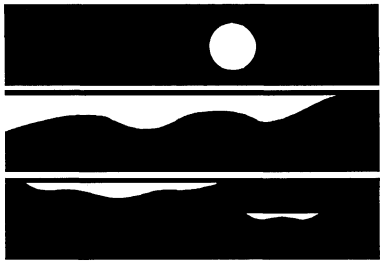

Earthworm Bioassay Protocol for Soil Toxicity Screening

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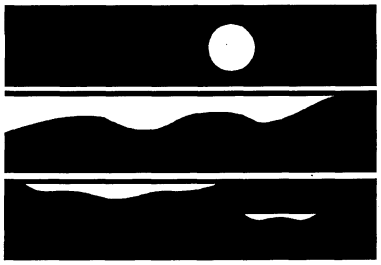

Earthworm Bioassay Protocol for Soil Toxicity Screening

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for the
Toxics Cleanup Program
Olympia, Washington 98504-7600

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Appendices

A: Specification for Type II Water

B: Laboratory Data Collection Cover Sheet

C: Laboratory Test Monitoring Data Sheet

Summary of 14-Day Earthworm Bioassay

Test Duration:	14 days
Temperature:	22±2°C
Photoperiod:	24-hours, using ambient laboratory light levels (400 lx or 7 uEm^{-2}s^{-1})
Test container:	1-pt standard glass canning jars or 500ml borosilicate glass beakers with sealable lid.
Total amount required (soil weight):	700g
Test amount/replicate:	200g
Test soil moisture content:	35 to 45%
Test Organism:	*Eisenia foetida*
Age of test organisms:	>2 months (i.e., fully clitellated)
Number of organisms/replicate:	10
Number of replicates:	3
Feeding regime:	Do not feed during test
Soil pH:	5.0 - 9.0, do not run test if outside acceptable pH range
Endpoints measured:	Survival, morphological and behavioral alterations
Positive control:	2-chloroacetamide
Negative control:	Artificial soil

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1. Introduction

1.1 Application And Background

Earthworm bioassays are a widely-recognized tool for evaluating the toxicity of contaminated soils (Becker *et al.*, 1991; Beyer, 1990; Greene *et al.*, 1988; Van Gestel *et al.*, 1988; Callahan *et al.*, 1985; Edwards, 1984; Porcella, 1983). The earthworm bioassay described in this document is intended to be used in screening-level assessments of soil toxicity at hazardous waste sites being investigated under the Washington Model Toxics Control Act (MTCA) Cleanup Regulation.

In this protocol, earthworms (*Eisenia foetida*) are added to field collected site soils. The primary test endpoint is earthworm mortality, recorded on day 14. It is not necessary to conduct a dilution series.

The testing procedures described in this document have been adapted from protocols developed by the following groups: United States Environmental Protection Agency (USEPA) Research Laboratory, Corvallis, Oregon (Green *et al.*, 1988); the Commission of the European Communities of the Organization for Economic Cooperation and Development (Edwards, 1984); and documents prepared for the *Soil and Plant Toxicity Assessment Short Course* (Linder *et al.*, 1991) at the 12th Annual Meeting of the Society of Environmental Toxicology and Chemistry in November 1991.

1.2 Health and Safety

Testing of materials from hazardous waste sites may involve significant risks to laboratory personnel. All persons potentially exposed to or involved with the material(s) to be tested should protect themselves by taking all necessary safety precautions to prevent physical harm. Procedures to prevent inhalation or dermal absorption of test materials should be observed. Each laboratory should be equipped with all necessary safety equipment prior to initiation of toxicity testing.

For guidance on safe practices when conducting toxicity tests, see laboratory and general industrial safety manuals, as well as Peltier and Weber (1985) and USEPA (1977).

2. Sample Requirements

2.1 Sample Size

Approximately 700g of soil per sample is required to perform this test. Each sample is tested using three replicates (a replicate is defined as one 200g aliquot of the bulk sample). The remainder of the sample (approximately 100g) is to be used for soil moisture and pH determinations. If chemical characterization of the soil, beyond the parameters listed in this protocol, is to be performed in conjunction with toxicity testing, additional soil must be collected. At the time of collection, sample containers should be filled completely to minimize headspace.

2.2 Sample Storage

Upon arrival at the laboratory facility, all soil samples should be stored in a dark, vented refrigerator at 4°C. Samples must be properly sealed and packaged when moving them from storage to work spaces as exposure to air may volatilize some substances. When the samples are being prepared for testing, they should be opened and dispensed in a fume hood. Sample preparation should begin as soon as possible, preferably within 24 hours of sample collection. Realizing that it is not always possible to initiate the test within 24 hours of collection, under no circumstances should the samples be held longer than 14 days from collection to test initiation. The toxicity of some samples may be affected when held beyond 14 days due to loss or degradation of contaminants present in the samples. This is especially true for volatile organics. It is recommended that standard Chain-of-Custody procedures be followed when handling and analyzing samples collected from hazardous waste sites.

3. Supplies

3.1 Equipment

The following equipment is required to perform the earthworm bioassay:

- Forceps
- pH meter
- Watch glasses
- Wash bottles for rinsing glassware and probes
- Water purification system that produces Type ll water (ASTM, 1983)
- Environmental chamber, incubator, or equivalent facility with temperature control (22±2°C), continuous light at a minimum of 400 lx (7 $uEm^{-2}s^{-1}$).
- Analytical balance, capable of weighing earthworms to 0.1g.
- Top loading balance, capable of weighing soil samples to 1.0g.
- Reference weights, Class S, for checking performance of balance. Weights should bracket the expected weights of the weighing pans as well as the weights of the pans plus the samples.
- Test chambers, standard 1 pt canning jars with lids and screw rings or 500ml glass laboratory beakers with sealable tops.
- Volumetric flasks and graduated cylinders, Class A, borosilicate glass or non-toxic plastic labware, 10 to 1000ml.
- Volumetric pipettes, Class A, 1 to 100ml.
- Serological pipettes, 1 to 10ml. graduated
- Pipette bulbs and fillers

- Bulb thermograph or electronic-chart type thermometers for continuous recording of temperature in the environmental chamber. Automated electronic data collection systems designed to monitor chamber conditions are also acceptable and in fact preferred.

- Glass thermometer

- Stainless steel spatulas or scoops

- Gloves, non-powdered

- Screen, 2 to 2.36 mm mesh (Tyler 8 mesh)

3.2 Materials

The following materials are required to perform the earthworm bioassay:

Soil Hydration Water

Water for soil hydration must, at a minimum, meet specifications for Type ll water (ASTM, 1983). Type II water is typically produced by distillation or deionization. However, any method of preparing the soil hydration water is acceptable provided that the requisite quality can be met. Specification for Type II water are included in Appendix A. Hydration water is considered to be of constant quality, if the monthly ranges of hardness, alkalinity and specific conductance vary by <10% of their respective averages, and if the monthly range of pH varies by <0.8 units of its average.

pH Buffers

Buffers for pH 4, 7, and 10 are required for standards and calibration checks (see USEPA Method 1501, USEPA 1979a).

Reference Toxicant

2-chloroacetamide, 99% purity (Heimbach and Edwards, 1983).

Artificial Soil

Specifications for soil composition (dry weight) are as follows: 10% peat moss, 20% Kaolin clay, 70% #70 grade silica sand, $CaCO_3$ (99% purity) to adjust pH to 7.0±0.5.

3.3 Test Organisms

Adult earthworms (*Eisenia foetida),* at least 2 months old (i.e., fully clitellated) from the same culture are used as the test species. The earthworms may be purchased from any reputable supplier. However, the species should be verified using appropriate systematic keys (Fender, 1985 or Reynolds, 1977). Although Jaenike (1982) presents some evidence for splitting of the species *Eisenia foetida* (Savigny) into subspecies, *Eisenia foetida foetida* and *Eisenia foetida andrei* (Bouche), there is currently little or no documented evidence of a difference in response to chemicals.

4. Preparation of Artificial Soil

4.1 Ingredients

- Peat moss - Canadian peat (*Sphagnum sp.*)
- Kaolin clay (particle size under 40 microns)
- Silica sand - #70 mesh grade
- $CaCO_3$ - 99% purity

Prepare the artificial soil by combining the ingredients listed above in the following percentages (dry weight): 70% - #70 mesh silica sand, 20% kaolin clay, 10% peat moss (screened with a 2.36 mm, Tyler 8 mesh or equivalent).

Once the materials are combined together, mix thoroughly, and add an amount of calcium carbonate ($CaCO_3$) equal to 0.40% of the combined total weight. This should adjust the pH of the mixture to 7±0.5. Check the pH of an aliquot using the procedure given in Section 5.2. Additional calcium carbonate may be added to the mixture if needed to adjust the pH to 7.0±0.5.

After pH adjustment, hydrate the artificial soil to 45% moisture content. This should take about 297ml of hydration water for 600g of soil (600g * 0.45= 270g = 270ml).

5. Preparation of Soil Samples

5.1 Moisture Fraction Determination

The initial moisture content of the bulk soil samples must be determined to calculate the appropriated amount of hydration water to add to each test sample.

To determine moisture content place a 25g aliquot of the bulk soil sample in a clean, weighing vessel and weigh it to obtain the initial wet weight for moisture content calculations. The combined weight of the sample and dish equals the initial wet weight.

Dry the sample at 103 - 105°C for 24 hours. After drying, place the sample into a desiccator to cool. After cooling, reweigh the dried sample. The combined weight of the dish and the dried sample equals the final dry weight.

Calculate the moisture fraction of the sample using the following formula:

MF= (I - F)/[A - (I - F)] * 100

where MF= Moisture fraction of bulk soil (in %)
I= Initial wet weight of sample + crucible (in g)
F= Final dry weight of sample + crucible (in g)
A= Initial aliquot weight (in g)

Example Calculation:

I= 40g
F= 35g
A= 25g

MF= (40g - 35g)/[25g - (40g - 35g)] * 100= 25%

5.2 Measurement of Soil pH

To measure soil pH, make a slurry of hydration water and soil in a 1:1 ratio. Combine 25g of soil and 25ml of hydration water in a 100ml beaker. For soils high in clay content, an additional 25ml of hydration water may need to be added so that a mixable slurry will be produced. Mix the slurry with a magnetic stir bar on a stirring plate for 5 minutes, then measure and record the pH of the slurry. Allow the slurry to settle for 30 minutes. Recheck and record the pH of the supernatant.

5.3 Hydration Of Soil Samples

Soil samples must be hydrated to between 35 to 45% of the bulk soil initial weight for use in testing (Dave Wilborn, 1993). Use the following formulas to determine the amount of water to add to each test sample to achieve a moisture content of 35%:

H= T - MF

where H= Hydration needed (in %)
T= Target moisture content (in %)
MF= Initial moisture fraction of bulk soil (in g)

Example Calculation:

T= 35%
MF= 25%

H= 35% - 25%= 10%

W= (B * H/100) * C

where W= Amount of water to add to sample (in ml)
B= Weight of bulk soil sample (in g)
H= Hydration needed (in %)
C= Conversion factor (1ml/1g)

Example Calculation:

B= 600g
H= 10%

W= (600g * 10%/100) * (1ml/1g)= 60ml

Hydration of the artificial soil should be completed prior to hydration of any test samples. This order allows comparison of the friability (soil crumbles when handled) between the artificial soil and the test samples. If the test sample, when hydrated to 35% of its initial weight, looks and feels similar to the artificial soil, do not hydrate the sample any higher. If the test sample seems drier than the artificial soil, then hydrate to 40% and recheck the test soil against the artificial soil. Continue to a maximum hydration of 45% in any test soil to achieve a comparable friable condition. Hydration of test soil should be performed in a hood. Record the amount of hydration water added to each test sample.

6. Reference Toxicant Testing (2-Chloroacetamide)

6.1 Preparation of Positive Controls

Reference toxicant testing must be conducted, at a minimum, monthly if an in-house worm culture is used for testing. If worms are purchased from an outside supplier, then reference toxicant testing should be performed for each new batch of worms received. Reference toxicant testing may be conducted either separately or concurrently with sample testing. However, it is recommended that acceptable earthworm response be established prior to testing samples.

At least three different concentrations of 2-chloroacetamide are required for the positive controls. The reported LC50 for earthworms using 2-chloroacetamide is 38.5mg/kg (Edwards, 1984). Concentrations of 19.25, 38.5, and 77.0mg/kg are suggested for the reference toxicant tests.

Calculations needed to determine the amount of 2-chloroacetamide stock solution to add to 600g of artificial soil (minimum amount needed for three replicates) for the reference toxicant test are shown below. The 38.5mg/kg concentration is used in the example below. Calculations for the other two concentrations of the positive controls are performed similarly.

Calculate the amount of 2-chloroacetamide powder to add to 600g of artificial soil

R= W * C * T

where R= amount of reference toxicant to add (in mg)
W= bulk sample weight used (in g)
C= conversion factor (1kg/1000g)
T= target concentration of reference toxicant (in mg/kg)

Example Calculation:

W= 600g
T= 38.5mg/kg

R= (600g) * (1kg/1000g) * (38.5mg/kg)= 23.1mg

This is the amount of 2-chloroacetamide powder required for 600g of artificial soil. However, a stock solution of 2-chloroacetamide will be used in the reference toxicant testing. The 2-chloroacetamide stock solution is typically made at a concentration of 22.5mg

2-chloroacetamide/ml water (Dave Wilborn, 1993). However, the laboratory may use any concentration for the stock solution that is appropriate to avoid waste.

If the laboratory is using a stock solution at 22.5mg/ml (4.5mg of 2-chloroacetamide powder in 200ml dilution water), then perform the calculation as follows:

V= R/S

where V= volume of stock solution to add (in ml)
R= amount of reference toxicant to add (in mg)
S= concentration of stock solution (in mg/ml)

Example Calculation:

R= 23.1mg
S= 22.5mg/ml

V= (23.1mg)/(22.5mg/ml)= 1.03ml

The artificial soil will need to be hydrated to a moisture content of 45%, which would require 270ml of hydration water (600g * 0.45= 270g = 270ml). Subtraction of the stock solution volume (1.03ml) produces a final volume of 269ml of hydration water (270ml - 1.03ml= 269ml) needed in combination with the reference toxicant to achieve a moisture content of 45%. The 2-chloroacetamide concentration must be measured precisely (mechanical pipette). However, the amount of water may be measured by mechanical pipette or volumetric flask. The water and stock solution are mixed together in a beaker prior to adding to the artificial soil. The final volumes of hydration water and reference toxicants used should be recorded on the sample sheets.

Mix the soil for hydration in a container (plastic bag, stainless steel bowl or equivalent). If plastic bags are selected, make certain they will not leach toxins into the soil samples. Hydrate the artificial soil in a fume hood. Follow the procedures described in Section 5.2 and 7.0 respectively, to measure the pH and prepare for testing.

6.2 Reference Toxicant Data Analysis

Calculate and report the 14-day LC50 and 95% confidence limits for 2-chloroacetamide of the positive control data and check the results graphically. If the Trimmed Spearman-Karber method is used, follow the methods described by Hamilton *et al.* (1977). If the Probit technique is used, follow the methods described by Weber *et al.* (1989). Any other statistical method(s) approved by USEPA may be substituted for the trimmed Spearman-Karber or Probit techniques.

7. Conducting the Test

7.1 Test Containers and Labeling

Two types of test containers are acceptable: standard one-pint canning jars with screw-on lids or glass beakers with sealable lids. The lids of the containers should have a small hole (1-2 mm) drilled or punched in their center to allow for air exchange, and yet prevent the worms from escaping. Prior to use, all test containers and lids should be decontaminated with hot water/laboratory grade detergent, rinsed, and then baked in a drying oven for a minimum of 1 hour. If it is not possible to remove visible contamination for either the container or lid they should be discarded. After cleaning, label the side of each container with a minimum of the sample #, replicate #, and date of test initiation.

7.2 Loading Samples

Working under a fume hood, place the appropriate amount of hydrated soil sample (200g) into each jar using a spatula or scoop. Measure a single initial temperature (using the procedure described below) in one jar. This procedure assumes that all soils have been held together long enough for temperature equilibration to have occurred.

1. Place a glass thermometer in the selected jar (at mid depth in the test soil) and allow the thermometer to equilibrate for 10 min before reading the temperature.

2. If the initial temperature is outside the range of 22±2°C, place the test soils in the environmental chamber for 1 hour to equilibrate to the test range.

3. Recheck the temperature.

7.3 Negative Control

Use artificial soil without the addition of any toxicant for the negative control. Because the artificial soil is dry, hydrate it to 45% moisture content (600g x 0.45 = 270g = 270ml of water) prior to testing. Three replicates of the negative control are required.

7.4 Selection of Test Organisms

1. While wearing gloves, gently sift through the culture tray, examining each worm for condition and maturation (i.e., clitellate).

2. Place the worms in a central holding tray. Repeat this procedure until enough worms have been collected to perform the test.

3. Tare the bottom half of a disposable petri dish or beaker on an analytical balance.

4. Take ten worms at random from the culture tray and weigh them in the dish. It may not be possible to remove all the culture tray bedding material from each individual worm, but weigh as little bedding as possible.

5. Record their combined weight on the data collection sheet for the test container in which they are to be placed. Each batch of ten worms should weigh 3.0-6.0g.

6. Working under a fume hood, gently empty the petri dish with the group of ten worms in a random sequence onto the surface of the soil sample in a test jar. It is important not to damage the worms in the process. If any of the worms appear injured, they must not be used.

7. After the worms are placed in the test container, secure the screw ring or sealable lid to the test container.

Place the test containers in an environmentally controlled chamber or equivalent. Conduct the test at 22±2°C. A temperature monitoring device, located inside the chamber, should be used during the test. To promote burrowing, the worms must be exposed to continuous lighting, minimum of 400 lx (7 $uEm^{-2}s^{-1}$). Assign test containers to locations on the shelves of the chamber in a random sequence. Record the starting time of the test on the data collection sheet.

8. Test Monitoring

Although the test containers do not need to be opened during the test period, a visual inspection of them on day 7 of the test should occur. If the worms are on the surface and/or balled together, note this behavior on the test monitoring sheet.

8.1 Feeding Regime During Testing

The earthworms are not fed during the test period.

8.2 Test Termination, Survival Count, and Observations

The test duration is 14 days. Remove the temperature chart and check to see whether the temperature has remained constant. A photocopy of the chart should be attached to the paperwork for the test. Count the negative control first. Place a sorting tray (chemically-resistant material or glass) in a fume hood and empty the contents of a negative control replicate into it, keeping the emptied jar near the sorting tray. Retain the lid nearby for holding worms. Gently sort the soil with gloved fingers or with a flat stainless steel spatula. As the worms are located place them onto the lid, keeping them bunched. Remove dead worms from the soil and discard them.

When counting the worms, observe for altered behavior(s) or morphological changes, and record if present. Possible behavioral alterations include: lack of burrowing, coiling, "balling" together. Morphological changes include: contraction, rigidity, elongation, ulceration of the integument, mid-segmental swelling, segmental constriction, and/or segmental loss. On the data collection sheet, record the number of altered behaviors/morphological changes and dead worms counted in each test jar. A worm is defined as dead if it does not respond to a gentle mechanical stimulus to its anterior end.

Worms decay rapidly in moist soils, and if ten worms are not accounted for, the missing worms should be considered dead and completely decomposed. In addition, worms can lose a number of segments and still be able to move. Any worm that has lost a major portion of the body, yet remains capable of movement, is counted as alive. The alteration of body length should be noted under sublethal effects.

Mortality and morphological changes should seldom occur in the negative controls. After all worms are accounted for, push the soil off the sorting tray back into the test jar. Place the worms back into the jar on the surface and allow them to burrow into the soil.

If a reference toxicant test is being conducted, count the reference toxicant replicates next. Finally, count the worms in the test soil samples. If a toxicity gradient is suspected, begin with what is thought to be the lowest concentration, proceeding to increasing toxicity. Wash the

sorting tray with soap and water, and dry thoroughly with paper towels between each count. After counting and recording data, place worms from all replicates into a plastic bag for freezing and disposal.

Measure and record the final pH values of one replicate of each sample tested, including the positive and negative controls.

9. Data Analysis and Reporting

9.1 Cover Sheet

The cover sheet should contain specific information regarding the test including but not limited to the following (see appendix B for a sample sheet):

- Name of site where the soil was collected and other identifying information.
- Date and time the test is started.
- Name of person performing the test.
- Moisture fraction of artificial and test soils at test initiation
- Amount of water added to hydrate the soil samples.
- Any deviations/problems with conductance of the test.

9.2 Data Report

The final report should include all data collection, calculation, and observation sheets. The observation sheets should include all data obtained during the test that are suggestive of toxicity, including behavioral alterations (see Appendix C for sample sheet). If not provided elsewhere, also include the following information:

Data Handling

Calculate the percent survival for each replicate of the test and control samples at the end of the 14-day period and enter the results on the data collection sheet. Also report the average survival for each test sample and each control across replicates. Record any unusual behaviors or morphological changes that were observed. Calculate the percent of occurrence of these changes for each replicate of the test and control samples.

Statistical Analysis

List or describe all statistical procedures or/and software used for data analysis. Use Dunnett's Test (Weber *et al.,* 1989; Eirkson *et al.,* 1987; Zar, 1984) or other method with comparable power.

Control Charts

Prepare a control chart for the reference toxicant series by plotting successive toxicity values (LC50) and examining the results to determine whether they are within the prescribed limits. The mean and upper and lower control limits (±25%) are recalculated with each successive

point. Maintain a running plot for the toxicity values of successive tests with the reference toxicant. For further details, see Weber *et al.* (1989) or Greene *et al.* (1988).

Test Organisms

Report detailed information about the earthworms used including brood stock, scientific name and method of verification, age (i.e., clittela development), source, treatments, feeding history, and culture method.

Test Conditions

Report a description of the test conditions, especially if there was a deviation from this protocol. Report the soil preparation, addition of chemicals, culturing of the test species, lighting, pH, temperature, replicates, or the number of earthworms per jar.

Test Containers

Report a description of the test container used, its size, volume and weight of soil used for each replicate, and number of replicates per sample.

Chemical Analyses

Report the results of all moisture content, pH, and temperature measurements of the soil samples taken during the test. Report the source of the hydration water, the date and time of its collection, and any pretreatment (e.g., filtration).

Protocol Deviations and Problems

Report any deviations from the procedures outlined in this document and anything unusual about the test (e.g., equipment failure, fluctuations in temperature or other environmental conditions).

10. Quality Assurance/Quality Control (QA/QC)

10.1 Requirements and Specifications

Quality assurance (QA) practices for hazardous waste toxicity tests consist of all aspects of the test that affect data quality: (1) sample handling, (2) source and condition of the test organisms, (3) condition of equipment, (4) test conditions, (5) instrument calibration, (6) replication, (7) use of reference toxicants, (8) record keeping, and (9) data evaluation. The QA guidelines presented here are adapted from Green *et al.* (1988). For general guidance on good laboratory practices related to toxicity testing see FDA (1978), USEPA (1979b, 1980a and 1980b), and DeWoskin (1984).

Handling of Soil Sample

Soil samples collected for testing must be handled and stored as described in Section 2.0.

Facilities, Equipment and Test Chambers

Laboratory temperature control equipment must be adequate to maintain the required temperature (22±2°C) throughout the test.

Analytical Methods

Routine chemical and physical analyses must include established QA practices (USEPA 1979a,c).

Calibration and Standardization

Instruments used for routine chemical and physical parameters, such as pH and temperature, must be calibrated and standardized according to instrument manufacturers procedures.

Test Conditions

Soil temperatures must be maintained within the limits specified for each test. The pH of the soils must be checked at the beginning and the end of the test period.

Water for Soil Hydration

The hydration water used in the bioassay must meet the specifications for ASTM Type II water.

Test Organisms

The earthworm, *Eisenia foetida* (Bouche), is the test species used in this bioassay. This species has been used extensively in laboratory toxicity tests (Neuhauser and Callahan, 1990; van Gestel *et al.*, 1989; Heimbach, 1985, and Edwards 1984).

Earthworms should be purchased from the same commercial source to start cultures from a single gene pool. The supplier should be able to verify the species. Once verified, cultures should be maintained at the test facility. Records should be kept regarding the source of the initial stock and culturing techniques.

At a minimum, all organisms used in a particular test should originate from the same population. Changes in culture response can be monitored by using 2-chloroacetamide as a positive control, see Section 5.6. For a complete description of culture methods for *Eisenia foetida*, see Green *et al.* (1988).

Quality of Test Organisms

If the laboratory obtains earthworms from an outside source, the sensitivity of each batch of earthworms must be verified. Use the positive reference toxicant to evaluate sensitivity, and conduct the evaluation concurrently with the testing of soil samples from the hazardous waste site.

If the laboratory maintains in-house breeding cultures, the sensitivity of the offspring must be evaluated every month using a toxicity test with the reference toxicant. This evaluation may be performed prior to tests of soil samples.

Earthworms should not be used if they have been under stress from extremes (high or low) in food, moisture availability (Reinecke and Venter, 1987), temperature (Tomlin and Miller, 1980), pH (Satchell and Dottie, 1984), or crowding. Any of these conditions may adversely influence the health of the test organisms and subsequent test results.

Test Acceptability

Test results are unacceptable if negative control survival is <90%. An individual test may be conditionally accepted if temperature or light conditions fall outside specifications, depending on the degree of the departure and the objectives of the sampling and analysis plan.

Precision

The ability of the laboratory personnel to obtain consistent results must be demonstrated with the reference toxicant before attempting to measure toxicity of soils from hazardous waste sites. Overall laboratory precision conducting the earthworm bioassay should be determined by performing five or more tests with a reference toxicant. Precision can be described by the mean, standard deviation, and coefficient of variation (CV) of the calculated endpoints from the replicated tests.

Replication and Test Sensitivity

Test sensitivity (response at low concentrations) depends in part on the number of replicates, the probability level selected, and the type of statistical analysis conducted. A minimum of three replicates per sample are required.

Control Charts

Prepare a control chart for the reference toxicant by plotting successive toxicity values (LC50) and examining the results to determine whether they are within the prescribed limits. The mean and upper and lower control limits (±25%) are recalculated with each successive point. Maintain a running plot for the toxicity values of successive tests with the reference toxicant. For further details, see Weber *et al.* (1989) or Greene *et al.* (1988).

If the LC50 from a given test with the reference toxicant does not fall in the expected range for the earthworms when using the standard hydration water, then the sensitivity of the organisms and the overall credibility of the test system are suspect. In this case, the test procedure should be examined for defects and should be repeated with a different batch of earthworms.

Record Keeping

Proper record keeping is mandatory. Bound notebooks should be used to maintain detailed records of the test organisms such as species, source, age, date of receipt and other pertinent information relating to their history and condition. Additionally, information on the calibration of equipment and instruments, test conditions employed, and the test results must be recorded. Annotations should be kept current to prevent loss of information.

11. References

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Appendices

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Appendix A: Specifications* for Type II Water

Parameter	Specification
Total Matter, maximum (mg/l)	0.1
Specific Conductivity, maximum (umhos/cm @ 25°C)	1.0
Electrical Resistivity, minimum (megohm-cm @ 25°C)	1.0
Minimum color retention of potassium permanganate (minutes)	60
Maximum soluble silica	ND
Microbiological classification	A
Organic contaminants	B

*= ASTM, 1983
ND= Not detectable
A= When bacteria levels need to be controlled, reagent grade types should be further classified as follows: Max total bacteria count- A= 0/ml; B= 10/ml; and C= 100/ml
B= Type I water is intended for most analytical procedures and all procedures requiring water low in organics..

Appendix B: Laboratory Data Collection Cover Sheet

Name of Field Site _____

Date of Field Sample Collection _____

Chemical Analysis Results of Soil Sample:

Identified Contaminants _____

_____

_____

Suspected Contaminants _____

_____

_____

Laboratory Test Initiation Date_____ Time_____

Laboratory Test Termination Date _____ Time _____

Name of Laboratory Technician Conducting Test _____

pH of Sample Soil: Slurry start_____ end_____

Supernatant start_____ end_____

Deviations/Problems (list and describe below):

_____

_____

_____

_____

_____

_____

Appendix C: Laboratory Test Monitoring Data Sheet

Lab ID No.	Replicate No.	Soil Moisture Content Start (%)	Wt. of Worms	Slurry pH Start (s.u.)	Supernatant pH Start (s.u.)	Slurry pH Day-14 (s.u.)	Supernatant pH Day-14 (s.u.)	Temp. Start (EC)	Temp. Day-14 (EC)	No. of live worms Start	No. of live worms Day-14	Sublethal effects- (lethargy, balling, lack of burrowing) Day-14	Sublethal effects- as percent of total no. of worms Day-14

Any 7-day visual observations/comments- _____

_____

Test Termination Comments- _____

_____