Nematode Bioassay Protocol for Soil Toxicity Screening

August 10, 2004

Publication No. 04-09-044

printed on recycled paper

*The Department of Ecology is an equal opportunity agency
and does not discriminate on the basis of race, creed,
color, disability, age, religion, national origin,
sex, marital status, disabled veteran's status,
Vietnam Era veteran's status or sexual orientation.*

*If you have special accommodation needs or require this document
in alternative format, please contact the
Toxics Cleanup Program, Policy and Technical Support Section,
Chris Peredney at (360) 407-7188 (voice).
Ecology's telecommunications device (TDD) number at Ecology
Headquarters is TTY 711 or 1-800-833-6388.*

*For additional copies of this publication,
please contact:*

*Department of Ecology
Publications Distributions Office
P. O. Box 47600
Olympia, Washington 98504-7600
(360) 407-7472*

Refer to Publication Number 04-09-044

Nematode Bioassay Protocol for Soil Toxicity Screening

Edited by
Chris Peredney

Toxics Cleanup Program
Olympia, Washington 98504-7600

March 15, 2004

Publication No. 04-09-044
printed on recycled paper

Table of Contents

Page

Summary of 24 and 48-Hour Nematode Bioassay iii
Acknowledgements iv
1. Introduction 1
1.1 Application And Background 1
1.2 Health and Safety 1
2. Sample Requirements 2
2.1 Sample Size 2
2.2 Sample Storage 2
3. Supplies 3
3.1 Equipment 3
3.2 Materials 4
3.3 Test Organisms 7
4. Preparation of Artificial Soil 8
4.1 Ingredients 8
5. Preparation of Soil Samples 9
5.1 Moisture Fraction Determination 9
5.2 Measurement of Soil pH 9
5.3 Hydration of Soil Samples 10
5.4 Analytical 10
6. Reference Toxicant Testing (Cupric chloride) 11
6.1 Preparation of Positive Controls 11
6.2 Reference Toxicant Data Analysis 11
7. Conducting the Test 12
7.1 Test Containers and Labeling 12
7.2 Loading Samples 12
7.3 Negative Control 13
7.4 Selection of Test Organisms 13
8. Test Monitoring 14
8.1 Feeding Regime during Testing 14
8.2 Test Termination, Survival Count, and Observations 14
9. Data Analysis and Reporting 15
9.1 Cover Sheet 15
9.2 Data Report 15
10. Quality Assurance/Quality Control (QA/QC) 17
10.1 Requirements and Specifications 17
11. References 20

Appendices

A: Specification for Type II Water

B: Laboratory Data Collection Cover Sheet

C: Laboratory Test Monitoring Data Sheet

Summary of 24 or 48-Hour Nematode Bioassay

Test Duration:	24 hour or 48 hour
Temperature:	20±2°C
Lighting:	none
Test container:	35 X 10 mm Petri dishes
Total amount required (soil weight):	125g dry weight (adjust for percent moisture)
Test amount/replicate:	2.33g dry weight (adjust for percent moisture)
Test soil moisture content:	Field moisture for environmental samples (Test may be conducted at ~10% to 100% moisture) 35 to 45% of dry weight for laboratory spiked samples when comparing to Earthworm test
Test Organism:	*Caenorhabditis elegans* (or other species approved by Washington State Department of Ecology)
Age of test organisms:	3-4 day old
Number of organisms/replicate:	10
Number of replicates:	3
Feeding regime:	None for 24 hour exposure, for 48 hour OP50 strain of *E. coli* (Boyd et al., 2001)
Allowable Soil pH Range:	3.1-11.9(11.7 for 48-h) (Khanna et al. 1997)
Endpoints measured:	Mortality
Positive control:	$CuCl_2$
Negative control:	Reference soil
Test Acceptability:	≥80% nematode recovery from soil ≥90% control survival
Organism Recovery:	Colloidal Silica Floatation

Acknowledgements

This Document is a modification of Ecology Publication 96-327, *Earthworm Bioassay Protocol for Soil Toxicity Screening*.

The following individuals have made significant contributions to the final version of this document. The author would like to extend special thanks for their time and effort.

- Phillip L. Williams, Department of Environmental Health Science, University of Georgia, Athens, Georgia.
- Windy Boyd, Integrated Toxicology Program, Duke University, Durham, North Carolina
- Dale Norton, Environmental Assessment Program, Washington State Department of Ecology, Olympia, Washington
- Margaret Datin, Environmental Assessment Program, Washington State Department of Ecology, Olympia, Washington

1. Introduction

1.1 Application And Background

Bioassays provide an important tool for screening-level assessments of soil toxicity at hazardous waste sites being investigated under the Washington Model Toxics Control Act (MTCA) Cleanup Regulation. Until 2001 the only ASTM standardized soil test animal was the earthworm, *Eisenia fetida* (ASTM, 1997; Ecology, 1996). The earthworm bioassay is effective for many applications, but has limitations relating to soil conditions, soil volumes, and the length of time required to perform the test. Arid soil conditions, such as those found in Eastern Washington, preclude the use of earthworms as bioassay organisms. But, nematodes can survive in arid soils and are a natural inhabitant of these soils. The nematode can also tolerate a wider pH range than earthworms, expanding the application of soil bioassays. The 24-test duration allows petroleum testing to be completed within the prescribed sample holding period. The 24-hour nematode test results have been shown to be significantly similar to 14-day earthworm test results (Boyd et al 2001, Peredney and Williams 2000a, Williams et al 2000). This test is not meant to replace the earthworm protocol. It is meant to supplement the earthworm protocol where both protocols can be conducted and to provide an alternative where the earthworm protocol can not be performed due to environmental conditions such as soil moisture and pH.

In this protocol, nematodes (C*aenorhabditis elegans*) are added to field collected site soils. The primary test endpoint is nematode mortality, recorded at 24 or 48 hours. The standard test duration for the nematode bioassay is a period of 24 hours. The testing period can be extended to 48 hours in consultation with Ecology. It is not necessary to conduct a dilution series but is suggested in order to validate results. The soil volume required to conduct the test is 110 grams including analytical samples which is a significant reduction from the 700 grams required to conduct the earthworm test with additional volumes needed for analytical testing. This enables testing to be conducted with a small sample volume, which minimizes disposal, storage and shipping cost.

The testing procedures described in this document have been adapted from protocols standardized by the American Society of Testing and Materials (ASTM, 2001)

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 110g (dry weight) of soil per sample is required to perform this test. If percent moisture is not know at the time of sampling then the best policy is to err on the side of conservatism and collect more sample than required. For example if the sample is at 45% moisture and you need 110g (dry weight) then you would need to collect 169 g wet weight. Each sample is tested using three replicates (a replicate is defined as one 2.33g dry weight aliquot of the bulk sample). Small test amounts allow for increased recovery of test organisms. The small sample volume, low cost and rapid turn around allows for more tests to be conducted, providing better site characterization. The remainder of the sample (approximately 100g dry weight) 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 nematode bioassay:
The selection of material (type of plastic or glass) will be determined by the nature of the contaminates believed to be in the soil being tested.

- Platinum wire
- pH meter
- 100mm high wall Petri dishes for sorting
- Glass or plastic filling funnel
- Wash bottles for rinsing glassware and probes
- Water purification system that produces Type II water (ASTM, 1983)
- Environmental chamber, incubator, or equivalent facility with temperature control (20±2°C)
- Analytical balance, capable of weighing to 0.001g
- 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. *A documented calibration program can substitute for reference weights*
- Test chambers, standard 35 X 10 mm Petri dishes (the material that the dishes are composed of will be determined by the nature of the sample)
- 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)

- Stereo Zoom Microscope with top and bottom light sources

- Centrifuge capable of achieving 700Gs

- 50 ml centrifuge tubes

- Vortex mixer

- Bunsen burner or alcohol lamp

- Autoclave

3.2 Materials

The following materials are required to perform the nematode bioassay:

(DI-$H_2$0 is de-ionized/distilled water)

Soil Hydration Water

Water for soil hydration must, at a minimum, meet specifications for Type II 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 1979).

Reference Toxicant

Cupric chloride 99.99% (Freeman et al. 1999)

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.

K-medium (Williams and Dusenbery, 1990)

2.36g KCl
3.0g NaCl
1000ml DI-H_2O
Autoclave

M9 Buffer (Cox et al., 1981)

1.5g KH_2PO_4
3.0g Na_2HPO_4
2.5g NaCl
500ml DI-H_20
Autoclave, then add:
0.5 ml of 1M $MgSO_4$*7H_2O

K Agar Plates (Williams and Dusenbery, 1988)

1 L Di-H_2O
2.36g KCl
3.0g NaCl
2.5g Bacto-peptone
17.0g Bacto-agar
Autoclave and allow to cool to touch, add:
1.0ml cholesterol solution (01.g cholesterol /10ml un-denatured ethanol)
1.0ml 1M $CaCl_2$
1.0ml 1M $MgSO_4$
Pour plates and allow to solidify
Inoculate plates with 4-5 Drops OP50 Broth and spread with a flame sterilized glass rod. Incubate plates at 35ºC for ≥24 hours

OP50 Broth (Stock Solution for OP50 plates) (Brenner, 1974)

0.25g NaCl
0.5g Bacto-peptone
50ml DI-H_2O
Inoculate with *E.coli* strain OP50

1% SDS

1.0g SDS (Sodium Dodecyl Sulfate)
100ml DI-H_2O

Colloidal silica

Ludox® AM or equivalent

Silica (as SiO_2), wt%	12
SiO_2/Na_2O (by wt)	125
Viscosity (25°C), cP (mPa s)	7
Specific gravity (25°C)	121

3.3 Test Organisms

Three to four day old nematodes (*Caenorhabditis elegans*) from the same culture are used as the test species. The use of four day old, as opposed to three day old nematodes, may effect response and should be held consistent through out the procedure, and specified in result reporting. *C. elegans*, wild type strain N2, is obtained from the *Caenorhabditis* Genetics Center, Minneapolis, MN. Obtaining cultures from the *Caenorhabditis* Genetics Center ensures genetic consistency. Modified strains of C. elegans, such as stains with luciferase markers are also available from the *Caenorhabditis* Genetics Center. Once cultures are obtained they can be maintained in the lab using the procedures described in Annexe A-1 of *Standard Guide for Conducting Laboratory Soil Toxicity Test with the Nematode Caenorhabditis elegans* ASTM Standard E 2172-01 (ASTM 2001). This test protocol would also be appropriate for other free living bacterial feeding nematode species (Order Rhabditida) such as the genera *Panagrellus, Caenorhabditis* and *Rhabditis.*

Nematodes, including *C. elegans,* are natural inhabitants of soil and may be present in field collected samples. If nematodes are found in samples drying of the soil to remove natural nematode populations (followed by re-wetting) may be required prior to testing. The effect of this wetting and drying must be considered in uncertainties. Methods have been developed for incorporation of a fluorescent gene into *C. elegans* which differentiate it from indigenous nematodes without significantly affecting its response. If genetically modified organisms are used proper methods must be used to insure that modified organisms are not released to the environment. Autoclaving of all equipment that has been in contact with genetically modified nematodes for 15 minutes at 115ºC should be sufficient to kill adult, dauer larvae, and eggs. If bulk soil samples are autoclaved the center of the sample must reach 115ºC for 15 minutes. Large soil volumes should be divided into to smaller volumes for autoclaving.

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 56ml of hydration water for 125g of soil (125g * 0.45= 56.25g = 56.25ml).

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 appropriate amount of hydration water to add to each test sample to achieve or maintain desired field conditions.

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

Nematodes have been shown to enter an anhydrobioic state when soil moisture was less than 2.5% (Freckman 1978). When nematodes enter into anhydrobiotic states respiration ceases therefore toxic effects may not be expressed until soil moisture reaches a point where the nematodes come out of anhydrobiosis. Studies on the effect of moisture were conducted (Williams et al. *unpublished data*) showed that 20% moister was necessary to prevent moisture related mortality. Therefore, soils that have moisture content at or below 20% should be tested at a soil moisture of 20%. All other soils should be tested at field moisture.

Hydration of the reference soil should be completed prior to hydration of any test samples. This order allows comparison of the friability (soil crumbles when handled) between the reference soil and the test samples. Hydration of test soil should be performed in a hood. Record the amount of hydration water added to each test sample.

5.4 Analytical

The remainder of the soil sample can be used for analytical procedures or retained for retesting or archival needs.

6. Reference Toxicant Testing (Cupric chloride)

6.1 Preparation of Positive Controls

If bioassays are conducted on a regular basis reference toxicant tests must be conducted, at a minimum, monthly if an in-house nematode culture is used for bioassays. If nematodes are received 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 bioassays. If bioassays are infrequent, reference toxicity test should be conducted concurrently with bioassays, with minimum of two reference toxicity test per year regardless of organism source and frequency of bioassays. It is recommended that acceptable nematode response be established prior to performing bioassays.

At least three different concentrations of $CuCl_2$ are required for the positive controls. The purpose of the reference toxicity test is to insure a consistent response from the test organism or in other words check the health of the culture. Due to this fact, reference toxicity tests can be conducted in an aquatic media. Freeman et al. (1999) established a protocol for conducting QA procedures for aquatic toxicity testing. The protocols for reference toxicity testing are the same for soil testing as in aquatic testing. Individual labs should perform ongoing reference toxicity testing to insure the consistency of response. Reference toxicity tests should fall within 2 standard deviations of the mean response. If the response is outside of this range a new culture should be obtained. Freeman et al (1999) published an aquatic LC50 value of 63 mg/ L (41-97) for copper, in K-medium (Williams and Dusenbery, 1990). Preferably four concentrations of $CuCl_2$ solution should be prepared in K-medium to bracket the anticipated Cu LC50 value of ~63mg/ L; the use of three concentrations would be acceptable if the response of the population is consistent. Suggested concentrations would be 10, 50, 100, 250 mg /L Cu, as $CuCl_2$. Nematodes are placed in a 20±2ºC incubator in darkness for 24-hours.

6.2 Reference Toxicant Data Analysis

Calculate and report the 24 hour LC50 and 95% confidence limits for copper, as cupric chloride, 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

This Test is conducted in accordance with the procedures detailed in *Standard Guide for Conducting Laboratory Soil Toxicity Test with the Nematode Caenorhabditis elegans* ASTM Standard E 2172-01. (ASTM 2001).

7.1 Test Containers and Labeling

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. The standard test containers are 35 mm polystyrene tissue culture dishes. These dishes come pre-cleaned and sterilized and do not require preparation. If the soil tested is thought to contain substances incompatible with the standard polystyrene dishes, such as certain organics, glass dishes should be used. Prior to use, reusable test containers and lids should be decontaminated with hot water/laboratory grade detergent, acid washed, 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 soil sample (2.33g dry weight) into each dish using a spatula or scoop. If the sample was air dried, and needs to be re-hydrated, the process needs to commence 7 days prior to the initiation of testing to allow for equilibration of the solid and liquid phases. If the soil is at field moisture then a correction should be applied to account for water weight. Care should be taken to avoid moisture loss during field sampling and storage. If the soil has not lost significant moisture during transit and storage then re-hydration to field conditions can be accomplished 24 hours prior to test initiation.

Nematodes are introduced to the test containers after the equilibration period. Groups of ten nematodes are assigned randomly to the individual test containers. The nematodes are placed on the surface of the soil. The test organisms are transferred from their agar plates to the surface of the soil with a flame-sterilized platinum wire (Freeman et al, 1999) and allowed to burrow. The test containers must be placed into the test chamber randomly.

7.3 Negative Control

Tests should be conducted with an uncontaminated site reference soil that has similar physical-chemical properties. This allows for a distinction between effects related to poor tolerance of site conditions and those related to the toxicant. If the average survival in the reference soil replicates is less than 90% then the test would be considered invalid.

7.4 Selection of Test Organisms

Take ten worms at random from the culture dish place them in the dish containing the soil sample. Worms are transferred by a flamed platinum wire; they are gently lifted by the wire and placed in the test medium. Worms should be selected based on demonstrated motility and apparent good health. The amount of culture medium that is transferred with the worm should be minimized. It is important not to damage the worms in the process. If any of the worms appear injured, they must not be used. After the worms are placed in the test container, place the lid on the test container.

Place the test containers in an environmentally controlled chamber or equivalent. Conduct the test at 20±2°C. A temperature monitoring device, located inside the chamber, should be used during the test. 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

8.1 Feeding Regime during Testing

The nematodes are not fed during the 24 hour test. For test greater than 24 hour the nematodes are fed 1 ml of re-suspended *Escherichia coli* strain OP50 in K-media once at the initiation of the test (Boyd et al. 2001). To prepare the food source, fifty ml of an L-broth solution with *Escherichia coli* (OP50 strain) is centrifuged at 750 *g* for 10 minutes. The supernatant is removed and the pellet was re-suspended in 10 ml of K-medium for a 5:1 (L-broth to K-medium) concentration (Donkin and Williams 1995).

8.2 Test Termination, Survival Count, and Observations

The test duration is 24 or 48 hours. 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 (Reference Soil) first. The recovery of the worms is accomplished in accordance with the procedures detailed in ASTM E-2172 (ASTM, 2001). After exposure worms are rinsed into 50ml centrifuge tubes with colloidal silica and centrifuged at 700 *g's* for 2 min. Tubes are allows to sit for 15 min to allow worms to rise to the surface. Then tubes are then poured into 100-mm glass Petri dishes and viewed under a light microscope. Worms are removed from the dish with a platinum wire and placed on a K-agar plate with OP50 bacterial lawn and examined under a dissecting microscope. If a worm does not responded to a gentle probing with the platinum wire it is considered dead.

When counting the worms, observe for altered behavior or morphological changes, and record if present. Possible behavioral alterations include lack of burrowing and lack of movement. Morphological changes include contraction, rigidity, and elongation. 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 when placed on a K-agar plate with a bacterial lawn. Worms decay rapidly in moist soils, and if ten worms are not accounted for, the missing worms should be considered dead and completely decomposed. 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 sub lethal effects.

Start by counting negative controls Mortality and morphological changes should seldom occur in the negative controls if mortality is >10% the test is considered invalid. 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. To avoid cross contamination of low concentration samples from high concentration samples.

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
- Date and Time Test terminated
- Name of person performing the test
- Moisture fraction of reference 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 test 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 (±2 SD) 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 nematodes used including brood stock, scientific name and method of verification, age, 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 nematodes per sample.

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 (EPA 1997), 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 (20±2°C) throughout the test.

Analytical Methods

Routine chemical and physical analyses must include established QA practices (USEPA 2001).

Calibration and Standardization

Instruments used for routine chemical and physical parameters, such as pH, temperature, weight and volume must be calibrated and standardized according to instrument manufacturer's 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 Nematode *Caenorhabditis elegans* is the primary test species used in this bioassay. *C. elegans,* wild type strain N2, is obtained from the *Caenorhabditis* Genetics Center, Minneapolis, MN. This species has been used extensively in laboratory toxicity tests (Donkin and Dusenbery 1993, 1994; Freeman et al. 1999; Peredney and Williams 2000a,b; Boyd et al., 2001; Black and Williams 2001). Research is currently underway on the application of other nematode species for bioassay. These other species may be determined suitable by Ecology for bioassays when significant data have been published in the peer review literature or amendments are made to ASTM standards. Additionally, work is currently underway for the incorporation of marker genes in C. elegans that would allow for easy identification and differentiation from indigenous nematodes. This method would allow for use of genetically modified test organisms if adequate verification is provided that the effects of the modifications on response are understood.

A stock solution of the dauerlarval stage of *C. elegans* in M9 buffer (Cox et al. 1981) 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 (Williams and Dusenbery, 1988) with an established lawn of *Escherichia coli* strain OP50 (Brenner, 1974) as described in Donkin and Williams (1995) and ASTM (2001). The plates are incubated for three days at 20°C. Eggs and worms are washed from the plates with liquid K-medium (Williams and Dusenbery, 1990), 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 (Freeman et al. 1999).

Quality of Test Organisms

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

Nematodes should not be used if they have been under stress from extremes (high or low) in food, moisture availability, temperature, and pH. 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% and recovery of nematodes is <80%. 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 nematode 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 (±2 S.D.) 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 nematodes in K-medium, 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 nematodes.

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

ASTM, 1983. *Standard Specifications for Reagent Grade Water.* D 193-77 American Society for Testing and Materials, Philadelphia, PA.

ASTM, 1997. *Standard Guide for Conducting Laboratory Soil Toxicity or Bioaccumulation Tests With the Lumbricid Earthworm Eisenia Fetida*. E1676-97. American Society for Testing and Materials, West Conshohocken, PA.

ASTM, 2001. *Standard Guide for Conducting Laboratory Soil Toxicity Test with the Nematode Caenorhabditis elegans*. E 2172-01. American Society for Testing and Materials, West Conshohocken, PA.

Beyer, W.N., 1990. *Evaluating Soil Contamination.* Biological Report 90(2). U.S. Fish and Wildlife Service, Arlington, VA.

Boyd, W.A., V.A. Stringer, and P.L. Williams. 2001. "Metal LC50s of a Soil Nematode Compared to Published Earthworm Data". *Environmental Toxicology and Risk Assessment: Science, Policy, and Standardization-- Implications for Environmental Decisions, 10th Volume ASTM STP1403*. B.M. Greenburg, R. N. Hull, M. H. Roberts, Jr., and R.W. Gensemer, Eds., American Society for Testing and Materials, West Conshohocken, PA,.

Brenner, S., 1974 "The Genetics of *Caenorhabditis elegans*," *Genetics*, Vol 77, pp. 71-94,.

Cox, G. N., H. Kusch., and R. S. Edgar., 1981. "Cuticle of *Caenorhabditis elegans*: It's Isolation and Partial Characterization," *Journal of Cell Biology*, Vol 90, pp. 7-17.

DeWoskin, R.S., 1984. *Good Laboratory Practice Regulations: a Comparison*. Research Triangle Institute, Research Triangle Park, NC.

Donkin, S. G., and D. B. Dusenbery., 1993. "A Soil Toxicity Test Using the Nematode *Caenorhabditis elegans* and an Effective Method of Recovery," *Archives of Environmental Contamination and Toxicology*, Vol 25, pp. 145-151.

Donkin, S. G., and D. B. Dusenbery, 1994. "Using the Nematode *Caenorhabditis elegans* Soil Toxicity Test to Identify Factors Affecting Toxicity of Four Metal Ions in Intact Soil," *Water, Air and Soil Pollution*, Vol 78, pp. 359-373.

Donkin, S. G., and P. L. Williams, 1995. "Influence of Developmental Stage, Salts and Food Presence on Various End Points Using *Caenorhabditis elegans* for Aquatic Toxicity Testing," *Environmental Toxicology and Chemistry*, Vol 14, No. 12, pp. 2129-2147.

Ecology, 1996. "Earthworm Bioassay Protocol for Soil Toxicity Screening" Washington State Department of Ecology. Publication No. 96-327

Eirkson, C., M. C. Harrass, C.M. Osborne, P.G. Sayre and M. Zeeman, 1987. *Environmental Assessment Technical Assistance Document*, Publ. #4.07. PB87-175345. Food and Drug Administration, Washington, D.C.

Freckman, D. W. 1978. "Ecology of anhydrobiotic soil nematodes". In *Dried Biological Systems.* J. Crowe and J. Clegg, eds. Academic Press, New York.

Freeman, M. N., C. L. Peredney, and P. L. Williams., 1999. "A Soil Bioassay Using the Nematode *Caenorhabditis elegans*," *Environmental Toxicology and Risk Assessment: Standardization of Biomarkers for Endocrine Disruption and Environmental Assessment: Eighth Volume, ASTM STP 1364*, D. S. Henshel, M. C. Black, and M. C. Harrass, Eds., American Society for Testing and Materials, West Conshohocken, PA, pp. 305–318.

Greene, J.C., C.L. Bartels, W.J. Warren-Hicks, B.R. Parkhurst, G.L. Linder, S.A. Peterson and W.E. Miller, 1988. *Protocols for Short-term Toxicity Screening of Hazardous Waste Sites.* EPA/600/3-88/029. Environmental Research Laboratory, USEPA, Corvallis, OR.

Hamilton, M.A., R.C. Russo and R.V. Thurston, 1977. "Trimmed Spearman-Karber Method for Estimating Median Lethal Concentrations in Toxicity Bioassays" *Environmental Science and Technology*. 11:714-717. Correction: Ibid. 12:417.

Khanna, N.; C. Cressman III, C. Tatara, and P.L. Williams, 1997. "Tolerance of the Nematode *Caenorhabditis elegans* to pH, Salinity, and Hardness in Aquatic Medium." *Archives of Environmental Contamination and Toxicology*. 32(1):110-114.

Peltier, W. and C.I. Weber, 1985. *Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms.* Third Edition. EPA 600/4-85-013 Environmental Monitoring and Support Laboratory, USEPA, Cincinnati, OH.

Peredney, C. L., and P. L. Williams, 2000a. "Utility of *Caenorhabditis elegans* for Assessing Heavy Metal Contamination in Artificial Soil" *Archives of Environmental Contamination and Toxicology*, Vol 39, pp.113-118.

Peredney, C. L., and P. L. Williams. 2000b. "Comparison of the Toxicological Effects of Nitrate versus Chloride Metallic Salts on *Caenorhabditis elegans* in Soil," *Environmental Toxicology and Risk Assessment, Vol 9—ASTM STP 1381*,. Price FT, Brix KV, Lane NK, Eds., American Society for Testing and Materials, West Conshohocken, PA, USA. pp. 256-268.

Thomas, J.M., J.R. Skalski, J.F. Cline, M.C. McShane, J.C. Simpson, W.E. Miller, S.A. Peterson, C.A. Callahan and J.C. Greene, 1986. "Characterization of Chemical Waste site

Contamination and Determination of its Extent Using Bioassays" *Environmental Toxicology and Chemistry* 5:487-501.

USEPA, 1977. *Occupational Health and Safety Manual.* Office of Planning and Management, USEPA, Washington, D.C.

USEPA, 1979. *Methods for Chemical Analysis of Water and Wastes.* EPA\600\4-79\020. Environmental Monitoring and Support Laboratory, USEPA, Cincinnati, OH.

USEPA, 2001 *Good Laboratory Practice Standards*. 40 CFR 792.

USEPA, 1980b. *Physical, Chemical and Persistence, and Ecological Effects Testing: Good Laboratory Practice Standards (proposed rule).* 40CFR772. Federal Register 45:77353-77365.

Williams et al 2004. Unpublished data. Data for Ecology Contract C0400246. Presented as a poster at the 2004 Society of Environment Toxicology and Chemistry World congress. Will be published in a journal, to be decided.

Williams, P. L., and D. B Dusenbery., 1988. "Using *Caenorhabditis elegans* to Predict Mammalian Acute Lethality to Metallic Salts," *Toxicology and Industrial Health*, 4: 469-478.

Williams, P.L., and D. B. Dusenbery, 1990. "Aquatic Toxicity Testing Using the Nematode *Caenorhabditis elegans*" *Environmental Toxicology and Chemistry*, Vol 9, pp. 1285-1290.

Williams, P. L., G. L. Anderson, J. L. Johnstone, A. D. Nunn, M. F. Tweedle and P. Wedeking (2000). "*Caenorhabditis elegans* as an alternative animal species." J Toxicol Environ Health A 61(8): 641-7.

Weber, C.I., W.H. Peltier, T.J. Norberg-King, W.B. Horning II, F.A. Kessler, J.R. Menkedick, T.W. Neiheisel, P.A. Lewis, D.J. Klem, Q.H. Pickering, E.L. Robinson, J.M. Lazorchak, L.J. Wymer and R.W. Freyberg, 1989. *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Surface Waters to Freshwater Organisms.* Second Edition. EPA-600/2-89/001. Environmental Monitoring Systems Laboratory, USEPA, Cincinnati, OH.

Zar, J.H., 1984. *Biostatistical Analysis.* Second Edition. Prentice-Hall, Inc., Englewood Cliffs, NJ.

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 _________________________________

Sample ID#: ____

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 (%)	Slurry pH Start (s.u.)	Supernatant pH Start (s.u.)	Slurry pH 24h (s.u.)	Supernatant pH 24 h (s.u.)	Temp. Start (EC)	Temp. 24-h (EC)	No. of live worms Start	No. of live worms 24-h	Sublethal effects- (lethargy, balling, lack of burrowing) 24-h	Sublethal effects- as percent of total no. of worms 24-h

Any_visual_observations/comments- ____

____

Test Termination

Comments- ____

____