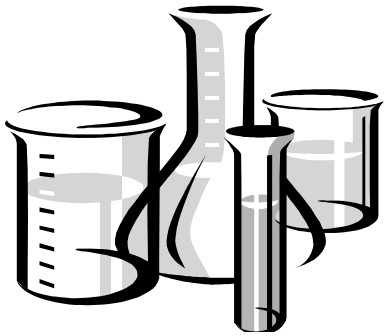
United States
Environmental Protection
Agency

Office of Chemical Safety
and Pollution Prevention
(7101)

EPA 712-C-016
January 2012

Ecological Effects Test Guidelines

OCSPP 850.3100: Earthworm Subchronic Toxicity Test



NOTICE

This guideline is one of a series of test guidelines established by the United States Environmental Protection Agency's Office of Chemical Safety and Pollution Prevention (OCSPP) for use in testing pesticides and chemical substances to develop data for submission to the Agency under the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601, et seq.), the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) (7 U.S.C. 136, et seq.), and section 408 of the Federal Food, Drug and Cosmetic (FFDCA) (21 U.S.C. 346a). Prior to April 22, 2010, OCSPP was known as the Office of Prevention, Pesticides and Toxic Substances (OPPTS). To distinguish these guidelines from guidelines issued by other organizations, the numbering convention adopted in 1994 specifically included OPPTS as part of the guideline's number. Any test guidelines developed after April 22, 2010 will use the new acronym (OCSPP) in their title.

The OCSPP harmonized test guidelines serve as a compendium of accepted scientific methodologies and protocols that are intended to provide data to inform regulatory decisions under TSCA, FIFRA, and/or FFDCA. This document provides guidance for conducting the test, and is also used by EPA, the public, and the companies that are subject to data submission requirements under TSCA, FIFRA, and/or the FFDCA. As a guidance document, these guidelines are not binding on either EPA or any outside parties, and the EPA may depart from the guidelines where circumstances warrant and without prior notice. At places in this guidance, the Agency uses the word "should." In this guidance, the use of "should" with regard to an action means that the action is recommended rather than mandatory. The procedures contained in this guideline are strongly recommended for generating the data that are the subject of the guideline, but EPA recognizes that departures may be appropriate in specific situations. You may propose alternatives to the recommendations described in these guidelines, and the Agency will assess them for appropriateness on a case-by-case basis.

For additional information about these test guidelines and to access these guidelines electronically, please go to http://www.epa.gov/ocspp and select "Test Methods & Guidelines" on the left side navigation menu. You may also access the guidelines in http://www.regulations.gov grouped by Series under Docket ID #s: EPA-HQ-OPPT-2009-0150 through EPA-HQ-OPPT-2009-0159, and EPA-HQ-OPPT-2009-0576.

OCSPP 850.3100: Earthworm subchronic toxicity test.

(a) **Scope—**

(1) **Applicability.** This guideline is intended to be used to help develop data to submit to EPA under the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601, et seq.), the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, et seq.), and the Federal Food, Drug, and Cosmetic Act (FFDCA) (21 U.S.C. 346a).

(2) **Background**. The source materials used in developing this harmonized OCSPP test guideline include the OPPT guideline under 40 CFR 795.150 Earthworm Toxicity Test (proposed in the Federal Register of June 25, 1991 (56 FR 29155)) and ASTM E 1676, Standard Guide for Conducting Laboratory Soil Toxicity or Bioaccumulation Tests with the Lumbricid Earthworm *Eisenia fetida*. This guideline was formerly Public Draft OCSPP 850.6200 (April, 1996).

(b) **Purpose.** This guideline is intended for use in developing data on the toxicity of chemical substances and mixtures ("test chemicals" or "test substances") subject to environmental effects test regulations. This guideline sets forth the procedures for a toxicity test using earthworms which are maintained in direct contact with an artificial (formulated) soil, allowing earthworms to ingest contaminated soil *ad libitum*. The Environmental Protection Agency will use data from this test in assessing the hazard of a chemical to earthworms in the soil environment.

(c) **Definitions**. The definitions in OCSPP 850.3000 apply to this guideline. In addition, the more specific definitions in this paragraph also apply to this guideline:

Artificial (formulated) soil means a defined dry weight mixture of 70 percent (70%) of number 70 mesh silica sand, 20% kaolin clay, 10% sphagnum peat moss, and calcium carbonate (to adjust the pH). These ingredients are weighed and mixed in the stated proportions and moistened to 35–45% (by weight) with reagent water.

Behavioral symptoms are indicators of toxicity to earthworms such that a distinct difference in position in the test container can be identified, *e.g.*, below surface or on the surface; writhing on the surface; stiffened and shortened on the surface or elongated and pulsing; or inactive below surface in a ball.

Clitellum means a glandular portion of the anterior epidermis, appearing as saddle-shaped or annular, usually differentiated externally by color. It is the most visible feature of an adult earthworm and secretes the cocoon into which eggs and sperm are deposited.

Mature or adult worms mean a condition of the worm exhibiting a clitellum in the anterior of the body.

Mortality means the lack of movement by the test organism in response to a definite tactile stimulus to the anterior end. Also, because earthworms tend to disintegrate rapidly after death, the absence of organisms in the enclosed soil test container is considered to mean death has occurred.

Pathological symptoms mean toxic effects, such as surface lesions and mid-segmental swellings or general ulcerated areas on the surface of the earthworm.

Test mixture means the test substance/artificial soil mixture(s) which the earthworms are exposed to during the test.

(d) **General considerations—**

(1) **Summary of the test.** This toxicity test is conducted by placing acclimated earthworms in test chambers containing artificial (formulated) soil which has been spiked with the test substance. Earthworms are allowed to ingest this test mixture soil *ad libitum*. Mortality and other effects are examined on a weekly basis for 28 days. The results are expressed as the 28-d LC_{50} (median lethal concentration). Sublethal effects may also be determined, and expressed as EC_{50}, (experimentally derived concentration of a test substance in a test matrix (*e.g.*, growth medium, soil sediment, feed) that would be expected to cause a defined adverse effect in 50% of a group test organisms under specified exposure conditions), NOEC (no observed effect concentration), LOEC (lowest observed effect concentration), and MATC (maximum acceptable toxicant concentration) values.

(2) **General test guidance.** The general guidance in OCSPP 850.3000 applies to this guideline except as specifically noted herein.

(3) **Range-finding test.** A range-finding test is usually conducted to establish the appropriate test concentrations to be used in the definitive test. The earthworms should be exposed (for at least 28 days) to a series of widely spaced concentrations of the test substance (*e.g.*, 0.1, 1.0, 10, 100, 1,000 milligrams per kilogram (mg/kg) dry weight artificial soil). Treatment replication is not needed and nominal concentrations of the chemical are acceptable. The number of test organisms used and details of observations do not have to be the same as definitive testing. However, the range-finding test will be more useful the greater the similarity between the range-finding test and the definitive test. Results of range-finding tests should be reported along with the results of the definitive test, if range-finding tests are conducted.

(4) **Definitive test**. The goal of the definitive test is to determine the concentration-response curve for mortality and the LC_{50} (95% confidence limits and standard error) at 28 days, as well as the slope of the concentration-response curve, its associated standard error and 95% confidence interval. Where sufficient data warrant, these values are also calculated for the 7, 14, and 21-day concentration response-curves for mortality. An additional desired endpoint is the weight loss EC_{50} (95% confidence interval) and the LOEC, NOEC and MATC values at each sampling time. For this determination, a minimum of five concentrations of the test chemical, plus appropriate controls, are tested. Analytical confirmation of test concentrations is performed as described in OCSPP 850.3000. A summary of test conditions is provided in Table 1 and validity elements for an acceptable definitive test are listed in Table 2 of this guideline.

(5) **Limit test.** In some situations, it is only necessary to ascertain that the 28-d LC_{50} is above a certain limit, and at the limit there is also no observable adverse growth effect. In a limit test, at least 20 earthworms are exposed to a single "limit concentration," with the same number of organisms in appropriate control(s). The multiple-concentration definitive test may be waived if the following conditions are met. First, if one or fewer mortalities occur at the limit concentration (*i.e.* the LC_{50} > limit concentration), and

second, the limit treatment responses are not significantly reduced (or inhibited) as compared to the control response (*i.e.*, NOEC > limit concentration). For most chemicals, 1,000 mg test substance (based upon 100% active ingredient for pesticides) per kg dry weight of artificial soil is considered appropriate as the limit concentration. Except for the number of treatment groups and number of test organisms, an acceptable limit test follows the same test procedures and is the same duration as the definitive test (see Table 1 of this guideline).

(e) **Test standards—**

(1) **Test substance**. The substance to be tested should be technical grade unless the test is designed to test a specific formulation, mixture, or end-use product. For pesticides, if more than one active ingredient constitutes a technical product, the technical grade of each active ingredient should be tested separately, in addition to the combination, if applicable. OCSPP 850.3000 lists the type of information that should be known about the test substance before testing, and discusses methods for preparation of test substances.

(2) **Test duration**. The test duration is 28 days.

(3) **Test organism—**

(i) **Species**. The test species is the earthworm *Eisenia fetida andrei* (Bouche). Some taxonomists consider these to be two separate species, *Eisenia fetida* (Savigny 1826) and *Eisenia andrei* (Andre 1963); see the references under paragraph (j)(1), (j)(5) and (j)(11) of this guideline for additional information about the taxonomy of the *Eisenia fetida* complex. These organisms naturally inhabit high organic environments such as compost and manure piles. They are easily bred in the laboratory and have been used commonly in laboratory experiments. The species identity of the test organism should be verified using appropriate taxonomic keys as described by Fender (paragraph (j)(4) of this guideline), or an equivalent method.

(ii) **Source.**

(A) Earthworms used in toxicity tests should be purchased from a commercial source that can verify the species. Once verified, cultures should be maintained at the test facility. Information about culturing procedures can be found in the reference in paragraph (j)(1) of this guideline. Records should be kept regarding the source of the initial stock and culturing techniques. All organisms used for a particular test should have originated from the same population (culture).

(B) All newly acquired earthworms should be quarantined and observed for at least 14 days prior to use in a test.

(iii) **Age and size.** Adult earthworms are used to start the test. The weight of the individual adult earthworms should be between 300 mg and 600 mg each. All organisms used in a test should be as uniform as possible in the state of maturity and weight.

(iv) **Acclimation**. It is recommended that earthworms be cultured at the test temperature. In any event, earthworms should be held for a minimum of 7 days in uncontaminated soil at the test temperature prior to testing. Any changes in soil temperature should not exceed 3 degrees Celsius (^{o}C) per day or 1 ^{o}C per hour.

(v) **Health status.** Earthworms should not be used if they have been under stress from too much moisture or a lack of moisture as described by Reinecke and Venter (see paragraph (j)(13) of this guideline); excessive or inadequate food or temperature as described by Tomlin and Miller (paragraph (j)(17) of this guideline); pH variation as described by Satchell and Dottie (paragraph (j)(14) of this guideline); or crowding. Any of these conditions will produce earthworms that may not be healthy.

(vi) **Care and handling**. Adult earthworms should be handled with care. Sufficient numbers of earthworms should be harvested and sorted to insure that healthy individuals are used for the test. For a satisfactory test, any animals that appear to be injured are discarded and not used in the test. Additionally, any organisms that touch dry surfaces or are dropped or injured during handling are discarded.

(vii) **Diet and feeding—**

(A) **Culture Period.** Substrate food for culturing *Eisenia fetida andrei* should be commercial alfalfa (*Medicago sativa*) pellets, saturated with reagent water at a ratio of approximately 1 gram (g) of dry pellets per 2 milliliters (mL) water and aged for two weeks in a covered container. Worms are fed once or twice per week and the bedding turned by hand to inspect the general condition of the culture.

(B) **Test Period.** The earthworms are not fed during the test period.

(4) **Administration of test substance**. The test is started by introducing adult earthworms, which have been acclimated to the test conditions, into test chambers which contain artificial (formulated) soil (see paragraph (e)(7)(vii) of this guideline) to which the test substance has been added. Earthworms should be introduced into test soils on the surface of the test medium so as to evaluate burrowing behavior. The jar capped and secured without making an airtight seal.

(i) **Preparation of treated soil.**

(A) Appropriate portions of artificial soil are mixed thoroughly with appropriate amounts of test substance (see paragraph (e)(4)(i)(B) of this guideline). Hand mixing or mechanical mixing may be used. Test concentrations should be prepared on day -1 and allowed to equilibrate overnight before adding test organisms.

(B) Test substances may be added directly to the artificial soil on a dry weight basis, but are typically dissolved in reagent water (preferably) or a vehicle to form a stock solution. Aliquots of the stock solution, or

dilutions thereof, are then added to the artificial soil. If a solvent is used, the opened chambers are placed in a hood for 24 hours to evaporate the solvent prior to adding the earthworms.

(ii) **Test concentrations.** For the definitive test, a minimum of five concentrations of the test chemical, plus appropriate controls, are tested. Test concentrations should be chosen in a geometric series in which the ratio is between 1.5 and 2.0 (*e.g.*, 2, 4, 8, 16, 32, and 64 mg/kg). All test concentrations should be based on milligram of test chemical (100% active ingredient for pesticides) per kilogram of artificial soil (air-dry weight).

(5) **Controls**.

(i) Every test should include a negative control consisting of the same artificial (formulated) soil, conditions, procedures, and earthworms from the same group as used with the test substance, except that none of the test substance is added. In addition if a vehicle is used, a vehicle control is tested, using the same vehicle batch as used to make the stock solution and at the highest concentration used to prepare test concentrations. See OCSPP 850.3000 for additional information on preparation of test substances using a vehicle.

(ii) A test is not acceptable if average survival of either the negative or solvent control earthworms was less than 80% at the end of the test or the total mean weight of the earthworms in either the negative or solvent control containers declined significantly during the test (*i.e.*, by 30%).

(6) **Number of test organisms and replicates—**

(i) **Definitive test.** In the definitive test, there is a minimum of three replicates for each treatment concentration and control group, with a minimum of 10 earthworms per replicate. The earthworms are selected from the culture randomly into groups of 10. These groups are then randomly assigned to the test chambers and then weighed to verify they do not differ more than ± 10% among the replicates. Each test chamber contains 200 g (dry weight) of artificial soil. Each test chamber should contain an equal amount of test soil and an equal number of earthworms. For a satisfactory test, replicate test chambers are to be physically separated, since the test chamber is the experimental unit. In addition, test chambers within the testing area are positioned in a random manner or in a way in which appropriate statistical analyses can be used to determine the variation due to placement.

(ii) **Limit test.** In a limit test, at least 20 earthworms are exposed to a single "limit concentration," with the same number of organisms in appropriate control(s).

(iii) **Loading**. The number of earthworms placed in a test chamber should not be so great as to affect the results of the test.

(7) **Facilities, apparatus and supplies**. Normal laboratory equipment and supplies, and items especially listed in (e)(7)(i) through (e)(7)(vii).

(i) **Facilities.**

(A) Facilities should be well ventilated and free of fumes and disturbances that may affect the test organisms.

(B) Ventilation hood for evaporating solvent from test soil when a solvent is used.

(ii) **Instruments and general equipment.**

(A) Suitable balances to measure soil mixtures.

(B) A properly calibrated balance with sufficient accuracy should be used to weigh the worms.

(iii) **Culturing.** Polyethylene containers (*e.g.*, rectangular dish pans measuring 32.5 by 27.5 by 12.5 centimeters) for culturing earthworms.

(iv) **Environmental control equipment.**

(A) Apparatus for providing continuous lighting.

(B) A mechanism (*e.g.*, environmental chamber) for controlling and maintaining the artificial soil temperature and relative humidity during the culturing, holding, acclimation, and test periods. Relative humidity should be maintained above 85%. An open pan of water can be used for this purpose to prevent moisture loss from the containers.

(v) **Test containers and exposure system.**

(A) Chambers for exposing test earthworms to the test substance. Glass canning jars, 1-pint capacity, or their equivalent, should be used for testing. The lids should be reversed (*i.e.*, turned upside down), loosely capped and secured without making an airtight seal to reduce evaporation and permit air exchange.

(B) Construction materials and equipment that contact test mixtures should not contain substances that can be leached or dissolved into artificial soil in quantities that can affect the test results. Construction materials and equipment that contact test mixtures should be chosen to minimize sorption of test substances.

(vi) **Cleaning.** The test chambers should be cleaned before each test following standard laboratory procedures. Hard glass jars are preferable and should be heated in an ashing oven between tests. For a satisfactory test, if soft glass is used it should be used only once and then be discarded. Refer to OCSPP 850.3000 for additional information.

(vii) **Artificial soil.**

(A) For each test substance concentration and control tested, enough artificial soil is prepared by recipe to yield 270 g of artificial soil (wet weight) per replicate. The constituents identified in this paragraph are mixed together on a dry weight basis: 10% Canadian sphagnum peat moss (that portion passing through a 2.36 millimeter (mm) screen); 20% kaolin clay (97% kaolinite with a particle size under 40 micrometers (μm)); and 70% silica sand (grade 70, 97.1% particle size of 0.053 to 0.3 mm).

(B) After these materials are mixed together, an amount of calcium carbonate (99% purity) is added to the mixture to adjust the soil pH to 6.5 ± 0.5. The amount of calcium carbonate used will depend on the pH of the peat moss used.

(C) An appropriate amount of reagent water (*e.g.*, 70 g per 200 g of dry soil) is added to the artificial soil and mixed with the artificial soil to raise the artificial soil moisture level to 35% by weight.

(8) **Environmental conditions**. Environmental parameters during the test should be maintained as follows:

(i) **Temperature**. The test soil temperature should be 22 ^{o}C and it should be constant within ± 2 ^{o}C during the test, as described by Edwards (paragraph (j)(3) of this guideline), or using an equivalent method.

(ii) **Lighting and photoperiod.** Replicates should be illuminated continuously with incandescent or fluorescent lights as described by Edwards (paragraph (j)(3) of this guideline), or using an equivalent method. Light intensity should be about 5.7 micromoles per square meter per second (μmol/m^2/s) (for cool-white lights, this is equivalent to approximately 420 lux) measured at the artificial soil surface.

(9) **Observations**. Earthworms in the test chambers are observed periodically during the test; the dead worms are removed and the findings recorded. Soil temperature and pH, and the concentration of test substance in the treated soil are measured at specified intervals. Data collected during the test are used to develop concentration-response curves and LC_{50} values for the test substance.

(i) **Measurement of test substance.** Analytical confirmation of test concentrations in artificial soil is to be performed using validated analytical methods, as described in OCSPP 850.3000. A 10-g sample should be removed from each replicate prior to addition of earthworms to measure pH and test substance concentrations. The homogeneity of the test substance in treated soil should be determined.

(A) For stable test substances, monitor test concentrations at the beginning and at the end of the test. However, for volatile, unstable substances, those subject to ready degradation, or where there is uncertainty in the

analytical measurements, it may be necessary to measure test concentrations more often during the test, *e.g.* every seven days.

(B) In addition to analyzing samples of artificial soil, at least one reagent blank, containing all reagents used, should also be analyzed.

(C) The measured concentration of the test substance in artificial soil in any chamber during the test should not vary more than 50% from the measured concentration prior to initiation of the test; concentration measurements should be as described by Neuhauser *et al.* (paragraphs (j)(9) and (j)(10) of this guideline), or an equivalent method.

(D) The mean measured concentration of test substance in artificial soil (dry weight) should be used to plot all concentration-response curves and to calculate all LC_{50}, EC_{50}, LOEC, and NOEC values.

(ii) **Environmental conditions—**

(A) **Temperature.** Temperature should be measured and reported at the beginning of the test and on days 7, 14, 21, and 28 in all test chambers. The temperature should be measured at least hourly in one test container.

(B) **Light intensity.** Light intensity should be measured at least once during the test at the surface of the container and checked weekly in the test chambers.

(C) **Total organic carbon (TOC) concentration.** The TOC concentration in soil at test initiation should be determined as measured by the method of Plumb (paragraph (j)(12) of this guideline), or an equivalent method.

(D) **Soil pH.** The pH should be measured at test initiation and 7 day intervals (days 7, 14, 21, 28) thereafter in each test chamber.

(iii) **Organisms—**

(A) **Mortality, behavior, and other adverse effects.** At test initiation burrowing behavior of earthworms placed on the surface of the test medium is recorded for each test chamber. Each test and control chamber should be checked for dead or affected earthworms and observations recorded 7, 14, 21, and 28 days after the beginning of the test or within 1 hour of the designated times in all test chambers. Missing earthworms should be considered to have died. Mortality is assessed by emptying the test medium on a glass or other inert surface, sorting earthworms from the test mixture and testing their reaction to a gentle mechanical stimulus. Any adverse effects (*e.g.*, behavioral or pathological symptoms) are noted and should be reported. The medium is returned to each container.

(B) **Weight loss.** Total biomass of test organisms per test chamber is measured at the beginning and end of the test. The worms should be washed and purged before weighing. To accomplish this, the bedding is rinsed from the worms with reagent water; the worms are placed in petri dishes with wet filter paper, and allowed to purge the gut contents for 24 hours. Before weighing worms, excess surface water may be removed by placing the worms between layers of an absorbent towel. However, it is very important not to dry the surface of the worms which might stress the worms unduly.

(f) **Treatment of results—**

(1) **Descriptive summary statistics—**

(i) **Environmental conditions.** The soil characteristics and TOC content should be described. Temperature and light intensity results should be summarized in tabular form, showing the range, mean and standard deviation during the test. The pH in each test chamber should be summarized in tabular form, showing the range, mean, and standard deviation.

(ii) **Test substance soil concentration.** Soil concentrations should be summarized in tabular form by treatment and sample time, showing mean concentrations and standard deviations. The treatment mean soil concentration should be calculated.

(iii) **Mortality.** Death is the primary effect used in this test guideline to evaluate the toxicity of the test substance. The number of initial earthworms at each treatment and control and the number of dead earthworms should be summarized in tabular form by observation time (*e.g.* day 7, 14, 21, and 28) treatment, and replicate.

(iv) **Sublethal effects and growth.** The sublethal effects and growth (*i.e.*, fresh weight) data should be used to determine concentration-response curves, calculate EC_{50} values, and determine LOEC, NOEC, and MATC values. Number of earthworms with abnormal appearance or behavior symptoms should be summarized in tabular form by symptom, time of observation, treatment, and replicate. Mean total biomass at the beginning and end of the test for each treatment replicate should be summarized in tabular form and the mean and standard deviation in weight loss or gain calculated by treatment.

(2) **Percent—**

(i) **Mortality**. Calculate the percent mortality at each treatment level and control by observation time.

(ii) **Appearance and behavior.** Calculate the percent effected at each treatment level and control by symptom, and observation time.

(3) **Limit test—**

(i) **LC_{50} value.** At test termination (28 days), if one or fewer earthworms are dead at the limit concentration, the LC_{50} is considered to be greater than the limit concentration. This is because the Binomial Theorem predicts that when 20 earthworms are tested, the probability of seeing $\leq$1 dead earthworm if the true 28-d LC_{50} is at or below the limit concentration is <0.001. Conversely the probability of seeing 2 or more dead earthworms if the true 28-d LC_{50} is at or below the limit dose is >0.999. Therefore, if $\leq$1 mortality occurs among the 20 earthworms tested, the 28-d LC_{50} is reported as greater than the limit concentration (*i.e.*, 28-d LC_{50} >1,000 mg/kg dry wt in soil).

(ii) **Proportion of mortality ($\hat{p}$).** The Binomial Theorem also can provide both an estimate of the true proportion of mortality ($\hat{p}$) in the population as well as confidence bounds on that estimate (see Table A4 of the reference in paragraph (j)(2)). For small sample sizes the interval may be large. For no mortalities in 20 earthworms ($\hat{p} = 0$), the upper 99% confidence bound on the estimate of $\hat{p}$ is 0.24 (95% upper confidence bound is 0.17), this means that the estimate of the true proportion of mortality may actually be as high as 0.24 when no earthworms die out of 20 tested (or as high as 0.17 for the 95% confidence estimate). For one mortality in 20 earthworms ($\hat{p} = 0.05$), the upper 99% confidence bound on the estimate of $\hat{p}$ is 0.32 (95% upper confidence bound is 0.29). For assessing risks, the confidence in the proportion impacted is considered in determining acute effects at environmental exposure doses.

(iii) **NOEC.** To ascertain that there is no observable adverse growth effect at the limit treatment (*i.e.*, NOEC $\geq$ limit dose), the limit treatment response is compared to the control treatment response using a one-sided two-sample parametric or nonparametric test for a reduction in growth from the control (see OCSPP 850.3000). The minimum significant difference detectable by the test or a similar estimate of the sensitivity of the test should be determined and reported.

(iv) **Multiple-concentration definitive testing.**

(A) At test termination, if two or more mortalities occur among the 20 earthworms tested or growth was significantly reduced from the control, a definitive dose-response test should be conducted.

(B) Multiple-concentration definitive testing may be waived if at test termination (28 days), one or fewer earthworms are dead at the limit concentration, and at the limit treatment there is also no observable adverse growth effect.

(4) **Multiple-concentration definitive test—**

(i) **Concentration-response curve, slope and LC_{50}.** Statistical procedures are employed to calculate the 28-d LC_{50} (standard error and 95% confidence interval) based upon mortality. If a concentration-response model (*e.g.*, probit) was fit to

the data to determine the LC_{50}, the model parameters (*e.g.*, slope) and their uncertainty estimates (*e.g.*, standard error) should be recorded.

(ii) **Concentration-response curve, slope and EC_{50}.** Statistical procedures are employed to calculate the 28-d EC_{50} (standard error and 95% confidence interval) based upon biomass weight loss. If a concentration-response model was fit to the data to determine the EC_{50}, the model parameters (*e.g.*, slope) and their uncertainty estimates (*e.g.*, standard error) should be recorded.

(iii) **NOEL.** Appropriate statistical methods (*e.g.*, one-way analysis of variance and multiple comparison tests) should be used to test for significant differences between treatment means and determine LOEC, NOEC and MATC for mortality, biomass weight loss, and other sublethal effects.

(iv) **Statistical methods.** Statistical procedures for modeling quantal and continuous data are available and should be used. Additional discussion about measurement endpoints and statistical procedures is found in OCSPP 850.3000.

(g) **Tabular summary of test conditions.** Table 1 lists the important conditions that should prevail during the definitive test. Except for the number of test organisms per concentration, number of replicate chambers, and test concentrations, Table 1 also lists the important conditions that should prevail during a limit test. Meeting these test conditions will greatly increase the likelihood that the completed test will be acceptable or valid.

Table 1.—Summary of Test Conditions for Earthworm Subchronic Toxicity Test

Test type	Spiked formulated soil
Test duration	28 days
Temperature	22 °C (constant during test within ± 2 °C)
Light quality	Incandescent or fluorescent lights
Light intensity	5.7 µmol/m^2/s (approximately 420 lux)
Photoperiod	Continuous light
Test chamber size	Glass canning jars, 1 pint, or equivalent
Substrate	Artificial (formulated) soil
Substrate amount per replicate	270 g wet weight (200 g dry weight)
Age/size of test organisms per replicate	Adult earthworms, 300 - 600 mg
Number of test organisms per concentration	30 (minimum)
Number of organisms per test chamber	10
Number of replicate chambers per concentration	3 (minimum)
Loading	300 - 600 mg per 270 g of soil
Feeding regime	No feeding during test
Test concentrations	Minimum of 5 test concentrations chosen in a geometric series plus a control, and a vehicle control if a vehicle is used
Measures of Effect or Measurement Endpoints	LC_{50} based upon mortality at 28 days at a minimum; EC_{50}, NOEC, LOEC and MATC based upon sublethal effects (weight change)

(h) **Test validity elements.** This test would be considered to be unacceptable or invalid if one or more of the conditions in Table 2 occurred. This list should not be misconstrued as limiting the reason(s) that a test could be found unacceptable or invalid. However, except for the conditions listed in Table 2 and in OCSPP 850.3000, it is unlikely a study will be rejected when there are slight variations from guideline environmental conditions and study design unless the control organisms are significantly affected, the precision of the test is reduced, the power of a test to detect differences is reduced, and/or significant biases are introduced in defining the magnitude of effect on measurement endpoints as compared to guideline conditions. Before departing significantly from this guideline, the investigator should contact the Agency to discuss the reason for the departure and the effect the change(s) will have on test acceptability. In the test report, all departures from the guideline should be identified, reasons for these changes given, and any resulting effects on test endpoints noted and discussed.

Table 2.—Test Validity Elements for Earthworm Subchronic Toxicity Test

1. All test chambers were not identical.
2. Treatments were not randomly assigned to individual test chamber locations or individual test organisms were not impartially or randomly assigned to test chambers.
3. The negative and vehicle control [when a vehicle was used] were not included in the test.
4. Average survival of either the negative or solvent control earthworms was less than 80% at the end of the test.
5. The total mean weight of the earthworms in the control containers declined significantly during the test (*i.e.*, by 30%).

(i) **Reporting—**

(1) **Background information.** Background information to be supplied in the report consists at a minimum of those background information items listed in paragraph (j)(1) of OCSPP 850.3000.

(2) **Guideline deviations.** Provide a statement of the guideline or protocol followed. Include a description of any deviations from the test guideline or any occurrences which may have influenced the results of the test.

(3) **Test substance.**

(i) Identification of the test substance: common name, IUPAC and CAS names, CAS number, structural formula, source, lot or batch number, chemical state or form of the test substance, and its purity (*i.e.* for pesticides, the identity and concentration of active ingredient(s)). If radiolabeled substance was used provide the radio purity and location(s) of the label.

(ii) Storage conditions of the test chemical or test substance and stability of the test chemical or test substance under storage conditions if stored prior to use.

(iii) Methods used for preparation of artificial soil, its age (*i.e.*, prepared 1 day or 7 days prior to use), and storage conditions if stored prior to use. If artificial soil is not used describe the source, collection method, handling and preparation of soil used in the test, its composition (percent sand, silt, and clay), and textural classification.

(iv) Source and amount of hydration water added to test soil and the moisture content for the test mixture at test initiation.

(v) Methods of preparation of the test substance and the treatment concentrations used in the range-finding and definitive test, or limit test: description of test chemical introduction into the test medium (*e.g.*, as a powder, stock solution), the mass and volume of soil and test substance used for each treatment, how test

substance was mixed into the soil, number of treatments and nominal test substance concentration in soil in each treatment at the beginning of the test.

(vi) If a vehicle (*e.g.*, solvent) is used to prepare stock or test substance provide: the name and source of the vehicle, the nominal concentration(s) of the test substance in the vehicle, in stock solutions, or mixtures, and the vehicle concentrations used in the treatments.

(vii) Description of stock solution preparation.

(4) **Test organisms.**

(i) Scientific and common names and method of verification, and source.

(ii) Culture method and conditions, and feeding history.

(iii) Health status of culture used for collection of test earthworms (*e.g.*, any prophylactic or preventative treatments, quarantine period).

(iv) Collection method and date of collection.

(v) Acclimation period.

(vi) Age and weight (by treatment and replicate) in tabular form at test initiation.

(5) **Test system and conditions.** Description of the test system and conditions used in the definitive or limit test, any preliminary range-finding tests, and any positive control tests.

(i) Description of the test chambers: type, size, volume, material, and conditioning method.

(ii) Statement verifying that earthworms were not fed during the test.

(iii) Volume and weight of soil used in each test container.

(iv) Time between introduction and mixing of the test substance with the soil and introduction of the earthworms.

(v) Number of worms per test container.

(vi) Number of test containers per concentration.

(vii) Methods used for test container and treatment randomization as well as methods for impartial assignment of earthworms to test containers.

(viii) Method of introducing earthworms to test containers.

(ix) Test duration.

(x) Methods and frequency of environmental monitoring performed during the definitive or limit study for lighting, soil temperature and soil pH.

(xi) Method, type and frequency of observations made on earthworms during the test.

(xii) For the definitive or limit test, all analytical procedures and preservation methods should be described. The accuracy of the method, method detection limit, and limit of quantification should be given.

(6) **Results.**

(i) Environmental monitoring data results (lighting, soil temperature and pH) in tabular form (provide raw data for measurements not made on a continuous basis), and descriptive statistics (mean, standard deviation, minimum, maximum).

(ii) For preliminary range-finding test(s), if conducted, the number of dead and intoxicated earthworms at each concentration level and in the control(s).

(iii) For the definitive and limit test, test substance concentration in the soil at test initiation (day 0), during (if needed, at day 7, 14, 21) and test conclusion (day 28), in tabular form and descriptive statistics (time weighted average, standard deviation, minimum, maximum).

(iv) The total organic carbon (TOC) content of the soil mixture.

(v) For the definitive and limit test, the number of dead earthworms at each observation period at each treatment level and control(s) (provide the raw data).

(vi) For the definitive and limit test, the burrowing behavior of earthworms observed at test initiation: description of behavior, duration, severity, and number affected at each treatment level and control(s) (provide the raw data).

(vii) For the definitive and limit test, a description of all observations made on the test earthworms that are suggestive or predictive of toxicity and all concomitant gross toxicological manifestations include time of onset, duration, severity, and number affected at each treatment level and control(s) (provide the raw data).

(viii) For the definitive and limit test, the weight, in tabular form, at each test concentration (including controls), in each test chamber at test initiation and termination.

(ix) For the definitive test, the LC_{50} value, and its standard error and 95% confidence interval should be reported for 7, 14, 21, and 28-day periods. The slope of the concentration-response curve, its standard error, and 95% confidence interval should also be reported.

(x) For the definitive test, the EC_{50} for weight with its standard error and 95% confidence interval, and the LOEC, NOEC, and MATC for day 28.

(xi) Description of statistical method used, including software package, for determining LC_{50} EC_{50}, LOEC, NOEC, and MATC values and the concentration-response model parameters and the basis for the choice of method. Provide results of any goodness-of-fit tests and determination of minimum significant differences.

(j) **References**. For additional background information on this test guideline, the references in this paragraph should be consulted:

(1) American Society for Testing and Materials. ASTM E 1676-04, Standard guide for conducting laboratory soil toxicity or bioaccumulation tests with the lumbricid earthworm *Eisenia fetida* and the enchytraeid potworm *Enchytraeus albidus*. Current edition approved April 1, 2004. In Annual Book of ASTM Standards, Vol. 11.06, West Conshohocken, PA.

(2) Conover, W. 1980. Practical Nonparametric Statistics, 2nd Edition. John Wiley & Sons, Inc., New York, NY. 493 pp.

(3) Edwards, C.A., 1984. Report of the second stage in development of a standardized laboratory method for assessing the toxicity of chemical substances to earthworms. The Artificial Soil Test. DG X1/AL/82/43, Revision 4.

(4) Fender, W.M., 1985. Earthworms of the Western United States. Part 1. Lumbricidae, Megadrilogica 4: 93-129.

(5) Jaenicke, J., 1982. *"Eisenia foetida"* is two biological species. Megadrilogica 4:6-8.

(6) Greene, J.C., Bartels, C.L., Warren-Hicks, W.J., Parkhurst, B.R., Linder, G.L., Peterson, S.A., and Miller, W.E., 1989. Protocols for Short-Term Toxicity Screening of Hazardous Waste Sites, EPA/600/3-88/029, U.S. Environmental Protection Agency, Environmental Research Laboratory, Corvallis, OR.

(7) Hartenstein, R. *et al.*, 1979. Reproductive potential of the earthworm *Eisenia foetida.* Oecologia *43*:329-340.

(8) Meier, J.R., Chang, L.W., Jacobs, S., Torsella, J., Meckes, M.C. and Smith, M.K., 1997. Use of plant and earthworm bioassays to evaluate remediation of soil from a site contaminated with polychlorinated biphenyls. Environmental Toxicology and Chemistry 16(5): 928-938.

(9) Neuhauser, E.F. *et al.*, 1986. Contact and artificial soil tests using earthworms to evaluate the impact of wastes in soils, In: Hazardous and Industrial Soil Waste Testing: Fourth_Symposium, ASTM STP 886. J.K. Petros, Jr. and R.A. Conway, eds., American Society for Testing and Materials. Philadelphia, PA pp. 192-203.

(10) Neuhauser, E.F. *et al.*, 1985. The toxicity of selected organic chemicals to the earthworm *Eisenia fetida*. *Journal of Environmental Quality* 14: 383-388.

(11) Oien, N. and J. Stenerson, 1984. Esterases of earthworm - III. Electrophoresis reveals that *Eisenia foetida* (Savigny) is two species. Comparative Biochemistry and Physiology 78c (2):277-282.

(12) Plumb, R.H., Jr., 1981. Procedures for handling and chemical analysis of sediment and water samples. Technical Report EPA/CE-81-1, prepared by Great Lakes Laboratory, State University College at Buffalo, Buffalo, NY., for the U.S. Environmental Protection Agency/Corps of Engineers Technical Committee on Criteria for Dredged and Fill Material. U.S. Army Engineer Waterways Experiment Station, CE, Vicksburg, MS.

(13) Reinecke, A.J. and Venter, J.M., 1987. Moisture preferences, growth and reproduction of the compost worm *Eisenia fetida* (Oligochaeta). Biology and Fertility of Soils 3: 135-141.

(14) Satchell, J.E. and Dottie, D.J., 1984. Factors affecting the longevity of earthworms stored in peat. Journal of Applied Ecology 21: 285-291.

(15) Stafford, E.A. and Edwards, C.A., 1985. Comparison of heavy metal uptake by *Eisenia faetida* with that of other common earthworms. Final Technical Report. Entomology Department, Rothamsted Experiment Station, Harpenden, Herts. ALS 2JQ, U.K. U.S. Army Contract DAJA 45-84-0027.

(16) Spurgeon, D.J. and Hopkin, S.P., 1995. Extrapolation of the laboratory-based OECD earthworm toxicity test to metal-contaminated field sites. Ecotoxicology 4:190-205.

(17) Tomlin, A.D. and Miller, J.J., 1980. Development and fecundity of the manure worm, *Eisenia faetida* (Annelida:Lumbricidae), under laboratory conditions. In: D.L. Dindal (ed.), Soil Biology as Related to Land Use Practices. Proc. 7th Internat. Soil Zool. Coll. of ISSS. EPA, Washington, DC., pp. 673-678.

(18) vanGestel, C.A.M., and van Dis, W.A., 1988. The influence of soil characteristics on the earthworm *Eisenia fetida andrei* (Oligochaeta). Biology and Fertility of Soils 6:262-265.