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

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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.

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[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 and health practices and determine the applicability of regulatory requirements prior to use.* While some safety considerations are included in this guide, it is beyond the scope of this standard to encompass all safety requirements necessary to conduct soil toxicity tests. Specific precautionary statements are given in Section 8.

2. Referenced Documents

2.1 *ASTM Standards:*[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*—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 *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.

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

3.2.5 *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 3.2.18); 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*—artificial or field collected soil to be used for evaluating the acceptability of a test.

3.2.7 *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.

3.2.8 *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.

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

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

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

3.2.14 *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

[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.

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

3.2.17 *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.

3.2.18 *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 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.

12.2 Concentrations should be measured for (*1*) chemicals of interest in batches of soil; (*2*) test materials in stock solutions; and (*3*) chemicals of interest 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 The precision and bias of each analytical method used should be determined in an appropriate matrix, that is, soil. When appropriate, reagent blanks, recoveries, and standards should be included when samples are analyzed.

13. Acceptability of Test

13.1 Table 1 provides conditions that should be met for the test to be considered to be acceptable. Listed below are other situations that may lead to the rejection of test data.

13.1.1 All test containers were not identical (see 7.4 and 11.1).

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

13.1.3 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).

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

13.1.5 The concentration of solvent in the range used affected the survival of the test organisms (see Guide E1676 or E1706).

13.1.6 All animals in the test population were not obtained from the same source, were not all of the same species and age, or were not of acceptable quality (see Section 10).

13.1.7 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).

TABLE 1 Test Conditions for the 24-h Nematode Toxicity Test

Type	Static
Test Duration	24 h to 48h
Temperature	20°C
Light	None
Testing Containers	35 × 10 mm petri dishes
Soil Quantity	2.33 g
Solution Volume	35–45 % of dry weight
Organism Age	3–4 days
Number of Organisms per Treatment	10
Food Source	None for 24 h exposures, for 48 h exposures, OP50 strain of *E. coli* **(6)**
Allowable pH Range	3.1–11.9
Organism Recovery	Colloidal silica flotation
Biological Endpoint	Mortality
Test Acceptability	≥80 % Recovery ≥90 % Control survival

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

13.1.9 The temperature was not within the acceptable range of the test.

13.1.10 The negative control soil organisms did not survive as required for the test species (see 9.2).

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 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 See Guide E1706 for additional statistical guidance and Guide E1295 for specific guidance on regression based statistics.

14.3 Most 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 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 affected 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.

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.

14.4.1 All exposure concentration effects (or field stations) can be compared with the control effects by using mean separation techniques, orthagonal contrasts, Fisher's methods, Dunnett's procedure, or Williams' method.

15. Report

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

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

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

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

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

15.1.5 Identity and concentration of any solvent used.

15.1.6 Source and quality of hydration and test water.

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

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

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

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

15.1.11 Definition(s) of the effects used to calculate LC50 or EC50, biological endpoints for tests, and a summary of general observations of other effects.

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

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

15.1.14 Summary of general observations on other effects or symptoms.

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

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

ANNEXES

(Mandatory Information)

A1. *Caenorhabditis elegans*

A1.1 *Significance—C. elegans* has many desirable characteristics for a test species: (*1*) it is easily cultured in the laboratory; (*2*) it has a short life-cycle; (*3*) it is hermaphroditic in nature; (*4*) it is tolerant to wide pH ranges in various commonly used aquatic test media (**11**); (*5*) it has an alternate dauerlarval stage of suspended development; and (*6*) its complete genome and nervous system have been extensively mapped (**13**).

A1.2 *Life History*—The life cycle of *C. elegans* is rapid, consisting of a 14-h embryongenesis and 36-h postembryonic development through four larval stages, L 1–L 4, to the adult at 20°C (**3**). On average, hatching to reproduction takes 3 days (**14**). The hermaphrodite form of *C. elegans* produces sperm in the late L 4 larval stage. Structurally, the adult is a female producing oocytes. The sperm previously produced is stored in its spermathecae. Mating with male *C. elegans* stimulates production of oocytes. A single hermaphrodite has the ability to produce more than 1000 progeny when mated; however, with the hermaphrodite the size of the brood is limited by the number of sperm produced previously (**15**). Without limiting environmental factors such as food availability, adult *C. elegans* males and hermaphrodites can live up to 17 days (**14**).

A1.3 *Taxonomy*

A1.4 *Culture of Test Organisms*—A stock solution of the dauerlarval stage of *C. elegans* in M9 buffer (**16**) is kept at 20°C and is renewed monthly. Age-synchronized adult worms to be used in soil toxicity testing are generated from these dauers. Several hundred dauers are placed onto K-agar plates (**12**) with an established lawn of *Escherichia coli* strain OP50 (**17**) as described in Donkin and Williams (**18**). The plates are incubated for three days at 20°C. Eggs and worms are washed from the plates with liquid K-medium (**19**), and treated for 10-15 min in a 10 % clorox solution (NaOH) to kill adult worms and isolate the eggs. After the eggs are isolated, they are placed onto K-agar plates with an OP50 lawn, and incubated at 20°C for four days to yield age-synchronized adult worms (**3**).

A1.5 *Obtaining Brood Stock—C. elegans,* wild type strain N2, is obtained from the *Caenorhabditis* Genetics Center, Minneapolis, MN.

A1.6 *Handling—C. elegans* should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly as possible, so that the worms are not stressed unnecessarily. Any worms that are dropped or injured during handling should be discarded.

A1.7 *Age*—Tests with *C. elegans* should be started with age-synchronized adult worms (**3**).

A1.8 *Acclimation*—It is recommended that the test organisms be cultured and tested at the same temperature (see A1.4).

A1.9 *Test Specifications:*

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

A1.9.1.1 *Test Duration*—24 h to 48 h.

A1.9.2 *Test Containers*—35 mm polystyrene tissue culture dishes (**3**).

A1.9.3 *Week Prior (Day -7) to Initiation of Test:*

A1.9.3.1 Test soils are hydrated and mixed well into batches, separated into replicates, and placed into test containers that are placed into the test chamber (see 11.2). No standing water should be present in the test containers. If a site, reference, or artificial soil is spiked with chemicals of compounds in solution, the solution is used as part of the hydration water.

A1.9.3.1.1 *Tests With Whole (100 %) Site or Reference Soil*—If the negative control is artificial soil, it is hydrated to 35 to 45 % of its dry weight. The site and reference soils are also hydrated to 35 to 45 % of their dry weight. Since most soils collected in the field contain some moisture, this moisture content is obtained and used for determining how much additional water to add to the soils to gain a hydration level of 35 to 45 %.

NOTE A1.1—Hydrating soils to a standard level is problematic. Due to the variation in water holding capacity (influenced by factors such as soil texture, structure, and organic matter content) between soils, one soil may appear very wet and even have standing water on the surface after hydration to 45 % of its dry weight as a standard. The site and reference soils can be hydrated to a level approximating the appearance of the artificial soil. Another alternative is to measure the water holding capacity of the soil and hydrate the soil to 75 % of the water holding capacity value. Measuring the water potential, for example, using a tensiometer, of the soil may prove to be a better method of hydrating soils. The water potential of artificial soil hydrated to 35 to 45 % of its dry weight could be determined. Soils could be hydrated to the water potential value obtained for the artificial soil using this as a standard.

NOTE A1.2—A sediment can be defined as particulate material that usually lies below water (see Guide E1706). The definition of a soil as defined within this guide (see Section 3) indicates that a soil is not usually covered by water. It is sometimes difficult to distinguish between a soil and a sediment that has been dried out or deposited on dry land.

A1.9.3.1.2 *Tests With Site Soil Diluted With Artificial Soil*—The artificial soil portion of each concentration is hydrated to 35 to 45 % of its dry weight. The site soil portion of each concentration is hydrated as in A1.9.3.1.1. These two portions are then mixed together and allowed to equilibrate to form the batch for each concentration from which the replicates are taken.

A1.9.3.1.3 *Tests With Artificial Soil Spiked With Compounds*—If a series of concentrations is prepared by spiking artificial soil with solutions of compounds, the artificial soil is hydrated to 35 to 45 % of its dry weight with test water and the chemical solution combined to make the necessary amount of hydration. If a series of concentrations is prepared by spiking artificial soil with dry chemicals, the chemical is first mixed into the artificial soil very well. The artificial soil is then hydrated with test water, and the batch is mixed very well before being separated into replicates.

A1.9.4 Nematodes are introduced to the test containers after the equilibration period (see 11.2). Groups of ten nematodes are assigned randomly to the individual test containers. The nematodes are placed on the surface of the soil (see 11.3). The test containers must be placed into the test chamber randomly.

A1.9.5 *Feeding*—Organisms tested for greater than 24 h in soil toxicity tests require food (**6**). The food source used in the 48-h soil toxicity test is *Escherichia coli* strain OP50. Organisms used in the 24-h soil toxicity test do not require food (**5**).

A1.9.5.1 *Preparation of Food Source*— The food source used is a saturated culture of *E. coli* strain OP50 in L-broth (**18**). L-broth is prepared in a 5:1 ratio or 5 parts L-broth to 1 part K-medium. For example, if a test required 5 mL K-medium, 25 mL of L-broth is centrifuged at 3,000 rpm for 10 min, after which the bacterial pellet is resuspended in 5 mL 100 % K-medium.

A1.9.5.2 *Delivery of Food Source*— Immediately prior to loading organisms into the test containers, prepared food (see A1.9.5.1) is added to the surface of the soil.

A1.10 *C. elegans* soil test of 24-h duration should be considered unacceptable if the mean survival of all negative control containers is less than 90 % and recovery of nematodes in all treatment groups is less than 80 % (see Section 13). These recovery rates have been reported with soils used in published studies (**3**). However, if the soils are high in organic matter (such as some artifical soils) or have very small particle size or if exposure duration is greater than 24 h, obtaining these recovery rates may be problematic.

A1.11 *Test Measurements:*

A1.11.1 *pH*—If a concentration series is being tested, the initial pH should be checked in the high and low concentrations at a minimum. For pH measurement, 23.33 g of soil and 15 mL of spiking solution were equilibrated for 7 days. The pH was measured in the supernatant (**5**). If a number of different undiluted site soils are being tested, pH should have already been measured in each soil (see 9.4.3). pH should also be measured in the negative control (and reference soil, if used). Initial pH is measured in a subsample taken from the batch preparation for each treatment.

A1.11.1.1 At the conclusion of a test with a series of concentrations, the pH is checked in subsamples of soil from one of the replicates of the control (and reference soil, if used), high and low concentrations.

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

subsamples taken from the batch preparation for each treatment and is determined gravimetrically.

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

A2. ARTIFICIAL SOIL COMPOSITION

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

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

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

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