Designation: E2172 – 22

Standard Guide for Conducting Laboratory Soil Toxicity Tests with the Nematode *Caenorhabditis elegans*[1]

This standard is issued under the fixed designation E2172; 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 chemicals associated with soil to nematodes from soil toxicity tests. This standard is based on a modification to Guide E1676. The methods are designed to assess lethal or sublethal toxic effects on nematodes in short-term tests in terrestrial systems. Soils to be tested may be (*1*) references soils or potentially toxic soil sites; (*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 *Caenorhabditis elegans* (see Annex A1). Methods described in this guide may also be useful for conducting soil toxicity tests with other terrestrial species, although modifications may be necessary.

1.2 *Summary of Previous Studies*—Initial soil toxicity testing using the free-living, bacterivorous soil nematode *Caenorhabditis elegans* was developed by Donkin and Dusenbery (**1**).[2] Following the development of an effective method of recovery of *C. elegans* from test soils, the organism was used to identify factors that affect the toxicity of zinc, cadmium, copper, and lead (**2**). Freeman et al. further refined the nematode bioassay by decreasing the quantity of soil and spiking solution volumes, determining test acceptability criteria, and developing control charts to assess worm health using copper as a reference toxicant (**3**). More recently, the toxicological effects of nitrate and chloride metallic salts in two natural soils were compared (**4**). LC50 values for *C. elegans* exposed for 24-h to nitrate salts of cadmium, copper, zinc, lead and nickel in an artificial soil (see Annex A2) were found to be similar to LC50 values for the earthworm, *Eisenia fetida* (**5**). Increasing the exposure time to 48-h resulted in much lower LC50 values (**6**). However, longer exposure times necessitate the addition of food and lead to lower recovery percentages in soils high in organic matter. A modification of the recovery method has also been used with a transgenic strain of *C. elegans* used as a soil biomonitoring tool to assess sub-lethal effects of metal exposures in soil (**7**). A variety of sub-lethal endpoints have been developed using *C. elegans* in aquatic media and may prove useful for assessing soil exposures (**8**).

1.3 Modification of these procedures might be justified by special needs. The results of tests conducted using typical 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 tests with terrestrial worms.

1.4 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. 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. These methods might also be useful for conducting bioaccumulation tests.

1.5 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).

1.6 This guide is arranged as follows:

[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.

Current edition approved Aug. 1, 2022. Published September 2022. Originally approved in 2002. Last previous edition approved in 2014 as E2172–01(2014). DOI: 10.1520/E2172-22.

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

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

1.8 *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.

1.9 *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:*[3]

D4447 Guide for Disposal of Laboratory Chemicals and Samples

E943 Terminology Relating to Biological Effects and Environmental Fate

E1295 Guide for Conducting Three-Brood, Renewal Toxicity Tests with *Ceriodaphnia dubia*

E1676 Guide for Conducting Laboratory Soil Toxicity or Bioaccumulation Tests with the Lumbricid Earthworm *Eisenia Fetida* and the Enchytraeid Potworm *Enchytraeus albidus*

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.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *artificial soil, n*—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, n*—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 *concentration, n*—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.

3.2.4 *diluent soil, n*—the artificial or reference soil used to dilute site soils.

3.2.5 *hydration water, n*—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 3.2.18); however, depending on the nature of the test being implemented, site surface water or ground water may also be utilized for hydration.

3.2.6 *negative control soil, n*—artificial or field collected soil to be used for evaluating the acceptability of a test.

3.2.7 *reference soil, n*—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.

3.2.8 *sampling station, n*—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.

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

3.2.10 *sediment, n*—particulate materials that usually lie below water. Formulated particulate material that is intended to lie below water in a test.

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

3.2.12 *site soil, n*—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 contaminants.

3.2.13 *soil, n*—solid particles produced by the physical and chemical disintegration of rocks, which may or may not contain organic material.

[3] 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.

3.2.14 *spiking, v*—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 carder, the soil is mixed thoroughly to distribute the test material evenly throughout the soil.

3.2.15 *test chamber, n*—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.

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

3.2.17 *test soil, n*—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.

3.2.18 *test water, n*—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 should be deionized 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 *Toxicity of Test Soils is 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 for 24 h exposures to the terrestrial nematodes *C. elegans*.

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, nematodes have a number of characteristics that make them appropriate organisms for use in the assessment of potentially hazardous soils. Bacterial-feeding nematodes such as *C. elegans* feed on soil microbes and contribute to the breakdown of organic matter. They are also of extreme importance in the cycling and degradation of key nutrients in soil ecosystems (**9**). Soil nematodes also serve as a source of prey and nutrients for fauna and microflora such as soil nematophagous fungi (**10**). A major change in the abundance of soil invertebrates such as nematodes, 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 Results from soil tests might be an important consideration when assessing the hazards of materials to terrestrial organisms.

5.3 The soil 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.

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

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

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

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

5.6.2 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 tests, geochemical analyses, and community structure are taken simultaneously from the same grab of the same site.

5.7 Soil toxicity 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 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 test, but it may be necessary to feed the test organisms in long-duration tests (tests greater than 24 h in duration).

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 Although the natural geochemical properties of soil have not been fully examined with *C. elegans*, it is anticipated that some test soils collected from the field might not be within the tolerance limits of the test species. Of these properties, pH tolerance in aquatic media has been examined and the organism can survive a pH range varying from 3.1 to 11.9 for 24 h and 3.2 to 11.8 for 96 h (**11**).

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

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

7. Apparatus

7.1 *General Facilities*—The facility should include separate constant temperature areas (chambers) for culturing and testing to reduce the possibility of contamination by test materials and other substances, especially volatile compounds. Culture containers should not be in a room (chamber) in which tests are conducted, stock solutions or test solutions are prepared, or equipment is cleaned. The facilities should be well ventilated and free of fumes. See Guide E1706 for additional detail.

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. 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. See Guide E1706 for additional detail.

7.3 *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.

7.4 *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.

7.4.1 All test containers used in a soil test must be identical. The test containers should be covered with a lid.

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

7.5 *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 nitric acid); (*6*) rinse at least twice with distilled, deionized, or reagent grade water; and (*7*) dried at room temperature or in a low-temperature (up to 90°C) air-drying oven. Care should be taken to avoid the use of "plastics" that may break down 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.

7.5.1 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.

7.5.2 Many organic solvents leave a film that is insoluble in water. A dichromate-sulfuric acid cleaning solution can generally be used in place of both the organic solvent and the acid, but the solution might leave chromium residues on glass.

7.5.3 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).

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

7.6 *Acceptability*—Before a 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 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, and recommended handling procedures 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. Wearing rubber boots, disposable safety gear, appropriate gloves, and an appropriate cartridge respirator might minimize exposure to workers. 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 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. Since nematodes are naturally found in the soil, field collected samples may contain nematodes similar to (or including) *C. elegans*. Such situations may require the drying of the soil to remove natural nematode populations (followed by re-wetting) prior to performing the testing. Soil testing procedures are detailed in Section 11.

9.2 *Negative Control and/or Reference Soil*—A negative control soil is used for the following: (*1*) to yield a measure of the acceptability of the test, (*2*) to provide evidence of the health and relative quality of the test organisms, (*3*) to determine the suitability of the test conditions and handling procedures, and (*4*) to provide a basis for interpreting data obtained from the test soils. A reference soil is used to describe the matrix effects of a test. Every test must have a negative control of artificial or reference soil and may also have a reference soil if the negative control is an artificial soil. A reference soil should be collected from the field in a clean area and represent the test soil as much as possible in soil characteristics (for example, percent organic matter, particle size distribution, and pH). This provides a site-specific basis for comparison of toxic and nontoxic conditions. The same conditions, procedures, and organisms must be used with the negative control and reference soil as are used in the other treatments, except that contaminated soil or test materials are not added.

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

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. 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. Observations concerning habitat and type of vegetation and measurements such as soil temperature and moisture should be taken in the field.

9.4.2 *Storage*—Soil samples should be utilized as soon as possible in accordance with Test Methods E1706.

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 105°C. Information on moisture content is necessary to determine the amount of hydration water to add to the test soils. 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). See Guides E1676 and E1706 for a description of spiking procedures.

9.5.1 *Test Concentrations:*

9.5.1.1 If the test is intended to allow the calculation of an LC50, the test concentrations should bracket the predicted LC50. The prediction might be based on the results of a test on the same or a similar test material on the same or a similar species. The LC50 of a particular compound may vary, depending on physical and chemical soil characteristics. If a useful prediction is not available, it is desirable to conduct a range-finding test in which the organisms are exposed to a control and three or more concentrations of the test material that differ by a factor of ten.

9.5.1.2 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.

10. Test Organism

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

10.2 *Age*—All organisms should be as uniform as possible in the state of maturity and weight class. The state of maturity or weight class for a particular test species should be chosen so that the sensitivity to test materials is not affected by age, reproduction, or other intrinsic life-cycle factors. Three- to four-day old worms from age-synchronized cultures should be used for the tests described in this study. See Annex A1 for additional information.

10.3 *Source*—All organisms in a test must be from the same source. Laboratory cultures can be started from organisms obtained from the *Caenorhabditis* Genetics Center in Minneapolis, MN. Laboratory cultures may be the best source of test species because laboratories can provide organisms whose history, age, and quality are known

10.4 *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. Nematodes should be cultured at the same temperature as that used for testing (see Annex A1).

10.5 *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 with a microscope. Any organisms that touch dry surfaces or are dropped or injured during handling should be discarded.

10.6 *Reference Toxicity Tests*—Reference toxicity tests should be conducted regularly to insure the health and stability of the *C. elegans* culture used in soil toxicity tests. The reference toxicity test procedures and control charts have been published(**9**).

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.

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. The test container is the experimental unit (see 7.4). 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*—Seven days before the test is started (Day -7), the soil to be tested, negative control, and reference soil (if used) are mixed and hydrated, the soils are placed into test containers. A 7-day equilibration period is required to provide time for most liquid/solid phase equilibration reactions (**5**). The minimum amount of soil to mix and hydrate should be enough for three replicates, a pH sample, and to account for soil adhering to the sides of the mixing chamber. This mixed and hydrated soil is called a batch. Extra batch soil may be mixed and hydrated if a sample is to be removed for chemical analysis or for any other purpose. Site soil has been mixed previously during processing.

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

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 Prior to adding organisms, the test containers are placed into the test chamber, for a sufficient period of time, 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 7-day equilibration; (see 11.2) this constitutes the beginning of the test (Day 0). The test organisms are transferred from their agar plates to the surface of the soil with a flame-sterilized platinum wire (**3**) and allowed to burrow.

11.3.1 Ten organisms are loaded into each test container containing 2.33 g soil (**3-5**).

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 *Recovery of Test Organisms*—After exposure, the soil and worms are rinsed from dishes into 50-mL centrifuge tubes with Ludox®, a colloidal silica suspension. Each tube is vortexed to ensure thorough mixing of the soil suspension. After centrifuging at 700 × *g* for 2 min, tubes are set aside for ~ 15 min to allow time for the worms to buoy to the top of the solution. The solution is then poured into 100-mm glass petri dishes and viewed under a light microscope. Worms are removed from the solution with a platinum wire, placed on a K-agar plate with a food source (**12**) and examined under a microscope. If worms do not respond to gentle probing with a platinum wire, they are scored as dead (**5**). Live worms are either obviously moving before or after probing. Unrecovered worms are scored as dead. For 24-h exposures, worms are not expected to decompose and recovery of organisms should be > 80% (**3**). For exposures greater than 24 h, dead worms can decompose and a lower recovery rate may be observed(**6**).

11.6 *Test Measurements:*

11.6.1 Temperature should be monitored for the duration of the test.

11.6.2 pH should be measured 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.

11.6.3 Percent moisture may be measured at the beginning and end of the test from subsamples, as noted in 11.6.2.

11.7 *Chemical Analyses:*

11.7.1 *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.

11.7.2 *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.

12. Analytical Methodology

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