of the sediment.

13.2 *Static Testing*—Overlying water should be added to the test chambers at the volume specified by the experimental design. Watch glasses should be used to cover the test chambers and overlying water gently aerated. Aeration can be provided to each test chamber through a 1-mL glass pipet that extends between the beaker spout and the watch glass cover to a depth not closer than 2 cm from the sediment surface. Air should be bubbled into the test chambers at a rate that does not cause turbulence or disturb the sediment surface. To allow any suspended sediments to settle, the test organisms should not be introduced into the test system for between 12 and 24 h. Water quality characteristics should be measured prior to the addition of the test organisms (see 13.11).

13.2.1 Water lost to evaporation or splattering should be replaced as needed with temperature acclimated de-ionized water or overlying water. The water quality of the overlying water in static sediment toxicity tests (water hardness, alkalinity, total dissolved solids, and dissolved oxygen) might be altered by the presence of sediment (4) or by the addition of food to the test chamber (3). These changes in water quality characteristics might influence the availability of contaminants to the test organisms (see Section 6, Interferences).

13.3 *Flow-Through Testing*—The water-delivery system should be turned on before a test is started to verify that the system is functioning properly. The water flow to each test chamber should not differ by more than 10 % (see 8.3.1). The total volume flow per hour for continuous flow diluters should be recorded.

13.3.1 After the sediment has been added (Day −1), overlying water is added to the test chambers (see 13.2). After aliquots are removed for water quality determinations (Day 0), overlying water flow is started prior to the addition of the test organisms and food (4).

13.4 *Duration of Test*—The test begins when test organisms are first placed in the test chambers (Day 0) and continues for the duration specified in the experimental design for a specific test organism (see Annexes A1 through A4).

13.5 *Dissolved Oxygen*—The dissolved oxygen concentration in each test chamber should be measured in at least one test chamber in each treatment (*a*) at the beginning and end of the test and at least weekly (if possible) during the test, (*b*)

whenever there is an interruption of the flow of air (static tests) or water (flow-through tests), and (*c*) whenever the behavior of the test organisms indicates that the dissolved oxygen concentration might be too low (for example, emergence from the sediment). A measured dissolved oxygen concentration should be >40 % and ≤100 % saturation (12.4.2 of Guide E 729).

13.6 *Overlying Water Quality Measurements*—Conductivity, hardness, pH, and alkalinity should be measured in all treatments at the beginning and end of a short-term test, and at least weekly during a long-term test, using appropriate ASTM standards when possible.

13.7 *Temperature*—Test temperature depends upon the species used (see Annexes A1 through A4). Other temperatures may be used to study the effect of temperature on survival, growth, or reproduction of test organisms, and contaminant related properties (for example, bioavailability). The daily mean test temperature must be within ±1°C of the desired temperature. The instantaneous temperature must always be within ±3°C of the desired temperature.

13.8 *Feeding*—Recommended food, ration, method, and frequency of feeding test organisms are contained in Annexes A1 through A4. The food used should be analyzed for the test material and other possible contaminants. A batch of food may be used if it will support normal function. Detailed records on feeding rates and appearance of the sediment should be made daily.

13.9 *Debris*—Any floating debris may be skimmed from the test chambers before test organisms are added. This can be accomplished with a piece of fine nylon screen or other suitable material. If more than 0.1 g of floating debris is removed, an analysis should be performed to determine the amount of chemical removed from the system (25).

13.10 *Light*—For sediment toxicity tests various light:darkness regimes can be used depending on the species being tested (see Annexes A1 through A4) and various experimental designs.

13.11 *Acclimation*—Test organisms should be acclimated if they are cultured in water different from the overlying water or temperature (4) (see Annexes A1 through A4).

13.12 *Biological Data*—Effects indicating toxicity of test sediment include mortality and sublethal effects on growth, maturation, behavior, and reproduction. Test chambers should be observed at least daily. At the end of the exposure period, recovery of the test organisms from sediments should be accomplished following the methods outlined for each species (see Annexes A1 through A4).

13.13 *Other Measurements:*

13.13.1 *Field Sediment*—Sediment samples should be collected from the same grab for analysis of sediment physical and chemical characterizations. A separate sample for benthic faunal analyses may be desirable (see Guide D 4387).

13.13.2 *Laboratory Spiked Sediments*—At the beginning and at the end of the experiment, measurement of the concentration of the test material(s) in both stock solutions and sediment is desirable. To monitor changes in sediment or interstitial water chemistry during the course of the experiment, separate sediment chemistry chambers should be set up and sampled at the start and end of the experiment. It is not necessary to add test organisms to these chambers at the beginning of the test, but for later sampling, test organisms should be added after the initial sample is taken.

13.13.2.1 Concentration of test material(s) in overlying water, interstitial water, and sediment should be measured at several concentrations and as often as practical during the test. If possible, the concentration of the test material in overlying water, interstitial water and sediments should be measured at the start and end of the test. Measurement of test material(s) degradation products might also be desirable.

13.13.2.2 Measurement of test material(s) concentration in water can be accomplished by pipeting water samples from a point midway between top, bottom and sides of the test chamber. Overlying water samples should not contain any surface scum, any material from the sides of the test chamber, or any sediment.

13.13.2.3 Measurement of test material(s) concentration in sediment at the end of a test can be taken by siphoning the overlying water without disturbing the surface of the sediment, then removing appropriate aliquots of the sediment for chemical analysis.

13.13.2.4 Interstitial water can be sampled by using the water that (*a*) comes to the surface in a mixing apparatus, (*b*) is on the surface of the sediment after it settles, (*c*) is separated from the sediment particles by centrifuging a sediment sample, (*d*) is filtered through an apparatus to extract interstitial water, (*e*) has been pressed out of the sediment, or (*f*) by using an interstitial water sampler. Care should be taken to ensure that contaminants do not transform, degrade, or volatilize during the interstitial water sample preparation (see Guide E 1391).

14. Analytical Methodology

14.1 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 (29).

14.2 Concentrations should be measured for contaminants in bulk sediment, test material(s) in the interstitial water, test material(s) in the overlying water, and test material(s) in the stock solution. In addition, measurement of either the apparent dissolved or undissolved substances of the test material(s) is desirable. The apparent dissolved material is defined and determined as that which passes through a 0.45 µm membrane filter.

14.2.1 If samples of 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 (30) (see Section 10, Sediments).

14.3 Methods used to analyze food or test organisms should be obtained from appropriate sources (31).

14.4 The precision and bias of each analytical method used should be determined in an appropriate matrix: that is, sediment, water, tissue. When appropriate, reagent blanks, recoveries, and standards should be included when samples are analyzed.

15. Acceptability of Test

15.1 A sediment toxicity test should be considered unacceptable if one or more of the following occurred, except, for example, if temperature was measured numerous times, a

deviation of more than 3°C (see 13.6) in any one measurement might be inconsequential. However, if temperature was measured only a minimal number of times, one deviation of more than 3°C might indicate that more deviations would have been found if temperature had been measured more often.

15.1.1 All test chambers (and compartments) were not identical (see 8.4.1, 12.3),

15.1.2 The overlying water was not acceptable to the test organisms (see 9.1),

15.1.3 Test organisms were not acclimated to the appropriate overlying water or temperature (if they were cultured in water different from the overlying water or temperature),

15.1.4 The natural geochemical properties of test sediment collected from the field was not within the tolerance limits of the test species (see 10.3.5),

15.1.5 Appropriate negative and solvent controls, or reference sediment, were not included in the test (see 10.4.3),

15.1.6 The concentration of solvent in the range used affected survival, growth, or reproduction of the test organisms (see 10.4.4),

15.1.7 All animals in the test population were not obtained from the same source, were not all of the same species, or were not of acceptable quality (see 11.3),

15.1.8 Treatments were not randomly assigned to individual test chamber locations and the individual test organisms were not impartially or randomly assigned to test chambers or compartments (see 12.3),

15.1.9 Each test chamber did not contain the same amount of sediment, determined either by volume or by weight,

15.1.10 Temperature, dissolved oxygen, and concentration of test material were not measured, or were not within the acceptable range (see 13.7 and Annexes A1 through A4),

15.1.11 The negative control or reference sediment organisms did not survive, grow or reproduce as required for the test species (see Annexes A1 through A4), or

15.1.12 Average survival in any negative control chamber was less than acceptable limits (see Annexes A1 through A4).

16. Calculation

16.1 The calculation 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 the advantages and disadvantages of various alternative procedures, and appropriate preliminary tests, such as those for outliers and for heterogeneity.

16.2 For each set of data, the LC50 or EC50 and its 95 % confidence limits should be calculated (when appropriate) on the basis of (*a*) the measured initial concentrations of test material, if available, or the calculated initial concentrations for static tests, and (*b*) the average measured concentrations of test material, if available, or the calculated average concentrations for flow-through tests. If other LC or ECs are calculated, their 95 % confidence limits should also be calculated (see Guide E 729).

16.3 Most toxicity tests produce quantal data, that is, counts of the number of responses in two mutually exclusive categories, such as alive or dead. A variety of methods (32) can be used to calculate an LC50 or EC50 and 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 effected is between zero and 100, but the most widely used are the probit, moving average, Spearman-Karber and Litchfield-Wilcoxon methods. The method used should appropriately take into account the number of test organisms per chamber. The binomial test can also be used to obtain statistically sound information about the LC50 or EC50 even when less than two concentrations kill or affect between zero and 100 percent. The binomial test provides a range within which the LC50 or EC50 should lie.

16.4 When samples from field sites are independently replicated, the sites effects can be statistically compared by *t*-tests, analysis of variance (ANOVA) or regression type analysis. Analysis of variance is used to determine whether any of the observed differences among the concentrations (or samples) are statistically significant. This is a test of the null hypothesis that no differences exist in the effects at all of the concentrations (or samples) and at the control. If the *F*-test is not statistically significant ($P > 0.05$), it can be concluded that the effects observed in the test material treatments (or field sites) were not large enough to be detected as statistically significant by the experimental design and hypothesis test used. Nonrejection does not mean that the null hypothesis is true. The NOEC based on this end point is then taken to be the highest test concentration tested (33, 34). The amount of effect that occurred at this concentration should be considered.

16.4.1 All exposure concentration effects (or field sites) can be compared with the control effects by using mean separation techniques such as those explained by Chew (35) orthagonal contrasts, Fisher's methods, Dunnett's procedure or Williams' method. The lowest concentration for which the difference in observed effect exceeds the statistical significant difference is defined as the LOEC for that end point. The highest concentration for which the difference in effect is not greater than the statistical significant difference is defined as the NOEC for that end point (33).

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 available documents:

17.1.1 Name of test and investigator(s), name and location of laboratory, and dates of start and end of test,

17.1.2 Source of negative control, reference or test sediment, method for collection, handling, shipping, storage and disposal of sediment,

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

17.1.4 Source of overlying water, its chemical characteristics, and a description of any pretreatment, and results of any demonstration of the ability of a species to survive, grow or reproduce in the water,

17.1.5 Source, history and age of test organisms; source,

history and age of brood stock, culture procedures; and 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 weight and lengths, observed diseases or unusual appearance, treatments, holding and acclimation procedures,

17.1.6 Source and composition of food, concentrations of test material and other contaminants, procedure used to prepare food, feeding methods, frequency and ration,

17.1.7 Description of the experimental design and test chambers (and compartments), the depth and volume of sediment and overlying water in the chambers, lighting, number of test chambers and number of test organisms per treatment, date and time test starts and ends, temperature measurements, dissolved oxygen concentration (as percent saturation) and any aeration used prior to initiating a test and during the conduct of a test,

17.1.8 Methods used for, and results of (with standard deviations or confidence limits), physical and chemical analyses of sediment,

17.1.9 Definition(s) of the effects used to calculate LC50 or EC50s, biological endpoints for tests, and a summary of general observations of other effects,

17.1.10 A table of the biological data for each test chamber for each treatment including the control(s) in sufficient detail to allow independent statistical analysis,

17.1.11 Methods used for, and results of, statistical analyses of data,

17.1.12 Summary of general observations on other effects or symptoms, and

17.1.13 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 methodology used and the quality of the results.

ANNEXES

(Mandatory Information)

A1. *HYALELLA AZTECA*

A1.1 *Significance—Hyalella azteca* (Saussure), Amphipoda, has many desirable characteristics of a test species: short generation time, easily collected from natural sources or cultured in the laboratory in large numbers, and data on survival, growth, and reproduction can be obtained in toxicity tests (36). Landrum and Scavia (37), Nebeker et al. (22), and Ingersoll and Nelson (4) have successfully used *H. azteca* in sediment toxicity testing and have shown it to be a sensitive indicator of the presence of contaminants associated with sediments. Ingersoll and Nelson (4) report *H. azteca* to have a wide tolerance of sediment grain size. Sediment ranging from >90 % silt- and clay-size particles to 100 % sand-size particles did not reduce survival or growth in the laboratory.

A1.2 *Life History and Life-Cycle*—The life-cycle of *H. azteca* can be divided into three distinct stages according to Cooper (36): (*1*) an immature stage, consisting of the first 5 instars; (*2*) a juvenile stage, including instars 6 and 7; and (*3*) an adult stage, the 8th instar and older. The potential number of adult instars is large and growth is indeterminate such that old adults can be much larger than younger adults (38). DeMarch (39) indicates that juvenile *H. azteca* can complete a life-cycle in 27 days or longer depending on temperature.

A1.2.1 *H. azteca* is an epibenthic detritivore and will burrow in the sediment surface, and Hargrave (40) has demonstrated in laboratory experiments that *H. azteca* digests bacteria and algae from ingested sediment particles (<65 μm), further illustrating sediment interactions by *H. azteca*.

A1.2.2 Sexual dimorphism occurs in *H. azteca*; the adult male is larger than females and has larger second gnathopods (41).

A1.2.3 DeMarch (41) indicates that the number of young produced per adult female is optimum at temperatures of between 26 and 28°C. Whereas, Cooper (36) and Strong (38) report that maximum brood size is more dependent on the size of the adult amphipods than on temperature.

A1.3 *Obtaining Test Organisms*—The following culture procedures are adapted from deMarch (41), Nebeker et al. (22), and Ingersoll and Nelson (4). *H. azteca* can be reared in 10- or 20-L aquaria under flowing water conditions with a 16 to 8 h ratio of light to darkness photoperiod at 20 ± 2°C, and about 500 fc (5382 lx). For static cultures, the water should be gently aerated and about 25 to 30 % of the water volume should be replaced weekly. In flow-through cultures, water delivery can be at a low rate (100 mL/min) (4).

A1.3.1 *H. azteca* can be cultured with a variety of foods. Dried maple, alder, birch or poplar leaves, presoaked for several days and tannins flushed out with water, then can be added weekly as the primary substrate and food. Rabbit pellets,[9] ground cereal leaves,[10] fish food flakes,[11] frozen or newly hatched brine shrimp, or heat-killed young *Daphnia* can be used to feed *H. azteca*. In addition, Strong (38) demonstrated success in culturing *H. azteca* yielding the best survivorship and consistently the largest clutches by feeding the amphipods filamentous green algae (*Oedogonium cardiacum*) and homogenized rotting spinach ad libitum.

A1.3.2 To clean the culture tanks or reduce populations of animals, half of the leaf substrate containing a portion of the animals should be transferred to a sorting tray, discarding

[9] Rabbit pellets, such as Purina Rabbit Pellets, available from Purina Mills, Inc., 1401 Hanley St., St. Louis, MO 63144, have been found suitable for this purpose.

[10] Ground cereal leaves, such as Cerophyl, available from Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178, has been found suitable for this purpose.

[11] Fish food flakes such as Tetra-Min and Tetro Conditioning Food, available from many pet foot distributors, have been found suitable for this purpose.

the remainder of the old contents and returning the leaf substrate and animals to the chamber. The number of amphipods should be reduced periodically as the population expands rapidly.

A1.4 *Collection—H. azteca* can be found in permanent lakes, ponds and streams throughout the entire American continent (**41, 42**). Methods used by Landrum and Scavia (**37**) indicate that the amphipods can be collected from a natural freshwater source. Pennak (**42**) suggests using a dip-net to collect aquatic vegetation and bottom debris containing amphipods. Sites with stony bottoms might require collecting with forceps or the use of a small aquarium net. Live specimens can be maintained in aquaria if they are well supplied with aquatic vegetation (**42**). Collection procedures for *H. azteca* by deMarch (**41**) indicate that rinsing aquatic vegetation is effective if a 200 to 550 μm mesh net is used to catch the amphipods. Up to 200 amphipods can be transported in a large plastic bag containing 1 L of water from the collection site, with the remainder of the bag filled with air or oxygen and then placed into a cooler (**41**). For verification and accurate identification of field collected *H. azteca*, it is important that mature males and females be used (**42**).

A1.5 *Brood Stock*—Brood stock can be obtained from the wild, another laboratory or a commercial source. *H. azteca* brought into the laboratory should be acclimated to the culture water by gradually changing the water in the culture chamber from the water in which they were transported to 100 % culture water. *H. azteca* should be acclimated to the culture temperature by changing the water temperature at a rate not to exceed 2°C within 24 h, until the desired temperature is reached (**41**). Brood stock should be cultured so they are not unnecessarily stressed. To maintain *H. azteca* in good condition and avoid unnecessary stress, crowding and rapid changes in temperature and water quality characteristics should be avoided.

A1.6 *Handling—H. azteca* should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly as possible, so that the amphipods are not unnecessarily stressed. Amphipods should be introduced into solutions beneath the air-water interface (**4**). Any *H. azteca* that touch dry surfaces, are dropped, or injured during handling should be discarded. Removing animals from sieves may form air bubbles on body surfaces causing animals to float on the water surface. Any "floaters" should be gently placed into the water column using a probe. If the animals continue to float they should be removed and discarded.

A1.7 *Age*—Tests with *H. azteca* should be started with juvenile organisms (second or third instar) about 2 to 3 mm in length (**4, 22**). To obtain *H. azteca* for testing, amphipods should be separated from the leaf material by scooping up the leaves with clinging amphipods, and placing the leaves on a 5 to 10 mm mesh screen, which is placed over a collecting pan containing 2 cm of culture water. Culture water should be sprinkled on the leaves while turning and separating the leaves. Mixed age *H. azteca* should be washed from the leaves and dropped through the screen into a collecting pan (**22**). To separate the juvenile amphipods from the larger adults a sieve stack (U.S. Standard) #30 (600 μm), #40 (425 μm), and a #60 (250 μm) can be used (**4**). Culture water should be rinsed through the sieves and juvenile animals retained by the #60 sieve are washed into a collecting pan while the larger animals in the top sieves (#30 and #40) are returned to the culture. The juvenile amphipods are then placed in 1-L beakers containing culture water (about 200 amphipods/beaker) and kept in the dark at the temperature of the culture with gentle aeration. *H. azteca* can be isolated in the 1-L beakers up to 24 h prior to the start of the sediment toxicity test.

A1.7.1 Borgmann (**43**) recommends collecting uniform aged young (<1 week old) for experimental purposes using 2.5-L jars containing about 1 L of culture water and between 5 and 25 adult *H. azteca*. The jars are placed in an incubator at 16 to 8 h ratio of light to darkness photoperiod, about 500 fc (5382 lx). Each jar contains pieces of pre-soaked (in culture water) cotton gauze as a substrate. Once a week the animals should be removed from the gauze and collected by filtration through a 275 μm nylon mesh screen, then rinsed into petri dishes where the young and adults are sorted. Fresh culture water and food should be placed in the jars and the adults returned. Each jar should receive 0.02 g of fish food flakes[10] or more if required by larger animals.

A1.8 *Acclimation*—If amphipods are cultured in water different from the overlying water or temperature, an acclimation process is necessary. The water acclimation process used by Ingersoll and Nelson (**4**) is to first place animals for 2 h in a 50 to 50 mixture of culture water to overlying water, then for 2 h in a 25 to 75 mixture of culture water to overlying water, followed by a transfer into 100 % overlying water. At this stage the amphipods are considered acclimated to the overlying water and are ready for immediate use. *H. azteca* can then be randomly selected from the acclimation water with a pipette and placed into counting beakers (for example, 30-mL) that can be floated in the test chambers before the amphipods are introduced into the exposure system (**4**).

A1.9 *Toxicity Test Specifications:*

A1.9.1 *Experimental Design*—Decisions concerning the various aspects of experimental design, such as the number of treatments, number of test chambers and amphipods per treatment, and water quality characteristics, should be based on the purpose of the test and the procedure used to calculate results. Nebeker et al. (**22**) recommend two or more replicate 20-L aquaria per treatment with 100 juvenile *H. azteca* placed in each aquarium. Ingersoll and Nelson (**4**) recommend four replicate 1-L beakers per treatment, with 20 *H. azteca* per replicate, for a total of 80 amphipods per treatment. Duration of the test can range from a ≤10 day short-term test to a long-term test >10 days and continuing up to 30 days (**4, 22**). The number of young and adult survival (**4, 22**), growth, and development (**4**) can be used as the biological endpoints. A test duration up to 30 days can add potential reproductive capacity as another biological endpoint, measuring effects on reproductive behavior, appearance of secondary sex characteristics, egg production, and number of young produced. Tests with *H. azteca* have been conducted at 20°C (**4, 22**) and from 21 to 25°C (**37**), photoperiod 16 to 8 h ratio of light to darkness, about 50 fc (538 lx) (**4**).

A1.9.2 *Static and Flow-Through Tests*—Ingersoll and Nelson (**4**) and Nebeker et al. (**22**) recommend using borosilicate glass 1-L beakers to expose the *H. azteca* to the test material. These exposure chambers contain about 800

mL overlying water and 200 mL (2 cm) test sediment, in both the static and flow-through water systems. For the static tests cover watch glasses may be used to fit over the top, such that an aeration tip fits through the beaker pour spout and the cover (4). Nebeker et al. (22) suggest for the static long-term test, using 20-L aquaria with 2 to 3 cm of test sediment on the bottom overlaid with 15 cm water. For flowthrough testing, Ingersoll and Nelson (4) suggest using a 4 by 13 cm notch cut in the lip of the 1-L beaker. The notch should be covered with 0.33 mm U.S. Standard sieve size #50 screen, either made of stainless steel or polyethylene, using a silicone adhesive to attach the screen to the beaker.

A1.9.3 *Initiation of a Test*—Sediments should be homogenized and placed in the test chambers on the day prior to the addition of the test organisms (Day −1). Test chambers should be covered and overlying water aerated (4) or unaerated overnight but aerated for 30 min before *H. azteca* are added (22). The test begins when the juvenile *H. azteca* are introduced to the test chambers (Day 0). It is recommended that flow-through and static tests might need to be started on different days to assure that sufficient time is available to complete all tasks. Test chambers should be inspected <2 hours after amphipods are introduced to ensure that animals are not trapped in the surface tension of the water (4). These *floaters* might not survive well and should be replaced with new animals (see A1.6).

A1.9.4 *Feeding*—Ingersoll and Nelson (4) recommend rabbit pellets[9] to be used as a food for *H. azteca* in short and long-term sediment toxicity tests. Nebeker et al. (22) suggest feeding rabbit pellets[9] in a 28 day test. The pellets should be ground and dispersed in deionized water. A fluorocarbon plastic stir bar and a magnetic stir plate should be used to homogeneously resuspend the rabbit pellet[9] when aliquots are removed for feeding. If food collects on the sediment, a fungal or bacterial growth might start on the surface of the sediment, in which case feeding should be suspended for one or more days. A drop in dissolved oxygen to 40 % saturation might indicate that all of the food added in the water is not being consumed such that feeding might be suspended for the amount of time necessary to increase the dissolved oxygen concentration (4).

A1.9.4.1 In static tests Nebeker et al. (22) suggest a feeding regime twice weekly of 200 mg (0.5 mL dry volume) rabbit pellets[9] mixed in 100 mL distilled water for 100 juvenile *H. azteca* in a 20-L aquarium. Nelson and Ingersoll (4) recommend feeding *H. azteca* three times weekly 14 mg rabbit pellets per feeding for 20 young amphipods in a 1-L beaker. Lower feeding levels for flow-through and static tests may be used for *H. azteca*: three times weekly 6 mg rabbit pellets[9] per feeding for the first week of the test, and 12 mg per feeding for the following weeks.

A1.9.4.2 For flow-through testing, prior to starting a test, 20 mg rabbit pellets[9] should be added to each test chamber, and three times a week each test chamber should be fed 20 mg per feeding for 20 young *H. azteca* during the exposure (4).

A1.10 *Biological Data*—During the conduct of the test, observations should be made to assess behavior (for example, floaters, sediment avoidance) and reproductive activities (for example, amplexus). At the end of the test the *H. azteca* must be removed from the test chambers for survival (4, 22), observable behavior, any noticeable reproduction (for example, amplexus, gravid females, young present) and growth (4). According to Ingersoll and Nelson (4) without material above the sediment surface, such as the leaves used in culturing, *H. azteca* burrow in the top 1 cm sediment surface or are found swimming in the water column. Many of the surviving amphipods can be pipeted from the water column before sieving the sediments. At the end of the test the sediment should be screened using a #35 (500 μm) U.S. Standard size sieve (22). Ingersoll and Nelson (4) recommend using a #50 (300 μm) U.S. Standard size screen cup first by swirling the overlying water to suspend the upper 1 cm of sediment and pouring that slurry into the cup. Next, a stack of sieves #25 and #40 U.S. Standard size should be used to sieve the bulk sediment in order to collect and count the live animals remaining in the sediment. The *H. azteca* are rinsed from the screens into collecting pans and pipeted from the rinse water (4). It might be difficult to recover young *H. azteca* due to their small size. Material retained in the collecting pans may be preserved in a sugar formalin mixture for examination at a later date (4). The preserved material may be inspected using a low power binocular microscope to search for *H. azteca* missed the last day of the test.

A1.10.1 For quantifying growth, *H. azteca* body length (±0.01 mm) should be measured from the base of the first antenna to the tip of the third uropod along the curve of the dorsal surface (4). In addition, wet and dry weight measurements have been used to estimate growth for *H. azteca* (37).

A1.10.2 A *H. azteca* sediment toxicity test, independent of duration, is unacceptable if the average survival in any negative control chamber is less than 80 % (see Section 15, Acceptability of Test).

A2. *CHIRONOMUS TENTANS*

A2.1 *Significance—Chironomus tentans* Fabricius (Diptera: Chironomidae) has been used in sediment toxicity tests because it is a fairly large midge with a short generation time, is easily cultured in the laboratory, and the larvae have direct contact with the sediment by burrowing into sediment to build a case. *C. tentans* has been successfully used in sediment toxicity testing and is sensitive to many contaminants associated with sediments (22, 25, 44, 45, 46). The members of the genus are important in the diet of young and adult fish and surface feeding ducks (47).

A2.2 *Life History and Life-Cycle*—The classification of holometabolous insects, such as *C. tentans*, presents special difficulties because each life-stage often has different ecological requirements. Further detailed studies at the species level are needed to better understand the various physical, chemical, and biological factors that interact to produce a suitable environment for larval development (48). *C. tentans* has a holarctic distribution and is locally common in the mid-continental areas of North America (47, 49, 50). Sadler (51) describes the general biology of *C. tentans*. The larval stages

often inhabit eutrophic lakes and ponds. Qualitative observations indicate larvae occur most frequently in fine sediment and detritus; however larvae reportedly inhabit sediments with particles ranging from <0.15 mm to 2.0 mm (52). Chironomid larvae usually penetrate a few centimeters into sediment. In both lotic and lentic habitats with soft bottoms, about 95 % of the chironomid larvae occur in the upper 10 cm of substrate, very few larvae are found below 40 cm (48). Larvae are generally not found when hydrogen sulfide is greater than 0.3 mg/L (52). Larvae of *C. tentans* are found in the field at a temperature range between 0 and 35°C, pH range between 7 and 10, conductivity range between 100 and 4000 $\mu S\ cm^{-1}$, sediment organic carbon range between 2 and 15 %, and at dissolved oxygen concentrations as low as 1 mg/L (47, 52, 53). Sadler (51) reported that *C. tentans* will eat essentially any material of appropriate size.

A2.2.1 The biology of *C. tentans* facilitates laboratory culture since larvae are tolerant of a wide spectrum of conditions and adults mate even when confined (47). The life-cycle of *C. tentans* can be divided into three distinct stages: (*1*) a larval stage, consisting of the 4 instars; (*2*) a pupal stage; and (*3*) an adult stage. Midge egg masses hatch in 2 or 3 days after deposition in water at 19 to 22°C. Larval growth occurs in four instars of about one week each. Under optimal conditions larvae will pupate and emerge as adults after 24 to 28 days at 20°C. Adults emerge from pupal cases over a period lasting several days. Males are easily distinguished from females because males have large, plumose antennae and a much thinner abdomen with visible genitalia. Mating behavior has been described by Sadler (51) and others (54).

A2.3 *Obtaining Test Organisms*—The following is a description of culturing procedures adapted from Adams et al. (25), Nebeker et al. (22) and others (47, 54); these procedures should not be considered definitive, since procedures that work well in one laboratory sometimes work poorly in another laboratory: *C. tentans* can be reared in aquaria in static or flowing water with a 16 to 8 h light to darkness photoperiod at 20 to 23°C, at about 50 fc (538 lx). For static cultures the water should be gently aerated, and about 25 to 30 % of the water volume should be replaced weekly. Cultures should be maintained in an isolated area or room free of contamination and excessive disturbances. Adams et al. (25) recommends rearing midges in glass aquaria filled with water to a depth of 45 cm covered with nylon screen. The size of the aquaria may vary from a minimum of 3 L to a maximum of 19 L depending on the need for animals.

A2.3.1 *Chironomus tentans* require a substrate in which to construct a case. Shredded paper towels have been found to be well suited for this purpose. Strips cut from brown paper towels should be soaked overnight in acetone to remove impurities and are then rinsed in three changes of culture water until the acetone is removed. A kitchen blender should be used to shred the rinsed towels into a pulp. Care must be taken to avoid over blending and possibly shortening the wood fibers in the pulp. The pulp should be rinsed twice with culture water to remove extremely small fibers and refrigerated until needed. The paper toweling pulp should be placed into the water of a culture chamber to a depth of 3 cm. One gram of dry fish food flakes[11] should be mixed in 10 mL of culture water with a kitchen blender and refrigerated. This suspension should be fed twice daily to the cultures for optimum growth. The amount given depends on the number and size of the larvae. If after feeding the culture water does not clear in 3 to 4 h, the feeding level should be reduced. Overfeeding will lead to the growth of fungus in the aquaria and will necessitate more frequent water changes. Therefore, new cultures should receive 0.5 mL or less of this suspension per feeding. Nebeker et al. (22) suggest supplementing the fish food flakes[11] diet with ground cereal leaves.[10]

A2.4 *Brood Stock*—Brood stock can be obtained from the wild, laboratory or a commercial source. When midges are brought into the laboratory, they should be acclimated to the culture water by gradually changing the water in the culture chamber from the water in which they were transported to 100 % culture water. Midges should be acclimated to the test temperature by changing the water temperature at a rate not to exceed 2°C within 24 h, until the desired temperature is reached. Brood stock should be cultured so they are not unnecessarily stressed. To maintain midges in good health and avoid unnecessary stress, crowding and rapid changes in temperature and water quality characteristics should be avoided.

A2.5 *Age*—Tests with *C. tentans* can be started with second instar larvae according to Wentsel et al. (44), Adams et al. (25), Nebeker et al. (22) and Giesy (45). Tests started with first instar *C. tentans* larvae have met with limited success (22). Twelve to 16 days before a test is begun, at least 3 freshly laid midge egg cases should be placed in a clean 20 by 40 cm glass or enameled rearing pan filled with water to a depth of 3 cm. Egg cases should be isolated by aspirating adults into a 250-mL Erlenmeyer flask in the morning. In late afternoon, about 20 mL of culture water should be added to the flask. Egg cases are deposited overnight and first instar larvae begin to hatch after about 3 days at 20°C. No substrate is added to the pan before hatching. Fish food flakes[11] should be added at a rate of 50 mg/day suspended in water. Fresh water should be added as needed to make up for evaporation. The larvae in the rearing pans are presumed to be second instars on the 12th day from the time the eggs were laid (10 day old larvae). Most larvae will remain as second instars through the 16th day (14 day old larvae). Larvae ≥16 days old should not be used to start a test. To maintain a supply of second instar larvae for active toxicity testing, a rearing pan should be started every 4 days. Each pan can be expected to produce at least enough second instar larvae for one sediment toxicity test.

A2.6 *Handling*—Midges should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly, so that the midges are not unnecessarily stressed. Larvae should be transferred with a 7-mm inner diameter glass pipet. Midges should be introduced into solutions beneath the air-water interface. Any midges that touch dry surfaces, are dropped, or injured during handling should be discarded.

A2.7 *Acclimation*—If the midges are cultured in water different from the overlying water or temperature, an acclimation process is necessary. The water acclimation process used by Ingersoll and Nelson (4) is to first place animals for 2 h in a 50 to 50 ratio mixture of culture water to overlying water, then for 2 h in a 25 to 75 ratio mixture of culture

water to overlying water, followed by a transfer into 100 % overlying water. At this stage the midges are considered acclimated to the overlying water and are ready for immediate use. Midges should be randomly selected from the acclimation water with a pipette and placed into counting beakers, for example 30-mL, that can be floated in the test chambers before the midges are introduced into the exposure system (4).

A2.8 *Toxicity Test Specifications:*

A2.8.1 *Experimental Design*—Decisions concerning the various aspects of experimental design, such as the number of treatments, number of test chambers and midges per treatment, and water quality characteristics, should be based on the purpose of the test and the type of procedure that is to be used to calculate results. Tests with *C. tentans* have been conducted at temperatures between 20 and 23°C (22, 25, 44). Cooler test temperatures may reduce the growth of fungus on the sediment surface. Duration of the test can range from a ≤10 day test to >10 days and continuing up to 25 days (22, 25, 44, 45). Larval survival, growth, or adult emergence can be monitored as biological endpoints.

A2.8.2 *Static and Flow-Through Tests*—Wentsel et al. (44) recommend using 20 *C. tentans* in each 2-L exposure beaker containing 2 cm of sediment and 1.5 L of overlying water in static testing. Adams et al. (25) use 3-L aquaria constructed of glass and silicone rubber for either static or flow-through testing. These test chambers measure 20.5 by 12.5 by 14.5 cm with a 12.5 by 44.5 cm piece of fine mesh stainless steel screen positioned on the upper end of one side. This overflow screen prevents the escape of larvae and maintains an overlying water volume of 2 L with 100 g of test sediment and 25 *C. tentans* larvae per chamber. Nebeker et al. (22) recommend 20-L aquaria with 100 *C. tentans* larvae and 2 to 3 cm of test sediment on the bottom with 15 cm of overlying water in static tests. If less sediment is available for testing, 4-L glass jars can be used, but proportionally fewer animals and less food should be used. Adams et al. (25) and Giesy et al. (45) also describe a method to expose midges individually to contaminated sediment in static tests. Up to 15 *C. tentans* are placed in separate 50-mL plastic centrifuge tubes. Each tube contains one midge, 7.5 g of sediment and 47 mL of water. For 24 h after hatching, first instar midge larvae are often planktonic (55). If flow-through tests are started with first instar *C. tentans* larvae, water flow into the test chambers should not be started for at least 24 h after larvae are added. This will allow time for larvae to settle onto the sediment surface.

A2.8.3 *Initiation of a Test*—Sediments should be homogenized and placed in the test chambers on the day before addition of test organisms (Day −1). Test chambers should be covered and overlying water aerated overnight. The test begins when midges are introduced to the test chambers (Day 0). Larvae must be collected from at least three separate egg cases to start a sediment toxicity test. It is recommended that flow-through and static tests might need to be started on different days to assure that sufficient time is available to complete all tasks. Test chambers should be inspected <2 hours after midges are introduced to ensure that animals are not trapped in the surface tension of the water (4). These floaters do not survive well and should be replaced with healthy animals.

A2.8.4 *Feeding*—Adams et al. (25) recommend feeding animals in flow-through or static tests 50 mg fish food flakes[11] (dry weight, administered in a 0.5 mL suspension) daily to each 3-L test chamber containing 25 larvae. Nebeker et al. (22) suggest feeding animals in static tests a food mixture of 600 mg ground cereal leaves[10] (1.5 mL dry volume) and 100 mg (0.3 mL dry volume) of finely crushed fish food flakes[11] in water and feeding this amount of food to the 100 *C. tentans* larvae in each 20-L test chamber at the start of the test (Day 0) and on Day 8. On Day 14 they should be fed 800 mg (2.0 mL) ground cereal leaves[10] and 100 mg (0.3 mL) fish food flakes,[11] and on Day 18 they should be fed 1000 mg (2.5 mL) ground cereal leaves[10] and 100 mg (0.3 mL) fish food flakes.[11] Giesy et al. (45) recommend feeding a 0.1 mL suspension of 0.06 g/mL goldfish food[12] daily to each individual midge in each centrifuge tube. If food collects on the sediment, a fungal or bacterial growth might start on the surface of the sediment, in which case feeding may be suspended for one or more days. A drop in dissolved oxygen to 40 % saturation might indicate that all of the food added in the water is not being consumed such that feeding should be suspended for the amount of time necessary to increase the dissolved oxygen concentration.

A2.8.5 *Biological Data*—Several endpoints can be monitored in midge sediment toxicity tests. During the test, emergence of larvae from the test sediment can be monitored. Additionally, data on larval survival, growth, and adult emergence can be obtained.

A2.8.5.1 Larval survival and growth can be assessed by ending the tests on Day 10 to Day 14 when larvae hav reached the third or fourth instar (22, 25, 45). At this time, larvae can be removed from sediment using a #35 (500 μm) U.S. Standard size sieve (4). The midges can be rinsed from the sieve into collecting pans and pipeted from the rinse water. Growth determinations using dry weight (dried at 60°C to a constant weight) is preferable to length. Growth can also be estimated by measuring head capsule width, and also be used to determine instar development.

A2.8.5.2 Nebeker et al. (22) suggest conducting adult *C. tentans* emergence sediment toxicity tests for 25 days when tests are started with second instar larvae. The adult emergence exposure chambers are covered by screen to retain emerging adults. The adult *C. tentans* should begin emerging after 20 days; the test should be continued for at least 5 days to count all the adults emerging and monitor delayed development. A small vacuum pump with a 10-mm diameter plastic line running through an Erlenmeyer flask trap is used to collect adults and make daily count of adults emerging. The screen cover is slowly lifted off the container and the adults are vacuumed from the screen and inside walls of the container. Percent adult emergence is generally less than 60 % in these tests. Endpoints calculated in these adult emergence tests can include (*1*) percent emergence, (*2*) mean emergence time, or (*3*) day to first emergence. Egg hatching studies may also be conducted by covering the test chambers and confining the adults. Adults will emerge and

[12] Goldfish food, such as Tetra-Min, available from many pet food distributors, has been found suitable for this purpose.

lay eggs in these chambers. These egg masses can then be used to estimate effects of exposure on either the number of eggs produced or hatched.

A2.8.5.3 A *C. tentans* sediment toxicity test, independent of test duration, is unacceptable if the average survival in any negative control chamber is less than 70 % (see Section 15, Acceptability of Test).

NOTE A2.1—A low percent emergence of adults might not be the result of low survival; larvae or pupae might not have completed development.

A3. *CHIRONOMUS RIPARIUS*

A3.1 *Significance—Chironomus riparius* Meigen (Diptera: Chironomidae) has been used in sediment toxicity tests because it is a fairly large midge, has a short generation time, is easily cultured in the laboratory, and the larvae have direct contact with the sediment by burrowing into the sediment to build a case. *C. riparius* has been successfully used in sediment toxicity testing and is sensitive to many contaminants associated with sediments (**4, 56, 57, 58**). The members of the genus are important in the diet of young and adult fish and surface feeding ducks (**47**).

A3.2 *Life History and Life-Cycle*—The classification of holometabolous insects, such as *C. riparius*, presents special difficulties because each life-stage often has different ecological requirements. Further detailed studies at the species level are needed to better understand the various physical, chemical, and biological factors that interact to produce a suitable habitat for larval development (**47**). The distribution of the family is world wide. Most of the species in the family are thermophilous and adapted to living in standing water, although species do occur in cold habitats and in running water (**47**). *C. riparius* is a nonbiting midge. The tubiculous larvae frequently inhabit eutrophic lakes, ponds, and streams and reportedly live in mud-bottom littoral habitats to depths up to 1.0 m (**59**). Qualitative observations indicate larvae inhabit gravel, limestone, marl, plants, and silt (**53**). Ingersoll and Nelson (**4**) report *C. riparius* to have a wide tolerance of sediment grain size. Sediment ranging from >90 % silt- and clay-size particles to 100 % sand-size particles did not reduce larval survival or growth in the laboratory. Larvae of *C. riparius* larvae reportedly occur in the field at a temperature range between 0°C and 33°C, pH range between 5 and 9, and at dissolved oxygen concentrations as low as 1 mg/L (**53**). *C. riparius* tubes are of the type characteristic of bottom-feeding chironomid larvae (**59**). Larvae frequently extend their anterior ends outside of their tubes feeding on the sediment surface (**59**). Credland (**60**) reported *C. riparius* will eat a variety of materials of the appropriate size.

A3.2.1 The biology of *C. riparius* facilitates laboratory culture since larvae are tolerant of a wide spectrum of conditions and adults mate even when confined (**55, 58, 60**). The life-cycle of *C. riparius* can be divided into three distinct stages: (*1*) a larval stage, consisting of the 4 instars; (*2*) a pupal stage; and (*3*) an adult stage. Midge egg masses hatch in 2 or 3 days after deposition in water at between 19 and 22°C. Larval growth occurs in four instars of about 4 to 7 days each. Under optimal conditions larvae will pupate and emerge as adults after 15 to 21 days at 20°C. Adults emerge from pupal cases over a period lasting several days. Males are easily distinguished from females because males have large, plumose antennae and a much thinner abdomen with visible genitalia. Mating behavior has been described by Credland (**60**).

A3.3 *Obtaining Test Organisms*—The following is a description of culturing procedures adapted from Ingersoll and Nelson (**4**) and others (**51, 54, 58, 60**); these procedures should not be considered definitive, since procedures that work well in one laboratory sometimes work poorly in another laboratory: *C. riparius* can be reared in aquaria in either static or flowing water with a 16 to 8 hour ratio of light to darkness at 20 to 22°C, at about 50 fc (538 lx). For static cultures the water should be gently aerated and about 25 to 30 % of the water volume should be replaced weekly. Cultures should be maintained in an isolated area or room free of contamination and excessive disturbances. Ingersoll and Nelson (**4**) recommend rearing *C. riparius* in 30 by 30 by 30-cm polyethylene containers covered with nylon screen. Each culture chamber contains 3 L of culture water. At least three egg cases should be used to start a new culture. To start a culture, 200 to 300 mg of ground cereal leaves[9] is added to the culture chamber; additionally, green algae (*Selenastrum capricornutum* (**61**) is added ad libitum to maintain a growth of algae in the water column and on the bottom of the culture chamber. Cultures should be fed about 3 mL of a suspension of commercial dog treats (**62**) daily. This suspension should be prepared by heating and melting 15 g of dog treats in 150 mL of culture water. After refrigeration, the oily layer which forms on the surface should be removed. The rest should be used to feed the cultures. This suspension contains about 100 mg dry solid/mL. Overfeeding will lead to the growth of fungus in the aquaria and will necessitate more frequent water changes. To obtain egg cases and larvae, adults should be left in the culture chamber to mate and deposit eggs. Egg cases adhere to the side of the culture chamber and can be removed with a sharp blade. These egg masses can then be placed in individual 100 mL beakers containing 50 mL of culture water; hatching should start in about 3 days at 20°C. While removal of adults by aspiration into a 250 mL flask before mating works well with *C. tentans* (see Annex A2), this procedure has not been successful with *C. riparius*.

A3.4 *Brood Stock*—Brood stock can be obtained from the wild, another laboratory, or a commercial source. When midges are brought into the laboratory, they should be acclimated to the culture water by gradually changing the water in the culture chamber from the water in which they were transported to 100 % culture water. Midges should be acclimated to the test temperature by changing the water temperature at a rate not to exceed 2°C within 24 h, until the desired temperature is reached. Brood stock should be cultured so they are not unnecessarily stressed. To maintain midges in good health and avoid unnecessary stress, crowding and rapid changes in temperature and water quality characteristics should be avoided.

A3.5 *Age*—Tests with *C. riparius* can be started with

either larvae less than 24-h old (4) or with three day old larvae (56, 57). Freshly laid midge egg cases can be transferred from the culture into individual 100 mL beakers containing 50 mL of culture water. At 20°C larvae should begin to hatch within 3 days. Larvae must be collected from at least three separate egg cases to start a sediment toxicity test.

A3.6 *Handling*—Midges should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly as possible, so that the midges are not unnecessarily stressed. First instar midges should be transferred with a 2 mm inner diameter glass pipet (eye dropper). Older larvae should be transferred with a 7 mm inner diameter glass pipet. Midges should be introduced into solutions beneath the air-water interface. Any midges that touch dry surfaces, are dropped, or injured during handling should be discarded.

A3.7 *Acclimation*—If the midges are cultured in water different from the overlying water or temperature, an acclimation process is necessary. The water acclimation process used by Ingersoll and Nelson (4) is to first place animals for 2 h in a 50 to 50 ratio mixture of culture water to overlying water, then for 2 h in a 25 to 75 ratio mixture of culture water to overlying water, followed by a transfer into 100 % overlying water. At this stage the midges are considered acclimated to the overlying water and should be ready for immediate use. Midges should be randomly selected from the acclimation water with a pipette and placed into counting beakers (for example, 30-mL) that can be floated in the test chambers before the midges are introduced into the exposure system.

A3.8 *Toxicity Test Specifications:*

A3.8.1 *Experimental Design*—Decisions concerning the various aspects of experimental design, such as the number of treatments, number of test chambers and midges per treatment, and water quality characteristics, should be based on the purpose of the test and the type of procedure that is to be used to calculate results. Ingersoll and Nelson (4) recommend using 50 *C. riparius* in each 1-L exposure beaker containing 200 mL of sediment and 800 mL of overlying water in either static or flow-through testing. Lee (57) recommends using 13-L glass aquaria containing 130 *C. riparius* larvae, 2 L of sediment and 11 L of overlying water in static tests. Tests with *C. riparius* have been conducted at temperatures between 20 and 22°C (4, 56, 57). Cooler test temperatures might reduce the growth of fungus on the sediment surface. Duration of the test can range from a ≤10 day test to >10 days and continuing up to 30 days (4, 56, 57). Larval survival, growth, or adult emergence can be monitored as biological endpoints.

A3.8.2 *Static and Flow-Through Tests*—Ingersoll and Nelson (4) recommend that borosilicate glass 1-L beakers can be used to expose the *C. riparius* to the test material, in either static or flow-through tests. For the static tests, cover watch glasses may be used, such that an aeration line fits through the beaker pour spout and the cover. For flow-through testing, Ingersoll and Nelson (4) suggest using a 4 by 13 cm notch cut in the lip of the 1-L beaker. The notch should be covered with 0.33 mm U.S. Standard sieve size #50 screen, either made of stainless steel or polyethylene, using a silicone adhesive to attach the screen to the beaker. For 24 h after hatching, first instar midge larvae are often planktonic (55). Pittinger et al. (56) suggest not running water through the diluter for at least 24 h after larvae are added to the test chambers. This will allow time for larvae to settle onto the sediment surface.

A3.8.3 *Initiation of a Test*—Sediments are homogenized and placed in the test chambers the day before addition of test organisms (Day 1). Test chambers are then covered and overlying water is aerated overnight. The test begins when midges are introduced to the test chambers (Day 0). Ingersoll and Nelson (4) start sediment toxicity tests with 50 first instar *C. riparius* larvae per 1-L test chamber. Pittinger et al. (56) and Lee (57) suggest starting tests with 3 day old larvae (130 larvae per 13-L chamber (57)). It is recommended that flow-through and static tests might need to be started on different days to assure that sufficient time is available to complete all tasks. Test chambers should be inspected <2 hours after midges are introduced to ensure that animals are not trapped in the surface tension of the water. These floaters do not survive well and should be replaced with healthy animals.

A3.8.4 *Feeding*—Lee (57) recommends feeding animals in a static system 200 mg fish food flakes[11] every other day to each 13-L test chamber containing 130 larvae. Pittinger et al. (56) suggest feeding animals in a static renewal system trout food[13] and dehydrated cereal (5 to 1 w/w) and commercial dog treats daily to each test chamber containing 20 larvae. In flow-through and static toxicity tests, Ingersoll and Nelson (4) feed 50 *C. riparius* larvae in each 1-L test chamber a combination of ground cereal leaves[10] (suspended in water) a green algae (*S. capricornutum*) and commercial dog treats. In flow-through sediment toxicity tests, 75 mg of ground cereal leaves,[10] 30 mg of dog treats and 6×10^7 *S. capricornutum* algal cells should be added to each 1-L test chamber the day test starts (Day 0). From Day 1 to Day 6 of the test, 15 mg of ground cereal leaves[10] should be added to each test chamber; from Day 1 to Day 12, 30 mg of dog treats should be added to each test chamber and from Day 13 to the end of the test, 15 mg of dog treats should be added to each test chamber; 6×10^7 *S. capricornutum* algal cells should be added to each test chamber daily. In static sediment toxicity tests, 10 mg of ground cereal leaves,[10] 10 mg of dog treats and 3×10^7 *S. capricornutum* algal cells should be added to each 1-L test chamber on Day 0. From Day 1 to Day 6 of the test, 10 mg of ground cereal leaves[10] and 3×10^7 algal cells should be added to each 1-L test chamber; for the first two weeks of the test, 10 mg of dog treats should be added to each test chamber each Monday, Wednesday, and Friday and for the rest of the test 5 mg of dog treats should be added to each test chamber each Monday, Wednesday and Friday; from Day 7 until the end of the test 3×10^7 algal cells should be added to each test chamber each Monday, Wednesday and Friday. Lower feeding levels for flow-through tests might be used for *C. riparius* daily: 6×10^7 *S. capricornutum* algal cells, 10 mg dog treats, and 10 mg ground cereal leaves[10] on Days 0 through 6. If food collects on the sediment, a fungal or

[13] Trout food, such as Ralston Purina Trout Chow, available from Purina Mills, Inc., 1401 Hanley St., St. Louis, MO 63144, has been found suitable for this purpose.

bacterial growth might start on the surface of the sediment, in which case feeding should be suspended for one or more days. A drop in dissolved oxygen to 40 % saturation might indicate that all of the food added in the water is not being consumed such that feeding should be suspended for the amount of time necessary to increase the dissolved oxygen concentration (4).

A3.8.5 *Biological Data*—Several endpoints can be monitored in midge sediment toxicity tests. During the test, emergence of larvae from the test sediment can be monitored. Additionally, data on larval survival, growth, and adult emergence can be obtained.

A3.8.5.1 Larval survival and growth can be assessed by ending the tests on Day 10 to Day 14 when larvae have reached the third or fourth instar (4, 25, 45). At this time, larvae should be removed from sediment using a #35 (500 μm) U.S. Standard size sieve (4). The midges should be rinsed from the sieve into collecting pans and pipeted from the rinse water. Growth determination using dry weight (dried at 60°C to a constant weight) is preferable to length. Growth can also be estimated by measuring head capsule width, and also used to determine instar development.

A3.8.5.2 Ingersoll and Nelson (4), Pittinger et al. (56) and Lee (57) recommend conducting *C. riparius* sediment toxicity tests until the larvae pupate and emerge as adults. Cast pupal skins left by emerging adult *C. riparius* should be removed and recorded daily. These pupal skins remain on the water surface for over 24 h after the emergence of the adult. The test should be ended after the animals have been exposed for up to 30 days, when about 70 to 95 % of the control larvae should have completed metamorphosis into the adult form. Endpoints calculated in these adult emergence tests can include: (*1*) percent emergence, (*2*) mean emergence time, or (*3*) day to first emergence. Egg hatching studies may also be conducted by covering the test chambers and confining the adults. Adults will emerge and lay eggs in these chambers. These egg masses can then be used to estimate effects of exposure on either the number of eggs produced or hatched.

A3.8.5.3 A *C. riparius* sediment toxicity test, independent of duration, is unacceptable if the average survival in any negative control chamber is less than 70 % (see Section 15, Acceptability of Test).

NOTE A3.1—A low percent adult emergence might not be the result of low survival; larvae or pupae might not have completed development.

A4. *DAPHNIA* SP. AND *CERIODAPHNIA* SP.

A4.1 *Significance—Daphnia magna* and *Ceriodaphnia dubia* are easily cultured in the laboratory, have a short generation time, and survival and reproduction data can be obtained in toxicity tests, and a large data base has developed regarding their sensitivity to toxicants. Nebeker et al. (22), Prater and Anderson (63), Giesy et al. (64), Malueg et al. (65), and Burton et al. (66) and others (45, 67–75) have successfully used cladocerans in sediment testing and have shown them to be sensitive indicators of the presence of associated contaminants.

A4.1.1 In whole sediment toxicity tests, cladocera behave as nonselective epifaunal zooplankton. The organisms are frequently observed on the sediment surface and are likely exposed to both water soluble and particulate bound contaminants (through ingestion) in overlying water and surface sediments. These routes of exposure do not, however, mimic those of infaunal benthic invertebrates, which are exposed directly to sediment and interstitial water. One of the most important reasons for using cladocerans as toxicity test organisms is their importance in the food web of some systems (42, 76, 77). These assays have been useful at discriminating sediment contamination and allowing comparisons of relative sediment toxicity. Because they are not benthic organisms, their responses may not be indicative of in situ benthic community effects.

A4.2 *Life History and Life Cycle*—Pennak (78) recognizes four distinct periods in the life history of a cladoceran: egg, juvenile, adolescent, and adult. Unstressed populations consist almost exclusively of females producing diploid parthenogenetic eggs which develop into female young. Adult *Ceriodaphnia* can produce from four to 15 parthenogenetic eggs in each brood whereas *Daphnia* can produce five to 25 or more eggs (79). When a clutch of eggs is released into the brood chamber, segmentation begins promptly; the first juvenile instar is released into the surrounding water in approximately two days (78). There are only a few juvenile instars and the greatest growth occurs during these stages. The adolescent period is a single instar between the last juvenile instar and the first adult instar during which the first clutch of eggs reaches full development in the ovary. At the close of the adolescent instar, the animal molts and the first clutch of eggs is released into the brood chamber, while a second clutch is developing in the ovary. At the close of each adult instar, four successive events occur: the young are released from the brood chamber to the outside environment, molting occurs, with an increase in size, and there is release of a new clutch of eggs into the brood chamber.

A4.2.1 When populations are stressed (for example, low oxygen, crowding, starvation), males are produced from diploid parthenogenetic eggs. With the appearance of males, females produce haploid eggs which require fertilization. Following fertilization, the eggs are enclosed by the ephippium and shed at the next molt. The embryos lie dormant until suitable conditions arise upon which they become females producing diploid parthenogenetic eggs (80).

A4.3 *Obtaining Test Organisms*—The following culture procedures are adapted from Knight and Waller (81), while other appropriate methods include the U.S. Environmental Protection Agency (82, 83) and Guides E 729 and E 1295. Following Knight and Waller's (81) methodology, *D. magna* and *C. dubia* can be cultured in reconstituted hard water (160 to 180 mg/L $CaCO_3$) and fed a daily diet of a vitamin enriched *Selenastrum capricornutum* suspension. Cultures are maintained at 25 ± 1°C with a light:dark cycle of 16:8 h provided by overhead fluorescent lighting covered with opaque plastic to reduce light intensity to less than 20 lux. This reduces the photosynthetic activity of the algal food, which could alter water quality. *D. magna* mass cultures are

started by placing 10 neonates (less than 24-h old) into 1-L beakers containing 500 mL reconstituted hard water and a feeding suspension of *S. capricornutum* of approximately 240 000 algal cells/mL culture water. Cultures are fed 12 mL initially and on Day 1, 25 mL (500 000 cells/mL culture water) on Days 2 through 4, and 25 to 50 mL (100 000 cells/ml culture water) on Day 5 and thereafter. Using this culture method, *D. magna* typically will have first broods between Days 6 and 8 with successive broods hatching every 36 to 48 h thereafter. On days when hatches occur and young are not needed, adults are transferred to clean 1-L beakers containing 300 mL hard water, 200 mL old culture water, and 50 mL of food. When neonates are needed for testing, adults are isolated the night before by placing each adult into a separate 100-mL beaker containing 100 mL reconstituted hard water and 3 mL feeding suspension. See also Specification E 1193 for culture requirements. Neither first brood young nor young from females older than two weeks are used in toxicity testing or initiating new cultures. The *S. capricornutum* feeding suspension may also be supplemented with an approximate 6 % by volume addition of ground cereal leaves[10] preparation to the algal feeding suspension (Waller, personal communication). *C. dubia* mass cultures can be initiated by placing 20 neonates (less than 12 h old) into a 600 mL-beaker containing 360 mL reconstituted hard water and 12 mL of *S. capricornutum* feeding suspension. Cultures are fed 12 mL initially and on days one and two, and then 18 mL thereafter. When three distinct sizes are noted (generally day six), the largest organisms are isolated in 100 mL-beakers containing 60 mL of hard water and 2 mL feeding suspension. Third brood neonates, less than 12 h old are used in toxicity testing and initiating new mass cultures. Generally, the first brood is produced on day four, the second brood on day five and the third brood on day seven. See also Guide E 1295 for culture requirements.

A4.3.1 The U.S. Environmental Protection Agency (83) recommends culturing *D. magna* in reconstituted hard water at 20°C with ambient light intensity of 50 to 100 ft c (10 to 20 $\mu E/m^2/s$, or 538 to 1076 lux), and a light:dark cycle of 16:8 h. Culture vessels can be 3-L glass beakers containing 2.75 L reconstituted hard water and 30 *D. magna*. The *D. magna* can be fed on a daily diet of *S. capricornutum* (100 000 algal cells/mL culture water) or fed three times a week a feeding suspension consisting of trout chow, alfalfa, and yeast (TCY) (1.5 mL TCY/1000 mL culture water). This should supply approximately 300 young per week.

A4.3.2 The U.S. Environmental Protection Agency (82) procedures for *Ceriodaphnia* cultures are as follows. *Ceriodaphnia* are cultured in moderately hard water (80 to 90 mg/L $CaCO_3$) at 25 ± 1°C and receive a light:dark cycle of 16:8 h. Mass cultures are maintained as backup organism reservoirs and individual organisms are cultured as the source of neonates for toxicity tests. Mass cultures can be initiated in two 3-L beakers filled to three-fourths capacity with moderately hard water and 40 to 50 neonates/L of medium. The stocked organisms should be transferred to fresh culture media twice weekly for two weeks. At each renewal, the adults are counted and the offspring and old medium discarded. The adults are discarded after two weeks and new mass cultures initiated with neonates. Mass cultures are fed daily at the rate of 7 mL of a yeast, ground cereal leaves,[10] trout chow food preparation (YCT), and 7 mL of *S. capricornutum* concentrate (3.0 to 3.5 × 10^7 cells/mL). Individual *C. dubia* cultures are maintained in 30-mL plastic cups or beakers containing 15 mL of culture media. Cultures are fed daily at the rate of 0.1 mL YCT and 0.1 mL algal concentrate per 15 mL media and are transferred to fresh media at least three times a week. Adults are used as sources of neonates until 14 days of age. Cultures properly maintained should produce at least 15 young per adult in three broods (seven days or less). Goulden and Henry (79) list two other fresh water algal species which can be used for cladoceran food: *Ankistrodesmus falcatus* and *Chlamydomonas reinhardtii*. Winner (84) discusses the effects of four diets (*C. reinhardtii, Selenastrum capricornutum*, yeast, ground cereal leaves,[10] trout chow (YCT), and YCT plus *S. capricornutum*) and two reconstituted waters on the vitality of five to six lifespan generations of *C. dubia*. His results indicate that healthy populations can be maintained in reconstituted hard water containing only four salts as long as the food is nutritionally adequate and the water is reconstituted from an ultrapure base water.

A4.4 *Brood Stock—D. magna* and *C. dubia*.[14] Animals received from an outside source should be acclimated gradually to new culture media over a period of one to two days and taxonomy verified.

A4.5 *Background*—Experimental design, such as number of test chambers, number of treatments, animals per treatment, and water quality characteristics, should be based on the purpose of the test and the procedure used to calculate results. See Guides E 729 and E 1295, Test Method E 1297, and the preceding guide text for guidance. Nebeker et al. (85) recommended conducting 48 h sediment static tests in duplicate using 1-L beakers containing 200 mL of sediment and 800 mL of water (1:4). The sediment is allowed to settle overnight, followed by gentle aeration of overlying water for 30 min before introducing 15 *D. magna* per replicate. Malueg et al. (65) conducted recirculating sediment toxicity tests in a modified recycling device described by Prater and Anderson (63). The test chamber (23 cm long by 6.4 cm wide by 16 cm high) was positioned on a plexiglass plate over two 4-L jars. Twenty *D. magna* were placed in a vessel in the water column and five *Hexagenia* added to chamber sediment. Three to six replicates were used for each control and test sediment. Seven day (three brood) toxicity tests for aqueous media using cladocerans have been conducted (22, 86, 87) and variations of these methods used to assess sediment toxicity (22, 88).

A4.6 *Handling*—The cladocerans are delicate and should be handled as carefully and little as possible. They are transferred with a 5-mm bore pipet and released slowly beneath the water surface.

A4.7 *Experimental Design for Acute Toxicity Tests*—Sediments may be mixed, if appropriate for the study, by mixing with either a large plastic paddle, magnetic stirring bar or shaker table, before allocating to test chambers. See Test Method E 1297 and Guide E 1391 for guidance. Whole sediment assays use a 1:4 of sediment to water. Acute

[14] Starter cultures obtained from the Aquatic Biology Branch, Environmental Monitoring Systems Laboratory, USEPA, 3411 Church Street, Newtown, OH 45244 have been found suitable.

toxicity tests are conducted in triplicate using 250 or 100-mL beakers to which 30 mL of sediment (by weight) and 120 mL of reconstituted or site water are added (for 250-mL beakers). The weight of 30 mL of sediment is determined by initially calculating the average wet weight (g) of five, 5-mL aliquots of sediment obtained using a 10-cc syringe. The average weight of 5-mL is divided by five to obtain the weight of 1 mL of sediment. The weight of 1 mL is multiplied by 30 mL to obtain the number of grams to be weighed into each test beaker. When a syringe cannot be used to dispense sediments, sediment weight is used rather than volume, weighing 30 g (wet weight) into each test beaker. In addition, sediment dry weights are determined by weighing triplicate three 5-mL aliquots of wet sediment, drying at 100 to 105°C for 24 h and then reweighing the sediment. Percent dry weight is calculated by dividing the dry sediment weight (g) by the wet weight and multiplying by 100. Grams of dry weight per mL of wet sediment is determined by dividing the dry weight by the mL of wet sediment. Overlying water is gently added to each beaker, minimizing sediment resuspension. After a 1 to 2 h settling period, ten test organisms are randomly added to each beaker. Test chambers should be inspected less than 2 h after the addition of test organisms to check for any floaters. Floaters may not survive and are subjected to a different exposure, thus can be removed and replaced within the first 2 h. Floating may be caused by the sediment sample and may be considered a treatment effect in some cases. However, responses tend to be variable and are seldom dose proportional. Surface films which entrap *D. magna* can be reduced by wiping the surface with cellulose filter paper prior to organism addition.

A4.8 *Experimental Design for Short-term Chronic Toxicity Tests*—Test initiation, test conditions and monitoring are as described in A4.7, A4.9, and A4.9.1 with the following exceptions, and basically follow standard methods (22 and Guide E 1295). Tests are conducted in 30-mL beakers using 5 mL (or 5 g) sediment and 20 mL overlying water in replicates of ten. One organism (*D. magna* less than 24 h old or *C. dubia* less than 6 h old) is randomly added to each beaker, after the settling period. At each 24-h test interval, the adult is removed and placed in a beaker containing the control water, young are counted and discarded, and physicochemical measures made. Approximately 15 mL of overlying water is suctioned off and gently renewed. The culturing food (such as YCT or algal-ground cereal leaves mixture) is then added (0.1 mL) to each beaker. After feeding, the adult organism is returned to the test beaker. The test is terminated at seven days and/or when at least 60 % of the controls have produced their third brood.

A4.9 *Monitoring Data*—Test conditions and monitoring should follow standard methods (82, 83). Test beakers are maintained at 25 ± 1°C and receive a 16:8 h light:dark cycle (20 lux). Dissolved oxygen and temperature are monitored at 0, 24, and 48 h. Dissolved oxygen should not be allowed to drop below 40 % saturation. If it does, gentle bubbling should be used until adequate saturation is attained. The pH, hardness, and alkalinity are monitored at 0 and 48 h. Survival numbers were recorded at 24 and 48 h. Death of a test animal is judged as a result of observing no movement upon gentle prodding. Tests are considered valid when control mortality is ≤ 10 % (83). Control treatments consist of reconstituted water or reference site water, and a control or reference sediment, or both, with the overlying test water (reconstituted or reference site). See the preceding guide text for additional guidance on sediment characterization, controls, references, and data analyses.

A4.9.1 The seven-day, three-brood survival and reproduction test requires the daily counting of adult survivors and young production. Dissolved oxygen, temperature, and pH should be measured daily, before renewing overlying waters on two to three beakers in each treatment and control. Alkalinity and hardness are measured at test initiation and termination. For the test results to be acceptable controls must have 80 % survival with *C. dubia* controls averaging 15 young and *D. magna* averaging 20 young per surviving female (82, 86, 89).

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