Designation: E1676 – 12 (Reapproved 2021)

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:

1.6 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this 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 Jan. 1, 2021. Published February 2021. Originally approved in 1995. Last previous edition approved in 2012 as E1676–12. DOI: 10.1520/E1676-12R21.

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, health, and environmental 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.

1.8 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

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

[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.

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.

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

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

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 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

[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.

the soil (see 6.1.5). A surfactant should not be used in the preparation of a stock solution because it might affect the bioavailability, form, and toxicity of the test material.

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

9.5.3.3 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, orthogonal contrasts, Fisher's methods, Dunnett's procedure, or Williams' method. The lowest concentration for which the difference in observed effect exceeds the statistically significant difference is defined as the LOEC for that end point. The highest concentration for which the difference in effect is not greater than the statistically significant difference is defined as the NOEC for that end point.

14.6 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.