Designation: E1676 – 12

Standard Guide for Conducting Laboratory Soil Toxicity or Bioaccumulation Tests with the Lumbricid Earthworm *Eisenia Fetida* and the Enchytraeid Potworm *Enchytraeus albidus*[1]

This standard is issued under the fixed designation E1676; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This guide covers procedures for obtaining laboratory data to evaluate the adverse effects of contaminants (for example, chemicals or biomolecules) associated with soil to earthworms (Family Lumbricidae) and potworms (Family Enchytraeidae) 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) or on potworms in short to long-term tests (14 to 42 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) and for the species *Enchytraeus albidus* (see Annex A4). Methods described in this guide may also be useful for conducting soil toxicity tests with other lumbricid and enchytraeid 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), LOEC (lowest observed effect concentration) or as an ECx (concentration where x % reduction of a biological effect occurs. 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, 2x, 5x, 10x) (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] This guide is under the jurisdiction of ASTM Committee E50 on Environmental Assessment, Risk Management and Corrective Action and is the direct responsibility of Subcommittee E50.47 on Biological Effects and Environmental Fate.

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

Current edition approved Nov. 1, 2012. Published December 2012. Originally approved in 1995. Last previous edition approved in 2004 as E1676–04. DOI: 10.1520/E1676-12.

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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:*[2]

D653 Terminology Relating to Soil, Rock, and Contained Fluids

D4447 Guide for Disposal of Laboratory Chemicals and Samples

E380 Practice for Use of the International System of Units (SI) (the Modernized Metric System) (Withdrawn 1997)[3]

E943 Terminology Relating to Biological Effects and Environmental Fate

E1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses

E1383 Guide for Conducting Sediment Toxicity Tests with Freshwater Invertebrates (Withdrawn 1995)[3]

E1688 Guide for Determination of the Bioaccumulation of Sediment-Associated Contaminants by Benthic Invertebrates

E1706 Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Freshwater Invertebrates

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 E943 and Guide E1023. For an explanation of units and symbols, refer to Practice E380.

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 E1688.)

3.2.4 *bioaccumulation factor (BAF)*—the ratio of tissue residue to sediment or soil contaminant concentration at steady-state. (See Guide E1688.)

3.2.5 *bioaccumulation potential*—a qualitative assessment of whether a contaminant in a particular sediment or soil is bioavailable. (See Guide E1688.)

3.2.6 *bioconcentration*—the net assimilation of a substance by an organism as a result of uptake directly from aqueous solution. (See Guide E1688.)

3.2.7 *bioconcentration factor (BCF)*—the ratio of tissue residue to water contaminant concentration as steady-state. (See Guide E1688.)

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 E1688.)

3.2.9 *clitellum*—the fleshy "ring" or "saddle" of glandular tissue found on certain mid-body segments of oligochaete (Lumbricidae and Enchytraeidae) worms. It is the most visible feature of an adult earthworm or potworm 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 *enchytraeid*—potworm members of the Family Enchytraeidae of the Class Oligochaeta of the Phylum Annelida.

3.2.14 *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 3.2.27); however, depending on the nature of the test being implemented, site surface water or groundwater may also be utilized for hydration.

3.2.15 *lumbricid*—earthworm members of the Family Lubricidae of the Class Oligochaeta of the Phylum Annelida.

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

[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] The last approved version of this historical standard is referenced on www.astm.org.

3.2.17 *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.

3.2.18 *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.

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

3.2.20 *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.

3.2.21 *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.

3.2.22 *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 D653.)

3.2.23 *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.

3.2.24 *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.

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

3.2.26 *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.

3.2.27 *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 and the toxic effects on survival or sublethal endpoints for 4 to 42-day exposures to enchytraeids.

4.2 *Summary of Changes*—This current version of the standard is a revision of the E1676-97 version. Changes made since 1997 involve toxicity testing procedures for the Enchytraied potworm, *Enchytraeus albidus*. There has been an additional annex added (Annex A4) and the main document has been modified to include this species.

5. Significance and Use

5.1 Soil toxicity tests provide information concerning the toxicity and bioavailability of chemicals associated with soils to terrestrial organisms. As important members of the soil fauna, lumbricid earthworms and enchytraeid potworms have a number of characteristics that make them appropriate organisms for use in the assessment of potentially hazardous soils. Earthworms 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, 2**).[4] Enchytraeids contribute up to 5.2 % of soil respiration, constitute the second-highest biomass in many soils (the highest in acid soils in which earthworms are lacking) and effect considerably nutrient cycling and community metabolism (**3-5**). Earthworms and potworms accumulate and are affected by a variety of organic and inorganic compounds (**2-10, 11-14**). In addition, earthworms and potworms are important in terrestrial food webs, constituting a food source for a very wide variety of organisms, including birds, mammals, reptiles, amphibians, fish, insects, nematodes, and centipedes (**15, 16, 3**). A major change in the abundance of soil invertebrates such as lumbricids or enchytraeids, 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 and enchytraeid worms have been used in field and laboratory investigations in

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

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 **(7)** suggest that the selection of earthworm test species does not affect the assessment of a chemical's toxicity markedly. The sensitivity of various enchytraeid species has not been investigated in a comparable way so far, but ecological importance and practicability reasons favor strongly the selection of a species belonging to the genus *Enchytraeus*.

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 **(17)**; (*3*) has been studied extensively, producing a data pool on the toxicity and bioaccumulation of a variety of compounds **(2, 7, 8, 18-23)**; (*4*) has been approved for use in toxicity testing by the European Union (EU) and the Organization for Economic Cooperation and Development (OECD); and (*5*) has been used by the Environmental Protection Agency (EPA) for the toxicity screening of hazardous waste sites **(24)**.

5.2.2 The recommended enchytraeid test species is *Enchytraeus albidus* Henle 1837 (white potworm). *E. albidus* is one of the biggest (up to 15 mm) species of the oligochaete family Enchytraeidae and it is distributed world-wide **(25, 26)**. *E. albidus* is found in marine, limnic, and terrestrial habitats, mainly in decaying organic matter (seaweed, compost) and rarely in meadows **(4, 26)**. This broad ecological tolerance and some morphological variations might indicate that there are different races for this species. *E. albidus* is commercially available, sold as food for fish, can be bred easily in a wide range of organic waste materials and has a short life cycle (33 to 74 days; **27, 28**). *E. albidus* was studied in various tests, which covered a wide range of compounds **(28-30)**. In addition, it is currently under investigation for use in toxicity testing and soil quality assessment by the European Union (EU), the Organization for Economic Cooperation and Development (OECD), and the International Organization for Standardization (ISO). Other species of the genus *Enchytraeus* are also suitable, for example, *E. buchholzi* Vejdovsky 1879 or *E. crypticus* Westheide and Graefe 1992 (see Annex A4). Those species are true soil inhabitants and are smaller in size. Other species of *Enchytraeus* may be used, but they should be identified clearly and the rationale for their selection should be reported.

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 chemicals associated with soil by analysis of animal tissues for the chemicals being monitored. These results are useful for studying the biological availability of chemicals.

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.

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 chemical 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 chemicals 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, A1.9.5, and A4.10.8).

6.1.5 The addition of solvents to the test containers might obscure the adverse influence of chemicals 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 (that is, substances for which the Henry's constant or the air/water partition coefficient is greater than one, or substances for which the vapor pressure exceeds 0.0133 Pa at 25ºC) 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 (except in the case of the Enchytraeid Reproduction Test). 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 (except in the case of the Enchytraeid Reproduction Test).

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, Annex A3, and Annex A4.

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 D4447).

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 **(31-34)**, and recommended handling procedures **(35-39)** 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 D4447).

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 chemicals (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. In addition, a reference control (artificial or reference soil spiked with a compound with known toxicity at the concentrations(s) used) is desirable.

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 **(40)**, Eberhardt and Thomas **(41)**, Gilbert **(42)**, and ISO **(43)**.

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 chemicals 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 E1706 stored at 4 ± 2°C for no longer than eight 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. A 6.30-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 chemicals (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 grade[5] or better, unless technical or other grade material is specifically needed. Before a test is started, the following should be known concerning the test material: (*1*) identity and concentration of major ingredients and impurities; (*2*) water solubility in hydration water, log P_{ow}, and vapor pressure; (*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. Additional information on the fate of the test substance in soil is desirable.

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, can be 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 **(7, 21, 23, 44)** because of its high volatility and ability to dissolve many organic chemicals. Other water-miscible organic solvents, such as methanol or ethanol **(9)**, 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

[5] *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 *Analar 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.

amount of solvent is unacceptable if any treatment contained a concentration of solvent in that range.

9.5.3.3 For compounds insoluble in water and in organic solvents, 10 g of finely ground quartz sand should be mixed with the quantity of test substance to obtain the desired test concentration. This mixture of quartz sand and test substance should be added to the premoistened soil and thoroughly mixed by adding an appropriate amount of deionized water to obtain the moisture required as-described by OECD (**45**).

9.5.3.4 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 or a NOEC, the test concentrations should bracket the predicted LC50 or NOEC. 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 or NOEC 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.

9.5.4.3 If the test is intended to allow the calculation of the ECx (for example, EC_{10}, EC_{50}), the test concentrations should cover the whole range of potential effects. At least three replicates for each concentration and at least six replicates for the controls should be used. The spacing factor may vary, that is, less than two at low concentrations and more than two at high concentrations. 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 five concentrations of the test material that differ by a factor of ten.

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 and Annex A4); however, descriptions of additional species may be included in revisions of this guide. The use of these 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 and Annex A4).

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. Paragraphs A1.5 and A4.6 contain 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, but not potworms, should be cultured at the same temperature as that used for testing (see 11.5, A1.9.1.4, A4.5.2, and A4.10.7).

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 **(46)**. 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 moisture sample, 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 at least three replicates is hydrated with water, and replicates are placed into test containers (see Annex A1 and Annex A4).

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 **(9)**.

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 **(47)** found that temperature variations between 10 and 26°C had little influence on the toxicity of the chemicals. In the case of *E. albidus*, any temperature higher than 22°C should be avoided since reproduction can be affected. The test temperature depends on the species used (see Annex A1 and Annex A4). 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 seven-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 **(10, 19, 48)** or 48 h **(49, 50)** for a purging time period. Although Stafford and McGrath **(50)** 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 **(51)** 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 (except in the case of the Enchytraeid Reproduction 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, A1.9.5, and A4.10.8).

11.8 *Light*—To maximize exposure, continuous lighting **(21, 52)** 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 **(52)**. In the case of the Enchytraeid Reproduction Test, a controlled light-dark cycle of long-day conditions (preferabley 16 to 8 h at 400 to 800 lux in the area of the test vessels) is desirable.

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 and A4.10.13.2). Toxicity 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 chemical concentrations accumulated in earthworm tissues (see A3.8.3).

12. Analytical Methodology

12.1 Chemical and physical data for soil and tissue material should be obtained using appropriate ASTM International standards whenever possible. For those measurements for which ASTM International standards do not exist or are not sufficiently sensitive, methods should be obtained from other sources, for example, EPA **(53)**.

12.2 Concentrations should be measured for (*1*) chemicals in batches of soil, (*2*) test materials in stock solutions, and (*3*) chemicals 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 chemicals of concern should be obtained from appropriate sources **(54)**.

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) or the Enchytraeid Reproduction Test (see A4.10.7).

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) except in the case of the Enchytraeid Reproduction Test.

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 and A4.10.10).

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, A3.7, and A4.10.7).

13.1.11 The negative control soil organisms did not survive, grow, or reproduce as required for the test species (see 9.2, Annex A2, and Annex A4).

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 **(55-58)** 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-Karber, 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. In a case in which few data are available, the geometric mean (the root of the multiplication of LC0 and LC100) or a nonlinear interpolation may be used to determine the LC50 or EC50.

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 **(59)**. The amount of effect that occurred at this concentration should be considered.

14.5 All exposure concentration effects (or field stations) can be compared with the appropriate control effects (negative or solvent) by using mean separation techniques, orthagonal 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.

14.6 Bioaccumulation test results are reported as the magnitude of chemical 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 E1688). Analysis of field collected organisms is also an option.

14.7 Three designs are possible for the test performance (the concentrations should be spaced by a factor not exceeding two): *(1)* For determination of the NOEC, at least five concentrations in a geometric series should be used. Four replicates for each treatment plus eight controls are recommended. *(2)* For determination of the ECx (for example, EC_{10}, EC_{50}), twelve concentrations should be used. Two replicates for each treatment and six control replicates are recommended. The spacing factor may vary, that is, less than two at low concentrations and more than two at high concentrations. *(3)* For the mixed approach, eight concentrations in a geometric series should be used. Four replicates for each treatment plus eight controls are recommended. This combined approach allows for determination of both the NOEC and ECx.

14.8 The ECx approach can be used for the Enchytraeidae reproduction test described in Annex A4. To compute any ECx value, the per-treatment means are used for regression analysis after an appropriate dose-response function has been obtained. An ECx is calculated by inserting a value corresponding to x % of the control mean into the equation obtained by regression analysis. The 95 % confidence limits are calculated according to Fieller **(60)**. Alternatively, the results can be expressed as percentages of inhibition relative to the control. In these cases, the normal (logistic) sigmoid curve can often be fitted to the results by use of the probit regression procedure **(61)**. But if the hormesis phenomenon has been observed, probit analysis should be replaced, for example, by a four-parameter logistic or Weibull function fitted by a nonlinear regression procedure.

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); known chemical and physical properties; and, if necessary, application of the test compound.

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 chemicals, 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.

16. Keywords

16.1 bioaccumulation tests; earthworm; potworms; soil toxicity

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 (**62**); (*2*) it reproduces prodigiously (**63**); (*3*) it is collected easily from natural sources or cultured in the laboratory (**2, 52, 64, 65**) ; and (*4*) data on its survival, growth, and reproduction can be obtained in toxicity tests (**64, 66-71**). Stafford, et al (**72**) 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 (**6**), contaminated field soils (**8, 73**), activated sludge (**67**), sediment (**74**), and cow manure (**23**).

A1.2 *Life History*—The life-cycle of *E. fetida* can be divided into three distinct phases, according to Jefferies and Audsley (**75**): (*1*) the cocoon phase, consisting of an egg cocoon that can produce from one to eleven hatchlings under laboratory conditions (**76**); (*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 (**76**) 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 (**77**) 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 (**1, 78**) that rarely inhabits agricultural soils but is found in compost piles, manure piles, and other disturbed sites rich in organic matter (**18**). 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) (**8**).

A1.2.2 The specific sources of nutrition for *E. fetida* are not well understood, but Morgan (**79**) 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* (**80**).

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 (**76**) 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 (**78**) 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 (**81**) (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 (**82**) and Valembois, et al (**83**) demonstrated biochemical differences between the two forms. Oien and Stenersen (**84**) and Jaenike (**85**) conducted electrophoretic work that led them to consider the two forms as separate species, and Sheppard (**86**) 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 (**78**) 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 (**52**) and Greene, et al (**24**). *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 ± 3°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 (**87**), (*2*) dairy waste sludge cake (**88**), (*3*) horse manure (**10**), (*4*) activated sludge and horse manure (**89**), and (*5*) commercial alfalfa pellets (*Medicago sativa*) (**73**). 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 (**62**) 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 g/cm^3. For example, 350 adult worms weighing 700 mg each would be equal to 0.03 g/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 **(77)**. However, bait farms may contain mixtures of *E. f. andrei* and *E. f. fetida*. Reynolds **(77)** and Fender **(81)** report that *E. fetida* can be found in manure piles and usually not far from human activity. Fender **(81)** (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 **(81)** 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 **(8, 10, 24, 64, 73)**. 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 **(7)** 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 **(64)** 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 **(24)** 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 **(8)**. 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 **(22, 73, 90, 91)**, with an evaluation at seven days being optional. Tests investigating the bioaccumulation of xenobiotics in field-collected soils have been conducted for 56 days **(8)** 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, rigidness, and flaccidness can be used successfully in toxicity testing **(92-94)**.

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 **(68)**. 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 (**23**), Malecki, et al (**68**), Van Gestel, et al (**71**), and Venter and Reinecke (**95**) 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 (**96**).

A1.9.1.4 Although Heimbach and Edwards (**47**) 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 (**7, 21, 44, 64, 66, 73, 91, 92**). Van Gestel, et al (**96**) report that a temperature range from 20 to 25°C is optimal for *E. fetida*. Kaplan, et al (**89**) 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 (**47**) or above 29°C (**89**). (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 (**24**). 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 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. Because of 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 (**24**). Measuring the water potential (**97**), 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 (**8**) 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 (**47**) 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 E1383). 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

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

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 **(8)** 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 **(52)**. 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 **(9)**, segmental swelling **(9, 64, 94)** , lesions/ulcers **(9, 64, 94)**, coiling **(9, 64, 92)** , shortening/stiffening **(9, 64, 94, 98)** , flaccid/elongated **(9, 94)**, segmental constrictions **(92, 94)**, and tail end autotomy **(99)**. 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 **(52, 45)**. The following constituents are mixed together on a dry weight basis:

(*1*)	Canadian sphagnum (*Sphagnum*) 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 USING 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 (**100**). Earthworm bioassays are an important tool to determine soil toxicity, and potential bioaccumulation with respect to the chemical availability in soil. A method to determine chemical bioavailability and mobility using the earthworm *Eisenia fetida* has successfully evaluated the following chemicals; metals, PAHs, PCBs, pesticides, and butyltins (**2**, **16**, **101**, **102-106**, **107**). The bioaccumulation assay adds information on bioavailability and contaminant mobility of specific chemicals 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.

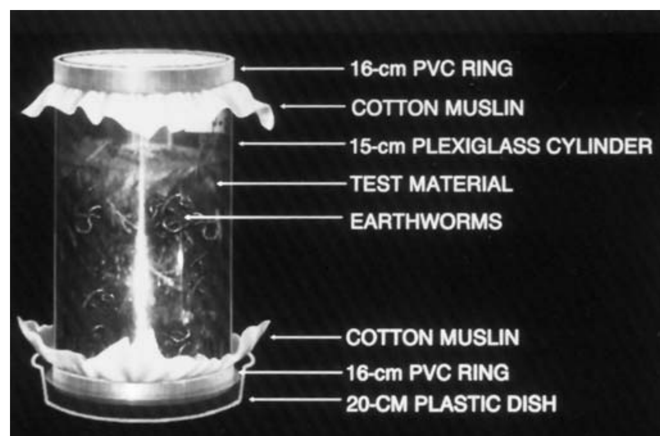

FIG. A3.1 Diagram of the Test Container for the Bioaccumulation Test (see A3.5.3)

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 (**108**).

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-μm 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 chemical(s) of concern as a Day 0 background tissue sample. The Day 0 background tissue sample is used to determine chemicals present in earthworms before 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 chemical(s) of concern are added to the test cylinder. Test containers have accommodated up to 30 g (~75 earthworms)/cylinder (**106**).

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 (**2**, **16**, **101**, **102**, **103**, **104**, **105**, **106**). Soils with less nutrients tested with this procedure may require added food due to test length (**109**). 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 (**24**). 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 chemical(s) of concern before 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 (**2**, **16**, **101**, **102**, **103**, **104**, **105**, **106**, **107**). Data are reported in tables comparing whether the chemical 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 chemical 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 E1688). 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 (**102**).

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 (**109**). This variation is conducted with Bermuda grass planted in the cylinders (**106**). 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 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.

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

A3.10.3 The more recent variation of the test (OECD Guideline 317, 2010 (**110**)) describes a method that consists of two phases: the uptake (exposure) phase and the elimination (post-exposure) phase. A single concentration is used. During the uptake phase, replicated groups of worms are exposed to soil which has been spiked with the test substance. In addition to the test animals, groups of control worms are held under identical conditions without the test substance. The dry weight and lipid content of the test organisms are measured. This can be done using worms of the control group. Analytical background values (blank) can be obtained by analyzing samples of the control worms and soil. For the elimination phase, the worms are transferred to a soil free of the test substance. An elimination phase is always required unless uptake of the test substance during the exposure phase has been insignificant. An elimination phase provides information on the rate at which the test substance is excreted by the test organisms. If a steady state has not been reached during the uptake phase, the determination of the kinetic parameters – kinetic bioaccumulation factor BAF_k, uptake and elimination rate constant(s) – should preferably be based on simultaneous fitting of the results of the uptake and elimination phases. The concentration of the test substance in/on the worms is monitored throughout both phases of the test. The minimum number of treated replicate vessels should be three per sampling point. The total number of replicates prepared should be sufficient to cover all sampling times during the uptake and the elimination phase.

(1) Feeding should be included in the tests using a soil with low total organic carbon content is used. When an artificial soil is used, a weekly feeding rate (that is, the worms should be fed once a week) of 7 mg of dried dung per g soil dry weight is recommended for earthworms, and a weekly rate of 2-2.5 mg of ground oat flakes per g soil dry weight is recommended for enchytraeids (Bruns et al., 2001a (**111**)). The first food ration should be mixed with the soil immediately before the test organisms are added.

(2) During the uptake phase, measurements are made at sampling times up to 14 days (enchytraeids) or 21 days (earthworms) until the steady-state is reached (Bruns et al., 2001a, b; (**111**, **112**), Sousa, et al., 2000 (**113**)). The steady state occurs when a plot of the concentration in worms against time is parallel to the time axis, and three successive concentration analyses made on samples taken at intervals of at least two days do not vary more than ± 20% of each other based on statistical comparisons (for example, analysis of variance, regression analysis).

(3) The elimination phase consists of transferring the test organisms to vessels containing the same substrate without the test substance. During the elimination phase, measurements are made at sampling times during 14 days (enchytraeids) or 21 days (earthworms) unless earlier analytical determination showed 90% reduction of the test substance residues in worms. The concentration of the test substance in the worms at the end

of the elimination phase is reported as non-eliminated residues. The steady state bioaccumulation factor (BAF_{ss}) is calculated preferably both as the ratio of the concentration in worms (C_a) and in the soil (C_s) at apparent steady state, and as a kinetic bioaccumulation factor, BAFK, as the ratio of the rate constant of uptake from soil (k_s) and the elimination rate constant (k_e) assuming first-order kinetics. If first-order kinetics is obviously not applicable, other models should be employed.

(4) The uptake rate constant, the elimination rate constant (or constants, where other models are involved), the kinetic bioaccumulation factor (BAF_K), and where possible, the confidence limits of each of these parameters are calculated from computerized model equations described in OECD Guideline 317(**110**). The goodness of fit of any model can be determined from, for example, the correlation coefficient or the coefficient of determination (coefficients close to one indicate a good fit) or chi-squared. Also the size of the standard error or confidence limit around the estimated parameters may be indicative of the goodness of fit of the model.

A4. ENCHYTRAEIDAE REPRODUCTION TEST

A4.1 *Scope*:

A4.1.1 This standard annex of Guide E1676 covers the additional or modified procedures required to perform an Enchytraeid Reproduction Test (ERT) from Guide E1676 for Conducting a Laboratory Soil Toxicity or Bioaccumulation Test with the Lumbricid Earthworm *Eisenia fetida*.

A4.2 *Significance* —*Enchytraeus albidus* (Henle, 1837), Oligochaeta, has been selected (together with other species of the genus *Enchytraeus*) as a test species for the following reasons (**28-30, 114**): *(1)* it has a short generation time; *(2)* it reproduces very well in the laboratory; *(3)* it can easily be kept and cultured in the laboratory; *(4)* data on it's survival, growth, and reproduction are available from the literature; *(5)* it is a representative of an ecologically relevant family of soil organisms, especially in acidic soils (**3**). *E. albidus* seems to be sensitive towards different anthropogenic stress factors like pesticides or heavy metals (**11-14**). It has been used successfully as a laboratory test organism in many testing media, for example, artificial soil (**28**), contaminated field soils (**115**), sediment (**116**), agar (**29**), and water (**117**). Basic information on the ecology and ecotoxicology of enchytraeids in the terrestrial environment can be found in Refs (**3, 4, 5, 28, 118, 119, 120**).

A4.3 *Life History* —Like *E. fetida*, the life cycle of *E. albidus* and other species of this genus can be divided into three phases: *(1)* the cocoon phase, *(2)* the juvenile (immature) phase, *(3)* and the adult (mature) phase (see A1.2). Its life cycle is short as maturity is reached between 33 days (at 18°C) and 74 days (at 12°C); that is, from freshly deposited cocoon through clitellate worm and deposition of the next generation of cocoons (**28, 29**). In the case of *E. albidus*, reproduction is strongly inhibited at temperatures higher than 22 to 25°C, whereas other, mainly smaller, species of the same genus produce cocoons at temperatures between 25 and 30°C (**121**). Despite the fact that *E. albidus* individuals have been kept under optimal laboratory conditions for more than 1.5 years, an age of less than one year is more usual in the field. The length of an adult *E. albidus* is usually 15 mm, but can vary, depending on nutrition, between 10 and 35 mm.

A4.3.1 *E. albidus* is found in marine, limnic, and terrestrial habitats worldwide, mainly in decaying organic matter (seaweed, compost) and rarely in meadows (**4, 122**). The worms can be kept for up to four days in water (**117**). In general, many species of the genus *Enchytraeus* are known to be among the first enchytraeids colonizing new biotopes or belong to the dominant species at disturbed sites (for example, urban soils) (**122**), while others can be found in all other terrestrial habitats as well as in limnic and marine sediments (**4, 25, 26**).

A4.3.2 The rate of organic matter consumption in the laboratory for *E. albidus* is not known. The specific sources of nutrition for this species are not well understood. It is known that the worms can take up amino acids directly from the surrounding aquatic phase (**43**), that they feed on microorganisms (especially bacteria) from decaying organic material (including dead earthworms), and that they are even able to divide leaves and digest this nearly intact plant material (**123**). Often mineral debris is taken up along with the organic material.

A4.4 *Taxonomy* —The test species *E. albidus* belongs to the genus *Enchytraeus* sp. (order Oligochaeta, class Clitellata, phylum Annelida). Henle (1837) scientifically described it as the first member of the new family Enchytraeidae. In the meantime, approximately 116 species have been described in the genus *Enchytraeus* sp. worldwide, but many of these descriptions are not valid. The taxonomic status of nearly all *Enchytraeus* species has to be revised. Species determination is only possible morphologically, if at all, with adult animals since juveniles do not have sexual organs like sperm ducts. *E. albidus* is not only the type species for the whole family but also the best known species, which has been used in ecotoxicology, physiology, biochemistry, and genetics for more than 50 years.

A4.4.1 Some morphological features of *E. albidus* can be quite variable, especially the spermatheca. So, considering its wide geographical and ecological range, it has been proposed that it is actually not one but a group of closely related species. However, no evidence of this has been found up to now, e.g. by means of biochemical or genetic methods. *E. albidus* shows some morphological features which are easy to detect even for

those not experienced in enchytraeid taxonomy. This species can be distinguished quite easily from all other species in the genus *Enchytraeus*: *(1)* it is the largest species of this genus (except some subantarctic species), *(2)* it is the only terrestrial species having four setae per bundle in at least some segments (usually in the head region), and *(3)* it has a very unique and quite and has an obviously long seminal duct, which extends through the clitellum region and several segments beyond (**27, 28**).

A4.4.2 When in cultures, because of slight differences in their ecological demands, *E. albidus* is outnumbered by other, usually smaller and faster reproducing species of this genus. Such animals can only be determined by specialists, since very often not only morphological but also enzymatic parameters are necessary. Therefore, when another species has to be selected for testing purposes, only worms from a well-defined source should be used for this purpose. Some helpful guidance on species determination can be found in Nielsen and Christensen (**26**) and Bougouenec and Giani (**121**).

A4.5 *Culture of Test Organisms*—Since the beginning of this century, at least, *E. albidus* was bred as fish food to be used in aquaria(**114**). Even cultures on a "field scale" were recently considered in Canada, Russia, and France (for example, **124**).

A4.5.1 *E. albidus* (as well as other *Enchytraeus* species) can be bred in large plastic boxes (for example, 30 by 60 by 10 cm) filled with a mixture of artificial soil and natural, uncontaminated garden soil. Compost material should be avoided since it could contain toxic substances like heavy metals. Fauna should be removed from the breeding soil before use. Pure artificial soil can also be used but the reproduction rate could be slower compared to that obtained with mixed substrates. The substrate should have a pH of 6.0 ± 0.5.

A4.5.2 The culture should be kept in an incubator at a temperature of 15 ± 2°C without light. A temperature higher than 23°C should be avoided. The artificial/natural soil moisture should be moist but not wet. When the soil is gently pressed by hand, only small drops of water should appear. In any case, anoxic conditions should be avoided (e.g. if a lid is used, the number of lid holes should be high enough). The breeding soil can be aerated by carefully mixing it once per week.

A4.5.3 The worms can be fed approximately twice a week with a proper amount of oatmeal flakes (rolled oats) which are strewn on the soil surface or carefully mixed into the substrate at least every two weeks. If food from the last feeding date remains on the soil surface, the amount of food given should be adjusted accordingly. If fungi grow on the remaining food, it should be replaced by a new quantity of rolled oats. From time to time, the rolled oats can be supplemented with commercially purchased vitamins, milk and cod liver oil. After three months, the animals can be transferred into a freshly prepared culture or breeding substrate. No carrying capacities have been calculated so far.

A4.5.4 The rolled oats, which should be stored in sealed vessels, can be autoclaved or heated before use to avoid infections by flour mites (for example, *Glyzyphagus* sp., Astigmata, Acarina) or predaceous mites (for example, *Hypoaspis* (*Cosmolaelaps*) *miles*, Gamasida, Acarina). None of these animals in low numbers appears to be a problem for healthy worms. After this procedure, the food can be ground up so that it can easily be strewn on the soil surface. Another possible food source is baker's yeast or the fish food "Tetramin."

A4.5.5 In general, the culturing conditions are sufficient if worms *(a)* do not try to leave the substrate, *(b)* move quickly through the soil, *(c)* exhibit a shiny outer surface without soil particles clinging to it, *(d)* are more or less whitish colored, and *(e)* if worms of different ages are visible. Generally, worms can be considered to be healthy if they reproduce continuously.

A4.6 *Obtaining Brood Stock*—*E. albidus* starter cultures can be obtained from *(1)* laboratories or universities working in soil ecology and *(2)* local aquarium stores. In the latter case, an expert should confirm species determination.

A4.7 *Age*—The animals used in the tests should be adult worms. They should have eggs (white spots) in the clitellum region, and they should have approximately the same size (≈1 cm). Synchronization of the breeding culture is not necessary.

A4.8 *Handling* —*E. albidus* 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.

A4.9 *Selection and Acclimatization*—Before testing, the enchytraeids should be acclimated to the soil used for the tests under the test conditions (including feeding) for at least 24 h. A higher number of adult worms than that needed for performing the test is used. At the end of the acclimation period, only worms with eggs and showing no behavioral anomalies (for example, trying to escape from the soil) should be selected for the test. The selected worms are placed in a petri dish filled with a small amount of water to be observed with a stereomicroscope and the animals that have no eggs are discarded. Freshwater is preferred to demineralized water or tap water (possible copper contamination) which could be harmful to the enchytraeids. The other organisms living in the cultures such as mites, should also be removed.

A4.10 *Toxicity Test Specifications*:

A4.10.1 *Introduction*—This test is designed to assess the effects of chemicals on the reproductive output of the enchytraeid worm (*Enchytraeus albidus*). It is based principally on a method developed by the Umweltbundesamt, Germany (**28**). Other methods for testing the toxicity of chemicals to Enchytraeidae and other earthworms have also been considered (**29, 30**). Adult enchytraeid worms are exposed to a range of concentrations of the test substance mixed in an artificial soil. The test can be divided into two steps: *(a)* a range-finding test in which mortality is the main endpoint assessed after two weeks exposure and *(b)* a definitive reproduction test in which the total number of juveniles produced by parent animals and the survival of parent animals are assessed. The test duration is

six weeks. After the first three weeks the adult worms are removed and morphological changes (for example,. open wounds) are recorded. After an additional three weeks, the number of offspring, hatched from the cocoons, is counted. The reproductive output of the animals exposed to the test substance is compared to that of the control(s) to determine the no observed effect concentration (NOEC). As far as possible, the data are also analyzed using a regression model to estimate the concentration that would cause a x % reduction in reproductive output, that is, ECx (for example, EC_{10}, EC_{50}).

A4.10.2 *Design for the Range-Finding-Test*—When necessary, a range-finding test should be conducted with five concentrations of the test substance. One replicate for each treatment and the control is desirable. The main endpoint is mortality.

A4.10.2.1 The test duration is two weeks. At the end of the test, mortality of the worms should be assessed by carefully searching the substrate for surviving individuals (for example, using a spatula). An animal is recorded as dead if it does not respond to a gentle mechanical stimulus to the front end. Moreover, changes in behavior (for example, inability to dig into the soil; lying motionless against the glass wall of the test vessel) and in morphology (for example, open wounds), should be recorded. Likewise, the presence of juveniles can be observed by using the staining method (see A4.10.9). This will help select the test concentrations for the definitive test.

A4.10.2.2 Probit analysis **(61)** should be applied to determine the LC_{50}. In case of failure (for example, if data from less than three concentrations with partial kills are available), alternative methods can be used such as moving averages **(60)** or simple interpolation (for example, geometrical mean of LC_0 and LC_{100}, as computed by the square root of LC_0 multiplicated by LC_{100}).

A4.10.2.3 The LC_{50} should be used to determine the concentration range for the definitive test. The NOEC or the EC_{10} for reproduction are assumed to be lower than the LC_{50} by a factor up to ten. However, this is an empirical relationship and it might be different in a given case. Therefore, additional endpoints or observations or both in the range-finding test, such as the occurrence of juveniles, can help refine the test concentration range to be used for the definitive test.

A4.10.2.4 If a more accurate determination of the LC_{50} is required, the test should be performed using eight concentrations of the test substance, with four replicates for each test concentration and eight replicates for the controls.

A4.10.3 *Definitive Reproduction Test*—The endpoint is fecundity (for example, the number of juveniles produced). As in the range-finding test, all other harmful signs should be recorded. Three options for the design for the definitive reproduction test are described in 14.7.

A4.10.3.1 Ten adult worms per test vessel should be used. The animals are fed at the beginning of the test and then once a week. After 21 days, living adult worms are counted and changes in behavior (for example,. inability to dig into the soil; lying motionless against the glass wall of the test vessel) and in morphology (for example, open wounds) should also be recorded. Then, all adult worms are removed as in A4.10.2.1. The test soil (that is, without the parent worms and containing the cocoons laid down) is incubated for three additional weeks under the same test conditions, including food supply until Day-28.

A4.10.3.2 After six weeks, the newly hatched worms are isolated and counted using Bengalred staining (see A4.10.9; 125). Wet (but not heat) isolation techniques have proved to be suitable **(28, 45, 119)** and may also be used. However, the method using Bengalred is preferred since the wet isolation from a soil substrate is hampered by the clay particles that make the water turbid.

A4.10.3.3 If no effects are observed at the highest concentration in the range-finding test (that is, 1000 mg/kg), the reproduction test can be performed as a limit test, using 1000 mg/kg to demonstrate that the NOEC or the EC_{10} for reproduction is greater than this value. The number of replicates should be eight for both the test concentration and control.

A4.10.4 *Equipment*—The test vessels should be made of glass or other chemically inert material. The test vessels are glass jars with glass lids (volume: 0.20 to 0.25 L; diameter: ≈6 cm). The lids allow for air exchange and they also reduce water evaporation. Normal laboratory equipment and especially the following should be used: drying cabinet; stereomicroscope; pH and lux meters; suitable accurate balances; adequate equipment for temperature control; adequate equipment for humidity control; incubator or small room with air conditioner; jewelers tweezers, hooks, or loops; and photo basins with ribbed bottoms.

A4.10.5 *Test Substrate*—Other potential test substrates are *(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.

A4.10.5.1 The composition of artificial soil is described in detail in Annex A2 **(45)**. The dry constituents of the soil are mixed thoroughly (for example, in a large-scale laboratory mixer). This should be done about one week before starting the test. The mixed soil should be stored for at least two days to equilibrate/stabilize the acidity. For the determination of pH, a mixture of soil and 1M KCl solution in a 1:5 ration is used. If the pH value is not within the required range (6.0 ± 0.5), a sufficient amount of $CaCO_3$ is added or a new batch of soil is prepared.

A4.10.5.2 The maximum water-holding capacity (WHC) of the artificial soil should be determined. One or two days before starting the test, the dry artificial soil is moistened by adding enough deionized water to obtain approximately half of the final water content, that is, 40 to 60 % of the maximum WHC (corresponding to 50 ± 10 % moisture dry mass). At the start of the test, the premoistened soil should be divided into as many batches as the number of test concentrations and controls used for the test, and the moisture content should be adjusted to 40 to 60 % by using the solution of the test substance or by adding distilled or deionized water or both. The moisture content should be determined at the beginning and at the end of the test (at 105°C). It is be optimal for the worms' life (the moisture can also be checked as follows: when the soil is gently squeezed in the hand, small drops of water should appear between the fingers).

A4.10.5.3 *Effect of Grain Size, Organic Carbon, and Moisture on the Test Organisms*—The potential effects of these soil properties on test organisms are not known. This limitation is especially important when using field-collected soils for which no reference control soil (that is, an uncontaminated soil having the same properties as the test soil) is used.

A4.10.6 *Test Groups and Controls*—For each test concentration, an amount of test soil corresponding to 20-g dry weight should be placed into the test vessel. Controls, without the test substance, are also prepared. Food is added according to A4.10.8. In each test vessel, ten worms should be placed carefully on the soil surface (for example, using jeweler's tweezers, hooks, or loops). The collected worms are randomly allocated to test vessels. The number of replicates for test concentrations and for controls depends on the test design used. All test vessels should be randomly placed in the incubator and they should be moved every week.

A4.10.6.1 If a solvent is used for application of the test substance, one control series containing the solvent should be run in addition to the test series. The solvent or dispersant concentration should be the same as that used in the test vessels containing the test substance (see 9.5.3.4). Alternativly, only the highest solvent concentration can be tested.

A4.10.7 *Test Conditions*—The test temperature should be 20 ± 2°C. To avoid worms escaping from the soil, the tests are carried out under controlled light-dark cycle of long-day conditions (preferably 16 to 8 h at 400 to 800 lux in the area of the test vessels).

A4.10.7.1 The vessels should be covered with glass lids which help reduce water evaporation. To check the soil humidity, the vessels should be weighed at the beginning of the test and furthermore once a week, and the weight loss should be replenished with the appropriate amount of deionized water. Loss of water can also be diminished by keeping a high air humidity (>80 %) in the test incubator.

A4.10.7.2 The moisture content and the pH should be measured at the beginning and the end of both the range-finding test and the definitive test. This should be done using an additional sample of the test soil containing no worms. The same amount of food as in the other vessels should be added to these additional vessels at the beginning of the test; indeed, the measured parameters may be influenced by the soil microbial activity. It is not necessary to add food to these vessels during the test.

A4.10.8 *Feeding*—Any food capable of maintaining the enchytraeid population can be used. Commercially purchased rolled oats, preferably autoclaved before use to avoid microbial contamination (heating is also appropriate), were found to be suitable. For each test vessel, the first feeding should be made by mixing 50 mg of ground rolled oats with the soil containing the test substance before placing the worms. Afterwards, weekly food supplies, consisting of 25 mg of ground rolled oats per vessel, should be given, except after 28 days (feeding is not necessary since the juveniles are too small), by putting the food on the surface of the soil taking care not to injure the worms. To reduce fungal growth, the oats flakes should be sunk into the soil (for example, small pieces of soil can be moved to the top of the oat flakes). The flakes should not be completely incorporated, since this procedure might harm the worms. In case the worms do not consume the whole food provided, food supply should be reduced accordingly to avoid fungal growth or molding.

A4.10.9 *Isolating Techniques for Juvenile Worms:*

A4.10.9.1 *Staining with Bengalred*—This method, originally developed in limnic ecology, was first proposed for the counting of juvenile enchytraeids in the enchytraedae reproduction test by W. de Coen (**125**) Independently, a modified version (Bengalred mixed with formaldehyde instead of ethanol) was developed by RIVM Bilthoven (**30**). At the end of the definitive test (thati is, after six weeks), the artificial soil in the test vessels should be transferred to a shallow container (for example, a Bellaplast vessel or to a photo basin with ribbed bottom) and the juveniles are fixed with ethanol (approximately 5 mL per replicate/vessel). Then the vessels should be filled with water up to a layer of 1 to 2 cm. Afterwards, a few drops (200 to 300 mL) of Bengalred (1 % solution in ethanol) should be added (0.5 % eosin might be an alternative) and the two components are mixed carefully. After 12 h, the worms are completely reddish colored. Now it is very easy to count them because they are lying on the surface of the substrate. Another possibility is to press the substrate/alcohol mixture through a sieve (mesh size: 0.250 mm) before counting the worms. The kaolinite, the peat, and some sand grains are lost and the reddish colored worms are easier to see. The use of illuminated lenses (lens size at least 100 by 75 mm; magnification factor 2 to 3×) also facilitates counting the already reddish juveniles. Thanks to this improvement, the counting time is reduced to a few minutes per vessel. Using the staining method, the vessels of one test can be assessed by a single person within one day (maximum two days) some hours or days after the end of the test.

A4.10.9.2 *Wet Removal of Juvenile Worms* (**118, 119**)—The removal of juvenile worms should be started immediately after the end of the test. The artificial soil of each test vessel should be placed into a common plastic or stainless steel sieve. The sieves are put in plastic bowls without touching the bottom. The bowls are carefully filled up with water until the samples in the sieves are completely under the water surface. To ensure a recovery rate of more than 90 %, the removal should occur within three days at 20 ± 2°C (that is, the worms have enough time to move from the soil through the sieve into the water). Once the worms are isolated, the sieves are removed and the water (except for a small amount) is slowly decanted. The sediment at the bottom of the bowls should not be disturbed. Then the plastic bowls are shaken slightly to suspend the soil in the overlying water, which is transferred to a petri dish. After clarification of the water (that is, the soil particles have settled), the enchytraeids can now be collected out of the petri dish under a stereomicroscope using a softsteel forceps.

A4.10.9.3 *Flotation*—Alternatively according to a note by R. Kuperman (U.S. Army), the following procedure is also possible (**126**): After fixing the content of a test vessel with ethanol, the artificial soil is flooded with Ludox (AM-30 colloidal silica, 30 wt. % suspension in water) up to 10 to 15 mm above the soil surface. After thoroughly mixing the soil

with the flotation agent, the juvenile worms floating on the surface can easily be counted after 2 to 3 min.

A4.10.10 *Test Acceptability Requirements*—For the test to be valid, the following performance criteria must be met in the controls: *(1)* the mortality does not exceed 20 % at the end of the range-finding test and after the first three weeks of the reproduction test, *(2)* the average number of juveniles is higher than 25 per test vessel at the end of the test, assuming that 10 adult worms per test vessel were used, and *(3)* the coefficient of variation around the mean number of juveniles is not higher than 50 % at the end of the reproduction test.

A4.10.11 *Reference Substance*—A reference substance should be tested once a year or possibly included in the test series. A suitable reference substance is carbendazim, which has been shown to affect survival and reproduction of enchytraeids (**14**). The EC_{50} for reproduction should be in the range of 1.2 ± 0.8 mg a.i./kg dry mass (**28**). If a positive toxic standard is included in the test series, one concentration is used and the number of replicates should be the same as that in the controls, that is, eight replicates. For carbendazim, the testing of 1.2 mg a.i./kg dry weight (tested as liquid formulation) is recommended.

A4.10.12 *Performance with Other Enchytraeus Species than E. albidus:*

A4.10.12.1 *Selection of Species*—Species other than *E. albidus* may be used but the test procedure and the validity criteria should be adapted to provide suitable test conditions. Many *Enchytraeus* species are readily available and can be satisfactorily maintained in the laboratory. Therefore, the most important criterion for selecting an *Enchytraeus* species other than *E. albidus* is ecological relevance and, additionally, comparable sensitivity. There may also be formal reasons for a change of species. In countries in which *E. albidus* does not occur and cannot be imported (for example, because of quarantine restrictions), other *Enchytraeus* species may be used. Potential candidates are listed in the following.

A4.10.12.2 *Enchytraes crypticus (Westheide & Graefe 1992)*—In recent years, this species has often been used in ecotoxicological studies because of the simplicity of its breeding and testing (**30, 115**). However, its individual size is small, which makes handling more difficult than with *E. albidus* (especially before implementation of the staining method). Additionally, it was only described from earthworm cultures. Since this species has not been found to exist with certainty in the field up to now, its ecological requirements are not known.

A4.10.12.3 *Enchytraeus buchholzi (Vejdovsky 1879)*—This name probably covers a group of closely related species which are morphologically difficult to distinguish. Therefore, its use is not recommended until the animals used in a test are clearly described. From an ecological standpoint, these animals are usually found in meadows and disturbed sites like roadsides.

A4.10.12.4 *Enchytraeus luxurious (Schmelz and Collado, 1999)*—U. Graefe (Hamburg) found this species for the first time in a meadow close to St. Peter-Ording (Schleswig-Holstein, Germany). Because of its size, it could be a good alternative to *E. albidus*.

A4.10.12.5 *Enchytraeus bulbosus (Nielsen and Christensen 1963)*—This species has hitherto been reported from German and Spanish mineral soils, where it is common but usually not very abundant. In comparison to other small species of this genus, it is relatively easy to determine. Additionally, *E. bulbosus* seems to be easy to culture (E. Belotti, personal communication). Up to now, however, nothing is known about its behavior in laboratory tests and about its sensitivity to chemicals.

A4.10.12.6 *Breeding Conditions*—All *Enchytraeus* species mentioned previously can be kept and bred in the same substrate as *E. albidus*. The size of the breeding vessels can be smaller. They can also be fed the same food (that is, rolled oats), but because of their smaller individual size, the amount of food per feeding should be adjusted. In general, it should be kept in mind that the lifecycle of these animals is shorter, which means, for example, that feeding should be done more often.

A4.10.12.7 *Test Conditions*—The conditions are the same as in the case of *E. albidus*, except for the following aspects: *(1)* the size of the test vessel may be smaller; *(2)* the duration of the reproduction test may be shorter, that is, four instead of six weeks; the duration of the Range-finding test should not be changed; *(3)* because of the small individual size of the juvenile worms the use of the staining method is strongly recommended for counting; and *(4)* the value for the validity criterion "number of juveniles per test vessel in the control" should be changed to "50."

A4.10.13 *Treatment of Results*—See Section 14.

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