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Standard Guide for Conducting Laboratory Soil Toxicity or Bioaccumulation Tests With the Lumbricid Earthworm *Eisenia Fetida*¹

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1. Scope

1.1 This guide covers procedures for obtaining laboratory data to evaluate the adverse effects of contaminants associated with soil to earthworms (Family Lumbricidae) from soil toxicity or bioaccumulation tests. The methods are designed to assess lethal or sublethal toxic effects on earthworms or bioaccumulation of contaminants in short-term tests (7 to 28 days) in terrestrial systems. Soils to be tested may be (1) reference soils or potentially toxic site soils; (2) artificial, reference, or site soils spiked with compounds; (3) site soils diluted with reference soils; or (4) site or reference soils diluted with artificial soil. Test procedures are described for the species *Eisenia fetida* (see Annex A1). Methods described in this guide may also be useful for conducting soil toxicity tests with other terrestrial species, although modifications may be necessary.

1.2 Modification of these procedures might be justified by special needs. The results of tests conducted using atypical procedures may not 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 soil toxicity and bioaccumulation tests with terrestrial worms.

1.3 The results from field-collected soils used in toxicity tests to determine a spatial or temporal distribution of soil toxicity may be reported in terms of the biological effects on survival or sublethal endpoints (see Section 14). These procedures can be used with appropriate modifications to conduct soil toxicity tests when factors such as temperature, pH, and soil characteristics (for example, particle size, organic matter content, and clay content) are of interest or when there is a need to test such materials as sewage sludge and oils. These methods might also be useful for conducting bioaccumulation tests.

1.4 The results of toxicity tests with (1) materials (for example, chemicals or waste mixtures) added experimentally

to artificial soil, reference soils, or site soils, (2) site soils diluted with reference soils, and (3) site or reference soils diluted with artificial soil, so as to create a series of concentrations, may be reported in terms of an LC50 (median lethal concentration) and sometimes an EC50 (median effect concentration). Test results may be reported in terms of NOEC (no observed effect concentration) and LOEC (lowest observed effect concentration). Bioaccumulation test results are reported as the magnitude of contaminant concentration above either the Day 0 tissue baseline analysis or the Day 28 tissues from the negative control or reference soil (that is, 2 \times , 5 \times , 10 \times) (see A3.9).

1.5 This guide is arranged as follows:

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

1.7 This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use. While some safety considerations are included in this guide, it is beyond the scope of this standard to encompass all safety requirements necessary to conduct soil toxicity tests. Specific precautionary statements are given in Section 8.

2. Referenced Documents

2.1 ASTM Standards:

¹ This guide is under the jurisdiction of ASTM Committee E47 on Biological Effects and Environmental Fate and is the direct responsibility of Subcommittee E47.02 on Terrestrial Assessment and Toxicology.

An ASTM guide is defined as a series of options or instructions that do not recommend a specific course of action.

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- D 653 Terminology Relating to Soil, Rock, and Contained Fluid²
- D 4447 Guide for the Disposal of Laboratory Chemicals and Samples³
- E 380 Practice for Use of the International System of Units (SI) (the Modernized Metric System)⁴
- E 943 Terminology Relating to Biological Effects and Environmental Fate⁵
- E 1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses⁵
- E 1383 Guide for Conducting Sediment Toxicity Tests with Freshwater Invertebrates⁵
- E 1688 Guide for Determination of the Bioaccumulation of Sediment-Associated Contaminants by Benthic Invertebrates⁵
- E 1706 Test Methods for Measuring the Toxicity of Sediment-Associated Contaminants with Fresh Water Invertebrates⁵

3. Terminology

3.1 Definitions:

3.1.1 The words “must,” “should,” “may,” “can,” and “might” have very specific meanings in this guide. “Must” is used to express an absolute requirement, that is, to state that the test must be designed to satisfy the specified condition, unless the purpose of the test requires a different design. “Must” is used only in connection with the factors that relate directly to the acceptability of the test (see Section 13). “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, the violation of several will often render the results questionable. Terms such as “is desirable,” “is often desirable,” and “might be desirable” are used in connection with less important factors. “May” is used to mean “is (are) allowed to,” “can” is used to mean “is (are) able to,” and “might” is used to mean “could possibly.” Thus, the classic distinction between “may” and “can” is preserved, and “might” is never used as a synonym for either “may” or “can.”

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

3.2 Definitions of Terms Specific to This Standard:

3.2.1 *artificial soil*—a synthetic soil, prepared with a specific formulation, designed to simulate a natural soil (see Annex A2). Artificial soil may be used as a diluent medium to prepare concentrations of site or reference soil and may be used as a negative control medium.

3.2.2 *batch*—the total amount of test soil prepared for each concentration in a test. A batch is any hydrated test soil ready for separation into replicates.

3.2.3 *bioaccumulation*—the net accumulation of a substance by an organism as a result of uptake from all environmental sources. (See Guide E 1688.)

3.2.4 *bioaccumulation factor (BAF)*—the ratio of tissue residue to sediment contaminant concentration at steady-state. (See Guide E 1688.)

3.2.5 *bioaccumulation potential*—a qualitative assessment of whether a contaminant in a particular sediment is bioavailable. (See Guide E 1688.)

3.2.6 *bioconcentration*—the net assimilation of a substance by an organism as a result of uptake directly from aqueous solution. (See Guide E 1688.)

3.2.7 *bioconcentration factor (BCF)*—the ratio of tissue residue to water contaminant concentration as steady-state. (See Guide E 1688.)

3.2.8 *biota-sediment accumulation factor (BSAF)*—the ratio of lipid-normalized tissue residue to organic carbon-normalized sediment contaminant concentration at steady state, with units of g-carbon/g-lipid. (See Guide E 1688.)

3.2.9 *clitellum*—the fleshy “ring” or “saddle” of glandular tissue found on certain mid-body segments of lumbricid earthworms. It is the most visible feature of an adult earthworm and secretes the cocoon into which eggs and sperm are deposited.

3.2.10 *concentration*—the ratio of the weight of test materials to the weight of soil (artificial, reference, or site), usually expressed on a dry weight basis as percent or milligram/kilogram.

3.2.11 *depuration*—loss of a substance from an organism as a result of any active (for example, metabolic breakdown) or passive process.

3.2.12 *diluent soil*—the artificial or reference soil used to dilute site soils.

3.2.13 *hydration water*—water used to hydrate test soils to create an environment with a moisture level suitable for the species being tested. The water used for hydration is often test water (see 3.2.26); however, depending on the nature of the test being implemented, site surface water or groundwater may also be utilized for hydration.

3.2.14 *lumbricid*—earthworm members of the Family Lumbricidae of the Class Oligochaeta of the Phylum Annelida.

3.2.15 *negative control soil*—artificial or reference soil to be used for evaluating the acceptability of a test.

3.2.16 *reference soil*—a field-collected soil that has physicochemical and biological properties as similar as possible to the site soil but does not contain the potentially toxic compounds of the site soil. It is used to describe matrix effects on the test in question. It may be used as a diluent medium to prepare concentrations of site soil and may be used as a negative control medium.

3.2.17 *sampling station*—a specific location, within a site or sampling unit, depending on the field study design, at which soil is collected for chemical, physical, and biological evaluation.

3.2.18 *sampling unit*—an area of land within a site distinguished by habitat and topography.

3.2.19 *site*—a delineated tract of land that is being considered as a study area, usually from the standpoint of its being potentially affected by xenobiotics.

² Annual Book of ASTM Standards, Vol 04.08.

³ Annual Book of ASTM Standards, Vol 11.04.

⁴ Annual Book of ASTM Standards, Vol 14.02.

⁵ Annual Book of ASTM Standards, Vol 11.05.

3.2.20 *site soil*—a soil collected from the field to be evaluated for potential toxicity. A site soil may be a naturally occurring soil or one that has been influenced by xenobiotics.

3.2.21 *soil*—sediments or other unconsolidated accumulations of solid particles produced by the physical and chemical disintegration of rocks, and that may or may not contain organic material. (See Terminology D 653.)

3.2.22 *spiking*—the experimental addition of a test material to an artificial, site, or reference soil, such that the toxicity of the material added can be determined. After the test material is added, which may involve a solvent carrier, the soil is mixed thoroughly to distribute the test material evenly throughout the soil.

3.2.23 *test chamber*—an enclosed space or compartment in which environmental parameters such as temperature and lighting are controlled (for example, incubator or modified room). Test containers are placed in the test chamber for biological evaluation.

3.2.24 *test container*—the experimental unit; the smallest physical entity to which treatments can be assigned independently.

3.2.25 *test soil*—a soil prepared to receive a test organism. Site or reference soil mixed with artificial soil or reference soil mixed with site soil in known concentrations for evaluation are test soils. Artificial, site, or reference soils spiked with test materials such as chemicals, oils, or manufacturing products are test soils. Once a site, reference, or artificial soil is hydrated, even though it is not mixed with artificial or reference soil or spiked with a material, it may be called a test soil.

3.2.26 *test water*—water used to prepare stock solutions, rinse test organisms, rinse glassware, and apparatus or for any other purpose associated with the test procedures or culture of the test organism. Test water must be deionized or distilled water or better, such as reagent-grade water produced by a system of reverse osmosis, carbon, and ion-exchange cartridges.

4. Summary of Guide

4.1 The toxicity of test soils or the bioavailability of contaminants are assessed during the continuous exposure of terrestrial organisms. Soils tested may be the following: (1) soils collected from potentially contaminated sites, (2) soils collected from reference sites, (3) artificial soil (see Annex A2) spiked with compounds, (4) site soil spiked with compounds, (5) reference soil spiked with compounds, (6) site soil diluted with artificial soil, (7) site soil diluted with reference soil, or (8) reference soil diluted with artificial soil. A negative control of artificial or reference soil is used for the following: (1) to yield a measure of the acceptability of the test; (2) to provide evidence of the health and relative quality of the test organisms; (3) to determine the suitability of test conditions, food, and handling procedures; and (4) to provide a basis for interpreting data obtained from the test soils. Specified data are obtained to determine the toxic effects on survival or sublethal endpoints for 7 to 28-day exposures or containment bioaccumulation for 28-day exposures to terrestrial lumbricids.

5. Significance and Use

5.1 Soil toxicity tests provide information concerning the toxicity and bioavailability of contaminants associated with soils to terrestrial organisms. As important members of the soil fauna, lumbricid earthworms have a number of characteristics that make them appropriate organisms for use in the assessment of potentially hazardous soils. They may ingest large quantities of soil, have a close relationship with other soil biomasses (for example, invertebrates, roots, humus, litter, and microorganisms), constitute up to 92 % of the invertebrate biomass of soil, and are important in recycling nutrients (1-3).⁶ Earthworms accumulate and are affected by a variety of organic and inorganic compounds (3-8). In addition, earthworms are important in terrestrial food webs, constituting a food source for a very wide variety of organisms, including birds, mammals, reptiles, amphibians, fish, insects, and centipedes (9, 10). A major change in the abundance of soil invertebrates such as lumbricids, either as a food source or as organisms functioning properly in trophic energy transfer and nutrient cycling, could have serious adverse ecological effects on the entire terrestrial system.

5.2 A number of species of lumbricids have been used in field and laboratory investigations in the United States and Europe. Although the sensitivity of various lumbricid species to specific chemicals may vary, from their study of four species of earthworms (including *E. fetida*) exposed to ten organic compounds representing six classes of chemicals, Neuhauser, et al (5) suggest that the selection of earthworm test species does not affect the assessment of a chemical's toxicity markedly.

5.2.1 *E. fetida* is a species whose natural habitats are those of very high organic matter such as composts and manure piles. It was selected as the test species because it (1) is bred in the laboratory easily; (2) is the earthworm species used most commonly in laboratory experiments (11); (3) has been studied extensively, producing a data pool on the toxicity and bioaccumulation of a variety of compounds (3, 5, 6, 12-17); (4) has been approved for use in toxicity testing by the European Economic Community and the Organization for Economic Cooperation and Development; and (5) has been used by the Environmental Protection Agency (EPA) for the toxicity screening of hazardous waste sites (18).

5.3 Results from soil toxicity tests might be an important consideration when assessing the hazards of materials to terrestrial organisms.

5.4 Information might also be obtained on the bioaccumulation of contaminants associated with soil by analysis of animal tissues for the contaminants being monitored. These results are useful for studying the biological availability of contaminants.

5.5 The soil toxicity test might be used to determine the temporal or spatial distribution of soil toxicity. Test methods can be used to detect horizontal and vertical gradients in toxicity.

⁶ The boldface numbers in parentheses refer to the list of references at the end of this standard.

5.6 Results of soil toxicity tests could be used to compare the sensitivities of different species.

5.7 An understanding of the effect of these parameters on toxicity and bioaccumulation may be gained by varying soil characteristics such as pH, clay content, and organic material.

5.8 Results of soil toxicity tests may be useful in helping to predict the effects likely to occur with terrestrial organisms in field situations.

5.8.1 Field surveys can be designed to provide either a qualitative or quantitative evaluation of biological effects within a site or among sites.

5.8.2 Soil surveys evaluating biological effects are usually part of more comprehensive analyses of biological, chemical, geological, and hydrographic conditions. Statistical correlation can be improved and costs reduced if subsamples of soil for laboratory toxicity tests, geochemical analyses, and community structure are taken simultaneously from the same grab of the same site.

5.9 Soil toxicity and bioaccumulation tests can be an important tool for making decisions regarding the extent of remedial action necessary for contaminated terrestrial sites.

6. Interferences

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

6.1.1 The alteration of field samples in preparation for laboratory testing (for example, transport, screening, or mixing).

6.1.1.1 Maintaining the integrity of soils during their removal, transport, and testing in the laboratory is extremely difficult. The soil environment is composed of a myriad of microenvironments, redox gradients, and other interacting physicochemical and biological processes. Many of these characteristics influence soil toxicity and the availability of compounds to 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 Soils tested at temperatures other than those from the field in which they are collected might affect contaminant solubility, partitioning coefficients, and other physical and chemical characteristics.

6.1.2 Interaction among chemicals present in the soil.

6.1.3 The use of laboratory-spiked soils that might not be representative of contaminants associated with soils in the field.

6.1.4 The addition of food to test containers may affect the results of a toxicity test, but it may be necessary to feed the test organisms in long-duration tests (see 11.7, A1.9.1.2, and A1.9.5).

6.1.5 The addition of solvents to the test containers might obscure the adverse influence of contaminants associated with soil and affect soil quality characteristics.

6.1.6 The natural geochemical properties of test soil collected from the field might not be within the tolerance limits of the test species.

6.1.7 Field-collected soils may contain indigenous organisms including (1) the same or closely related species to that being tested and (2) microorganisms (for example, bacteria and molds) and algae species that might grow in or on the soil and test container surfaces.

6.2 Tests may not be applicable with materials that are highly volatile or rapidly transformed biologically or chemically. The dynamics of test material breakdown products should therefore be considered, especially in relation to assumptions of chemical equilibria.

7. Apparatus

7.1 *General Facilities*—The facility should include separate constant temperature areas (chambers) for culturing and testing to reduce the possibility of contamination by test materials and other substances, especially volatile compounds. Culture containers should not be in a room (chamber) in which toxicity tests are conducted, stock solutions or test solutions are prepared, or equipment is cleaned. The facilities should be well ventilated and free of fumes.

7.2 *Equipment and Apparatus*—Equipment and apparatus that contact stock solutions, test solutions, site soils, and test soils, into which test organisms will be placed, should not contain substances that can be leached or dissolved in amounts that affect the test organisms adversely. In addition, equipment and apparatus that contact soils or solutions should be chosen to minimize the sorption of test materials. Glass, Type 316 stainless steel, nylon, high-density polyethylene, polycarbonate, and fluorocarbon plastics should be used whenever possible to minimize leaching, dissolution, and sorption. Copper, brass, lead, galvanized metal, and natural rubber should not be used. Items made of neoprene rubber and other materials not previously mentioned should not be used unless it has been shown that their use will not affect the survival, growth, or reproduction of test organisms adversely.

7.3 *Test and Culture Chambers*—A test or culture chamber is an enclosed space or compartment in which temperature and lighting are controlled (for example, incubator or modified room). The ventilation of chambers, especially test chambers, is desired.

7.3.1 Test and culture chambers usually require continuous lighting. A timing device should be used to provide a light:dark cycle if a photoperiod other than continuous light is used.

7.3.2 Temperature-recording devices should be used to monitor the temperature of test and culture chambers. Both test and culture chambers should be at the same temperature.

7.4 *Culture Containers*—Containers used to culture test organisms should be made of materials that will not affect their survival, growth, or reproduction adversely. Consideration should be given to cleaning and organizational space. The size of culture containers may depend on the species being cultured.

7.5 *Test Containers*—Test containers should be made of materials that minimize the sorption and leaching of test compounds and do not affect the survival, growth, and reproduction of the test organism adversely. Glass is an ideal material.

7.5.1 All test containers used in a soil toxicity test must be identical. The test containers should be covered with a lid to prevent escape of the test organisms and help reduce drying of the test soil.

7.5.2 Species-specific information on test containers and test conditions is given in Annex A1 and Annex A3.

7.6 *Cleaning*—Test containers and equipment and apparatus should be cleaned before use. Items may be cleaned in the following manner: (1) scrub thoroughly with a scratch pad to remove visible soil and residue; (2) detergent wash; (3) water rinse; (4) organic solvent wash (for example, acetone); (5) acid wash (for example, 10 % concentrated hydrochloric acid); (6) tap water rinse; (7) rinse at least twice with distilled, deionized, or reagent grade water; and (8) dried at room temperature or in a low-temperature (up to 90°C) air-drying oven. Care must be taken to avoid the use of “plastics” that may breakdown in the presence of the solvent used or at prolonged exposures near 90°C. For acceptable items, the following steps may be used alternatively for cleaning: (1) scrub thoroughly with a scratch pad to remove visible soil and residue; (2) detergent wash; (3) water rinse; (4) acid wash (for example, 10 % concentrated hydrochloric acid); (5) tap water rinse; (6) rinse at least twice with distilled, deionized, or reagent grade water; and (7) bake in an oven at 350°C. Clean lids should be placed on test containers after the containers have cooled.

7.6.1 A laboratory dish-washing machine may be used to accomplish the detergent wash/water rinse and tap water rinse stages. If a dish-washing machine is used, a neutralizing rinse may be necessary after the acid wash to prevent acid damage to the machine’s metal parts.

7.6.2 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, but the solution might leave chromium residues on glass.

7.6.3 Upon completion of a test, all items to be reused should immediately be (1) emptied of soil, (2) rinsed with water, and (3) cleaned by the procedures previously outlined. Test organisms and soil should be disposed of using appropriate procedures (see Guide D 4447).

7.6.4 Test containers should be stored with their lids on to keep them clean.

7.7 *Acceptability*—Before a toxicity test is conducted in new test facilities, it is desirable to conduct a “non-toxicant” test, in which all test containers contain a negative control of artificial or reference soil. Survival, growth, or reproduction of the test species will demonstrate whether the facilities, hydration water, artificial soil, 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.

8. Safety Precautions

8.1 Many substances pose health risks to humans if adequate precautions are not taken. Information on the chemical and physical properties, toxicity to humans (19-22), and recommended handling procedures (23-27) of the test material should be studied and made available to all personnel involved before a test is begun. Contact with the test materials should be avoided.

8.1.1 Many materials can affect humans adversely if precautions are inadequate. Field-collected soils might contain toxic materials, and respiratory exposure and skin contact should be prevented or minimized. As much information as possible should be collected on the history of the site and the potential problems from human exposure. Exposure to workers might be minimized by wearing rubber boots, disposable safety gear, gloves, and a cartridge respirator. Information or directives on necessary precautions should be available from a site safety manager at some sites.

8.1.2 When screening, mixing, or distributing hazardous soils in the laboratory, proper handling procedures might include working (1) under a ventilated hood, wearing protective gloves, laboratory coats, aprons, and safety glasses; or (2) in a ventilated room, wearing rubber boots, disposable safety gear, gloves, and a full-face bottled air respirator. When initiating toxicity tests in the laboratory, procedures might include wearing appropriate protective gloves, laboratory coats, aprons, and safety glasses and working in a ventilated hood.

8.2 Careful consideration should be given to those chemicals that might biodegrade, transform to more toxic components, volatilize, oxidize, or photolyze during the test period.

8.3 Health and safety precautions and applicable regulations for the disposal of stock solutions, test organisms, and soils should be considered before beginning a test (see Guide D 4447).

8.4 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.

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

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

8.7 The use of ground fault systems and leak detectors is recommended strongly to help prevent electrical shocks.

9. Soil

9.1 *General*—Before the preparation or collection of soil, an approved, written procedure should be prepared for the handling of soils that might contain unknown quantities of toxic contaminants (see Section 8). All soils should be characterized and have at least the following determined: pH, percent organic matter, cation exchange capacity (CEC), total nitrogen, particle size distribution (percent sand, silt, and clay), and percent water content. In addition, chemical analyses should be performed for compounds suspected of occurring in the particular soil (for example, heavy metals and organics). Toxicological results might provide information directing a more intensive analysis. Soil toxicity testing procedures are detailed in Section 11.

9.2 *Negative Control and Reference Soil*—A negative control soil is used for the following: (1) to yield a measure of the acceptability of the test, (2) to provide evidence of the health and relative quality of the test organisms, (3) to determine the suitability of the test conditions and handling procedures, and

(4) to provide a basis for interpreting data obtained from the test soils. A reference soil is used to describe the matrix effects of a test. Every test must have a negative control of artificial or reference soil and may also have a reference soil if the negative control is an artificial soil. A reference soil should be collected from the field in a clean area and represent the test soil as much as possible in soil characteristics (for example, percent organic matter, particle size distribution, and pH). This provides a site-specific basis for comparison of toxic and nontoxic conditions. The same conditions, procedures, and organisms must be used with the negative control and reference soil as are used in the other treatments, except that contaminated soil or test materials are not added.

9.3 Field Sampling Design—A site is defined as a delineated tract of land that is being considered as the overall study area, usually from the standpoint of its being potentially affected by xenobiotics. The field collection is often conducted in areas in which little is known concerning contamination or contamination patterns. The object of a qualitative field sampling design is to identify sites that contain potentially toxic conditions that may warrant further study. The collection design might divide the site into sampling units based on habitat or topography to allow for maximum spatial coverage. Sampling stations may be set up within each unit (see 3.2). One sample is collected from each station. The lack of field replication at each station usually precludes statistical comparisons; however, the identification of samples for further study is possible, when survival, growth, or reproduction differ between sampling stations or sampling stations differ from a reference soil. Information on field sampling design is presented by Warren-Hicks, et al (28), Eberhardt and Thomas (29), and Gilbert (30).

9.3.1 If the object of the field sampling design is to test for statistically significant differences in the effects between negative control or reference soils and test soils from several sites or between sampling stations within a single site, a quantitative method is used that requires replicate sampling. The number of field replicates (that is, separate soil samples at a single sampling station) necessary per sampling station is a function of the need for sensitivity or power. A minimum of three field replicates from each station is recommended. These field replicates are each treated as a separate sample in the laboratory, that is, they are not mixed together. The field replicates from a single sampling station might be used (1) to test for within-sampling station variability, (2) to compare laboratory test procedures, or (3) to compare sensitivity among test species.

9.3.2 Sampling stations might be distributed along a known pollution gradient within a site or at random within sampling units. Comparisons can be made between both space and time if the sampling and testing take place during different times of the year.

9.4 Field-Collected Test Soil:

9.4.1 Collection—A shovel or auger (preferably stainless steel) should be used to collect soil samples (see Section 8). The surface of the location at which the sample is to be collected should be cleared of debris such as leaves and twigs. If the location is an area of grass or other plants, the plants

should be cut to ground level and removed before the sample is collected. The sample should be placed in a thick plastic bag (for example, 4 mil) and taped closed. This bag should then be placed in a second plastic bag, taped closed, and placed in a clean sample container with a lid (for example, plastic pail with O-ring seal). Direct sunlight should be minimized during collection if the contaminants associated with soils include compounds that photolyze readily. All soil samples should be placed in an ice chest and kept cold in the field. Field observations concerning habitat and type of vegetation and measurements such as soil temperature and moisture may be taken in the field.

9.4.2 Storage—Soil samples should be utilized as soon as possible in accordance with Test Methods E 1706 stored at $4 \pm 2^\circ\text{C}$ for no longer than two weeks before the start of the test. Freezing and longer storage times might change the soil properties and should be avoided. The soil may be stored in the sample containers in which it was collected in the field. It is desirable to avoid contact with metals and plastics.

9.4.3 Processing—The following procedures should be followed if a homogenous sample is needed. The samples should be screened to remove oversize material such as rocks. A6.30-mm mesh, stainless steel screen may be used. The soil should be mixed after screening (for example, in a stainless steel mixer) to ensure homogeneity (see Section 6). Subsamples of the processed soil should be removed for pH and moisture content determination. Moisture content is determined gravimetrically by drying a subsample for 24 h at 100°C . Information on moisture content is necessary to determine the amount of hydration water to add to the test soils (see A1.9.3). Each replicate is screened, mixed, and treated separately if a quantitative method of field sampling with replicates was used.

9.4.3.1 There may be some instances when an intact core sample needs to be tested, and no processing is therefore necessary.

9.4.4 Qualitative descriptions of the soil may include color, texture, or the presence of roots, leaves, and soil organisms. Monitoring the odor of soil samples should be avoided because of potentially hazardous volatile contaminants (see Section 8).

9.4.5 The natural geochemical properties (for example, pH) of test soil collected from the field should be within the tolerance limits of the test species, or controls for the variable should be run (for example, a pH-adjusted soil). Limits for the test species should be determined in advance (see 10.1).

9.5 Laboratory-Spiked Test Soil—Test soil can also be prepared in the laboratory by adding materials such as chemicals or waste mixtures to artificial, reference, or site soils (see 1.4).

9.5.1 Test chemicals should be reagent grade⁷ or better, unless technical or other grade material is specifically needed. Before a test is started, the following should be known

⁷ *Reagent Chemicals, American Chemical Society Specifications*, American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Anal. Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmaceutical Convention, Inc. (USPC), Rockville, MD.

concerning the test material: (1) identity and concentration of major ingredients and impurities; (2) water solubility in hydration water; (3) estimated toxicity to the test species and to humans; (4) precision and bias of the analytical method at the planned concentrations of the test material, if the test concentrations are to be measured; and (5) recommended handling and disposal procedures.

9.5.2 Stock Solutions—Test materials to be tested in artificial, reference, or site soil should be dissolved in a solvent (the preferred solvent is water) to form a stock solution. The stock solution itself, or dilutions of it, are then added to the soil. The concentration and stability of the chemical in the stock solution should be determined before beginning the test. The stock solution should be shielded from light both before and during the process of mixing into the soil if the chemical is subject to photolysis. Concentrations of the chemical in the solvent and soil should be monitored before the test begins.

9.5.3 Non-Water Solvents—If a solvent other than water is necessary, it should be one that is water-miscible and can be driven off (for example, evaporated), leaving only the test chemical on the soil. Both a solvent control and a negative control soil must be included in the test if a solvent other than water is used. The solvent control must contain the highest concentration of solvent added to the soil and must use solvent from the same batch used to make the stock solution. The same concentration of solvent should be used in all treatments.

9.5.3.1 Acetone is an organic solvent used for preparing stock solutions (**5, 15, 17, 31**) because of its high volatility and ability to dissolve many organic chemicals. Other water-miscible organic solvents, such as methanol or ethanol (**7**), may be used. Organic solvents may affect total organic carbon levels, introduce toxicity, or alter the geochemical properties of the soil (see 6.1.5). 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.

9.5.3.2 If the concentration of solvent is not the same in all test solutions that contain test material, a solvent test should be conducted to determine whether survival, growth, or reproduction of the test organisms are related to the solvent concentration over the range used in the toxicity test. If survival, growth, or reproduction are found to be related to solvent concentration, a soil toxicity test with that species in that amount of solvent is unacceptable if any treatment contained a concentration of solvent in that range.

9.5.3.3 The survival, growth, or reproduction of the organisms tested in the two controls should be compared if the test contains both a negative control and a solvent control. Only the solvent control may be used for meeting the acceptability of the test and as the basis for the calculation of results if a statistically significant difference in either survival, growth, or reproduction is detected between the two controls. The negative control might provide additional information on the general health of the organisms tested. The data from both controls should be used for meeting the acceptability of the test and as the basis for the calculation of results if no statistically significant difference is detected.

9.5.4 Test Concentrations:

9.5.4.1 If the test is intended to allow the 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 soil 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.

9.5.4.2 In some situations (for example, regulatory), it might be necessary to determine only (1) whether a specific concentration of test material is toxic to the test species or (2) whether the LC50 is above or below a specific concentration. When there is interest in a particular concentration, it might be necessary to test only that concentration and not to determine the LC50.

9.5.5 The addition of test materials to soil may be accomplished using various methods such as hand mixing or using a mechanical mixer (see 9.4.3).

9.5.5.1 If tests are repeated, mixing conditions such as the 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 a soil is distributed thoroughly and evenly within the soil. The homogeneity of laboratory-dosed material should always be determined prior to testing.

10. Test Organism

10.1 Species—Only one species is currently described in this guide (see Annex A1); however, descriptions of additional species may be included in revisions of this guide. The use of this species is encouraged to increase the comparability of results. The source and type of soil being tested or the type of test to be implemented might dictate the selection of a particular species. The species used should be selected based on (1) availability; (2) sensitivity to test materials; (3) tolerance to parameters such as temperature, pH, and grain size; and (4) ease of handling in the laboratory. The species used should be identified using an appropriate taxonomic key.

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

10.3 Source—All organisms in a test must be from the same source. Organisms may be obtained from laboratory cultures or natural populations from clean areas. Local and state agencies might require collecting permits. Laboratory cultures may be the best source of test species because laboratories can provide organisms whose history, age, and quality are known. State and federal institutions may have available laboratory cultures of test organisms. Commercial suppliers who have laboratory cultures of research and testing organisms may also be a source. It is important to obtain organisms that are of a known species or subspecies and not a mixture. Paragraph A1.5 contains additional information on possible sources of test organisms.

10.4 *Quality*—Chemical analysis of organisms collected from natural populations is desirable. It may be desirable to analyze for the test materials and other chemicals to which major exposure might have occurred.

10.5 *Care of Brood Stock*—Brood stock should be cared for properly to prevent unnecessary stress (see Annex A1). To maintain organisms in good condition and prevent unnecessary stress, they should not be crowded and should not be subjected to rapid changes in temperature or the quality of culturing medium. Earthworms should be cultured at the same temperature as that used for testing (see 11.5 and A1.9.1.4).

10.6 *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 test soils on the surface so as to evaluate burrowing behavior. Any organisms that touch dry surfaces or are dropped or injured during handling should be discarded.

11. Procedure

11.1 *Experimental Design of Laboratory Experiments*—Decisions concerning the various aspects of experimental design, such as the number of treatments and number of test containers and test organisms per container, should be based on the purpose of the test and the type of procedure that is to be used to calculate results (see Section 14). A test intended to allow the calculation of a specific endpoint such as an LC50 should consist of a negative control, a solvent control, if necessary, and several test concentrations (see 9.5.3).

11.1.1 The primary focus of the 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 assigned independently (32). The test container is the experimental unit (see 7.5). As the number of test containers per treatment increases, the number of degrees of freedom increases, and therefore the width of the fiducial interval on a point estimate, such as an LC50, decreases, and the power of a significance test increases (see Section 14). Because of factors that might affect the results within test containers and therefore the results of the test, (1) all test containers must be treated as similarly as possible, for example, temperature and lighting, and (2) each test container must be treated physically as a separate entity. The assignment of test organisms to test containers must be randomized, and test containers must be assigned randomly to individual test chamber locations.

11.2 *Soil Into Test Containers*—The day before the toxicity test is started (Day – 1), the soil to be tested, negative control, and reference soil (if used) are mixed, the moisture level is adjusted with hydration water, and the soils are placed into test containers. Paragraph A1.9.3 contains information on the hydration of test soils. If large interstitial spaces of air occur in the soil matrix, these spaces should be removed by pressing in the soil with a suitable utensil, for example, a spatula (see 7.2), while trying not to compact the soil. The minimum amount of soil to mix and hydrate should be enough for three replicates, a pH sample, and to account for soil adhering to the sides of the mixing chamber. This mixed and hydrated soil is called a batch. Extra batch soil may be mixed and hydrated if a sample

is to be removed for chemical analysis or for any other purpose. Site soil has been mixed previously during processing.

11.2.1 *Site Soil Sampler*—From each sample collected at a field station, soil sufficient for three replicates is hydrated with water, and replicates are placed into test containers (see Annex A1).

11.2.2 *Test Soils Prepared for a Concentration Series*—If site soil and artificial or reference soil are to be mixed in a concentration series, each concentration (treatment) is prepared as a batch from which replicates are placed into test containers. If site, reference, or artificial (see Annex A2) soil is to be spiked with chemicals, each concentration is prepared as a batch, and replicates are placed into test containers.

11.2.3 The test containers with soil are covered with a lid containing a very small hole to allow for air movement. The test containers are then placed into the test chamber, until the next day, to (1) allow the test containers to temperature equilibrate and (2) allow time for the test material to equilibrate with the soil. Each test container must contain the same amount of soil (specified in Annex A1) determined on a dry weight basis.

11.3 *Introduction of Test Organisms*—Test organisms are placed into the test containers after the overnight equilibration; this constitutes the beginning of the test (Day 0). The test organisms are placed on the surface of the soil and allowed to burrow because a lack of burrowing is considered a response possibly due to the presence of toxic compounds (7).

11.4 *Duration of Test*—The test begins when test organisms are first placed in the test containers and continues for the duration specified in the experimental design for a specific test organism.

11.5 *Temperature*—In toxicity tests with *E. fetida* in artificial soil with 2-chloroacetamide and benomyl, Heimbach and Edwards (33) found that temperature variations between 10 and 26°C had little influence on the toxicity of the chemicals. The test temperature depends on the species used (see Annex A1). Other temperatures may be used to study the effect of temperature on the survival, growth, or reproduction of test organisms and contaminant-related properties (for example, bioavailability).

11.6 Test Measurements:

11.6.1 Temperature should be monitored for the duration of the test. A continuous temperature recorder (or a continuous temperature/humidity recorder) with a 7-day chart can be placed in the test chamber and changed as necessary.

11.6.2 A rough measurement of the total biomass of test organisms per test container should be obtained at the beginning of the test. A rough measurement consists of weighing the worms after first removing any large fragments of bedding that may be adhering to them (see A1.7 and A1.7.1).

11.6.2.1 If weight loss is used as an endpoint, an accurate measurement of weight must be taken of the total biomass of test organisms per test container at the beginning and end of the test. The worms should be purged of their gut contents before weighing by placing them in petri dishes with wet filter paper. Bedding should be rinsed from the worms with test water before placing the worms in petri plates. Before weighing the

worms, excess surface water may be removed by placing the worms between layers of an absorbent towel. It is very important not to dry the surface of the worms, and consideration should be given to whether this step might stress the worms unduly. Researchers have commonly used 24 h (8, 13, 34) or 48 h (35, 36) for a purging time period. Although Stafford and McGrath (36) provided some evidence that some soil may still remain in the gut after 48 h, it is recommended that 24 h be used as a purging time. An excessively long period of starvation prior to initiating a lengthy test during which food is not added (see 11.7) may stress the test organisms.

11.6.2.2 Richards and Ireland (37) suggest that longer periods of starvation may result in the depuration of heavy metals from earthworm tissue. These factors need to be considered if bioaccumulation studies are to be performed, and an elimination study should be undertaken to determine the effect of purging on the concentration of the target compounds in the earthworms.

11.6.3 pH should be measured (see A1.11.1) at the beginning of the test in subsamples taken from the batch preparations and at the end of the test in subsamples from replicates of the various concentrations.

11.6.4 Percent moisture may be measured (see A1.11.2) at the beginning and end of the test from subsamples, as noted in 11.6.3.

11.6.5 Salinity should be measured (see A3.7) at the beginning and end of the test. This may be done in subsamples as noted in 11.6.3.

11.7 *Food*—It is recommended that food not be added to the test containers because it may affect the results of the test. In studies of longer duration, that is, over 28 days, the use of food may have to be reevaluated (see A1.9.1.2 and A1.9.5).

11.8 *Light*—To maximize exposure, continuous lighting (15, 38) using either a fluorescent or an incandescent light source must be used for testing. A minimum intensity of 37 fc (400 lux) is recommended for testing (38).

11.9 *Biological Data*—Effects indicating the toxicity of a test soil include mortality and may include sublethal effects on growth, behavior, reproduction, and physiological processes, as well as observations on external pathological changes, for example, segmental constrictions, lesions, or stiffness (see A1.10). Test containers may be observed on a weekly basis or only at the end of the test. Test soil and organisms are emptied onto a flat surface, and the organisms are removed and evaluated, at the end of the exposure period.

11.10 *Chemical Analyses:*

11.10.1 *Field-Collected Soils*—Soil samples for laboratory testing should be collected from the same grab as for chemical analysis. A subsample from the same grab may be used for faunal analyses.

11.10.2 *Artificial Soil and Field-Collected Soils Spiked in the Laboratory*—Measurement of the concentration of test materials in the batches of test soil is desirable at the beginning of the experiment. Chemical analyses at several concentrations of soil from the test containers may be made at the end of the test. To monitor changes in soil chemistry during the course of the experiment, separate test containers may be set up (including test organisms) and sampled as necessary or practical over

the duration of the experiment. The measurement of test materials degradation products might also be desirable.

11.10.3 *Tissue Analysis*—Contaminant bioavailability is indicated by the contaminant concentrations accumulated in earthworm tissues (see A3.8.3).

12. Analytical Methodology

12.1 Chemical and physical data for soil should be obtained using appropriate ASTM standards whenever possible. For those measurements for which ASTM standards do not exist or are not sufficiently sensitive, methods should be obtained from other sources, for example, EPA (39).

12.2 Concentrations should be measured for (1) contaminants in batches of soil, (2) test materials in stock solutions, and (3) contaminants in test containers. In addition, measurements for the presence of an apparently evaporated organic solvent may be desirable.

12.2.1 If samples of stock solutions or test soils are not to be analyzed immediately, they should be handled and stored appropriately (see 9.4.2).

12.3 Methods used for analyzing test organisms for contaminants of concern should be obtained from appropriate sources (40).

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

13. Acceptability of Test

13.1 A soil toxicity or bioaccumulation test should be considered unacceptable if one or more of the following situations occurred.

13.1.1 Continuous lighting had not been used during the test, if soil exposures were intended to be maximized (see 11.8), unless performing the bioaccumulation assay test variation with Bermuda grass (see A3.10).

13.1.2 All test containers were not identical (see 7.5 and 11.1).

13.1.3 Test organisms were not cultured at the same temperature as that used for testing (see 7.3.2, 10.5, and 11.5).

13.1.4 The natural geochemical properties of test soil collected from the field was not within the tolerance limits of the test species (see 9.4.5).

13.1.5 Appropriate negative and solvent controls were not included in the test (see 9.2 and 9.5.3).

13.1.6 The concentration of solvent in the range used affected the survival, growth, or reproduction of the test organisms (see 9.5.3.2).

13.1.7 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 Section 10).

13.1.8 Treatments were not assigned randomly to individual test chamber locations, and individual test organisms were not assigned randomly to test containers (see 11.1.1).

13.1.9 Each test chamber did not contain the same amount of soil, determined on a dry weight basis (see 11.2).

13.1.10 The temperature was not within the acceptable range (see A1.9.1.4 and A3.7).

13.1.11 The negative control soil organisms did not survive, grow, or reproduce as required for the test species (see 9.2 and Annex A2).

14. Calculation of Results

14.1 The calculation procedures and interpretation of the results should be appropriate to the experimental design. Procedures used to calculate the results of toxicity tests can be divided into two categories: those that test hypotheses and those that provide point estimates. No procedure should be used without careful consideration of (1) the advantages and disadvantages of various alternative procedures and (2) appropriate preliminary tests, such as those for outliers and heterogeneity.

14.2 The LC50 or EC50 and its 95 % fiducial limits should be calculated (when appropriate) for each set of data on the basis of the measured initial concentrations of test material, if available, or the calculated initial concentrations. If other LC or ECs are calculated, their 95 % fiducial limits should also be calculated.

14.3 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 (41-44) can be used to calculate an LC50 or EC50 and 95 % fiducial limits from a set of quantal data that is distributed binomially and contains two or more concentrations at which the percent dead or effected is between 0 and 100, but the most widely used are the probit, moving average, Spearman-Kärber, and Litchfield-Wilcoxon methods. The method used should take into account appropriately the number of test organisms per container. The binomial test can also be used to obtain statistically sound information concerning the LC50 or EC50 even when fewer than two concentrations kill or affect between 0 and 100 %. The binomial test provides a range within which the LC50 or EC50 should lie.

14.4 When samples from field stations are replicated independently, the effects at those stations can be compared statistically by *t*-tests, analysis of variance (ANOVA), or regression-type analysis. The ANOVA is used to determine whether any of the observed differences among the samples (or concentrations) are statistically significant. This is a test of the null hypothesis that no differences exist in the effects among the samples (or concentrations) and 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 stations) were not large enough to be detected as statistically significant by the experimental design and hypothesis test used. Non-rejection 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 (45). The amount of effect that occurred at this concentration should be considered.

14.4.1 All exposure concentration effects (or field stations) can be compared with the control effects by using mean separation techniques, orthogonal contrasts, Fisher's methods, Dunnett's procedure, or Williams' method. The lowest concentration for which the difference in observed effect exceeds the statistically 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 statistically significant difference is defined as the NOEC for that end point.

14.5 Bioaccumulation test results are reported as the magnitude of contaminant concentration above either the Day 0 tissue baseline analysis or the Day 28 tissues from the negative control or reference soil (that is, 2×, 5×, 10×) (see A3.9). Other approaches for evaluating data include kinetics studies with estimate uptake, depuration rates, and time to steady state, lipid normalization and normalizing soil concentrations of non-ionic organics to TOC (see Guide E 1688). Analysis of field collected organisms is also an option.

15. Report

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

15.1.1 Name of the test and investigator, name and location of the laboratory, and dates of the start and end of the test.

15.1.2 Source of the negative control, reference, or test soil.

15.1.3 Method of the collection, handling, shipping, storage, and disposal of soil.

15.1.4 Source of the test material; lot number, if applicable; composition (identities and concentrations of major ingredients and impurities, if known); and known chemical and physical properties.

15.1.5 Identity and concentration of any solvent used.

15.1.6 Source and quality of hydration and test water.

15.1.7 Source, history, and reproductive status of the test organisms; scientific name, name of person who identified the test organism, and taxonomic key used; culture procedures and any observed diseases, unusual appearance, or treatments; source of culture and date the culture stock was obtained; and biomass of test organism per test container.

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

15.1.9 Description of the experimental design and test chambers; weight (dry weight basis) of the test soil in each test container; amount of hydration water added to the test soil; type and intensity of lighting in the test chamber; number of test containers and number of test organisms per container and per treatment; date and time the test started and ended; temperature measurements during the test; pH values of test soils at the start and end of the test; and any other measurements taken.

15.1.10 Methods used for, and results (with standard deviations or fiducial limits) of, the physical and chemical analyses of site soil, test soil, and stock solutions.

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

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

15.1.13 Methods used for, and results of, the statistical analyses of data.

15.1.14 Summary of general observations on other effects or symptoms.

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

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

ANNEXES

(Mandatory Information)

A1. EISENIA FETIDA

A1.1 *Significance*—*Eisenia fetida* (Savigny, 1826), Oligochaeta, has many desirable characteristics for a test species: (1) it has a short generation time (46); (2) it reproduces prodigiously (47); (3) it is collected easily from natural sources or cultured in the laboratory (3, 38, 48, 49); and (4) data on its survival, growth, and reproduction can be obtained in toxicity tests (48, 50-55). Stafford, et al (56) indicated that *E. fetida* was the most sensitive species, of those examined, for indicating heavy metal availability from soils and dredged sediments. *E. fetida* has been used successfully as a laboratory test organism in many testing mediums, for example, artificial soil (4), contaminated field soils (6, 57), activated sludge (51), sediment (58), and cow manure (17).

A1.2 *Life History*—The life-cycle of *E. fetida* can be divided into three distinct phases, according to Jefferies and Audsley (59): (1) the cocoon phase, consisting of an egg cocoon that can produce from one to eleven hatchlings under laboratory conditions (60); (2) the young (immature) phase, during which the hatchlings grow physically but cannot produce cocoons; and (3) the adult (mature) phase, which is reached when the worms become capable of producing cocoons. Adult worms may still grow physically. Tomlin and Miller (60) report a life-cycle for *E. fetida* to vary from a mean of 51.5 days at 25°C to more than 166 days at 13°C, that is, from freshly deposited cocoon through clitellate worm and deposition of the next generation of cocoons. Reynolds (61) indicates that *E. fetida* has a maximum life expectancy of 4 to 5 years, although between 1 and 2 years is more usual.

A1.2.1 *E. fetida* is an epigeic species, that is, they live and feed on the surface (2, 62) that rarely inhabits agricultural soils but is found in compost piles, manure piles, and other disturbed sites rich in organic matter (12). The rate of soil consumption in the laboratory for *E. fetida* has been estimated at 16 mg soil/individual/day (300 mg, live weight individuals) (6).

A1.2.2 The specific sources of nutrition for *E. fetida* are not well understood, but Morgan (63) found that *E. fetida* was capable of using both the microorganisms found in organic wastes and simple nutrients for growth. Worms grew well on pure cultures of four species of fungi and on low concentrations of glucose and sucrose, but they died or lost weight on pure cultures of various bacteria and protozoa species. Worms confined with a single food source may have been exposed to the buildup of toxic metabolites produced by the microorganisms. More work needs to be performed in this area.

A1.2.2.1 Worms digest the microorganisms from ingested soil and organic debris, which illustrates their interactions with the soil environment. This occurs independently of whether mineral matter or fibrous organic material was ingested. Approximately 2.5 h were required at 25°C for passage of ingesta from mouth to anus for *E. fetida* (64).

A1.2.3 Although an increase in temperature within the range from 13 to 25°C reduces the amount of time needed for a life cycle, Tomlin and Miller (60) report that an increase in temperature within this range reduces the number of hatchlings per cocoon.

A1.3 *Taxonomy*—The taxonomic status of what Bouché (62) calls the *E. fetida* complex is unclear in the literature. Some authors consider this complex to consist of two subspecies, *E. fetida fetida* and *E. fetida andrei*, while other authors consider the complex to consist of two separate species, *E. fetida* and *Eisenia andrei*. This guide chooses to use the subspecies designations. The dorsal surface of *E. f. andrei* is uniformly reddish, while *E. f. fetida* is striped or banded. Fender (65) (classifying the two earthworms as different species instead of subspecies) describes *E. fetida* as having pigment covering only the center two thirds or so of the dorsal half of each segment, presenting a strongly banded appearance. He describes *E. andrei* as having pigment covering at least nine tenths of the length of each segment dorsally, giving it a nearly solid color. He indicates that the taxonomy in the literature is submerged in that of “*E. fetida*,” making it unclear which of the two forms is being discussed.

A1.3.1 Roch, et al (66) and Valembois, et al (67) demonstrated biochemical differences between the two forms. Oien and Stenersen (68) and Jaenike (69) conducted electrophoretic work that led them to consider the two forms as separate species, and Sheppard (70) added research indicating that ecological differences exist between the two forms. It is important to know which form is being used as a test organism for these reasons.

A1.3.2 Bouché (62) states that the *andrei* form is relatively homogeneous, while *fetida* may be multispecific. It is recommended that the *andrei* form be used as the test organism, that is, *E. f. andrei*.

A1.4 *Culture of Test Organisms*—The following culture procedures are adapted from Edwards (38) and Greene, et al (18). *E. fetida* can be reared in a bedding of sphagnum (*Sphagnum*) peat moss pH adjusted to 7.0 with pure calcium

carbonate and hydrated with test water, for example, distilled, deionized, or reverse osmosis. Plastic trays measuring approximately 34 by 28 by 14 cm can hold 700 g (dry weight) of peat moss hydrated with approximately 2300 mL of reagent water. The trays need to be covered, for example, with plastic, to prevent drying. Moisture should be monitored on a weekly basis. The trays should be maintained so that there is no standing water in the bottom of the trays and so that the surface of the bedding is not dry. Placing a piece of material such as plywood over the plastic will keep it in place. The trays are held under continuous lighting at $22 \pm 3^\circ\text{C}$ (see A1.9.1.4).

A1.4.1 *E. fetida* have been cultured with a variety of foods, for example: (1) cellulose and activated sludge (71), (2) dairy waste sludge cake (72), (3) horse manure (8), (4) activated sludge and horse manure (73), and (5) commercial alfalfa pellets (*Medicago sativa*) (57). Alfalfa pellets saturated with test water (at a ratio of approximately 1 g of dry pellets per 2 mL test water) and aged for two weeks in a covered container are consumed readily by *E. fetida*. Alfalfa pellets may be less likely to contain unknown compounds than the other feeds and are therefore recommended.

A1.4.1.1 The worms should be fed once or twice per week, depending on the number of individuals in a tray. Any remaining food is removed and discarded at feeding time. The bedding is then turned by hand to inspect the general condition of the worms and the bedding. If any dead worms are noticed, they should be removed. The tray should be set aside for more frequent evaluation, or it should be discarded, if many dead or stressed-appearing worms are found. Test water is added, and the bedding is turned again, if the bedding needs more moisture. Food is sprinkled over the surface of the bedding in an amount that has been determined will be consumed by the next feeding time.

A1.4.1.2 Some of the pests associated with the culture of worms are fungus gnats, soil mites, Collembola (small insects, commonly called springtails, which are abundant in moist leaf mold, soil, and rotten wood), and enchytraeids (small, white worms belonging to the Class Oligochaeta). None of these pests in low numbers appears to be a problem for the culture of healthy worms. Gnats are seasonal and are mostly a nuisance for the caretaker of the worms. Large numbers of mites and enchytraeids appear to compete for food with the worms, and mites have been observed on dead or dying worms. Biocides are not used for the control of pests because of their potential effect on earthworm health or testing sensitivity. The control of pests consists of removal by hand or by disposal of infected trays. Different geographical regions may have their own distinct types of pests.

A1.4.2 Earthworms should be cultured so they are not stressed unnecessarily. To maintain *E. fetida* in good condition and prevent unnecessary stress, the cultures should be kept at a constant temperature, the pH should be maintained near 7.0, feeding should be on a regular schedule, the moisture level of the bedding should remain adequate as described in A1.4, and crowding (see A1.4.2.1) should be prevented.

A1.4.2.1 Neuhauser, et al (46) calculated carrying capacities for *E. fetida*, in a volume of 300 cm^3 with a surface area of 78 cm^2 , to range from approximately 6 to greater than 23 g of

worm, depending on the type of food source and substrate. This is approximately 0.02 to 0.08 g of worm/ cm^3 of substrate. The number of worms that a tray holds is a function of the size and age of the worms. Adult worms have distinct, fully developed clitella and weigh a minimum of approximately 300 mg. Sub-adult worms have visible, but not fully developed, clitella and are approximately 150 to 300 mg in weight. Juvenile (young) worms do not have clitella and are usually less than 150 mg in weight. For optimal reproduction, it is recommended that the trays containing 9000 cm^3 of bedding hold a maximum of 245 g of worm, that is, $0.03\text{ g}/\text{cm}^3$. For example, 350 adult worms weighing 700 mg each would be equal to $0.03\text{ g}/\text{cm}^3$. To reduce the population of worms in a crowded tray, first prepare a new tray of bedding. Half of this new bedding is removed and placed on a piece of plastic sheeting. Half of the bedding containing a portion of worms from the crowded tray is placed into the new tray, and the bedding is mixed by hand. The half tray of new bedding on the plastic sheet is then added to the old tray of bedding and mixed.

A1.4.3 A tray will periodically need to have its bedding changed, even if it is not overcrowded. Prepare a new tray of bedding, and place the contents of the old tray of bedding on top of the new bedding. Allow this tray to sit uncovered in the continuously lighted culture chamber for two days, and allow the worms to burrow into the new bedding. Remove the old bedding from the top of the new bedding and discard. This procedure does not recover the cocoons, and some of the worms will still be in the old bedding.

A1.4.3.1 If it is critical to save each individual worm and cocoon, old bedding that needs changing can be spread onto a sheet of plastic, and every worm and cocoon can be picked by hand and placed into a new tray of bedding. Cocoons should be buried in the new bedding, but worms can be placed on the surface of the new bedding and allowed to burrow.

A1.5 *Obtaining Brood Stock*—*E. fetida* has been reared on earthworm farms and sold in every Canadian province and American state for fish bait (61). However, bait farms may contain mixtures of *E. f. andrei* and *E. f. fetida*. Reynolds (61) and Fender (65) report that *E. fetida* can be found in manure piles and usually not far from human activity. Fender (65) (classifying the two earthworms as different species instead of subspecies) states further that if the two earthworms are found in the same manure pile, *E. andrei* is usually found in dryer areas than *E. fetida* and is often most abundant in or below the soil contact region. Starter cultures might also be obtained from various institutions, laboratories, and biological firms, although it is important to ensure a pure culture. Field-collected *E. fetida* should be identified using adult worms. The taxonomic key of Fender (65) may be useful for this purpose.

A1.6 *Handling*—*E. fetida* 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 worms are not stressed unnecessarily. Any worms that are dropped or injured during handling should be discarded.

A1.7 *Age*—Tests with *E. fetida* should be started with sexually mature, fully clitellate adults (6, 8, 18, 48, 57). The

biomass of earthworms in each test container should be obtained.

A1.7.1 Worms are selected randomly and removed by hand from a culture tray and weighed in groups of ten (see A1.9.1) for each test container. Worms are purged of their gut contents prior to weighing only if weight loss is used as an endpoint (see 11.6.2.1).

A1.8 *Acclimation*—It is recommended that the test organisms be cultured and tested at the same temperature (see 11.5, A1.4, and A1.9.1.4) so that a period of acclimation to temperature is not necessary.

A1.9 *Toxicity Test Specifications:*

A1.9.1 *Experimental Design*—Decisions concerning the various aspects of experimental design, such as the number of concentrations and number of test containers and earthworms per concentration, should be based on the purpose of the test and the procedure used to calculate the results.

A1.9.1.1 Neuhauser, et al (5) used a minimum of five concentrations, with four replicates for each test concentration and ten worms per test container, for a definitive test in artificial soil. Each test container consisted of a glass dish 6.5 cm in height and 12.5 cm in diameter (0.8 L) that contained 400 g (dry weight) of test soil. Haque and Ebing (48) used five concentrations, with three replicates for each concentration and six worms per container, for a definitive test in artificial soil. Test containers were 1-L glass jars and held 500 g (dry weight) of test soil. Greene, et al (18) recommended a minimum of five concentrations, with three replicates per concentration and ten worms per container, for a definitive test in site soil mixed with artificial soil to make a “dilution” series. Test containers were 473-mL glass jars that held 200 g (dry weight) of test soil.

(1) It is recommended that a minimum of five concentrations, with a minimum of three replicates per concentration, be used for a definitive test. Ten worms per container is recommended.

(2) Using the data on the rate of consumption of soil given in A1.2.1 and assuming that a 600-mg individual would consume twice as much soil as a 300-mg individual, a 28-day test with ten worms weighing 600 mg each would consume only 9 g of soil. High stocking densities, that is, gram earthworm/gram soil, may increase the possibility that earthworms would ingest soil more than once, which may affect the uptake (and therefore toxicity) of compounds (6). Under high stocking densities, the death of an individual earthworm during a test may also be more likely to influence the remaining individuals adversely. It is recommended that each test container hold 200 g (dry weight) of test soil. This amount is well above the potential amount that ten earthworms would process in 28 days. If hazardous waste soils are being evaluated in a laboratory setting, it is important to try to reduce the amount of soil being transported from field to laboratory and the amount of waste generated by the laboratory, both from an economical and environmental viewpoint.

A1.9.1.2 The duration of the test, with mortality as the endpoint, is typically 14 days (16, 57, 74, 75), with an evaluation at seven days being optional. Tests investigating the bioaccumulation of xenobiotics in field-collected soils have

been conducted for 56 days (6) without the addition of food, but consideration should be made for the possible effect of a lack of food for time periods of this length (see A1.9.1.3 and A1.9.5).

(1) Loss in body weight and behavioral and morphological endpoints such as coiling, segmental swellings, segmental constrictions, lesions, rigidity, and flaccidity can be used successfully in toxicity testing (76-78).

A1.9.1.3 Growth and reproduction can be used as biological endpoints in tests with *E. fetida* of longer duration, for example, 140 days (52). The use of food must be considered in long-term growth and reproduction studies (see A1.9.1.2 and A1.9.5). The growth of young worms, rate of clitellum development, number of cocoons produced, cocoon mass, number of hatchlings per cocoon, and biomass of hatchlings have all been used as endpoints in research by Reinecke and Venter (17), Malecki, et al (52), Van Gestel, et al (55), and Venter and Reinecke (79) with xenobiotics. The importance of controlling environmental factors such as pH, temperature, and moisture content in growth and reproduction tests has been demonstrated by Van Gestel, et al (80).

A1.9.1.4 Although Heimbach and Edwards (33) tested *E. fetida* successfully within the range from 10 to 26°C (see 11.5), the majority of the testing with *E. fetida* has been conducted within the temperature range from 18 to 25°C (5, 15, 31, 48, 50, 57, 75, 76). Van Gestel, et al (80) report that a temperature range from 20 to 25°C is optimal for *E. fetida*. Kaplan, et al (73) report that *E. fetida* survived best over the temperature range from 20 to 29°C and that mortality was produced at 5 and 33°C. A temperature range from 19 to 25°C is highly recommended for testing, but the temperature range must not fall below 10°C (33) or above 29°C (73). (See Table A1.1.)

A1.9.1.5 *E. fetida* has been tested under continuous lighting and with a photoperiod of 12 h light and 12 h dark. A continuous lighting regimen is recommended in order to help keep the photosensitive earthworms burrowing. When measured, lighting intensity has been reported for toxicological testing with *E. fetida* from 37 to 100 fc (400 to 1080 lx). A minimum of 37 fc is recommended for testing (see Table A1.1).

A1.9.2 *Test Containers*—Glass testing containers have been used by most researchers with *E. fetida*. Glass, 473-mL canning jars are convenient and have been used successfully with 200 g (dry weight) of test soil (18). Canning jar lids may be used for a cover and held in place with the screw ring. A small (1 to 2 mm) hole should be placed in the center of the lid to allow for air exchange.

A1.9.3 *Day Prior (Day – 1) to Initiation of Test:*

A1.9.3.1 Test soils are hydrated and mixed well into batches, separated into replicates, and placed into test containers that are placed into the test chamber for overnight equilibration (see 11.2). No standing water should be present in the

TABLE A1.1 Test Specifications for the 14-Day *Eisenia fetida* Toxicity Test

Test Duration	14 days
Biological Endpoint	Mortality
Temperature	19–25°C
Photoperiod	24 h/400 to 1080 lx
Test Containers	473-mL glass jars

test containers. If a site, reference, or artificial soil is spiked with chemicals or compounds in solution, the solution is used as part of the hydration water.

(1) *Tests With Whole (100 %) Site or Reference Soil*—If the negative control is artificial soil, it is hydrated to 35 to 45 % of its dry weight, for example, 660 g (dry weight) would be hydrated with 231 to 297 mL of water. The site and reference soils are also hydrated to 35 to 45 % of their dry weight. Since most soils collected in the field contain some moisture, this moisture content is obtained and used for determining how much additional water to add to the soils to gain a hydration level of 35 to 45 %.

(a) Hydrating soils to a standard level is problematic. Due to the variation in water holding capacity (influenced by factors such as soil texture, structure, and organic matter content) between soils, one soil may appear very wet and even have standing water on the surface after hydration to 45 % of its dry weight, and another soil may appear considerably dryer after the same level of hydration. An alternative method for hydrating site and reference soils is to use the artificial soil when hydrated at 45 % of its dry weight as a standard. The site and reference soils can be hydrated to a level approximating the appearance of the artificial soil. Another alternative is to measure the water holding capacity of the soil and then hydrate the soil to 75 % of the water holding capacity value (18). Measuring the water potential (81), for example, using a tensiometer, of the soil may prove to be a better method of hydrating soils. The water potential of artificial soil hydrated to 35 to 45 % of its dry weight could be determined. Soils could be hydrated to the water potential value obtained for the artificial soil using this as a standard. Some variation in the moisture content between soils being evaluated may be acceptable based on the results of the research noted immediately below. Studies by Stafford and Edwards (6) with *Eisenia fetida* and five different soils found that a variation in moisture content of 25 to 45 % (presumably moisture content on a wet weight basis) made little difference in the rate of weight loss in the earthworms. Using 2-Chloroacetamide and Benomyl in artificial soil with *Eisenia fetida*, Heimbach and Edwards (33) found that changes in the water content of the artificial soil from 17.5 to 51 % of its dry weight had little influence on the toxicity of the chemicals.

(b) A sediment can be defined as a naturally occurring particulate material that 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 (see Guide E 1383). The definition of a soil as defined within this guide (see Section 3) indicates that a soil is not usually covered by water. It is sometimes difficult to distinguish between a soil and a sediment that has been dried out or deposited on dry land. Although earthworms can survive in a sediment for the duration of the test if the dissolved oxygen content is adequate, earthworms are not recommended for the evaluation of sediments, that is, sediments taken from below a body of water.

(2) *Tests With Site Soil Diluted With Artificial Soil*—The artificial soil portion of each concentration is hydrated to 35 to 45 % of its dry weight. The site soil portion of each concentration is hydrated as in (1) above. These two portions are then

mixed together to form the batch for each concentration from which the replicates are taken.

(3) *Tests With Artificial Soil Spiked With Compounds*—If a series of concentrations is prepared by spiking artificial soil with solutions of compounds, the artificial soil is hydrated to 35 to 45 % of its dry weight with test water and the chemical solution combined to make the necessary amount of hydration. If a series of concentrations is prepared by spiking artificial soil with dry chemicals, the chemical is first mixed into the artificial soil very well. The artificial soil is then hydrated with test water, and the batch is mixed again very well before being separated into replicates.

A1.9.4 Earthworms are introduced to the test containers the day after the equilibration period (Day 0). Groups of ten earthworms must be assigned randomly to the individual test containers. Earthworms are removed from the culture trays and weighed in groups of ten to obtain the total biomass per container. The earthworms are placed on the surface of the soil in the container and allowed to burrow (see 11.3). The test containers must be placed into the test chamber randomly.

A1.9.4.1 The worms are purged before weighing if weight loss is to be an endpoint (see 11.6.2.1).

A1.9.5 *Feeding*—It is recommended that food not be added to test containers for tests ≤ 28 days in duration (see 11.7). Stafford and Edwards (6) suggest that the results of a test may be affected by the addition of food due to potential binding properties of the feed and potential selective feeding by the earthworms. In tests longer than 28 days, the use of food may have to be reevaluated, depending on the purpose and endpoints of the test (see A1.9.1.2 and A1.9.1.3).

A1.10 *Biological Data*—Observations may be made at 24 h to evaluate burrowing or non-burrowing without opening the test containers. Mortality and sublethal evaluations may be evaluated on a weekly basis. At the end of the test, the test containers are emptied onto a flat surface, and the earthworms are accounted for and evaluated (see 11.9). Mortality is defined as a lack of response to a gentle mechanical stimulus, for example, touch with a small spatula or glass rod, to the anterior end of the worm (38). Earthworms may die and decompose within a 14-day testing period, so if all of the individuals are not accounted for at the end of the test, it may be assumed that they died and decomposed completely. Surviving worms may be rinsed with test water and evaluated for behavioral and external pathological endpoints. The following endpoints have been used in various studies: non-burrowing (7), segmental swelling (7, 48, 78), lesions/ulcers (7, 48, 78), coiling (7, 48, 76), shortening/stiffening (7, 48, 78, 82), flaccid/elongated (7, 78), segmental constrictions (76, 78), and tail end autotomy (83). Other endpoints may be developed.

A1.10.1 If weight loss is being used as an endpoint, the surviving earthworms should be washed and purged (see 11.6.2.1) before weighing.

A1.10.2 An *E. fetida* soil toxicity test, independent of duration, is unacceptable if the mean survival of all negative control containers is less than 90 % (see Section 13).

A1.11 *Test Measurements:*

A1.11.1 *pH*—If a concentration series is being tested, the initial pH should be checked in the high and low concentrations at a minimum. If a number of different undiluted site soils are being tested, pH should have already been measured in each soil (see 9.4.3). pH should also be measured in the negative control (and reference soil, if used). Initial pH is measured in a subsample taken from the batch preparation for each treatment.

A1.11.1.1 At the conclusion of a test with a series of concentrations, the pH is checked in subsamples of soil from one of the replicates of the control (and reference soil, if used), high and low concentrations. It is preferable that a replicate without any mortality be used for pH because the process of decay may alter the pH. If a test with undiluted site soils has been terminated, a sample for pH is taken from one replicate of each soil plus the control (and reference soil, if used). Care should be exercised to avoid a sample of soil containing dead worms.

A1.11.2 *Percent Moisture*—If a concentration series is being tested, the initial moisture content may be measured in the high and low concentrations. If a number of different undiluted site soils are being tested, moisture content measurements will have already been measured on the site soils (see 9.4.3). Moisture content may also be measured in the negative control (and reference soil, if used). Initial moisture is measured in subsamples taken from the batch preparation for each treatment and is determined gravimetrically.

A1.11.2.1 At the end of the test, moisture may be measured in one of the replicates of the high and low concentrations and the negative control (and reference soil, if used).

A1.11.3 *Temperature*—A copy of the temperature graph (or temperature/humidity graph) may be attached to the paperwork at the termination of the test (see 11.6.1).

A2. ARTIFICIAL SOIL COMPOSITION

A2.1 The artificial soil (AS) used in this test was developed with the advice of pedologists to overcome the variability between different soil types and has an adsorptive capacity resembling typical loam soils (38). The following constituents are mixed together on a dry weight basis:

(1) Canadian sphagnum (<i>Sphagnum</i>) peat moss (that portion passing through a 2.36-mm screen)	10 %
(2) Kaolin clay (97 % kaolinite with a particle size under 40 μm)	20 %
(3) Silica sand (Grade 70, 97.1 % particle size of 0.053 to 0.3 mm)	70 %

A2.1.1 After these materials are mixed together, an amount of calcium carbonate (99 % purity) equal to approximately 0.4 % of their total weight is added to the mixture to adjust the pH to 7.0 ± 0.5 . The exact amount of calcium carbonate used will depend on the pH of the peat moss used. For example, 50 kg of AS would have 200 g of calcium carbonate added to it. The materials and source of the materials need to be standardized as much as possible.

A3. BIOACCUMULATION TESTING UTILIZING EISENIA FETIDA

A3.1 *Scope:*

A3.1.1 This annex covers the additional procedures required to perform an *Eisenia fetida* bioaccumulation test.

A3.1.2 *Significance*—*Eisenia fetida* bioaccumulation testing. Bioavailability can not be determined from chemical analysis of the soil alone (84). Earthworm bioassays are an important tool to determine soil toxicity, and potential bioaccumulation with respect to the contaminant availability in soil. A method to determine contaminant bioavailability and mobility utilizing the earthworm *Eisenia fetida* has successfully evaluated the following contaminants; metals, PAHs, PCBs, pesticides, and butyltins (3, 10, 85, 87-91, 94). The bioaccumulation assay adds information on bioavailability and contaminant mobility of specific contaminants from soil to the soil dwelling earthworms, and the potential for contaminant movement to higher organisms (birds, mammals, fish, amphibians, reptiles, and insects) linked to worms in the food web.

A3.2 *Culture of Test Organisms*—Earthworms are obtained through either culture procedure (see A1.9.1.4) or ordering earthworms. A recent study has shown that reasonable control charts have been maintained with earthworms from an outside source (86).

A3.3 *Age*—Tests with *E. fetida* should use sexually mature fully clitellate earthworms (see A1.7).

A3.4 *Acclimation*—See 11.5, A1.4, and A1.9.1.4).

A3.5 *Test Specifications:*

A3.5.1 *Experimental Design*—Decisions concerning the various aspects of experimental design, such as the number of replicates, the number of test containers, and the mass of earthworms, should be based on the amount of tissue material needed for chemical analysis.

A3.5.2 *Test Material*—Test materials used have been primarily enriched dredged material. Soils used in this method are the following: soils collected from potentially contaminated sites, reference soils collected from uncontaminated sites, and a negative control material such as earthworm culture media for use in evaluating test acceptability.

A3.5.3 *Test Containers*—Test material is placed in transparent plexiglass cylinders 30 cm deep and 15 cm in diameter. The cylinder ends are closed with a 17-cm in diameter PVC and either 340 μ Nytex mesh or cotton muslin cloth. The bottom end is then placed in a 20-cm diameter plastic dish of test water

to allow water movement into the substrate and allow earthworms to move into areas of optimum moisture. (See Fig. A3.1.)

A3.5.4 Day (0) Test Initiation—A random sample of earthworms should be analyzed for the contaminant(s) of concern as a Day 0 background tissue sample. The Day 0 background tissue sample is used to determine contaminants present in earthworms prior to the test and should not be confused with any negative control or reference tissue samples which are exposed to test cylinders for the full 28 days and serve to determine test acceptability. If greater than 10 % mortality is seen in a negative control or reference test containers than that test is considered invalid and is rerun. If the test fails a second time it is assumed that the earthworms can not survive in the given soil and therefore contaminant bioaccumulation in the earthworm is not a concern. Prior to testing, earthworms are rinsed with test water, and placed on paper towels to remove excess water. On Day 0 the mass of earthworms needed for the particular chemical analysis procedures for the contaminant(s) of concern are added to the test cylinder. Test containers have accommodated up to 30 g (~75 earthworms)/cylinder (91).

A3.5.5 Day (28) Test Breakdown—On Day 28, earthworms are removed, rinsed with test water, blotted, counted, and weighed. Depuration of the earthworms is then recommended for 24 h on moist filter paper. Earthworms are then rinsed, reweighed, and frozen in preparation for chemical analysis.

A3.6 Feeding—Test materials used have been primarily enriched dredged material, therefore, not requiring an additional food source (3, 10, 85, 87, 88, 89, 90, 91). Soils with less nutrients tested with this procedure may require added food due to test length (92). Any food added would need to be chemically analyzed for concentrations of contaminant(s) of concern. (See A1.9.1.2 and A1.9.1.3)

A3.7 Quality Control Parameters—Temperature, pH, percent moisture, and salinity should be controlled and monitored throughout the test. Ideally these parameters should be the same as in the field, and within the range of the earthworms temperature, and pH requirements. Acceptable temperature range is from 10 to 29°C with a recommended range of 19 to

25°C. Acceptable pH range is between 4 and 10 (18). Recommended photoperiod is 24 h within 100 to 1080 lx. This is the same photoperiod suggested for the toxicity test. It is recommended to prevent earthworm escape, encourage maximum exposure to test material, and to discourage contact with container sides. (See Table A3.1.)

A3.8 Chemical Analysis:

A3.8.1 Test Material Analysis—All test materials should be analyzed for the contaminant(s) of concern prior to test initiation.

A3.8.2 Tissue Analysis—A random baseline tissue analysis is performed on Day 0 and all tissues exposed to test cylinders are analyzed on Day 28.

A3.8.3 Analytical Methodology—See Section 12.

A3.9 Test Evaluation—This bioassay has been used successfully in evaluating contaminant bioavailability and mobility on several projects (3, 10, 85, 87, 88, 89, 90, 91, 94). Data are reported in tables comparing whether the contaminant concentrations of Day 28 tissue exposed to the test soil are significantly different from the Day 0 tissue baseline analysis, and the Day 28 tissue exposed to the reference soil. If significantly different, the Day 28 tissue contaminant data are discussed as the magnitude above either the Day 0 tissue baseline analysis or the Day 28 tissues from the reference soil (that is, 2×, 5×, 10×). Other approaches for evaluating data include kinetics studies with estimate uptake, depuration rates, and time to steady state, lipid normalization and normalizing soil concentrations of non-ionic organics to TOC (see Guide E 1688). Analysis of field collected organisms is also an option.

A3.10 Test Variations—Variations on the above procedure have also been successfully used.

A3.10.1 An in-situ bioassay using the same procedure as above with a 7.5.1 polyethylene bucket with screen-covered holes in the base and lid to allow air and water but not earthworm exchange. Test containers were implanted 25 cm deep (soil level) and filled with the material removed from the hole (87).

A3.10.2 Another variation was developed with the recommendations to add a more realistic approach to field disposal site conditions by considering effects of natural site vegetation (93). This variation is conducted with Bermuda grass planted in the cylinders (91). The procedure differs as follows; On day 0, 1 gm of Bermuda grass seeds are spread over the cylinder surface. Seeds are covered with 1 mm of peat moss and lightly watered with RO water. Each cylinder received 125 mL of a

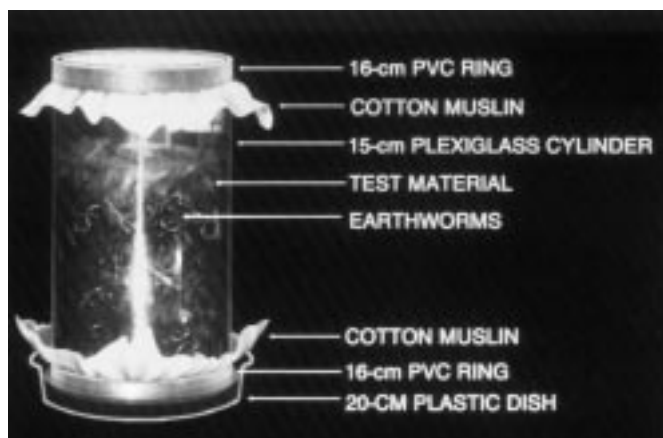


FIG. A3.1 Diagram of the Test Container for the Bioaccumulation Test (see A3.5.3)

TABLE A3.1 Test Specifications for the 28-Day *Eisenia fetida* Bioaccumulation Test

Test Duration	28 days
Biological Endpoint	contaminant accumulation
Temperature	same as field condition if within 10 to 29°C
Photoperiod	24 h/100 to 1080 lx
pH	same as field condition if within 4 to 10
% moisture	same as field condition
Salinity	same as field condition
Test Containers	plexiglass cylinders

dilute (600 mg/L of water) solution of soluble plant food (13-13-13), during the first two weeks to enhance seed sprouting. Excess water collecting in plastic trays was poured off. On Day 30 earthworms are added. On Day 60 Bermuda grass is harvested, earthworms are counted, weighed, and both are

prepared for chemical analysis. The following alterations are made in the temperature and lighting test conditions to promote grass growth: temperature 22°C (night) to 29°C (day), acceptable lighting for this study is 400 lux illumination for a period of 14 h light/10 h dark.

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