UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
REGION 5
77 WEST JACKSON BOULEVARD
CHICAGO, IL 60604-3590

February 3, 1999

DW-8J

Ms. Christine Freeman
Environmental Protection Department
Code 095
Naval Surface Warfare Center
300 Highway 361
Crane, Indiana 47522

REPLY TO THE ATTENTION OF

RE: Worm Toxicity Test NOD
Bioremediation Facility
Naval Surface Warfare Center
Crane, Indiana

Dear Ms. Freeman:

The United States Environmental Protection Agency (U.S. EPA) Regional Lab has evaluated the Worm Toxicity Testing Procedure for the Bioremediation of explosives contaminated soils. Our comments and the U.S. EPA Standard Operating Procedure are attached. Please revise the toxicity testing portion of the work plan to address these comments and our previous comments that were mailed separately. An important reference for you to use is EPA/600/3-88/029, "Protocols for Short Term Toxicity Screening of Hazardous Waste Sites" .

We apologize for the delays in sending out this review due to staff commitments and software problems. It is our understanding that you wish to get this procedure and test approved prior to the change in your contractors. In that event, sampling for Mine Fill B, and any required resampling for Mine Fill A would need to occur by mid-March at the latest. We will make every attempt to settle this issue with the highest priority. If you have any questions regarding this matter, please contact me at (312) 886-6146.

Sincerely,

Carol Witt-Smith
Corrective Action Expert
WMB, IL/IN/MI Section

Enclosure
Filename: wormnod2.usn

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ATTACHMENT

Quality Assurance Project Plan For Full-Scale Operations at the Bioremediation Facility NSWC Crane, Crane, Indiana

Review and Comments on Appendix G, dated 05/13/98
Pertaining to the Proposed Earthworm Toxicity Testing

Following are comments and review of the earthworm toxicity methods and documents found in Appendix G, in the QAPP for bioremediation activities proposed for the NSWC Crane, RCRA Corrective Action site, located in Crane, Indiana.

General Comments:

There are many important earthworm toxicity details missing and/or not appropriately explained or detailed in the QAPP Appendix G. Some of these range from needing more details about goals and objectives to number of test replicates and test organisms, from sampling techniques and sample collection containers to endpoints being tested for and how critical calculations will be made to draw conclusions.

To help in understanding these comments and to provide examples, the following documents should be referenced:

1. EPA/600/3-88/029 Protocols for Short Term Toxicity Screening of Hazardous Waste Sites.

2. The Region 5 SOP for Acute Solid Waste Toxicicty Testing, using the common earthworm (coy enclosed).

3. The Region 5 SOP for Laboratory Culture of the common earthworm (copy enclosed).

4. Reference toxicant test results and bench sheets from example reference tests we run here in the lab monthly (copy enclosed).

If you have any questions, would like additional copies of the above documents, or would like further information, please don't hesitate to call Peg Donnelly, from our Regional Lab, directly at (312) 353-9467.

The U.S. EPA is also considering a lab audit and split sampling in order to speed the approval of this method. If the Navy would like to consider the split-sampling option, we should set up a conference call as soon as possible.

Specific Comments:

1. Section 1.0 INTRODUCTION

Page G-3, paragraph 1 — "...document that the treated soil meets toxicity remedial goals." —

b. What are the stated goals and objectives of these earthworm toxicity tests?

c. What are the toxicity levels that you will allow for no further action, versus what levels will indicate that further clean-up measures must be taken?

d. These should typically be stated and determined/agreed upon before analytical testing begins.

4. Section 2.0 METHODS

Page G-4, table G-1

Collection of soil samples for earthworm toxicity tests is recommended to be in 3.5 gallon HDPE plastic containers lines with two plastic bags of approximately 4mm. This is for two reasons:

1) to assure there is no leaching of metals into the sample; and

2) to make sure all sample that will be used for a test is contained in the same container, thus validating that different mortality rates for different concentrations is not due to possible contamination of some sample containers versus others from the same SWMU.

5. Section 3.0 REMEDIAL GOALS

Page G-5, paragraph 2 — "For the earthworm toxicity the objective will be to determine if the compost samples show significant mortality when compared to the reference soil."

Please clarify with specifics what the term "reference soil" is meant to describe here. Does this refer to the artificial soil that will be made at the laboratory (typically, from 10% peat, 20% clay, and 70% sand) and used as dilution soil as well as soil for control organism exposures? Or, is the term "reference soil" meant to refer to non-contaminated soil from the site (plus straw and chicken manure) that will be used for dilution soil as well as control media?

6. Section 4.0 QUALITY CONTROL PROCEDURES

Page G-6 — Some of this page was not transmitted in a legible fashion, so comments provided here may be ambiguous. Clarifications for above and below comments may be obvious after viewing the page G-6, and reviewer apologizes for this/ these possible missing pieces of information. Please provide with the appropriate clean and legible copy of page G-6.

7. Section 4.2 ANALYTICAL TESTING QUALITY CONTROL

a. Page G-6, paragraph 2 — Must mention reference toxicant studies that are conducted, what reference toxicant is used, how often the reference toxicant tests are conducted, at what concentrations and temperature, what percent of control organisms must survive for a test to be valid, etc.

b. Also, there needs to be some mention as to what concentrations will be used for the actual sample tests.

c. How many replicates will be run per test concentration?

d. What types of deviations will be allowable?

e. What type of water will be used to saturate test media?

. What mortality rates in control replicates is allowed for test to be considered valid?

g. How will LC50 values be calculated?

8. Section 5.2 LABORATORY SHIPMENTS AND CONTACTS

Page G-8, paragraph 2 — Overall, we must see copies of more complete and precise SOPs from Aqua Survey, of Flemington, NJ (or incorporate some of the testing specifics into the QAPP). SOPs requested include earthworm culturing, (if culturing not taking place, then provide information on source of test organisms and age verificaion procedures, as well as culture information from the worm source/ supplier), reference toxicant testing (including laboratory LC50 charts from approximately the last dozen reference tests), earthworm toxicity testing, resumes of personnel involved in culture and toxicity testing performed at the facility. Also, SOPs should be provided for measuring pH, determining calculations for soil hydration capacity, and other determinants that take place during culture and testing, that might not already be provided in the earlier mentioned requested SOPs.

ATTACHMENT A, LABORATORY STANDARD OPERATING PROCEDURES

1. SOP/ PRP/ 004 (Aqua Survey, Inc., Flemington, NJ); PREPARATION OF SEDIMENT POREWATER SAMPLES FOR USE IN TOXICITY TESTS OR MONITORING POREWATER AMMONIA IN CONCENTRATIONS IN TEST SEDIMENTS

    a. Page A-1 -- Is this SOP for use in any phase or step of the Earthworm toxicity testing? If so, at what stage in the procedure and why is it used?

2. SOP/SED/201 (Aqua Survey, Inc., Flemington, NJ); SOIL TOXICITY / BIOACCUMULATION TEST USING THE EARTHWORM *EISENIA FOETIDA*

    a. Page A-5, paragraph II.A and B. — Please provide specific information as to source of organisms.

    b. Do you assure that test organisms were cultured in the same/ similar media as will be used for testing?

    c. Do you assure that culture temperature was the same as test temperature?

    d. Do you have information verifying the species, age and determining characteristics of purchased test organisms?

    e. Do you run a reference toxicant test concurrently with the all analytical tests using the same batch of purchased organisms, to be sure they are of good quality and not stressed?

3. Page A-5, paragraph II.C. — "Artificial Soil" -- Measurements and adjustments for pH should be made of all artificial or dilution soils to be used (as well as the test soils, as mentioned in III.Q). The desired range and how it is adjusted for pH should be included in SOP.

4. Page A-5, paragraph III.A. — "Preparation of Test Substance for Exposure" -- The test soil should be sieved before being used to initiate test. Procedure for how this takes place, either in the field at time of sample collection, or in laboratory should be provided either in this SOP, or in a necessary SOP on sample collection.

5. Page A-5, paragraph III.B. — "Test Concentrations" — The actual concentrations being used to determine earthworm toxicity for this site should be provided somewhere in the QAPP, or the SOPs. What is the dilution factor, how many concentrations will be provided for each test, and what are those pre-determined concentrations? (Also, see next comment.)

6. Page A-6, paragraph III.C. — "Preparation of Test Vessels" — One replicate is not sufficient. Commonly used methods and guidance materials suggest at least three replicates be used for each concentration, including controls. For this project, it will probably be best to have at least five test concentrations as well as control vessels. Four liter test vessels may be too large for conducting a test that uses 10 organisms per vessel and thus 200 g of test media per vessel. Same sized test vessels need to be used for all tests, all concentrations, and all reference tests for data to be most useful. Include the size of the actual test vessels that will be used, how they are cleaned and prepared, etc.

7. Page A-6, paragraph III.E. — "Number of Organisms" — Exactly how many test organisms will be used per replicate and per test. These numbers must be standard throughout this entire project (all sample analyses and reference tests), and stated specifically somewhere in the QAPP or SOP. As commented above, at least three replicates should be considered for each of five different concentrations, plus a control concentration with three organisms. It is common practice to expose ten organisms per test replicate.

8. Page A-6, paragraph III.G. — "Test Endpoint" — What status of an organism is considered not "surviving"? How are the LC50 values calculated, i.e. what statistical methods and computer programs are used to assist the analyst in obtaining these values?

9. Page A-6, paragraph III.H. — "Test Duration" — What is the duration of the test for this project? You must state the actual agreed upon test length somewhere in the QAPP and/ or SOPs, not simply state a range of possible test lengths.

10. Page A-7, paragraph III.N. — "Reference Toxicant" — Provide details on reference toxicant tests either in this SOP or a separate one that addresses reference toxicant tests for earthworms. Include test concentrations, number of replicates, number of organisms per replicate, test duration, vessel size, amount of test media per replicate, endpoint, etc.

11. Page A-8, paragraph III.R. and Page A-9 — "Preparation of Specimens for Bioaccumulation Analyses" — Is this an endpoint being tested for in this project? If so, further information may be needed. For example, how it will be reported, calculated and measured, what parameters, etc?

12. Page A-8 — DATA HANDLING — As mentioned above, what types of statistical analyses will be conducted? Will bench sheets be included in reports? What types of computer programs will be used?

13. FECAL COLIFORM TEST FOR SLUDGE (Pure Earth Environmental Laboratory, Pennsauken, NJ) — Page A-34-36 — the SOPs are brief and lack of.

ESAT REGION V STANDARD OPERATING PROCEDURES FOR BIOLOGICAL SUPPORT ACTIVITIES

TITLE:

STANDARD OPERATING PROCEDURE FOR THE LABORATORY CULTURE OF THE COMMON 'REDWORM' EARTHWORM, FAMILY LUMBRICIDAE

ESAT will follow this SOP:

Allison Harvey
Lockheed ESAT Region V
Biological Group Lead

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Team Manager

STANDARD OPERATING PROCEDURE FOR THE LABORATORY CULTURE OF THE COMMON 'REDWORM' EARTHWORM, FAMILY LUMBRICIDAE

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STANDARD OPERATING PROCEDURE FOR THE LABORATORY CULTURE OF THE COMMON 'REDWORM' EARTHWORM, FAMILY LUMBRICIDAE

1. SCOPE AND APPLICATION

The necessity to have a continuous source of test organisms of the proper age, size, quality, and condition precludes the need for a culture system. Worms procured from an outside source may not always be available in a suitable condition or quantity to meet test standards.

2. PURPOSE

To maintain a continuous and healthy supply of common 'redworm' earthworms, family Lumbricidae.

3. SAFETY AND WASTE HANDLING

All used soil is double-bagged and labeled as to its contents and the date the waste collection was initiated. All waste is stored in the Waste Accumulation Area. All waste containers should be kept in a secondary container (i.e. plastic tub). Proper segregation is needed to minimize the consequences of accidental mixing of incompatible waste by spillage, breakage, or fire.

4. SUMMARY OF METHOD

4.1 Distribution, Life Cycle, and Taxonomy

4.1.1 Taxonomy

'Redworms' are members of the Phylum Annelida (segmented worm), Class Oligochaeta, Order Haplotaxida, Family Lumbricidae. Most oligochaetes are burrowers. All members of the class Oligochaeta possess bristle-like appendages or hair-like structures, called setae (chaetae), growing out of their bodies which aid in locomotion. Members of this order also live mostly in fresh water or moist soils. The Lumbricidae family includes the various terrestrial earthworms often relatively large, with well-developed and complex reproductive systems; most are direct feeders (Brusca & Brusca, 1990). In the Lumbricidae particularly, the position of the clitellum is relatively constant within all species, and thus can be used for identification purposes.

4.1.2 Distribution

Lumbricidae can be found worldwide in its specific habitat. In nature, lumbricidae are litter dwellers; that is, they are found among masses of decaying vegetation such as fallen leaves, or manure piles, or under rotten logs. They are present in mineral soils

only when large amounts of organic materials are present.

4.1.3 Anatomy

An annelid worm is basically a fluid-filled cylinder, with the body wall comprising two sets of muscles, one circular, the other longitudinal. (Figure A.) The fluid-filled cavity (the coelom) is effectively a hydrostatic skeleton (fluid cannot be compressed) upon which the muscles work antagonistically to produce changes in width and length of the animal. The gut, vascular system and nervous system run from one end of the segmental chain to the other and coordinate the whole body. The series of segments is bounded by a nonsegmental structure at each end; the prostomium and a pygidium. (Figure B) The mouth opens into the gut immediately behind the prostomium, while the gut terminates at the anus on the pygidium. Worms have no eyes but they are sensitive to light, particularly at their prostomium (front end). Sensory cells in a worm's skin are less sensitive to red light than to light of mixed wavelengths. If you want to observe worms you can use red cellophane or an amber bread wrapper over your light source.

4.1.4 Locomotion

An earthworm moves by waves of muscular contraction and relaxation which pass along the length of the body, so that a particular region is alternately thin and extended or shortened and thickened. A good grip on the walls of the burrow is aided by the spiny outgrowths of the body wall called chaetae (setae) which project from the body wall. (Figure B.)

4.1.5 Digestion

The earthworm feeds either on leaves pulled into the burrow with the aid of its suctional pharynx or by digesting the organic matter it swallows when burrowing in earth otherwise too firm to penetrate. A muscular gizzard near the front end of the gut serves to break up compacted soil particles into smaller ones with the result that digestion and absorption of the organic material within the intestine is more efficient. Undigested matter is extruded from the anus on the surface of the soil as the familiar worm casts. The habit of producing casts at the burrow entrance results in the gradual accumulation of surface mold.

4.1.6 Life Cycle

Worms are very prolific; a single worm produces two to five cocoons per month, each with several worms. The life span of mature lumbricids in the field is probably quite short, often no more than a few months, although it has been calculated that their

potential longevity was 4-8 years. In protected culture conditions however, individuals of E. foetida have been kept for 4½ years (Edwards & Lofty, 1977).

Each worm is hermaphroditic, having both ovaries and testes limited to a few front segments. Male and female segments are separate, with segments containing testes always in front of those segments containing ovaries. The swollen region about ⅓ of the distance between the head and tail of a worm is the clitellum, sometimes known as the girdle or band. For members of the Lumbricidae, the clitellum commences after the 15th segment. The presence of the clitellum indicates that a worm is sexually mature. When two worms mate, they meet and overlap one another to about ⅓ to ¼ of their length, with their heads facing in the opposite direction and their undersides in contact. (Figure C.)

Two worms join head to tail by mucus secreted from their clitella, sperm then pass from each worm to the sperm storage sacs in the other worm and then they separate. Sometime after the worms separate, the clitellum secretes a second substance, a material containing albumin. The albuminous material hardens on the outside to form a cocoon in which eggs are fertilized and from which baby worms emerge. As the adult worm backs out of this hardening band, it deposits eggs from its own body and the stored sperm from its mate. Sperm fertilize the eggs inside this structure, which closes off at each end as it passes over the first segment. Sometimes called an egg case, this home for the developing worms is more properly called a cocoon. Cocoons continue to be formed until all the stored seminal fluid has been used up.

Cocoons are lemon-shaped objects about the size of a matchhead or a small grain of rice. They change color as the baby worms develop, starting as a luminescent pearly white, becoming quite yellow, then light brown. When the hatchlings are nearly ready to emerge, cocoons are reddish. It takes at least three weeks of development in the cocoon before one to several baby worms hatch. The time to hatchling is highly dependent upon temperature and other conditions.

Newly emerged worms are whitish and nearly transparent, although the blood vessels cause a pink tinge. They start to turn pink in 2-3 days, and turn red by the time they are ½ inch long. They are self-sufficient when hatched. Newly born earthworms generally bore deeper into the bedding when first hatched, and after several days, move to the top to start feeding. In 60-90 days, the new earthworms will start to develop their clitellums. These can be seen easier if the earthworm is turned over and poked at from the underside, where the clitellum first shows as a slight white swelling. When the clitellum is fully developed, the earthworm is ready for breeding. The onset of sexual maturity is signaled by the appearance of the clitellum - worms without this cannot reproduce. Subsequently, at the end of the reproductive life, worms become senescent and the clitellum regresses and may disappear completely.

A mature earthworm breeder can produce an egg capsule every 7 to 10 days. The egg capsule will hatch in approximately 21 to 28 days. NOTE: Laboratory culture at 15°C requires on average 5 weeks. Each egg capsule will hatch from two to twenty young earthworms with an estimated average of four. Each young earthworm will mature to breeding age in approximately 60 to 90 days, provided they receive proper feed and care. It generally requires several months to a year for an earthworm to reach full mature size, averaging 3-4 inches in length.

5. SAMPLE HANDLING AND PRESERVATION

Not applicable.

6. APPARATUS

6.1 Instrumentation

6.1.1 Environmental chamber capable of maintaining a uniform temperature of 15 ± 2 °C and 4300 ± 430 lux of light operating on a clock timer to control diurnal cycling.

6.1.2 Water purification system -- Millipore Milli-Q® or equivalent.

6.1.3 Balance, analytical, Sartorius® Research or equivalent -- capable of accurately weighing earthworms to 0.0001 g.

6.1.4 Balance, top-loading, Sartorius® Basic or equivalent -- capable of weighing soil samples to 1000.0 g.

6.1.5 Reference weights, Class S -- for checking performance of balance. Weights should bracket the expected weights of the weighing pans and the expected weights of the pans plus worms or soil.

6.1.6 National Bureau of Standards (NBS) certified thermometer (see EPA Method 170.1, EPA 1979b).

6.1.7 Magnetic stir plate with magnetic stir bars.

6.1.8 Magnetic stir bar retriever.

6.1.9 pH meter -- Orion Research® pH/millivolt meter or equivalent.

6.2 Glassware/Plasticware

6.2.1 Rubbermaid trays or equivalent -- 10 to 20 centimeters deep, 50 centimeters in length and 25 - 37.5 centimeters wide.

6.2.2 Black plastic or rubbermaid tray lids.

6.2.3 Large sheets of plastic for sorting.

6.2.4 Sample containers -- High Density Polyethylene (HDPE) plastic pails with covers. Consolidated Plastics Company, Part #33400AJ (1.0 Gal), #33404AJ (3.5 Gal) or equivalent.

6.2.5 One large plastic bag or equivalent container, capable of holding at least 1000.0 g.

6.2.6 Graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 10- to 1000- ml.

6.3 Other Supplies

6.3.1 Earthworm starter culture.

6.3.2 Disposable rubber gloves with approved protection factor (NIOSH).

6.3.3 Gummed labels.

6.3.4 Goose-neck or similar lamp with 100-watt bulb.

6.3.5 Newspaper

7. REAGENTS AND CONSUMABLE MATERIALS

7.1 Reagents

7.1.1 Reagent water -- defined as activated carbon-filtered, distilled or deionized water that does not contain substances toxic to the test organisms. A water purification system may be used to generate reagent water.

7.1.2 pH buffers 4.00 ± 0.01 @ 25 °C, 7.00 ± 0.01 @ 25 °C, and 10.00 ± 0.02 @ 25 °C (or as per instructions of instrument manufacturer) for standards and calibration check.

7.1.3 Topsoil -- light loamy soil, low clay or sand content.

7.1.4 Dry alfalfa pellets -- commercial rabbit feed (protein, minimum of 15%; fiber, maximum of 30%; ash, maximum of 12%; fat, minimum of 1%; and moisture, maximum of 12%) Can be purchased at any Feed store.

7.1.5 'Redworm' starter culture --

7.2 Alfalfa Chow

Fermented alfalfa pellets are used for food. Dry pellets (protein, minimum of 15%; fiber, maximum of 30%; ash, maximum of 12%; fat, minimum of 1%; and moisture, maximum of 12%) can be purchased at most feed stores. Worm food is prepared in 3.5 gallon (1 gal.) plastic "diaper pails". Two or more pails of food are prepared as needed. 1800 g (360 g) of pellets are placed into each pail. Add approximately 3600 ml (720 ml) of RO water. The mixture is then blended by hand. The container is sealed and allowed to ferment for two weeks before use. Batch numbers consists of the date the food was prepared. For example; feed prepared on October 25, 1992 would be assigned Batch #102592. Batch number, alfalfa weight, volume of water, and fermentation start date should be recorded on the Feed Log (ESAT-5-104.1). As feed is distributed to the earthworm beds the volume used is subtracted from the starting volume to determine the remaining volume. When the volume of feed reaches approximately 500 grams, this will signal the time to set up another pail of alfalfa chow to ferment. The exact volume is subjective and depends on the number of beds and the volume of food needed.

7.3 Culture Media

The culture media is topsoil; 70% composted pine bark, hardwood bark, peat fines, sand and ash.

8. PROCEDURE

8.1 GENERAL CULTURE CONDITIONS

8.1.1 Source of Organisms

Starter cultures of 'Redworms' can be obtained from Carolina Biological Supply Company, 2700 York Road, Burlington, North Carolina 27215 (1-800-334-5551).

8.1.2 Bedding

A loamy topsoil is the standard bedding used by Carolina Biological. Earthworm beds

should be crumbly moist, not soggy wet. If a handful of bedding is picked up, it should not be wet enough that water can be squeezed out of it, nor should it stick to the hand like mud. It should be moist to the point where it breaks into pieces and falls, or crumbles back into the bed.

The bedding is placed in a culture tray. Each tray will hold approximately 3500 grams of topsoil. A gummed label with a tray number is placed on the side of the tray. Once the tray is prepared, cover it with a piece of plastic or layer of wet newspaper to aid moisture retention. Caution: Do not seal earthworms in an airtight environment, they will die. Before utilizing bedding conduct a pH measurement and record on the Earthworm Culture Logsheet (ESAT-5-042.2). pH should fall between 5 and 8. If adjustments are necessary, follow the directions presented in section 8.1.6.

8.1.3 Ventilation

Worm trays need to be ventilated as an earthworm has no respiratory organs. Gaseous exchange occurs through its moist skin. Worms require gaseous oxygen from the air. The oxygen diffuses across the moist tissue of their skin, from the region of greater concentration of oxygen (the air) to that of lower concentration (inside the worm). When water has been sufficiently aerated, worms have been known to live under water for a considerable length of time. Carbon dioxide produced by the bodily processes of the worm also diffuses through its moist skin. A constant supply of fresh air throughout the bedding helps this desirable exchange of gases to take place (Appelhof, 1982). This can be accomplished by covering the culture trays with a piece of stiff black plastic cut slightly smaller than the bedding surface, a large garbage bag with airholes, a rubbermaid lid with airholes or wet newspaper. The covers function to retain moisture and exclude light while allowing air to circulate.

8.1.4 Moisture

All worms need moisture. They "breathe" through their skin which must be moist for exchange of air to take place. A worm's body consists of approximately 75% to 90% water. By preparing bedding with approximately the same moisture content as worm skin, the worm does not have to combat an environment which is either too dry or too moist. Basically, we want the bedding damp, but not soggy. If you squeeze a handful of bedding and produce three to four drops of water, it's probably all right; twenty drops or a stream of water is <u>too</u> wet.

8.1.5 Feeding: Quantity and Frequency

Feed consists of alfalfa pellets mixed with water and aged two weeks in a sealed container. A small trench is dug along the midline of the bedding tray and the aged

feed is dropped in and covered over to reduce the odor of the fermented alfalfa. Alfalfa pellets can be obtained from Agricultural Feed and Supply stores.

Food is dispensed with a glass beaker and measured by volume. Moldy alfalfa in the pail does not need to be removed before feeding and should be added to the trays. If the number of worms in a tray is low (e.g. 150 adults) feeding once per week is adequate. A tray holding approximately 200 adults may need 200 to 250 ml of food per week. Keep a record of volume (ml) of feed distributed on the Earthworm Culture Logsheet (ESAT-5-042.2).

Feed is added after the moisture content of the trays is examined and after the bedding is turned. Once a week, adjust the moisture level and turn the soil. Next, determine whether the tray needs more water. This is a subjective judgment. The bedding is turned over by hand, after water is added, to distribute the moisture and prevent the bedding at the bottom of the tray from becoming too compressed.

8.1.6 pH

While the ideal pH is between 6.8 and 7.2 (Gaddie & Douglas, 1977) a pH range of 5 to 8 is acceptable under laboratory conditions. Soil pH is measured by making a slurry of RO water and soil in a 2.5:1 basis; volume of water (1 ml water = 1 g): weight of soil. Ten grams of soil and twenty-five ml of RO water in a 50 ml beaker are used. The slurry is mixed with a magnetic stir bar on a magnetic stir plate until the reading stabilizes, at which time the pH is recorded in an Earthworm Culture Logsheet (ESAT-5-042.2).

Over-acidity (pH $\leq$ 5.0) can be a grave problem, causing the worms to crawl in an effort to escape, or even killing the entire crop. The best method for avoiding acid build-up is to avoid getting too much feed into the bedding. An acid bed with soured feed is an invitation to mites, often a real headache for the worm grower. One general bit of care will be useful in eliminating the CAUSE of many insect infestations, that is, the liberal use of crushed limestone in the tray (Shields, 1982).

Over-alkaline conditions (pH $\geq$ 9.0), though rare, will affect earthworms in much the same way as dehydration, or drying. The earthworm loses color and weight and may shrink, turn dark and become lifeless. Such a condition can be brought on by improper (too much) use of limestone or by use of the wrong type of lime product. The fastest remedy is to mix unleached dry peat moss into the bedding. Most peat moss has a pH of between 3 and 5 in its dry state.

All adjustments made to the bedding to bring the pH within the acceptable range should be noted in the commments field located at the bottom of the Earthworm

Culture Logsheet.

8.1.7 Temperature

An earthworm will die if exposed to freezing or low temperatures. Optimum feeding and conversion of food should occur within the temperature range of 13-25°C. Bedding temperatures above 29°C are lethal. Worm trays will be kept in an environmental chamber set at 15°C ± 2°C. In lieu of an environmental chamber, a well-ventilated room with no drafts and a room temperature of less than 20°C will suffice.

8.1.8 Illumination

Culture trays should be kept under continuous lighting (Harris, 1990) to induce the worms to remain in the trays.

8.1.9 Culture Vessels

Culture vessels should be shallow (no more than 8" to 12" deep) to accomodate the conditions listed below:

1. Red worms tend to be surface feeders.
2. Bedding can pack down in a deep container, compressing the air out of the bottom layers and make it more likely to develop foul-smelling anaerobic conditions.
3. Given bins of different shape but equal volume, the one with greater surface area provides better aeration (Appelhof, 1982).

Our cultures are grown in plastic plant trays measuring approximately 49.0 x 26.0 x 5.5 centimeters.

Disposable rubber gloves should be used when handling the worms and culture trays. Gloves are changed between each tray to reduce the possibility of spreading any disease from tray to tray.

8.1.10 Loading Factor

The number of worms that a tray holds is determined by the age and weight relationship of the worms.

8.2 CULTURE MAINTENANCE AND HARVEST

It is important that earthworm beds either be harvested or divided on a regular 60 or 90 day schedule, to prevent overcrowding. The smaller the culture tray, the more often the beds will need dividing. Earthworms which are overcrowded are generally very small, and are susceptible to "sour crop" or protein poisoning. Earthworms need room. When the young earthworms become too numerous in the bed, the larger earthworms eat what they need and then move to the bottom of the bed to get away from the smaller earthworms.

Indications of overcrowding include a high concentration of castings visible on the bed surface and large numbers of worms crawling to the sides and covers of the culture trays. Overcrowded trays become excessively wet over time even if they are not over-watered. This may be due to the moisture added in the fermented feed and/or to water as a respiration by-product. Crowding decreases the growth rate and reproductive efficiency. Peat moss also tends to become waterlogged with time and anaerobic conditions develop. Indications occur after two to three months and include (1) a change in color between the bottom bedding material and the upper one to two inches of material; and (2) development of a strong odor.

Earthworms mate at different levels in their bedding, rather than just upon the surface. Under proper conditions, they mate any time of the year. As the worms reproduce, the voracious young worms compete with their parents and all the other worms in the culture for the limited food available. Additionally, all the worms excrete castings, which have been shown to be toxic to members of their species. A casting is worm manure, the undigested material, soil, and bacteria excreted through the worm's anus after having moved through its digestive tract.

Once a tray of adult worms produces a large number (500+) of young worms, the tray needs to be split into two trays. Depending on the reasons for harvesting the worms, there are four alternative methods for recovering the worms.

8.2.1 One method is the Dump and Hand Sort (Figure D).

The tools needed for this procedure include a large sheet of heavy plastic, a goose-neck or similar lamp with a 100-watt bulb, a plastic dish pan or container for the worms and fresh bedding. Spread plastic on a flat surface, dump the entire contents of the worm tray on the plastic. Make about 9 cone-shaped piles. If the light is bright enough, the worms quickly move away from it towards the center of each pile of bedding. Leave piles alone for 5 to 10 minutes. Gently remove the outer surface of each pile and start hand-picking the worms gently scraping the 'dirt' off of them. As you do so, worms on newly exposed surfaces will again react to the light and retreat towards the interior. By following this procedure one pile at a time, you will find that when you return to the first pile, the worms will have disappeared again, and you can repeat the procedure. This method is used when conducting weight determination since it approaches 100%

worm recovery.

8.2.2 Let the Worms do the Sorting (Figure E)

A second method available is to pull all bedding and worms to one side of the box. Add new bedding to the vacant side, sprinkle with feed and cover with a sheet of black plastic while shining bright light over 'old' bedding. Worms move to the new bedding in search of food and to escape the light. Remove old bedding in layers over time, maybe 1 inch every 15 to 30 minutes. This method is most efficient when changing the bedding only and you have no desire to know the weight of the worms.

8.2.3 Divide and Dump (Figure F)

A third procedure is to simply remove two thirds of the bedding, dump it and add fresh bedding to fill in the culture tray. This method assumes that enough worms and cocoons will be in the retained third to populate the system for another cycle.

8.2.4 A fourth method would be to collect all cocoons and transfer them to another tray of fresh bedding.

If the adult worms are being harvested for use in a toxicity test, the tray is emptied onto a plastic-covered table and individual worms are hand-picked before they are able to burrow back into the soil.

8.3 Record-Keeping

Maintain a log recording dates of culture tray initiation, amounts of topsoil, water, pH, number of worms, adjustments, and all other relevant comments.

The Earthworm Culture Logsheet (ESAT-5-042.2) is designed to document feeding, harvesting, and bed changing schedule. Enter dates in column (1). In column (2) enter the amount of food (in mls) still remaining from the previous feeding. This amount will be a subjective guess based on the amount of food documented to have been fed in the previous feeding. In Column (3) enter whether the water holding capacity of the soil was checked and if water was added, the amount in mls that was added. For column (4) indicate by yes or no whether the soil was turned over to facilitate aeration and moisture distribution. Enter the amount of food in mls and the Food batch# into columns (5) & (6). Column (7) is available for all comments such as for example: Were any worms harvested and how many? Was the bedding changed? Were trays split to forestall overcrowding? etc.

9. CALCULATIONS

Not applicable.

10. QUALITY CONTROL

10.1 Competing Organisms

In general, organic residue is eaten by primary (1°) consumers such as mold and bacteria. These primary consumers, in turn, are eaten by secondary (2°) consumers such as earthworms, enchytraeids (nematodes), springtails and mold mites. (Figure G) These competitive organisms become a "problem" when their numbers become high as they discourage the earthworms from feeding.

10.1.1 Mites

Mites of one kind or another are a constantly recurring annoyance. They are apt to be prevalent wherever there are ground grain feeds. While they are harmless to the worms, they do consume some of the feed. However, the red or "fishworm" mite is apt to become a real menace, and a difficult to control. One general bit of care will be useful in eliminating the CAUSE of many insect infestations, is the liberal use of crushed limestone in the pits to keep the compost sweet. (Insect pests like it sour!) (Subphylum Cheliceriformes, Class Chelicerata, Subclass Arachnida, Order Acari).

Mites can be baited by placing slices of bread on the bedding and removing it after the mites concentrate on its underside.

10.1.2 Enchytraeids

Enchytraeids, also known as pot worms belong to the phyla Nematoda. Nematodes are characteristically small worms with thin, unsegmented bodies that are usually distinctly round in cross section. Enchytraeids are small, approximately 1/4 to 1 inch long and remain white in color throughout their lifetimes. Young earthworms and enchytraeids look virtually alike, but earthworms will turn reddish-pink within a week of emergence from their cocoons.

10.1.3 Springtails

Springtails are 1/16 mm long grey-white insects of the Subclass Oligoentomata, Order Collembola, Class Collembola. They are primitive insects with a pointed prong extending forward underneath their abdomen from the rear which allows them to

"spring". Springtails feed on molds and decaying matter.

REFERENCES CITED

1. Appelhof, Mary. Worms Eat My Garbage. Published by Flower Press, 10332 Shaver Road, Kalamazoo, MI, 49002. 1st Ed., 1982.

2. Bateman, Graham, ed., Aquatic Invertebrates; All the World's Animals. Published by Torstar Books, 300 E. 42nd Street, New York, NY 10017. 1987.

3. Brusca, Richard C. Invertebrates. Sinauer Associates, Inc. Sunderland, Massachusetts 01375. 1990.

4. Edwards, C.A., and J.R. Lofty. Biology of Earthworms. London: Chapman and Hall. Available in U.S. from Methuen, Inc., New York, N.Y. 2nd Ed. 1977. 333pp.

5. Gaddie, Ronald E., Sr., and Donald E. Douglas. Earthworms for Ecology and Profit. Vol. I: Scientific Earthworm Farming. Bookworm Publishing Co., Ontario, CA. 1975. 180pp.

6. Harris, Todd, Dr., Jay Glover, and Dr. Jim Maudsley. Region IV; SOP for Toxicity Testing Hazardous Waste Assessments.--Environmental Services Assistance Team, NSI Technology Services Corporation, U. S. EPA, ESD, College Station Road, Athens, Georgia. pp. XVII. 1-13.

7. Shields, Earl B. Raising Earthworms for Profit. Shields Publication, P.O. Box 669, Eagle River, WI. Original copyright, 1959, 18th Ed., 1982. 128pp.

8. Wallwork, J.A. Earthworm Biology. Published by Edward Arnold, 300 North Charles Street, Baltimore, Maryland, 21201. 1983. 55pp.

EARTHWORM CULTURE LOGSHEET
CULTURE TRAY # _____

Bed initiated/renewed on: _____ pH of bedding: _____
Media: g)_____ Proposed bed change date: _____

(1) Date	(2) Amt. Food Remaining	(3) Moisture Added	(4) Soil Turned	(5) Feed Added	(6) Food Batch#	(7) Remarks

Comments:

FEED LOG

BATCH ID: _____

Alfalfa wt: _____ g Water volume: _____ mls

Fermentation Start Date: _____

Total Start Volume: _____ mls

Date	Volume Used (mls)	Volume Remaining (mls)	Culture Tray ID:

ATTACHMENTS

Figure A. Major Elements of Earthworm (Gaddie and Douglas, 1975).

Figure B. Earthworm body (Brusca, 1990).

Figure C. Earthworm Mating and Cocoon Formation (Appelhof, 1982).

Figure D. Harvesting Techniques: Dump and Hand Sort (Appelhof, 1982).

Figure E. Harvesting Techniques: Let the Worms do the Sorting (Appelhof, 1982).

Figure F. Harvesting Techniques: Divide and Dump (Appelhof, 1982).

Figure G. Food Web of the Compost Pile (Appelhof, 1982).

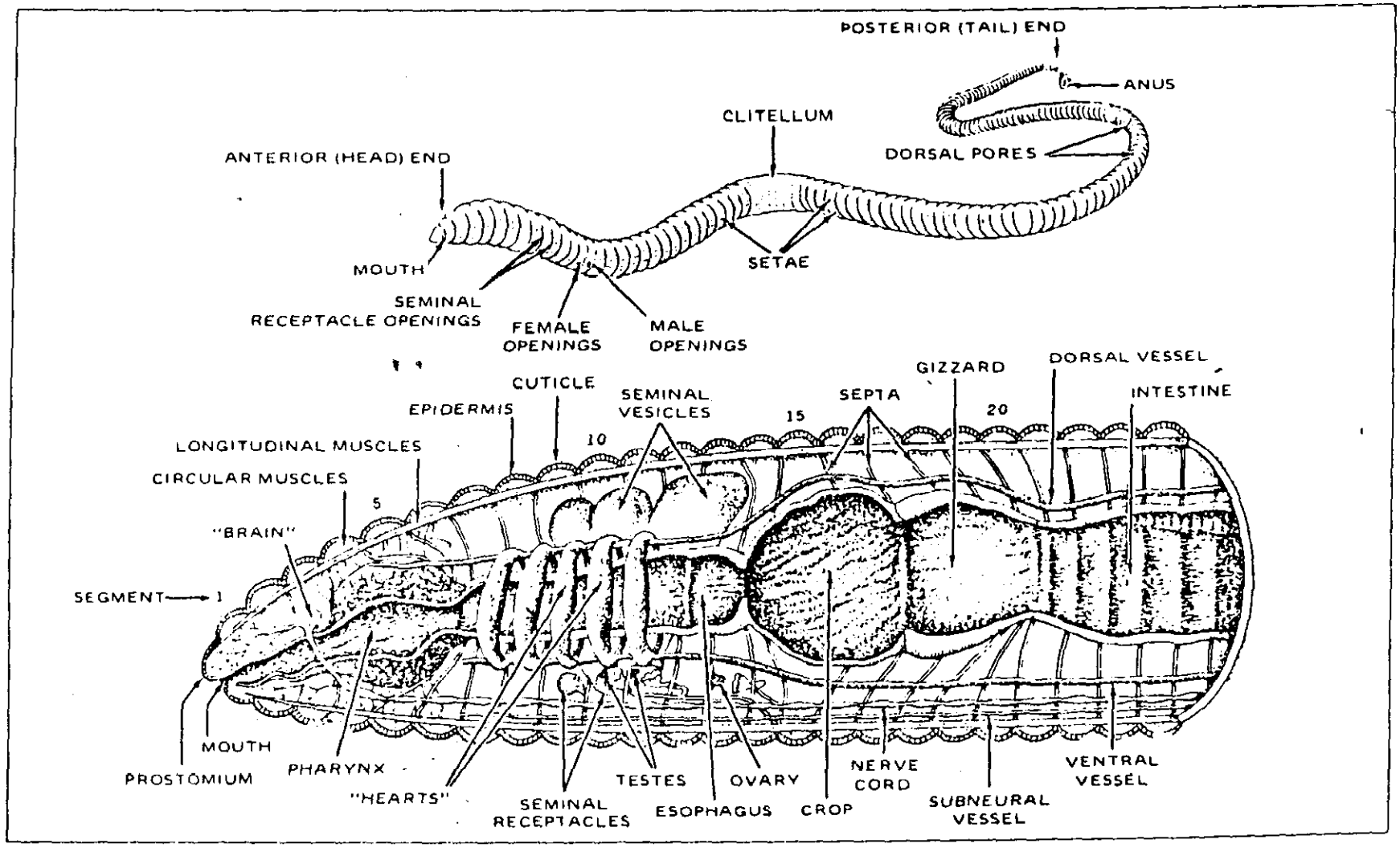
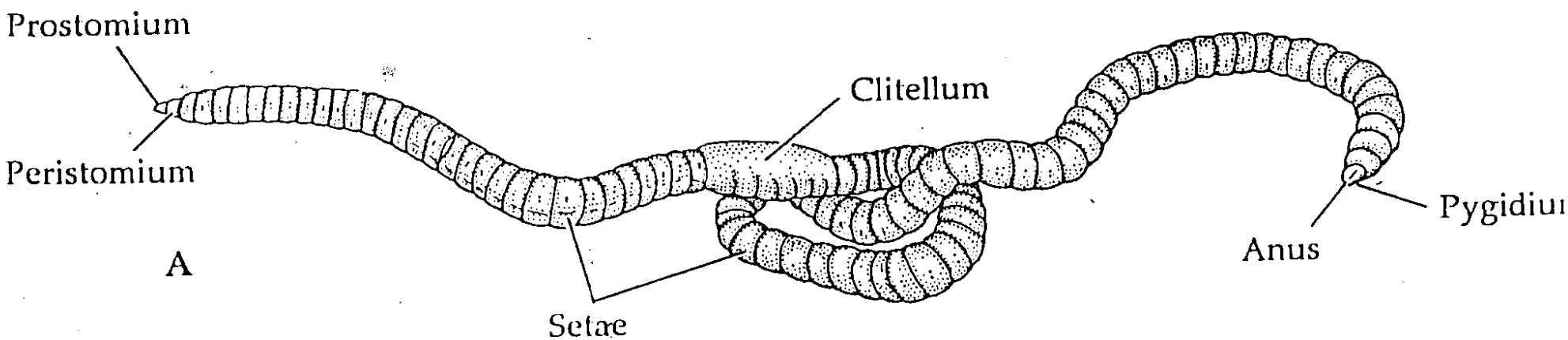


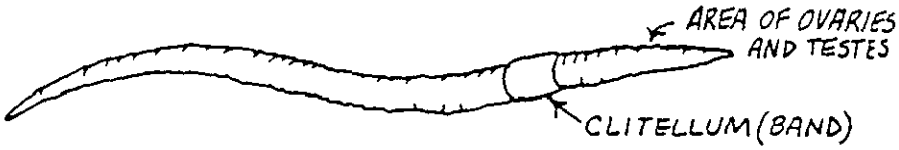
Figure 2-2. Major Elements of Earthworm (L. terrestris) Reproductive, Circulatory, Nervous, Digestive, Excretory, and Muscular Systems

FIGURE B.

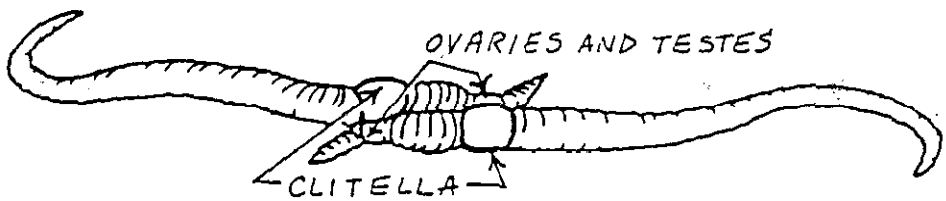


EARTHWORM MATING AND COCOON FORMATION

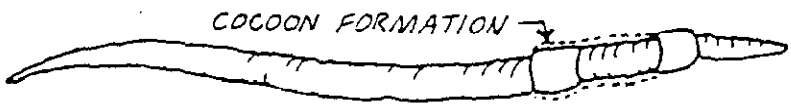
EACH WORM HAS BOTH OVARIES AND TESTES.



TWO WORMS JOIN BY MUCUS FROM THEIR CLITELLA. SPERM THEN PASS FROM EACH WORM TO THE SPERM STORAGE SACS IN THE OTHER WORM.

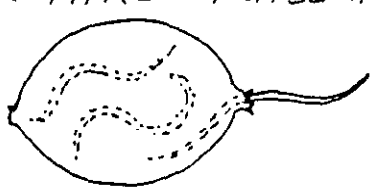


LATER, A COCOON FORMS ON THE CLITELLUM OF EACH WORM. THE WORM BACKS OUT OF THE HARDENING COCOON.



EGGS AND SPERM ARE DEPOSITED IN THE COCOON AS IT PASSES OVER OPENINGS FROM OVARIES AND SPERM STORAGE SACS.

AFTER BEING RELEASED FROM THE WORM, THE COCOON CLOSES AT BOTH ENDS. EGG FERTILIZATION TAKES PLACE IN THE COCOON.

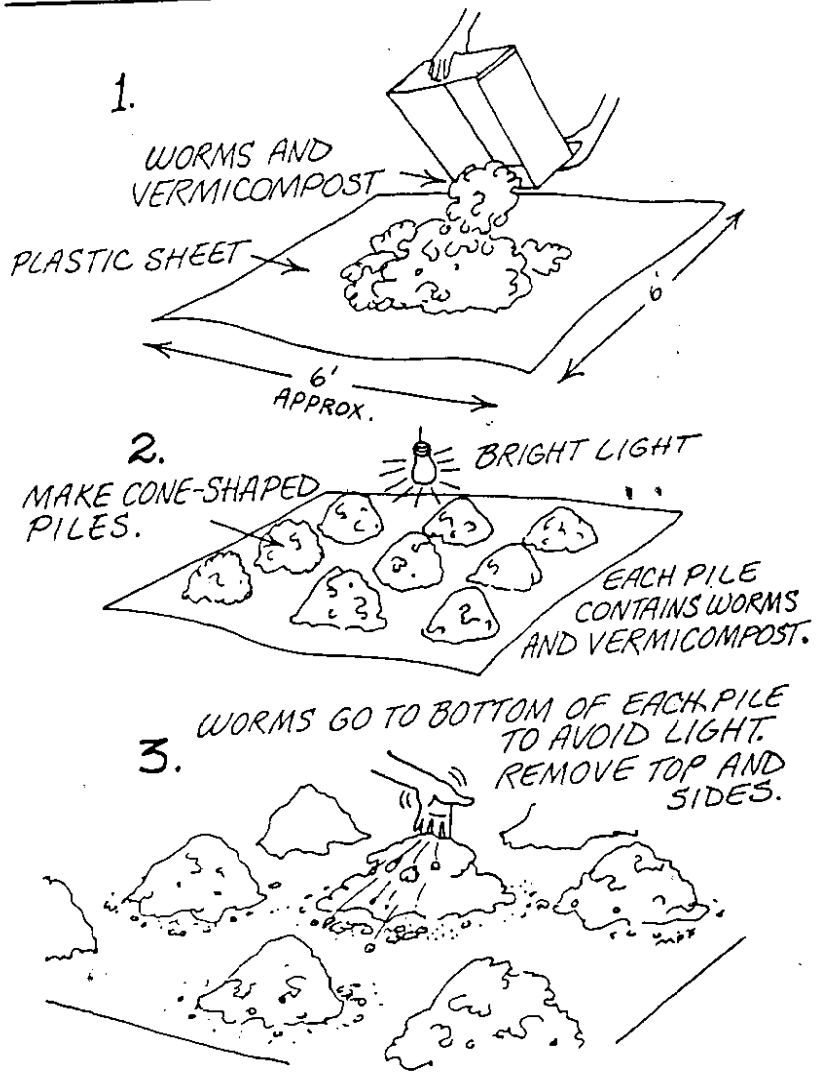


TWO OR MORE BABY WORMS HATCH FROM ONE END OF THE COCOON.

MEF.

FIGURE D.

HARVESTING TECHNIQUES: DUMP AND HAND SORT.



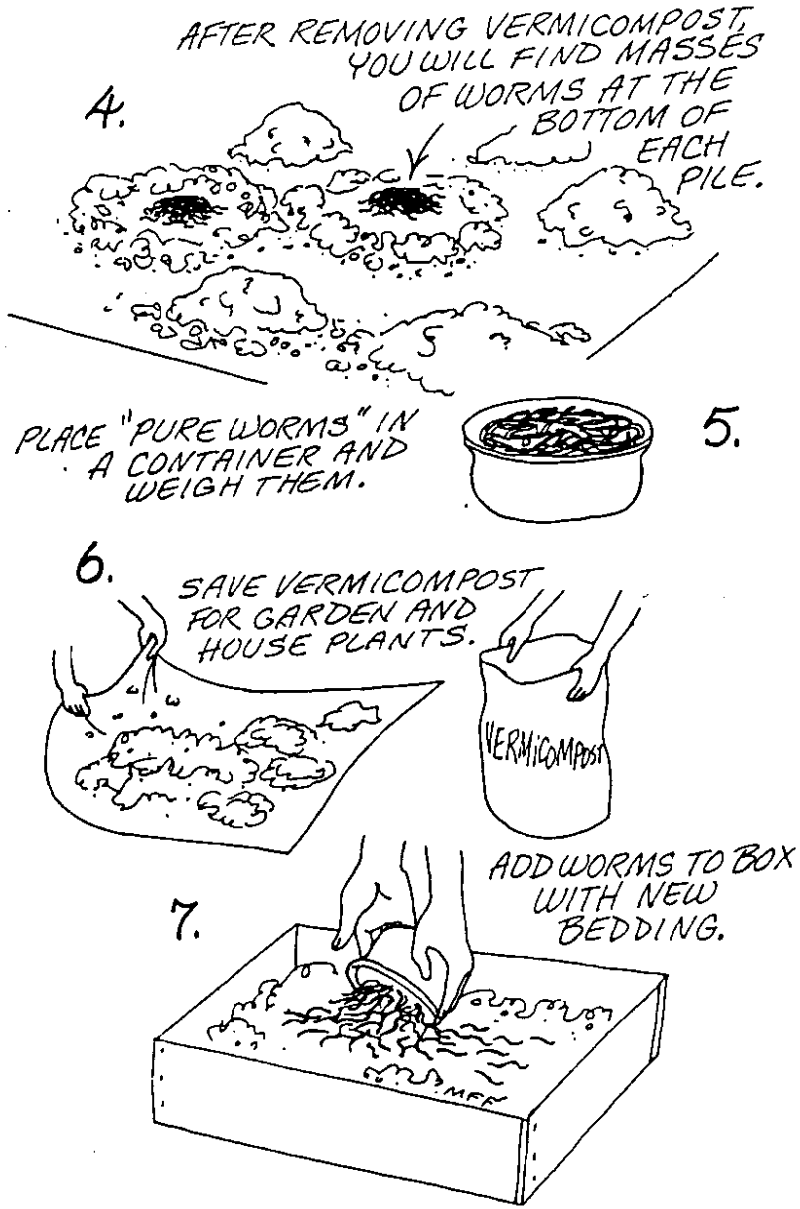
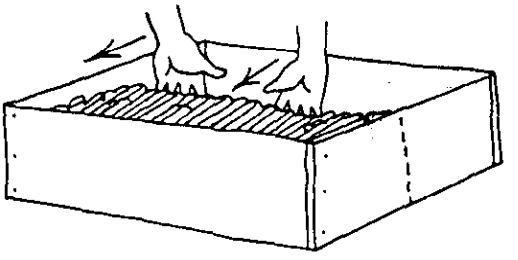
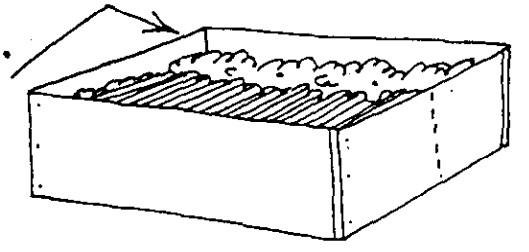


FIGURE E.

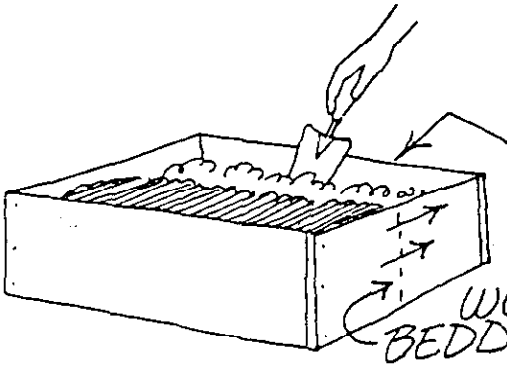
HARVESTING TECHNIQUE: LET THE WORMS DO THE SORTING.



1. PULL VERMICOMPOST AND WORMS TO ONE SIDE OF THE BOX.

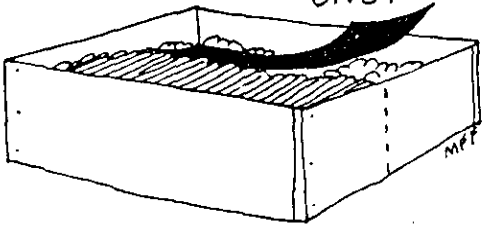


2. ADD NEW BEDDING TO VACANT SIDE.



3. BURY GARBAGE IN NEW BEDDING.

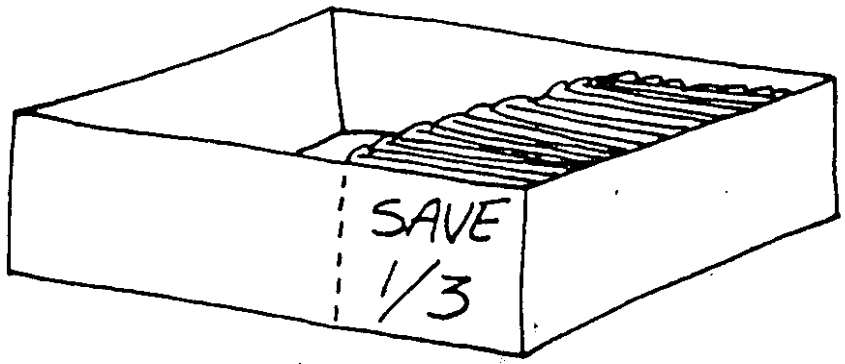
WORMS MOVE TO NEW BEDDING IN SEARCH OF FOOD.



4. REMOVE VERMICOMPOST IN 2-3 MONTHS AND REPLACE WITH NEW BEDDING.

FIGURE F.

HARVESTING TECHNIQUE: DIVIDE AND DUMP.



1.

TAKE OUT ALL BUT 1/3 OF WORMS AND VERMICOMPOST. ADD NEW BEDDING.

2.

ADD VERMICOMPOST TAKEN FROM BOX TO THE GARDEN...WORMS AND ALL.

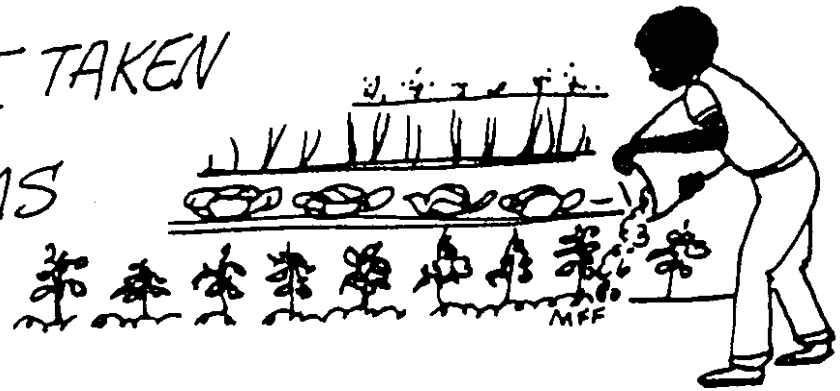


FIGURE G.

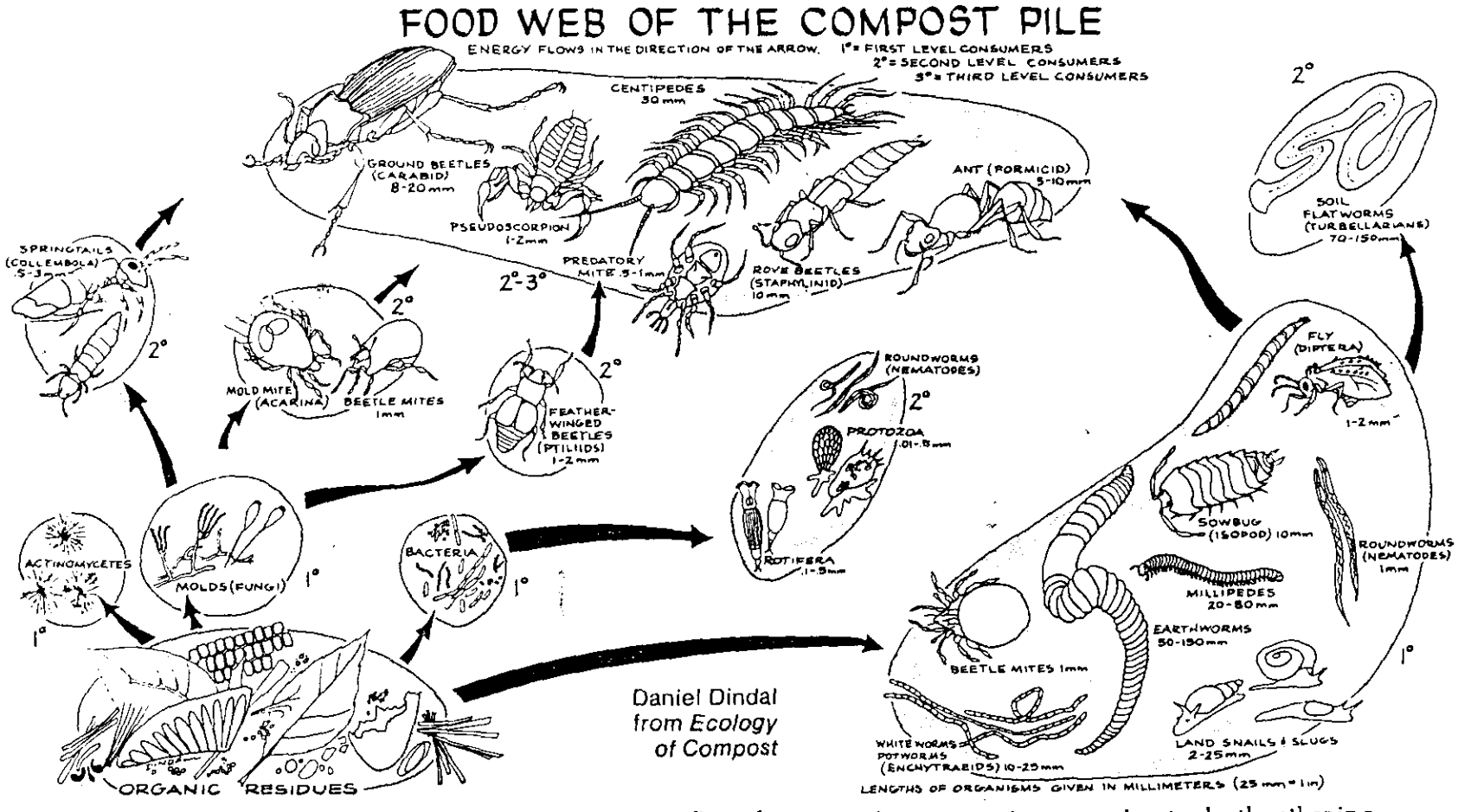


Figure 3. Organisms commonly found in compost. Energy flows from organism to organism as one is eaten by the other in a natural recycling system. Snails, beetles, millipedes, centipedes, and ants are less likely to find their way to worm bins set up with shredded corrugated and paper beddings.

SEED GERMINATION CONTROL CHART

EC50=	Mean = X-bar=	12.7	U.L.=
	Trials = n=	25	19.88641372
	Standard Deviation = SD=	3.594	L.L.=
	Coefficient of Variation = CV%=	28.31	5.508786282

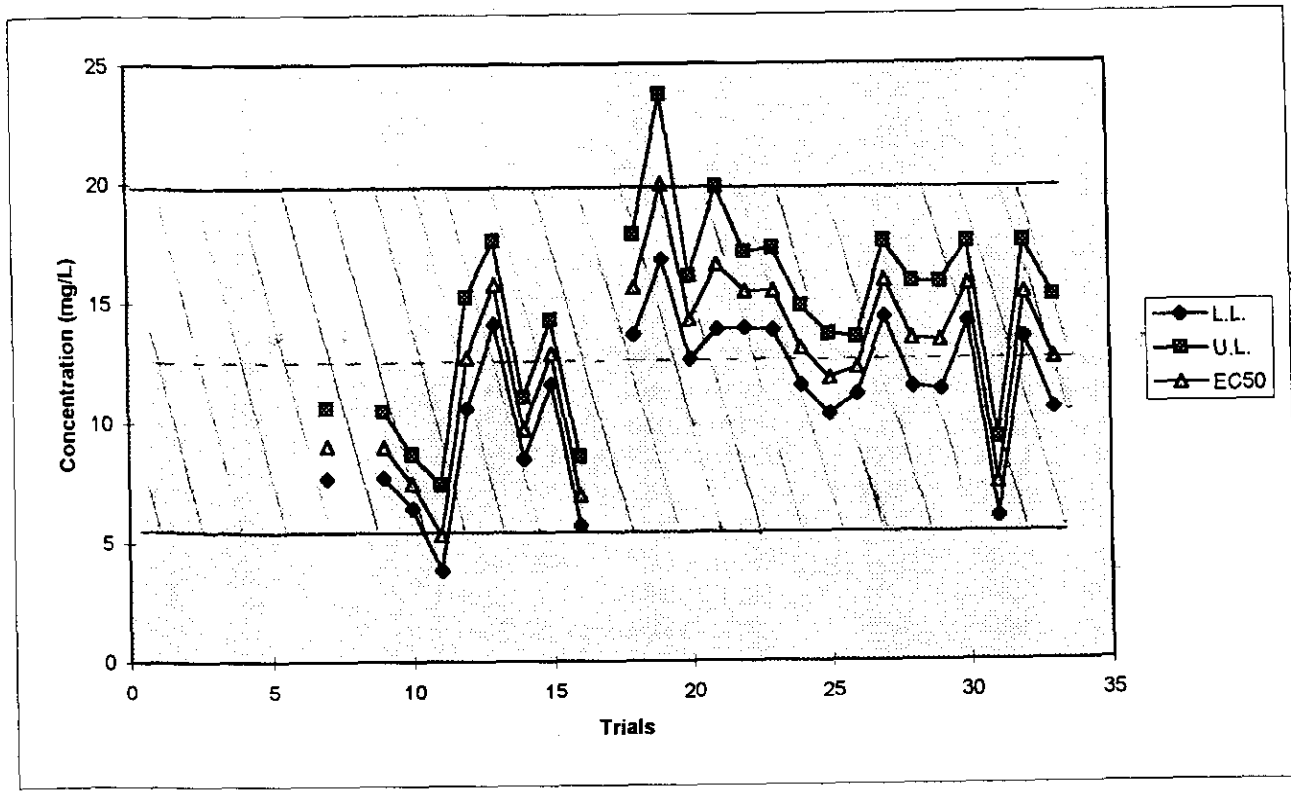
Dates	Tri	Trimmed-Spearman L.L.	U.L.	EC50	NOEC	LOEC	IC25	IC50	ChV	Probit
	1									
	2									
	3									
	4									
	5									
4/3/96	6									
4/10/96	7	7.69	10.65	9.05	15	30	5.07	17.61	21.21	
5/1/96	8									
5/8/96	9	7.72	10.48	8.99	7.5	15	6	11.25	10.61	10.15 (8.54-11.70)
6/12/96	10	6.46	8.69	7.49	3.75	7.5	5.24	7.26	5.3	9.20 (0.08-17.66)
7/3/96	11	3.81	7.42	5.32	7.5	15	3.24	9.96	10.61	10.03 (8.13-11.56)
8/7/96	12	10.6	15.25	12.72	15	30	10.06	18.45	21.21	17.21(14.89-19.29)
9/4/96	13	14.11	17.62	15.77	15	30	13.46	19.66	21.21	17.87(16.02-19.67)
10/2/96	14	8.5	11.05	9.69	7.5	15	8.64	11.62	10.61	11.42(9.82-12.76)
11/13/96	15	11.62	14.27	12.88	7.5	15	10.79	14.09	10.61	10.49
12/4/96	16	5.69	8.58	6.98	7.5	15	7.85	11.31	10.61	10.60(8.33-12.63)
1/8/97	17									non-compliant
1/29/97	18	13.64	17.87	15.61	7.5	15	12.7	19.86	10.61	18.50(16.20-20.66)
3/5/97	19	16.8	23.72	19.96	7.5	15	14.25	22.31	10.61	21.62(18.95-24.51)
3/27/97	20	12.65	16.11	14.28	7.5	15	11.34	16.39	10.61	17.34(14.79-19.76)
4/30/97	21	13.87	19.86	16.6	15	30	16.7	21.68	21.21	20.63(17.96-22.85)
6/4/97	22	13.93	17.13	15.45	7.5	15	12.38	18.33	10.61	16.97(15.16-18.67)
6/25/97	23	13.82	17.26	15.45	7.5	15	12.19	18.57	10.61	17.65(15.52-19.60)
7/31/97	24	11.55	14.83	13.09	3.75	7.5	9.49	14.34	5.3	14.16(12.61-15.64)
9/10/97	25	10.29	13.65	11.85	7.5	15	10.77	14.89	10.61	15.6
10/1/97	26	11.15	13.51	12.27	7.5	15	9.87	12.81	10.61	13.52(6.23-20.19)
11/12/97	27	14.39	17.58	15.91	7.5	15	12.19	18.04	10.61	16.73(15.14-18.29)
12/10/97	28	11.45	15.83	13.46	7.5	15	10.55	17.76	10.61	16.76
1/7/98	29	11.35	15.8	13.39	7.5	15	9.6	17.06	10.61	16.46(14.84-18.08)
1/28/98	30	14.14	17.51	15.73	7.5	15	12.88	19.38	10.61	17.69(15.78-19.49)
2/18/98	31	6.02	9.26	7.47	7.5	15	4.41	11.72	10.61	9.55(7.04-11.99)
4/1/98	32	13.48	17.54	15.38	7.5	15	13.75	19.65	10.61	17.76(12.59-28.18)
4/22/98	33	10.51	15.22	12.65	15	30	19.03	23.05	21.21	19.06(16.61-21.30)

Seed Germination Control Chart

SEED GERMINATION CONTROL CHART

EC50= .	Mean =	X-bar=	12.7	U.L.=	
	Trials =	n=	25		19.88641372
	Standard Deviation =	SD=	3.594	L.L.=	
	Coefficient of Variation =	CV%=	28.31		5.508786282

Dates	Tri	Trimmed-Spearman L.L.	U.L.	EC50	NOEC	LOEC	IC25	IC50	ChV	Probit



= 5.51 mg/L
= 19.89 mg/L
= 12.70 mg/L

ROOT ELONGATION CONTROL CHART

EC50=	Mean =	X-bar=	12.08	U.L.= 24.27859914
	Trials =	n=	57	
	Standard Deviation =	SD=	6.098	2(SD)= 12.196
	Coefficient of Variation =	CV%=	50.47	L.L.= -0.113686857

Dates	Tri	Trimmed-Spearman L.L.	U.L.	EC50	NOEC-G	LOEC-G	NOEC-L	LOEC-L	IC25-G	IC50-G	IC25-L	IC50-L	ChV-G	ChV-L	EC50 -Probit
	1														
	2														
	3														
	4														
	5														
	6														
	7														
	8														
	9														
2/3/94	10														
2/10/94	11	4.14	6.23	5.08	3.75	7.5	3.75	7.5	4.69	6.09	4.93	6.11	5.3	5.3	6.74 (2.96-9.25)
2/18/94	12	2.27	6.34	3.79	1.88	3.75	1.88	3.75	2.04	5.63	1.83	3.44	2.66	2.66	4.96 (2.00-8.01)
2/24/94	13	11.62	27.84	17.99	15	30	15	30	12.66	22.13	11.3	15.26	21.21	21.21	20.91 (11.00-39.11)
3/3/94	14	5.21	9.98	7.21	3.75	7.5	3.75	7.5	5.09	8.91	4.48	6.51	5.3	5.3	6.46 (1.99-26.02)
3/10/94	15	2.42	7.9	4.37	3.75	7.5	3.75	7.5	0.82	5.25	0.86	5.31	5.3	5.3	5.87 (2.65-8.04)
3/16/94	16	5.69	14.25	9.01	15	30	3.75	7.5	5.16	18.44	3.21	6.22	21.21	5.3	no convergence
3/24/94	17	6.56	14.03	9.59	7.5	15	7.5	15	8.13	12.5	8.15	11.06	10.61	10.61	12.79 (7.27-16.67)
3/31/94	18	6.57	13.99	9.59	3.75	7.5	7.5	15	5.16	11.25	7.64	10.55	5.3	10.61	7.71 (1.57-24.50)
4/7/94	19	5.46	11.94	8.07	3.75	7.5	3.75	7.5	6.7	10.63	3.76	8.55	5.3	5.3	10.13 (6.08-13.44)
4/13/94	20	3.97	10.16	6.35	7.5	15	3.75	7.5	5.83	8.75	4.94	7.04	10.61	5.3	no convergence
4/21/94	21														
5/5/94	22	7.03	15.73	10.53	7.5	15	7.5	15	10.1	13.65	6.08	10.17	10.61	10.61	7.87
5/12/94	23	6.11	13.38	9.04	15	30	7.5	15	5.39	19.22	1.58	8.53	21.21	10.61	no convergence
6/2/94	24	8.09	14.67	10.89	7.5	15	7.5	15	9.06	13.13	5.6	7.46	10.61	10.61	12.67 (7.56-16.44)
7/14/94	25	4.49	12.79	7.57	7.5	15	7.5	15	4.69	10.5	5.26	8.4	10.61	10.61	10.95 (5.8-14.98)
7/21/94	26														
8/18/94	27	12.22	19.3	15.35	15	30	15	30	17.45	21.63	9.54	13.77	21.21	21.21	no convergence
8/24/94	28														
9/8/94	29	4.48	8.29	6.1	3.75	7.5	3.75	7.5	2.93	9.38	2.58	6.29	5.3	5.3	6.68 (4.10-9.47)
10/6/94	30	4.77	11.66	7.46	7.5	15	7.5	15	6.09	12.66	7.68	10.63	10.61	10.61	11.78 (5.85-16.35)
11/3/94	31	4.17	6.74	5.3	3.75	7.5	3.75	7.5	4.42	6.16	4.93	6.68	5.3	5.3	7.17 (3.44-10.13)

ROOT ELONGATION CONTROL CHART

EC50= Mean = X-bar= 12.08 U.L.= 24.27859914
Trials = n= 57
Standard Deviation = SD= 6.098 2(SD)= 12.196
Coefficient of Variation = CV%= 50.47 L.L.= -0.113686857

Dates	Tri	Trimmed-Spearman L.L.	U.L.	EC50	NOEC-G	LOEC-G	NOEC-L	LOEC-L	IC25-G	IC50-G	IC25-L	IC50-L	ChV-G	ChV-L	EC50 -Probit
12/7/94	32	4.16	9.62	6.33	7.5	15	7.5	15	5.63	9.64	5.88	9.46	10.61	10.61	8.80 (4.5-11.75)
1/5/95	33	4.7	7.9	6.09	7.5	15	7.5	15	5.42	8.44	5.63	8.89	10.61	10.61	5.85 (2.32-15.07)
2/9/95	34	2.88	10.06	5.38	7.5	15	7.5	15	5	8.57	5.3	8.12	10.61	10.61	3.56 (2.26-5.26)
3/17/95	35	2.69	10.45	5.31	0.94	1.88	3.75	7.5	3.98	7.03	5	7.14	1.33	5.3	8.48 (3.23-13.00)
4/14/95	36	12.02	18.66	14.98	7.5	15	7.5	15	12.15	17.44	9.56	12.3	10.61	10.61	no convergence
5/17/95	37	7.53	14.31	10.38	15	30	7.5	15	8.59	12.81	5.66	7.65	21.21	10.61	12.12 (8.03-15.73)
6/22/95	38	7.96	12.89	10.13	7.5	15	3.75	7.5	7.5	11.43	4.6	6.94	10.61	5.3	10.51 (7.69-13.52)
7/20/95	39	7.87	12.59	9.95	7.5	15	3.75	7.5	7.97	11.25	4.74	6.38	10.61	5.3	10.66 (7.64-13.55)
8/3/95	40														
8/23/95	41														
9/6/95	42														
9/14/95	43	5.79	17.53	10.08	15	30	n/a	n/a	7.5	17.5	1.7	6.38	21.21	n/a	15.36 (6.17-22.10)
10/13/95	44	3.91	14.1	7.43	7.5	15	7.5	15	3.05	13.13	5.17	7.02	10.61	10.61	10.57 (2.68-19.03)
11/29/95	45	3.26	12.85	6.47	7.5	15	7.5	15	7.5	12	3.96	6.15	10.61	10.61	12.89 (3.91-19.37)
12/14/95	46	7.12	19.82	11.88	15	30	3.75	7.5	8.13	16.07	4.03	6.6	21.21	5.3	no convergence
1/17/96	47	4.63	12.28	7.54	7.5	15	3.75	7.5	8.82	11.47	3.33	6.22	10.61	5.3	no convergence
2/21/96	48	1.7	7.93	3.67	3.75	7.5	3.75	7.5	3.75	5.63	4.06	5.52	5.3	5.3	6.42 (2.21-9.35)
3/6/96	49	3.16	7.2	4.77	7.5	15	7.5	15	1.65	8.44	1.75	6.62	10.61	10.61	6.22 (2.38-11.33)
4/4/96	50	8.72	19.03	12.88	15	30	3.75	7.5	9.84	16.5	5.32	7.64	21.21	5.3	16.32 (7.92-24.01)
5/2/96	51	5.41	12.06	8.08	7.5	15	3.75	7.5	7.97	10.31	2.17	6.48	10.61	5.3	n/a
6/12/96	52				15	30	7.5	15	22.5	n/a	9.79	13.55	21.21	10.61	36.53 (24.73-189.95)
7/3/96	53	19.82	24.06	21.83	15	30	7.5	15	18.44	23.13	8.78	12.58	21.21	10.61	n/a
8/22/96	54	6.99	12.2	9.24	7.5	15	3.75	7.5	5.63	11.5	3.35	5.95	10.61	5.3	9.56 (6.63-12.96)
9/4/96	55	13.51	25.77	18.66	15	30	7.5	15	13.28	21.16	6.56	10.26	21.21	10.61	19.34(13.11-27.97)
9/20/96	56				n/a	n/a	7.5	15	n/a	n/a	9.28	12.74	n/a	10.61	no convergence
10/3/96	57	13.71	21.81	17.29	15	30	7.5	15	15.18	20.63	8.42	11.74	21.21	10.61	18.83(14.37-23.52)
11/14/96	58	13.92	21.1	17.14	7.5	15	7.5	15	13.36	19.58	8.28	11.89	10.61	10.61	17.74(13.09-22.30)
12/5/96	59	6.68	15.49	10.17	3.75	7.5	3.75	7.5	5.63	12.19	1.41	5.75	5.3	5.3	n/a
1/9/97	60	13.41	26.97	19.01	7.5	15	7.5	15	13.28	20.57	7.48	10.8	10.61	10.61	n/a
1/31/97	61				15	30	7.5	15	20.36	27.86	7.37	11.26	21.21	10.61	33.39(18.62-153.36)
2/13/97	62	13.47	22.4	17.37	15	30	7.5	15	16.02	21.14	8.65	11.58	21.21	10.61	n/a

ROOT ELONGATION CONTROL CHART

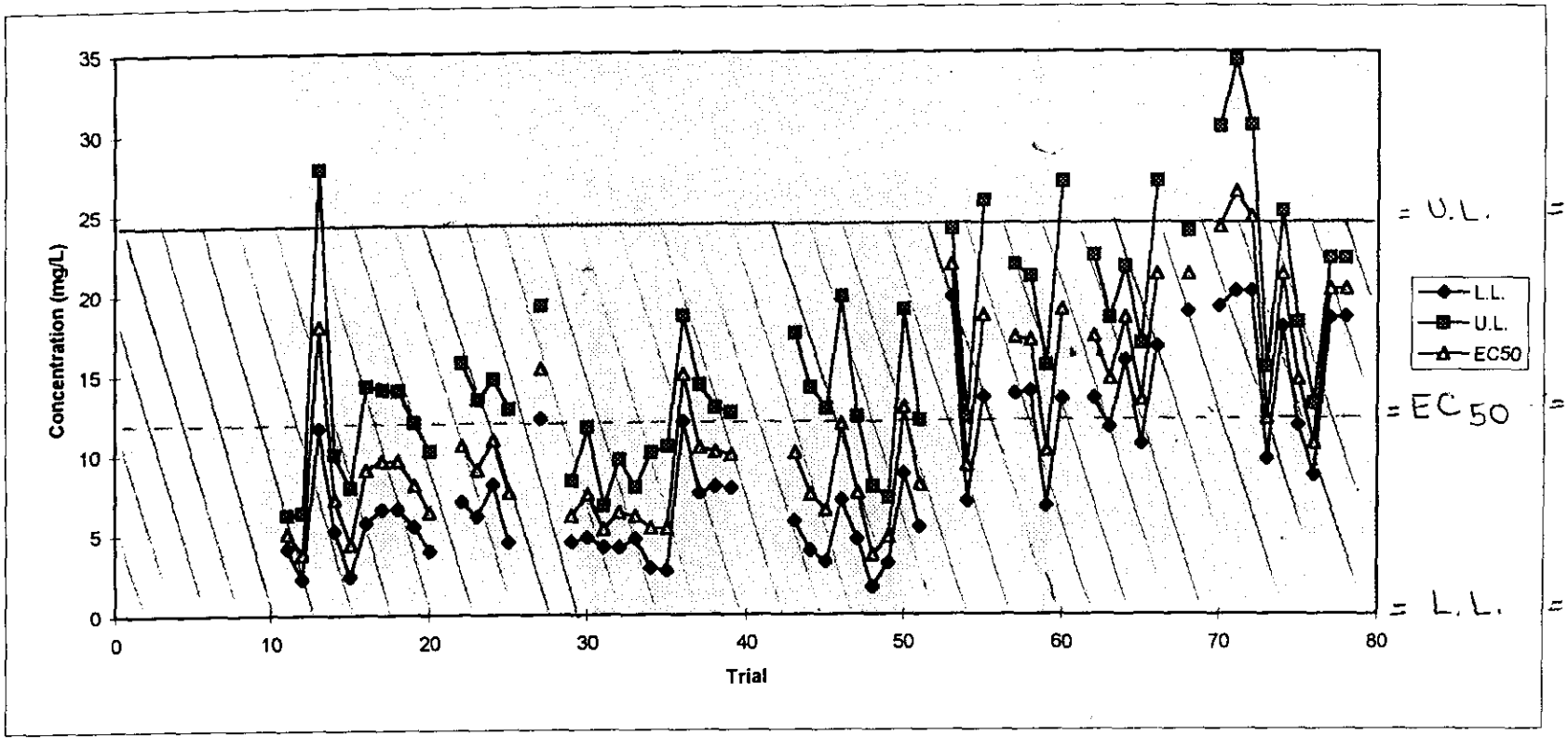
EC50= Mean = X-bar= 12.08 U.L.= 24.27859914
Trials = n= 57
Standard Deviation = SD= 6.098 2(SD)= 12.196
Coefficient of Variation = CV%= 50.47 L.L.= -0.113686857

Dates	Tri	Trimmed-Spearman L.L.	U.L.	EC50	NOEC-G	LOEC-G	NOEC-L	LOEC-L	IC25-G	IC50-G	IC25-L	IC50-L	ChV-G	ChV-L	EC50 -Probit
3/6/97	63	11.63	18.47	14.66	7.5	15	3.75	7.5	13.12	19.77	5.45	8.02	10.61	5.3	n/a
3/27/97	64	15.83	21.67	18.52	15	30	7.5	15	15.94	20.63	9.9	12.3	21.21	10.61	n/a
4/30/97	65	10.58	16.88	13.36	7.5	15	7.5	15	10.5	16.25	6.19	9.51	10.61	10.61	14.53(10.59-18.29)
6/5/97	66	16.69	26.96	21.21	7.5	15	7.5	15	14.49	22.5	9.63	12.32	10.61	10.61	n/a
6/26/97	67						7.5	15			9.59	13.4		10.61	n/a
7/16/97	68	18.86	23.85	21.21	15	30	7.5	15	17.39	22.5	7.58	11.02	21.21	10.61	n/a
8/1/97	69				15	30	7.5	15	14.46		8.95	11.65	21.21	10.61	n/a
9/12/97	70	19.22	30.37	24.16	15	30	7.5	15	19.22	25.94	6.53	10.39	21.21	10.61	26.1(16.1-39.9)
10/3/97	71	20.13	34.47	26.34	15	30	7.5	15	20.86	27.66	10.64	13.79	21.21	10.61	36.5
11/12/97	72	20.11	30.45	24.75	15	30	7.5	15	20.21	26.25	6.66	10.47	21.21	10.61	25.7(19.5-44.0)
12/10/97	73	9.65	15.39	12.19	7.5	15	3.75	7.5	10.43	13.83	2.99	5.72	10.61	5.3	11.8
1/7/98	74	17.92	25.11	21.21	15	30	15	30	17.92	23.61	3.61	12.39	21.21	21.21	23.7(8.3-30.5)
1/28/98	75	11.74	18.17	14.61	7.5	15	7.5	15	10.45	14.46	8.79	11.94	10.61	10.61	13.9
2/19/98	76	8.61	13.07	10.61	7.5	15	7.5	15	9.09	11.81	4.23	6.78	10.61	10.61	11.4(8.7-14.1)
4/2/98	77	18.48	22.2	20.26	15	30	7.5	15	18.56	22.37	7.07	12.76	21.21	10.61	n/a
4/22/98	78	18.53	22.15	20.26	15	30	7.5	15	17.95	21.96	6.39	11.16	21.21	10.61	n/a

ROOT ELONGATION CONTROL CHART

EC50=	Mean =	X-bar=	12.08		U.L.=	24.27859914
	Trials =	n=	57			
	Standard Deviation =	SD=	6.098	2(SD)= 12.196		
	Coefficient of Variation =	CV%=	50.47		L.L.=	-0.113686857

Dates	Tri	Trimmed-Spearman L.L.	U.L.	EC50	NOEC-G	LOEC-G	NOEC-L	LOEC-L	IC25-G	IC50-G	IC25-L	IC50-L	ChV-G	ChV-L	EC50 -Probit



Trials	Date	Water-Holding Capacity
A	3/5/98	-1 (Not Used)
B	3/5/98	30
C	3/11/98	28
D	3/11/98	24
E	3/11/98	30
F	3/11/98	29
G	3/11/98	30

Average =	28.5	Upper limit = Average plus 2(std dev)	33.19042
Standard deviation =	2.3452	Lower limit = Average minus 2(std dev)	23.80958
2(std dev)=	4.6904		

Artificial Soil WHC

Trials	Date	Water-Holding-Capacity
A	4/24/98	48.4
B	4/24/98	50.1
C	4/24/98	51.3
D	4/24/98	49.6
E	4/24/98	45.6

Average =	49	Upper limit = Average plus 2(std dev)	53.33
Standard deviation =	2.17		
2(std dev)=	4.33	Lower limit = Average minus 2(std dev)	44.67

Date: 03/05-06/98

Trial	pH of fine-grain Silica sand				
1	8.92	Average =	7.688	Upper limit =	9.584682
2	8.55			Average plus 2(std dev)	
3	7.31	Standard		Lower limit =	5.791318
4	7.32	deviation	0.948341	Average minus 2(std dev)	
5	9.54				
6	6.94	2(std dev)	1.896682		
7	7.16				
8	7.19				
9	6.87				
10	7.08				

pH Range = 5.8 - 9.6

SUMMARY OF TEST CONDITIONS FOR LETTUCE SEED (LACTUCA SATIVA) REFERENCE TOXICANT GERMINATION TEST.

1.	Test type:	Static
2.	Temperature (°C):	25 ± 2 °C
3.	Light quality:	Fluorescent
4.	Light intensity:	400 ± 40 ft-c
5.	Photoperiod:	Initial 48 hrs in total darkness followed by 72 hrs on a 16 hrs light:8 hrs dark diurnal cycle.
6.	Test vessel type and size:	Bottom halves of plastic petri dishes, 100-mm wide by 15-mm high, placed in gallon size GladLock® resealable bags.
7.	Test soil mass:	100 g silica sand.
8.	Test soil moisture:	85% of the Water-Holding-Capacity (WHC) of F95 silica sand. WHC = 28.2 mls/100 g dry sand 85% WHC = 24.0 mls/100 g dry sand
9.	Artificial soil:	F95 Natural grain silica sand
10.	Test soil pH:	Not applicable.
11.	Renewal of test materials:	None.
12.	Age of test organisms:	Seeds.
13.	Number of test organisms per chamber:	25
14.	Number of replicates per dilution:	3
15.	Positive Control:	2-Chloroacetamide
16.	Dilution factor:	0.5 for multiconcentration test.
17.	Test concentrations:	5.2 mls of 5000 mg/L 2-Chloroacetamide stock solution is dissolved in 162.8 mls of Milli-Q water to produce 168.0 mls of a 30 mg/L test concentration. This concentration is then serially diluted to generate 84 mls each of the following concentrations: 15 mg/L, 7.5 mg/L, 3.75 mg/L, and 1.88 mg/L.
18.	Test duration:	120 hours.
19.	Effect measured:	Germination
20.	Test acceptability:	Must have 80% (60 out of 75) germination of negative controls.

SEED GERMINATION TEST

Site Soil: Ref # 33
Sample Number:

Analyst: A. C. Harvey
Test Start Date: 04-22 thru 04-25-98
Start Time: 10:00 am

Site Soil (SS) pH:
Water-Holding-Capacity (WHC) of Dry Site Soil (SS):
Moisture content of Site Soil (SS):

Artificial Soil (AS) pH:
Water-Holding-Capacity (WHC) of Dry Artificial Soil (AS):
Moisture content of Artificial Soil (AS):

Hydration of Test Soil (TS):
Positive Controls: #1 #2 #3

25 No. seeds/Repl. 3 No. Repl./Conc. 75 No. seeds/Conc.

Concentrations	Rep 1	Rep 2	Rep 3	Total Germ.	% Germ.	# not Germ.	% Mort.
Positive Control #1:							
Positive Control #2:							
Positive Control #3:							
Negative Control:	17 .68	25 1.0	23 .92	65	87%	10	13%
Test Conc: 6.25% 1.88 mg/L	19 .76	21 .84	24 .96	64	85%	11	15%
Test Conc: 12.5% 3.75 mg/L	23 .92	23 .92	9 .36	55	73%	20	27%
Test Conc: 25% 7.5 mg/L	20 .80	22 .88	12 .48	54	72%	21	28%
Test Conc: 50% 15 mg/L	18 .72	9 .36	19 .76	46	61%	29	39%
Test Conc: 100% 30 mg/L	1 .04	0	4 .16	5	7%	70	93%

Comments:

Mesh size 18 seeds

ESAT-5-050.1

SEED GERMINATION CHEMICAL AND PHYSICAL CHARACTERIZATION DATA SHEET

Dataset: Ref # 33 Test dates: 4/22 thru 4/27/98

Site: Analyst: A.C. Harvey

Pos C #1: Pos C #2: Pos C #3:

Conc.	Pos C #1	Pos C #2	Pos C #3	Neg C	6.25% 1.88 mg/L	12.5% 3.75 mg/L	25% 7.5 mg/L	50% 15 mg/L	100% 30 mg/L
pH-Initial				5.0	5.0	5.0	5.0	5.0	5.0
pH-Final				5.3	5.3	5.3	5.3	5.3	5.3
Temp/ 0-hr				25°C	25°C	25°C	25°C	25°C	25°C
Temp/ 24-hr				↓	↓	↓	↓	↓	↓
Temp/ 48-hr				↓	↓	↓	↓	↓	↓
Temp/ 72-hr				↓	↓	↓	↓	↓	↓
Temp/ 96-hr				↓	↓	↓	↓	↓	↓
Temp/ 120-hr				26.5°C	27°C	27°C	26.5°C	26°C	27°C

Ecological Parameters (La Motte Soil Kit):

		Diluent	Sample
Available Phosphorous:			
Available Nitrogen:			
Available Potassium:	# drops		
pH:			
Texture: (Clay/Silt/Sand)			

pH readings taken with colorpHast pH 4.0 - 7.0

TRIMMED SPEARMAN-KARBER METHOD. MONTANA STATE UNIV

FOR REFERENCE, CITE:
HAMILTON, M.A., R.C. RUSSO, AND R.V. THURSTON, 1977.
TRIMMED SPEARMAN-KARBER METHOD FOR ESTIMATING MEDIAN
LETHAL CONCENTRATIONS IN TOXICITY BIOASSAYS.
ENVIRON. SCI. TECHNOL. 11(7): 714-719;
CORRECTION 12(4):417 (1978).

DATE: 4-22-98 TEST NUMBER: SEED#33 DURATION: 5 DAYS
CHEMICAL: 2-CHLOROACETAMIDE SPECIES: LACTUCA SAT

RAW DATA:

CONCENTRATION(MG/L)	1.88	3.75	7.50	15.00	30.00
NUMBER EXPOSED:	75	75	75	75	75
MORTALITIES:	11	20	21	29	70

SPEARMAN-KARBER TRIM: 14.67%

SPEARMAN-KARBER ESTIMATES: EC50: 12.65
95% LOWER CONFIDENCE: 10.51
95% UPPER CONFIDENCE: 15.22

EPA PROBIT ANALYSIS PROGRAM
USED FOR CALCULATING EC VALUES
Version 1.4

SEED GERMINATION

Conc.	Number Exposed	Number Resp.	Observed Proportion Responding	Adjusted Proportion Responding	Predicted Proportion Responding
Control	75	10	0.1333	0.0000	0.2054
1.8800	75	11	0.1467	-.0739	0.0000
3.7500	75	20	0.2667	0.0771	0.0000
7.5000	75	21	0.2800	0.0939	0.0024
15.0000	75	29	0.3867	0.2281	0.2347
30.0000	75	70	0.9333	0.9161	0.9144

Chi - Square Heterogeneity = 5.730

Mu = 1.280216
Sigma = 0.143906

Parameter	Estimate	Std. Err.	95% Confidence Limits	
Intercept	-3.896204	1.483947	(-6.804740,	-0.987667)
Slope	6.948987	1.112877	(4.767749,	9.130224)
Spontaneous esponse Rate	0.205406	0.023566	(0.159217,	0.251595)

SEED GERMINATION

Estimated EC Values and Confidence Limits

Point	Conc.	Lower 95% Confidence Limits	Upper 95% Confidence Limits
EC 1.00	8.8196	5.7395	11.1500
EC 5.00	11.0536	7.9246	13.3268
EC10.00	12.4677	9.3948	14.6826
EC15.00	13.5230	10.5261	15.6932
EC50.00	19.0641	16.6144	21.3002
EC85.00	26.8757	23.9386	31.6707
EC90.00	29.1505	25.7451	35.2653
EC95.00	32.8796	28.5340	41.5594
EC99.00	41.2083	34.3022	57.0507

SEED GERMINATION
File: SEED33 Transform: ARC SINE(SQUARE ROOT(Y))

Shapiro Wilks test for normality

D = 0.717

W = 0.896

Critical W (P = 0.05) (n = 18) = 0.897
Critical W (P = 0.01) (n = 18) = 0.858

Data PASS normality test at P=0.01 level. Continue analysis.

SEED GERMINATION
File: SEED33 Transform: ARC SINE(SQUARE ROOT(Y))

Bartletts test for homogeneity of variance

Calculated B statistic = 1.75
Table Chi-square value = 15.09 (alpha = 0.01)
Table Chi-square value = 11.07 (alpha = 0.05)

Average df used in calculation ==> df (avg n - 1) = 2.00
Used for Chi-square table value ==> df (#groups-1) = 5

Data PASS homogeneity test at 0.01 level. Continue analysis.

NOTE: If groups have unequal replicate sizes the average replicate size is used to calculate the B statistic (see above).

SEED GERMINATION
File: SEED33 Transform: ARC SINE(SQUARE ROOT(Y))

SUMMARY STATISTICS ON TRANSFORMED DATA TABLE 1 of 2

GRP	IDENTIFICATION	N	MIN	MAX	MEAN
1	CONTROL	3	0.970	1.471	1.241
2	1.88 MG/L	3	1.059	1.369	1.196
3	3.75 MG/L	3	0.644	1.284	1.071
4	7.5 MG/L	3	0.765	1.217	1.030
5	15 MG/L	3	0.644	1.059	0.905
6	30 MG/L	3	0.100	0.412	0.238

SEED GERMINATION
File: SEED33 Transform: ARC SINE(SQUARE ROOT(Y))

SUMMARY STATISTICS ON TRANSFORMED DATA TABLE 2 of 2

GRP	IDENTIFICATION	VARIANCE	SD	SEM
1	CONTROL	0.064	0.253	0.146
2	1.88 MG/L	0.025	0.159	0.092
3	3.75 MG/L	0.137	0.370	0.214
4	7.5 MG/L	0.055	0.236	0.136
5	15 MG/L	0.052	0.228	0.131
6	30 MG/L	0.025	0.159	0.092

SEED GERMINATION
File: SEED33 Transform: ARC SINE(SQUARE ROOT(Y))

ANOVA TABLE

SOURCE	DF	SS	MS	F
Between	5	2.027	0.405	6.782
Within (Error)	12	0.717	0.060	
Total	17	2.744		

Critical F value = 3.11 (0.05,5,12)
Since F > Critical F REJECT Ho:All groups equal

SEED GERMINATION
File: SEED33 Transform: ARC SINE(SQUARE ROOT(Y))

DUNNETTS TEST - TABLE 1 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	T STAT	SIG
1	CONTROL	1.241	0.867		

2	1.88 MG/L	1.196	0.853	0.228	
3	3.75 MG/L	1.071	0.733	0.856	
4	7.5 MG/L	1.030	0.720	1.060	
5	15 MG/L	0.905	0.613	1.684	
6	30 MG/L	0.238	0.067	5.028	*

Dunnett table value = 2.50 (1 Tailed Value, P=0.05, df=12,5)

SEED GERMINATION
File: SEED33 Transform: ARC SINE(SQUARE ROOT(Y))

DUNNETTS TEST - TABLE 2 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	NUM OF REPS	Minimum Sig Diff (IN ORIG. UNITS)	% of CONTROL	DIFFERENCE FROM CONTROL
1	CONTROL	3			
2	1.88 MG/L	3	0.438	50.6	0.013
3	3.75 MG/L	3	0.438	50.6	0.133
4	7.5 MG/L	3	0.438	50.6	0.147
5	15 MG/L	3	0.438	50.6	0.253
6	30 MG/L	3	0.438	50.6	0.800

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	1.88	3.75	7.5	15	30
Response 1	17	19	23	20	18	1
Response 2	25	21	23	22	90	0
Response 3	23	24	9	12	19	4

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: 2-CHLOROACETAMIDE
Test Start Date: 04-22-98 Test Ending Date: 04-27-98
Test Species: LACTUCA SATIVA
Test Duration: 5 DAYS
DATA FILE:

Conc. ID	Number Replicates	Concentration MG/L	Response Means	Std. Dev.	Pooled Response Means
1	3	0.000	21.667	4.163	24.333
2	3	1.880	21.333	2.517	24.333
3	3	3.750	18.333	8.083	24.333
4	3	7.500	18.000	5.292	24.333
5	3	15.000	42.333	41.284	24.333
6	3	30.000	1.667	2.082	1.667

The Linear Interpolation Estimate: 19.0257 Entered P Value: 25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 17.1430 Standard Deviation: 3.9154
Original Confidence Limits: Lower: 4.6875 Upper: 19.2773
Expanded Confidence Limits: Lower: -11.0846 Upper: 19.5541
Resampling time in Seconds: 0.00 Random_Seed: 632970167

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	1.88	3.75	7.5	15	30
Response 1	17	19	23	20	18	1
Response 2	25	21	23	22	90	0
Response 3	23	24	9	12	19	4

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: 2-CHLOROACETAMIDE
Test Start Date: 04-22-98 Test Ending Date: 04-27-98
Test Species: LACTUCA SATIVA
Test Duration: 5 DAYS
DATA FILE:

Conc. ID	Number Replicates	Concentration MG/L	Response Means	Std. Dev.	Pooled Response Means
1	3	0.000	21.667	4.163	24.333
2	3	1.880	21.333	2.517	24.333
3	3	3.750	18.333	8.083	24.333
4	3	7.500	18.000	5.292	24.333
5	3	15.000	42.333	41.284	24.333
6	3	30.000	1.667	2.082	1.667

The Linear Interpolation Estimate: 23.0515 Entered P Value: 50

Number of Resamplings: 80
The Bootstrap Estimates Mean: 22.6330 Standard Deviation: 0.8895
Original Confidence Limits: Lower: 20.5172 Upper: 23.7064
Expanded Confidence Limits: Lower: 17.7296 Upper: 24.4269
Resampling time in Seconds: 0.00 Random Seed: 207997655

SUMMARY OF TEST CONDITIONS FOR LETTUCE SEED (LACTUCA SATIVA) REFERENCE TOXICANT ROOT ELONGATION TEST.

1.	Test type:	Static
2.	Test duration:	120 hours.
3.	Temperature (°C):	25 ± 2 °C
4.	Light quality:	Dark
5.	Light intensity:	Dark
6.	Photoperiod:	Dark
7.	Test vessel type and size:	Plastic petri dishes, 100-mm wide by 15-mm high.
8.	Test solution volume:	4 mls
9.	Test solution pH:	≥ 5.0 but ≤ 10.0
10.	Dilution water:	20% DMW
11.	Renewal of test materials:	None
12.	Age of test organisms:	Seeds from a single lot not more than two years old.
13.	Number of test organisms per chamber:	5
14.	Number of replicates per dilution:	3
15.	Positive Control:	2-Chloroacetamide
16.	Dilution factor:	0.5 for multiconcentration test.
17.	Test concentrations:	5.0 mls of 5000 mg/L 2-Chloroacetamide stock solution is dissolved in 120.4 mls of Milli-Q water to produce 125.4 mls of a 30 mg/L test concentration. All dilutions are mixed in the same 50-ml volumetric flask, beginning with the lowest concentration first. 3.125% (0.03125 x 50.0 = 1.56 ml 30mg/L added to flask then brought up 50 mls volume with 20% DMW) 6.25% = 3.12 mls; 12.5% = 6.25 mls; 25% = 12.5 mls; and 50% = 25.0 mls.
18.	Effect measured:	Root Elongation
19.	Test acceptability:	Must have 80% (12 out of 15) germination of negative controls.

ROOT ELONGATION BIOASSAY DATA SHEET

Dataset: Ref #78
Site:
Sample Collection Date/Time:

Analyst: A. C. Harvey
Start-date/time: 04-22-98 / 1:30 pm
Stop-date/time: 04-27-98 / 1:30 - 2:30 pm
Sample Matrix:

EC50: 95% Confidence Limits:

Conc.		#1	#2	#3	#4	#5	ave. length	# germ	% germ	
Pos. C	1									
	2									
	3									
Neg. C	1	6	6	7	7	6	6.4	5	100%	100%
	2	9	5	6	4	4	5.6	5	100%	
	3	23	8	5	6	5	9.4	5	100%	
3.125% 0.94 mg/L	1	6	14	8	7	7	8.4	5	100%	100%
	2	4	7	5	6	5	5.4	5	100%	
	3	6	3	5	4	3	4.2	5	100%	
6.25% 1.88 mg/L	1	6	5	4	11	10	7.2	5	100%	100%
	2	4	8	5	5	6	5.6	5	100%	
	3	5	4	7	6	5	5.4	5	100%	
12.5% 3.75 mg/L	1	6	9	6	3	4	5.6	5	100%	100%
	2	11	7	5	4	6	6.6	5	100%	
	3	11	7	5	6	7	7.2	5	100%	
25% 7.5 mg/L	1	4	3	8	5	4	4.8	5	100%	100
	2	4	9	4	9	5	6.2	5	100%	
	3	3	3	5	5	4	4.0	5	100%	
50% 15 mg/L	1	3	1	2	3	4	2.6	5	100%	93
	2	3	2	3	4	1	2.6	5	100%	
	3	1	2	1	1	Ø	1.0	4	80%	
100% 30 mg/L	1	Ø	Ø	Ø	Ø	Ø	0.0	Ø	0%	
	2	Ø	Ø	Ø	Ø	Ø	0.0	Ø	0%	
	3	Ø	Ø	Ø	Ø	Ø	0.0	Ø	0%	

Size ~~15~~ 30 seeds

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ROOT ELONGATION CHEMICAL AND PHYSICAL DETERMINATION DATA SHEET

Dataset: Ref #78 Test dates: 4/22 thru 4/27/98
Site: Analyst: A.C. Harvey
Sample Collection Date/Time:
Sample Type: Soil/Water/Other (Circle One)

MOISTURE CONTENT CALCULATION:
Weight of empty aluminum boat = _____ (g) A

Initial weight of boat + wet soil = _____ (g) B

Grams Wet Weight utilized = _____ (g) B-A=C
Date/Time In: _____ Drying Temperature (°C): _____
Date/Time Out: _____ Drying Time (min. 24 hrs): _____

Final Weight of boat + dried soil = _____ (g) D

Weight of water lost = _____ (g) B-D=E

Moisture content = _____ % (E/C) x 100

ELUTION PREPARATION:
Elution Volume: _____ Rotation Start Date/Time: _____
(4 parts water:1 part soil) Rotation Stop Date/Time: _____
Settling Time: _____

Centrifuge Date: _____ Speed/Time: _____
Speed/Time: _____

Concentration / Parameters		Pos. Control	Neg. Control	3.125% 0.94 mg/L	6.25% 1.88 mg/L	12.5% 3.75 mg/L	25% 7.5 mg/L	50% 15 mg/L	100% 30 mg/L
Temperature			25°C	25°C	25°C	25°C	25°C	25°C	25°C
pH	Init.		5.79	5.81	5.92	5.96	5.98	5.95	5.96
	Final		8.82	8.53	8.49	8.26	8.40	7.74	8.30
Alkalinity			93						
Hardness			94						
Conductivity			215	172	174	151	151	156	166
Chlorine									

Comments:

Seeds sage 30 mesh

4.7
+20
94.0

7.3
+10
93

ESAT-5-053.1

TRIMMED SPEARMAN-KARBER METHOD. MONTANA STATE UNIV

FOR REFERENCE, CITE:
HAMILTON, M.A., R.C. RUSSO, AND R.V. THURSTON, 1977.
TRIMMED SPEARMAN-KARBER METHOD FOR ESTIMATING MEDIAN
LETHAL CONCENTRATIONS IN TOXICITY BIOASSAYS.
ENVIRON. SCI. TECHNOL. 11(7): 714-719;
CORRECTION 12(4):417 (1978).

DATE: 04-22-98 TEST NUMBER: ROOT#78 DURATION: 5 DAYS
CHEMICAL: 2-CHLOROACETAMIDE SPECIES: LACTUCA SAT

RAW DATA:

CONCENTRATION(MG/L)	.94	1.88	3.75	7.50	15.00	30.00
NUMBER EXPOSED:	15	15	15	15	15	15
MORTALITIES:	0	0	0	0	1	15

SPEARMAN-KARBER TRIM: .00%

SPEARMAN-KARBER ESTIMATES: EC50: 20.26
95% LOWER CONFIDENCE: 18.53
95% UPPER CONFIDENCE: 22.15

ROOT RLONGATION
File: ROOT78G Transform: ARC SINE(SQUARE ROOT(Y))

Shapiro Wilks test for normality

D = 0.038

W = 0.488

Critical W (P = 0.05) (n = 21) = 0.908
Critical W (P = 0.01) (n = 21) = 0.873

Data FAIL normality test. Try another transformation.

Warning - The two homogeneity tests are sensitive to non-normal data and should not be performed.

ROOT RLONGATION
File: ROOT78G Transform: ARC SINE(SQUARE ROOT(Y))

Hartley test for homogeneity of variance
Bartletts test for homogeneity of variance

These two tests can not be performed because at least one group has zero variance.

Data FAIL to meet homogeneity of variance assumption.
Additional transformations are useless.

ROOT RLONGATION
File: ROOT78G Transform: NO TRANSFORMATION

SUMMARY STATISTICS ON TRANSFORMED DATA TABLE 1 of 2

GRP	IDENTIFICATION	N	MIN	MAX	MEAN
1	CONTROL	3	1.000	1.000	1.000
2	0.94 MG/L	3	1.000	1.000	1.000
3	1.88 MG/L	3	1.000	1.000	1.000
4	3.75 MG/L	3	1.000	1.000	1.000
5	7.5 MG/L	3	1.000	1.000	1.000
6	15 MG/L	3	0.800	1.000	0.933
7	30 MG/L	3	0.000	0.000	0.000

ROOT RLONGATION
File: ROOT78G Transform: NO TRANSFORMATION

SUMMARY STATISTICS ON TRANSFORMED DATA TABLE 2 of 2

GRP	IDENTIFICATION	VARIANCE	SD	SEM
1	CONTROL	0.000	0.000	0.000
2	0.94 MG/L	0.000	0.000	0.000
3	1.88 MG/L	0.000	0.000	0.000
4	3.75 MG/L	0.000	0.000	0.000
5	7.5 MG/L	0.000	0.000	0.000
6	15 MG/L	0.013	0.115	0.067
7	30 MG/L	0.000	0.000	0.000

ROOT RLONGATION
File: ROOT78G Transform: NO TRANSFORMATION

ANOVA TABLE

SOURCE	DF	SS	MS	F
Between	6	2.526	0.421	221.000
Within (Error)	14	0.027	0.002	
Total	20	2.552		

Critical F value = 2.85 (0.05,6,14)
Since F > Critical F REJECT Ho:All groups equal

ROOT RLONGATION
File: ROOT78G Transform: NO TRANSFORMATION

DUNNETTS TEST - TABLE 1 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	T STAT	SIG

1	CONTROL	1.000	1.000		
2	0.94 MG/L	1.000	1.000	0.000	
3	1.88 MG/L	1.000	1.000	0.000	
4	3.75 MG/L	1.000	1.000	0.000	
5	7.5 MG/L	1.000	1.000	0.000	
6	15 MG/L	0.933	0.933	1.871	
7	30 MG/L	0.000	0.000	28.062	*

Dunnett table value = 2.53 (1 Tailed Value, P=0.05, df=14,6)

ROOT RLONGATION
File: ROOT78G Transform: NO TRANSFORMATION

DUNNETTS TEST - TABLE 2 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	NUM OF REPS	Minimum Sig Diff (IN ORIG. UNITS)	% of CONTROL	DIFFERENCE FROM CONTROL
1	CONTROL	3			
2	0.94 MG/L	3	0.090	9.0	0.000
3	1.88 MG/L	3	0.090	9.0	0.000
4	3.75 MG/L	3	0.090	9.0	0.000
5	7.5 MG/L	3	0.090	9.0	0.000
6	15 MG/L	3	0.090	9.0	0.067
7	30 MG/L	3	0.090	9.0	1.000

ROOT ELONGATION
File: ROOT78L Transform: NO TRANSFORMATION

Shapiro Wilks test for normality

D = 24.827

W = 0.956

Critical W (P = 0.05) (n = 21) = 0.908
Critical W (P = 0.01) (n = 21) = 0.873

Data PASS normality test at P=0.01 level. Continue analysis.

ROOT ELONGATION
File: ROOT78L Transform: NO TRANSFORMATION

Hartley test for homogeneity of variance
Bartletts test for homogeneity of variance

These two tests can not be performed because at least one group has zero variance.

Data FAIL to meet homogeneity of variance assumption.
Additional transformations are useless.

ROOT ELONGATION
File: ROOT78L Transform: NO TRANSFORMATION

SUMMARY STATISTICS ON TRANSFORMED DATA TABLE 1 of 2

GRP	IDENTIFICATION	N	MIN	MAX	MEAN
1	CONTROL	3	5.600	9.400	7.133
2	0.94 MG/L	3	4.200	8.400	6.000
3	1.88 MG/L	3	5.400	7.200	6.067
4	3.75 MG/L	3	5.600	7.200	6.467
5	7.5 MG/L	3	4.000	6.200	5.000
6	15 MG/L	3	1.000	2.600	2.067
7	30 MG/L	3	0.000	0.000	0.000

ROOT ELONGATION
File: ROOT78L Transform: NO TRANSFORMATION

SUMMARY STATISTICS ON TRANSFORMED DATA TABLE 2 of 2

GRP	IDENTIFICATION	VARIANCE	SD	SEM
1	CONTROL	4.013	2.003	1.157
2	0.94 MG/L	4.680	2.163	1.249
3	1.88 MG/L	0.973	0.987	0.570
4	3.75 MG/L	0.653	0.808	0.467
5	7.5 MG/L	1.240	1.114	0.643
6	15 MG/L	0.853	0.924	0.533
7	30 MG/L	0.000	0.000	0.000

ROOT ELONGATION
File: ROOT78L Transform: NO TRANSFORMATION

ANOVA TABLE

SOURCE	DF	SS	MS	F
Between	6	125:131	20.855	11.760
Within (Error)	14	24.827	1.773	
Total	20	149.958		

Critical F value = 2.85 (0.05,6,14)
Since F > Critical F REJECT Ho:All groups equal

ROOT ELONGATION
File: ROOT78L Transform: NO TRANSFORMATION

DUNNETTS TEST - TABLE 1 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	T STAT	SIG

1	CONTROL	7.133	7.133		
2	0.94 MG/L	6.000	6.000	1.042	
3	1.88 MG/L	6.067	6.067	0.981	
4	3.75 MG/L	6.467	6.467	0.613	
5	7.5 MG/L	5.000	5.000	1.962	
6	15 MG/L	2.067	2.067	4.660	*
7	30 MG/L	0.000	0.000	6.561	*

Dunnett table value = 2.53 (1 Tailed Value, P=0.05, df=14,6)

ROOT ELONGATION
File: ROOT78L Transform: NO TRANSFORMATION

DUNNETTS TEST - TABLE 2 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	NUM OF REPS	Minimum Sig Diff (IN ORIG. UNITS)	% of CONTROL	DIFFERENCE FROM CONTROL
1	CONTROL	3			
2	0.94 MG/L	3	2.751	38.6	1.133
3	1.88 MG/L	3	2.751	38.6	1.067
4	3.75 MG/L	3	2.751	38.6	0.667
5	7.5 MG/L	3	2.751	38.6	2.133
6	15 MG/L	3	2.751	38.6	5.067
7	30 MG/L	3	2.751	38.6	7.133

Conc. ID	1	2	3	4	5	6	7
Conc. Tested	0	0.94	1.88	3.75	7.5	15	30
Response 1	5	5	5	5	5	5	0
Response 2	5	5	5	5	5	5	0
Response 3	5	5	5	5	5	4	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: 2-CHLOROACETAMIDE
Test Start Date: 04-22-98 Test Ending Date: 04-27-98
Test Species: LACTUCA SATIVA
Test Duration: 5 DAYS
DATA FILE: 78G50.icp

Conc. ID	Number Replicates	Concentration MG/L	Response Means	Std. Dev.	Pooled Response Means
1	3	0.000	5.000	0.000	5.000
2	3	0.940	5.000	0.000	5.000
3	3	1.880	5.000	0.000	5.000
4	3	3.750	5.000	0.000	5.000
5	3	7.500	5.000	0.000	5.000
6	3	15.000	4.667	0.577	4.667
7	3	30.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 17.9464 Entered P Value: 25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 17.9248 Standard Deviation: 0.8339
Original Confidence Limits: Lower: 15.9375 Upper: 18.7500
Expanded Confidence Limits: Lower: 13.7277 Upper: 19.6339
Resampling time in Seconds: 0.05 Random_Seed: 1426974970

Conc. ID	1	2	3	4	5	6	7
Conc. Tested	0	0.94	1.88	3.75	7.5	15	30
Response 1	5	5	5	5	5	5	0
Response 2	5	5	5	5	5	5	0
Response 3	5	5	5	5	5	4	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: 2-CHLOROACETAMIDE
Test Start Date: 04-22-98 Test Ending Date: 04-27-98
Test Species: LACTUCA SATIVA
Test Duration: 5 DAYS
DATA FILE: 78G50.icp

Conc. ID	Number Replicates	Concentration MG/L	Response Means	Std. Dev.	Pooled Response Means
1	3	0.000	5.000	0.000	5.000
2	3	0.940	5.000	0.000	5.000
3	3	1.880	5.000	0.000	5.000
4	3	3.750	5.000	0.000	5.000
5	3	7.500	5.000	0.000	5.000
6	3	15.000	4.667	0.577	4.667
7	3	30.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 21.9643 Entered P Value: 50

Number of Resamplings: 80
The Bootstrap Estimates Mean: 21.9545 Standard Deviation: 0.4501
Original Confidence Limits: Lower: 21.3462 Upper: 22.5000
Expanded Confidence Limits: Lower: 20.6662 Upper: 23.0893

Conc. ID		1	2	3	4	5	6	7
Conc. Tested		0	0.94	1.88	3.75	7.5	15	30
Response	1	6.4	4.2	7.2	7.2	4.8	2.6	0
Response	2	5.6	5.4	5.6	6.6	6.2	2.6	0
Response	3	9.4	8.4	5.4	5.6	4.0	1.0	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: 2-CHLOROACETAMIDE
Test Start Date: 04-22-98 Test Ending Date: 04-27-98
Test Species: LACTUCA SATIVA
Test Duration: 5 DAYS
DATA FILE: 78L50.icp

Conc. ID	Number Replicates	Concentration MG/L	Response Means	Std. Dev.	Pooled Response Means
1	3	0.000	7.133	2.003	7.133
2	3	0.940	6.000	2.163	6.178
3	3	1.880	6.067	0.987	6.178
4	3	3.750	6.467	0.808	6.178
5	3	7.500	5.000	1.114	5.000
6	3	15.000	2.067	0.924	2.067
7	3	30.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 6.3856 Entered P Value: 25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 5.5234 Standard Deviation: 2.8375
Original Confidence Limits: Lower: 0.7281 Upper: 9.4940
Expanded Confidence Limits: Lower: -5.4951 Upper: 12.9133
Resampling time in Seconds: 0.05 Random_Seed: 234671290

Conc. ID		1	2	3	4	5	6	7
Conc. Tested		0	0.94	1.88	3.75	7.5	15	30
Response	1	6.4	4.2	7.2	7.2	4.8	2.6	0
Response	2	5.6	5.4	5.6	6.6	6.2	2.6	0
Response	3	9.4	8.4	5.4	5.6	4.0	1.0	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: 2-CHLOROACETAMIDE
Test Start Date: 04-22-98 Test Ending Date: 04-27-98
Test Species: LACTUCA SATIVA
Test Duration: 5 DAYS
DATA FILE: 78L50.icp

Conc. ID	Number Replicates	Concentration MG/L	Response Means	Std. Dev.	Pooled Response Means
1	3	0.000	7.133	2.003	7.133
2	3	0.940	6.000	2.163	6.178
3	3	1.880	6.067	0.987	6.178
4	3	3.750	6.467	0.808	6.178
5	3	7.500	5.000	1.114	5.000
6	3	15.000	2.067	0.924	2.067
7	3	30.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 11.1648 Entered P Value: 50

Number of Resamplings: 80
The Bootstrap Estimates Mean: 10.6703 Standard Deviation: 1.6606
Original Confidence Limits: Lower: 7.1147 Upper: 13.4302
Expanded Confidence Limits: Lower: 2.6597 Upper: 15.9222
Resampling time in Seconds: 0.00 Random Seed: -1698872910

SUMMARY OF TEST CONDITIONS FOR EARTHWORM (EISENIA FOETIDA) REFERENCE TOXICANT SURVIVAL TEST

1	Test type:	Static
2	Soil Temperature (°C):	18 ± 2°C
3	Light quality	Ambient laboratory light
4	Light intensity:	540 - 1080 lux
5	Photoperiod:	Near continuous illumination
6	Test vessel type and size:	1-pint glass canning jars with bands and lids. 1/16 inch air hole in lids
7	Test soil mass/volume:	200 g artificial soil
8	Renewal of test materials:	None
9	Age of test organisms:	≥ 60 days, 300 mg ≥ weight ≤ 600 mg, clitellate worms
10	Number of test organisms per chamber:	10
11	Number of replicates per concentration:	3 minimum
12	Number of test organisms per concentration:	30 minimum
13	Feeding/watering regime:	Do not feed
14	Test soil pH:	≥ 5.0 but ≤ 9.0
15	Test soil moisture content:	50% Water-Holding-Capacity WHC artificial soil = 49 ml/100 g; (50% WHC) (660 g) = 161.7 mls
16	Artificial soil/dilution media:	10% 2.36-mm screened sphagnum peat (66 g) 20% colloidal kaolinite clay (132 g) 70% F95 silica sand (462 g) 0.42% calcium carbonate (2.77 g)*
17	Test concentrations:	Minimum of 5 and a negative control 13.1 mls of 5000 mg/L 2-Chloroacetamide stock solution mixed with 148.6 mls Milli-Q water to produce 161.7 mls of a 80 mg/L concentration. Total test weight (soil + water); 660 g +161.78 mls = 821.7 g = 0.8217 kg 80 mg/kg X 0.8217 kg = 65.736 mg/5000 mg/L = 0.0131L = 13.1 mls 40 mg/kg; 6.6 mls stock + 129.4 mls water 20 mg/kg; 3.3 mls stock + 132.6 mls water 10 mg/kg; 1.6 mls stock + 134.2 mls water 5 mg/kg; 0.8 mls stock + 135 mls water
18	Dilution factor:	multiples of 5
19	Test duration:	14 days
20	Endpoint/Effect measured:	Death
21	Test acceptability:	90% of negative controls
22	Positive control:	2-Chloroacetamide

EARTHWORM SURVIVAL DATA

Site Soil: Reference toxicant

Analyst: A. C. Harvey

Sample Number:

Test Dates: 4/29 Thru 5/13

Repl. No.	Conc.	Init. Temp.	Init. pH	Biomass per Repl.	7 days Mort	7 days %E	Cumulative 14 days Mort	Cumulative 14 days %E	Final pH
1	80 mg/L	20°C	~~8.0~~ 7.5	4.06 g	10	100%	10	100%	7.5
2				4.61 g	10		10		
3				4.18 g	10		10		
1	40 mg/L	20°C	7.5	4.62 g	1	3%	~~2~~ 1	13%	7.5
2				4.32 g	Ø		2		
3				4.10 g	Ø		Ø		
1	20 mg/L	20°C	7.5	4.09 g	Ø	0%	1	23%	7.5
2				4.83 g	Ø		5		
3				4.41 g	Ø		1		
1	10 mg/L	20°C	7.5	4.37 g	Ø	0%	Ø	0%	7.5
2				4.05 g	Ø		Ø		
3				4.71 g	Ø		Ø		
1	5 mg/L	20°C	7.5	4.18 g	Ø	5%	~~8~~ 2	7%	7.5
2				4.47 g	Ø		~~10~~ Ø		
3				4.59 g	Ø		~~10~~ Ø		
1	Pos. C								
2									
3									
1	Neg. C	20°C	8.0	4.16 g	Ø	0%	~~10~~ Ø	3%	7.5
2				4.69 g	Ø		~~10~~ Ø		
3				4.23 g	Ø		~~7~~ 1		

Mort = Mortality
%E = Effective concentration

pH taken w/ Colorphast pH 7.5-14
Laboratory reared population

ESAT-5-046.1

EARTHWORM BIOASSAY OBSERVATION SHEETS

Site Soil: Reference toxicant Analyst: A. C. Harvey

Sample Number: Date: 4/29 thru 5/13

Observation Time: 24-hr Day 7 Day 14 Time: 10:00 am - 11:00 am

Conc.	Rep No.	Observations
80 mg/L	1	2 lying on surface 10 alive & alert 'elongated'
	2	5 lying on surface 10 alive & alert 'elongated'
	3	10 alive & alert, burrowing
40 mg/L	1	10 alive & alert, burrowing
	2	10 alive & alert, burrowing
	3	10 alive & alert, burrowing
20 mg/L	1	10 alive & alert, burrowing
	2	10 alive & alert, burrowing
	3	10 alive & alert, burrowing
10 mg/L	1	10 alive & alert, burrowing
	2	10 alive & alert, burrowing
	3	10 alive & alert, burrowing
5 mg/L	1	10 alive & alert, burrowing
	2	10 alive & alert, burrowing
	3	10 alive & alert, burrowing
Pos C	1	
	2	
	3	
Neg C	1	10 alive & alert, burrowing
	2	10 alive & alert, burrowing
	3	10 alive & alert, burrowing

alert = avoiding overhead light

ESAT-5-107.0

EARTHWORM BIOASSAY OBSERVATION SHEETS

Site Soil: Reference toxicant Analyst: A. C. Harvey

Sample Number: Date: 4/29 thru 5/13

Observation Time: 24-hr (Day 7) Day 14 Time: 10:00 am - 11:30 am

Conc.	Rep No.	Observations
80 mg/L	1	dead and liquefied on surface, all dead
	2	dead and liquefied, on surface
	3	dead and liquefied on surface
40 mg/L	1	1 dead on surface 4-mm moving segment 9 alive
	2	1 on surface ~~10 alive~~ #2 #3 10 alive, burrowing
	3	11 alive segment w/ constriction
20 mg/L	1	coiled, 10 alive, burrowing 1 elongated
	2	4 elongated resting on surface 10 alive
	3	coiled, 10 alive, 1 elongated
10 mg/L	1	11 alive, brown, burrowing
	2	10 alive, brown, burrowing
	3	10 alive, brown, burrowing
5 mg/L	1	10 alive, brown, burrowing
	2	10 alive, brown, burrowing
	3	10 alive, brown, burrowing
Pos C	1	
	2	
	3	
Neg C	1	10 alive, brown, burrowing
	2	10 alive, brown, burrowing
	3	10 alive, 1 - swelling/constriction - greyish hue

EARTHWORM BIOASSAY OBSERVATION SHEETS

Site Soil: Reference toxicant Analyst: A. C. Harvey

Sample Number: Date: 4/29 thru 5/13

Observation Time: 24-hr Day 7 Day 14 Time: 10:00am - 12:15 pm

Conc.	Rep No.	Observations						
	1							
	2							
	3							
40 mg/L	1	9	alive	biomass	3.48	lt maroon	2.5 cm fragment worm	
	2	8	alive	biomass	2.40	maroon	1 alive on surface	
	3	10	alive	biomass	3.43	maroon	3 cm fragment worm	
20 mg/L	1	9	alive	biomass	2.90	maroon	1 dead on surface	'burrowing'
	2	5	alive	biomass	1.96	maroon	fungus on surface	'burrowing'
	3	9	alive	biomass	2.92	elongated	1 alive, shrunk on surface	
10 mg/L	1	11	alive	biomass	3.64	lt maroon	1 alive curled on surface	no visible clitellum 25 cm worm
	2	10	alive	biomass	3.53	maroon	2 alive curled surface	'burrowing'
	3	10	alive	biomass	3.79	maroon	1 alive constricted	
5 mg/L	1	8	alive	biomass	2.91	maroon		'burrowing'
	2	10	alive	biomass =	4.24	maroon		'burrowing'
	3	10	alive	biomass =	3.81	maroon		'burrowing'
Pos C	1							
	2							
	3							
Neg C	1	10	alive	biomass	3.83g	healthy	maroon	'burrowing'
	2	10	alive	biomass	4.37g	healthy	maroon	'burrowing'
	3	9	alive	biomass	3.71g	healthy	maroon	'burrowing'

Need to set length standard to ~~ECO~~ distinguish worm fragments from whole worms.
increase moisture content

ESAT-5-107.0

TRIMMED SPEARMAN-KARBER METHOD. MONTANA STATE UNIV

FOR REFERENCE, CITE:
HAMILTON, M.A., R.C. RUSSO, AND R.V. THURSTON, 1977.
TRIMMED SPEARMAN-KARBER METHOD FOR ESTIMATING MEDIAN
LETHAL CONCENTRATIONS IN TOXICITY BIOASSAYS.
ENVIRON. SCI. TECHNOL. 11(7): 714-719;
CORRECTION 12(4):417 (1978).

DATE: 04-29-98 TEST NUMBER: WORMREF DURATION: 7 DAYS
CHEMICAL: 2-CHLOROACETAMIDE SPECIES: REDWORM

RAW DATA:

CONCENTRATION(MG/L)	5.00	10.00	20.00	40.00	80.00
NUMBER EXPOSED:	30	30	30	30	30
MORTALITIES:	0	0	0	1	30
SPEARMAN-KARBER TRIM:		.00%			

SPEARMAN-KARBER ESTIMATES: EC50: 55.28
95% LOWER CONFIDENCE: 52.82
95% UPPER CONFIDENCE: 57.85

DATE: 04-29-98 TEST NUMBER: WORMREF DURATION: 14 DAYS
CHEMICAL: 2-CHLOROACETAMIDE SPECIES: REDWORM

RAW DATA:

CONCENTRATION(MG/L)	5.00	10.00	20.00	40.00	80.00
NUMBER EXPOSED:	30	30	30	30	30
MORTALITIES:	2	0	7	4	30
SPEARMAN-KARBER TRIM:		3.33%			

SPEARMAN-KARBER ESTIMATES: EC50: 44.74
95% LOWER CONFIDENCE: 38.49
95% UPPER CONFIDENCE: 51.99

NOTE: MORTALITY PROPORTIONS WERE NOT MONOTONICALLY INCREASING.
ADJUSTMENTS WERE MADE PRIOR TO SPEARMAN-KARBER ESTIMATION.

EPA PROBIT ANALYSIS PROGRAM
USED FOR CALCULATING EC VALUES
Version 1.4

EARTHWORM SURVIVAL

Conc.	Number Exposed	Number Resp.	Observed Proportion Responding	Adjusted Proportion Responding	Predicted Proportion Responding
5.0000	30	2	0.0667	0.0667	0.0062

10.0000	30	0	0.0000	0.0000	0.0454
20.0000	30	7	0.2333	0.2333	0.1895
40.0000	30	4	0.1333	0.1333	0.4727
80.0000	30	30	1.0000	1.0000	0.7711

Chi - Square Heterogeneity = 42.428

```
*************************************************************
*                        WARNING                            *
*                                                           *
*   Significant heterogeneity exists.  The results reported *
*   for this data set may not be valid.  The results should *
*   be interpreted with appropriate caution.                *
*************************************************************
```

```
*************************************************************
*                         NOTE                              *
*                                                           *
*     Slope not significantly different from zero.          *
*     EC fiducial limits cannot be computed.                *
*************************************************************
```

Mu = 1.627525
Sigma = 0.371165

Parameter	Estimate	Std. Err.	95% Confidence Limits	
Intercept	0.615088	2.419615	(-7.084127,	8.314303)
Slope	2.694221	1.559613	(-2.268469,	7.656910)

Theoretical Spontaneous Response Rate = 0.0000

EARTHWORM SURVIVAL

Estimated EC Values and Confidence Limits

Point	Conc.	Lower 95% Confidence	Upper Limits
EC 1.00	5.8088		
EC 5.00	10.3991		
EC10.00	14.1854		
EC15.00	17.4924		
EC50.00	42.4155		
EC85.00	102.8490		
EC90.00	126.8264		
EC95.00	173.0032		
EC99.00	309.7176		

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	5	10	20	40	80
Response 1	10	10	10	10	9	0
Response 2	10	10	10	10	10	0
ponse 3	10	10	10	10	10	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: 2-CHLOROACETAMIDE
Test Start Date: 04-29-98 Test Ending Date: 05-06-98
Test Species: REDWORM
Test Duration: 7 DAYS
DATA FILE: WRM725.icp

Conc. ID	Number Replicates	Concentration MG/L	Response Means	Std. Dev.	Pooled Response Means
1	3	0.000	10.000	0.000	10.000
2	3	5.000	10.000	0.000	10.000
3	3	10.000	10.000	0.000	10.000
4	3	20.000	10.000	0.000	10.000
5	3	40.000	9.667	0.577	9.667
6	3	80.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 48.9655 Entered P Value: 25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 48.9534 Standard Deviation: 0.8976
Original Confidence Limits: Lower: 47.8571 Upper: 50.0000
Expanded Confidence Limits: Lower: 46.6379 Upper: 51.1379
Resampling time in Seconds: 0.22 Random_Seed: -1191039840

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	5	10	20	40	80
Response 1	10	10	10	10	9	0
Response 2	10	10	10	10	10	0
Response 3	10	10	10	10	10	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: 2-CHLOROACETAMIDE
Test Start Date: 04-29-98 Test Ending Date: 05-06-98
Test Species: REDWORM
Test Duration: 7 DAYS
DATA FILE: WRM750.icp

Conc. ID	Number Replicates	Concentration MG/L	Response Means	Std. Dev.	Pooled Response Means
1	3	0.000	10.000	0.000	10.000
2	3	5.000	10.000	0.000	10.000
3	3	10.000	10.000	0.000	10.000
4	3	20.000	10.000	0.000	10.000
5	3	40.000	9.667	0.577	9.667
6	3	80.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 59.3103 Entered P Value: 50

Number of Resamplings: 80
The Bootstrap Estimates Mean: 59.4648 Standard Deviation: 0.5359
Original Confidence Limits: Lower: 58.5714 Upper: 60.0000
Expanded Confidence Limits: Lower: 57.7586 Upper: 60.7586
esampling time in Seconds: 0.28 Random_Seed: -407853504

Conc. ID	1	2	3	4	5	6

Conc. Tested	0	5	10	20	40	80
Response 1	10	8	10	9	8	0
Response 2	10	10	10	5	8	0
Response 3	9	10	10	9	10	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: 2-CHLOROACETAMIDE
Test Start Date: 04-29-98 Test Ending Date: 05-06-98
Test Species: REDWORM
Test Duration: 14 DAYS
DATA FILE: WRM1425.icp

Conc. ID	Number Replicates	Concentration MG/L	Response Means	Std. Dev.	Pooled Response Means
1	3	0.000	9.667	0.577	9.667
2	3	5.000	9.333	1.155	9.667
3	3	10.000	10.000	0.000	9.667
4	3	20.000	7.667	2.309	8.167
5	3	40.000	8.667	1.155	8.167
6	3	80.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 44.4898 Entered P Value: 25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 41.2631 Standard Deviation: 8.6513
Original Confidence Limits: Lower: 18.5294 Upper: 48.1416
Expanded Confidence Limits: Lower: -10.0270 Upper: 52.1586
Resampling time in Seconds: 0.22 Random_Seed: -1713051296

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	5	10	20	40	80
Response 1	10	8	10	9	8	0
Response 2	10	10	10	5	8	0
Response 3	9	10	10	9	10	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: 2-CHLOROACETAMIDE
Test Start Date: 04-29-98 Test Ending Date: 05-06-98
Test Species: REDWORM
Test Duration: 14 DAYS
DATA FILE: WRM1450.icp

Conc. ID	Number Replicates	Concentration MG/L	Response Means	Std. Dev.	Pooled Response Means
1	3	0.000	9.667	0.577	9.667
2	3	5.000	9.333	1.155	9.667
3	3	10.000	10.000	0.000	9.667
4	3	20.000	7.667	2.309	8.167
5	3	40.000	8.667	1.155	8.167
6	3	80.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 56.3265 Entered P Value: 50

Number of Resamplings: 80
The Bootstrap Estimates Mean: 56.0154 Standard Deviation: 1.7427
Original Confidence Limits: Lower: 53.0233 Upper: 58.6667
Expanded Confidence Limits: Lower: 49.3897 Upper: 61.2408
Resampling time in Seconds: 0.27 Random_Seed: -1876549696

EARTHWORM SURVIVAL
File: WORM7 Transform: ARC SINE(SQUARE ROOT(Y))

Chi-square test for normality: actual and expected frequencies

.TERVAL	<-1.5	-1.5 to <-0.5	-0.5 to 0.5	>0.5 to 1.5	>1.5
EXPECTED	1.206	4.356	6.876	4.356	1.206
OBSERVED	0	1	15	2	0

Calculated Chi-Square goodness of fit test statistic = 15.8704
Table Chi-Square value (alpha = 0.01) = 13.277

Data FAIL normality test. Try another transformation.

Warning - The two homogeneity tests are sensitive to non-normal data and should not be performed.

EARTHWORM SURVIVAL
File: WORM7 Transform: ARC SINE(SQUARE ROOT(Y))

Shapiro Wilks test for normality

D = 0.018

W = 0.535

Critical W (P = 0.05) (n = 18) = 0.897
:itical W (P = 0.01) (n = 18) = 0.858

Data FAIL normality test. Try another transformation.

Warning - The two homogeneity tests are sensitive to non-normal data and should not be performed.

EARTHWORM SURVIVAL
File: WORM7 Transform: ARC SINE(SQUARE ROOT(Y))

Hartley test for homogeneity of variance
Bartletts test for homogeneity of variance

These two tests can not be performed because at least one group has zero variance.

Data FAIL to meet homogeneity of variance assumption.
Additional transformations are useless.

TITLE: EARTHWORM SURVIVAL
FILE: WORM7
TRANSFORM: ARC SINE(SQUARE ROOT(Y)) NUMBER OF GROUPS: 6

GRP	IDENTIFICATION	REP	VALUE	TRANS VALUE
1	CONTROL	1	1.0000	1.4120
1	CONTROL	2	1.0000	1.4120
1	CONTROL	3	1.0000	1.4120
2	5 MG/L	1	1.0000	1.4120
2	5 MG/L	2	1.0000	1.4120
2	5 MG/L	3	1.0000	1.4120
3	10 MG/L	1	1.0000	1.4120
3	10 MG/L	2	1.0000	1.4120
3	10 MG/L	3	1.0000	1.4120
4	20 MG/L	1	1.0000	1.4120
4	20 MG/L	2	1.0000	1.4120
4	20 MG/L	3	1.0000	1.4120
5	40 MG/L	1	0.9000	1.2490
5	40 MG/L	2	1.0000	1.4120
5	40 MG/L	3	1.0000	1.4120
6	80 MG/L	1	0.0000	0.1588
6	80 MG/L	2	0.0000	0.1588
6	80 MG/L	3	0.0000	0.1588

EARTHWORM SURVIVAL
File: WORM7 Transform: ARC SINE(SQUARE ROOT(Y))

SUMMARY STATISTICS ON TRANSFORMED DATA TABLE 1 of 2

GRP	IDENTIFICATION	N	MIN	MAX	MEAN
1	CONTROL	3	1.412	1.412	1.412
2	5 MG/L	3	1.412	1.412	1.412
3	10 MG/L	3	1.412	1.412	1.412
4	20 MG/L	3	1.412	1.412	1.412
5	40 MG/L	3	1.249	1.412	1.358
6	80 MG/L	3	0.159	0.159	0.159

EARTHWORM SURVIVAL
File: WORM7 Transform: ARC SINE(SQUARE ROOT(Y))

SUMMARY STATISTICS ON TRANSFORMED DATA TABLE 2 of 2

GRP	IDENTIFICATION	VARIANCE	SD	SEM
1	CONTROL	0.000	0.000	0.000
2	5 MG/L	0.000	0.000	0.000
3	10 MG/L	0.000	0.000	0.000
4	20 MG/L	0.000	0.000	0.000
5	40 MG/L	0.009	0.094	0.054
6	80 MG/L	0.000	0.000	0.000

EARTHWORM SURVIVAL
File: WORM7 Transform: ARC SINE(SQUARE ROOT(Y))

ANOVA TABLE

SOURCE	DF	SS	MS	F
Between	5	3.866	0.773	523.971
Within (Error)	12	0.018	0.001	
Total	17	3.884		

Critical F value = 3.11 (0.05,5,12)
Since F > Critical F REJECT Ho:All groups equal

EARTHWORM SURVIVAL
File: WORM7 Transform: ARC SINE(SQUARE ROOT(Y))

DUNNETTS TEST - TABLE 1 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	T STAT	SIG
1	CONTROL	1.412	1.000		
2	5 MG/L	1.412	1.000	0.000	
3	10 MG/L	1.412	1.000	0.000	
4	20 MG/L	1.412	1.000	0.000	
5	40 MG/L	1.358	0.967	1.732	
6	80 MG/L	0.159	0.000	39.957	*

Dunnett table value = 2.50 (1 Tailed Value, P=0.05, df=12,5)

EARTHWORM SURVIVAL
File: WORM7 Transform: ARC SINE(SQUARE ROOT(Y))

DUNNETTS TEST - TABLE 2 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	NUM OF REPS	Minimum Sig Diff (IN ORIG. UNITS)	% of CONTROL	DIFFERENCE FROM CONTROL
1	CONTROL	3			
2	5 MG/L	3	0.030	3.0	0.000
3	10 MG/L	3	0.030	3.0	0.000
4	20 MG/L	3	0.030	3.0	0.000
5	40 MG/L	3	0.030	3.0	0.033
6	80 MG/L	3	0.030	3.0	1.000

ıRTHWORM SURVIVAL
.le WORM14 Transform: ARC SINE(SQUARE ROOT(Y))

ıi-square test for normality: actual and expected frequencies

ITERVAL	<-1.5	-1.5 to <-0.5	-0.5 to 0.5	>0.5 to 1.5	>1.5
:PECTED	1.206	4.356	6.876	4.356	1.206
;SERVED	0	5	6	7	0

ılculated Chi-Square goodness of fit test statistic = 4.2237
ıble Chi-Square value (alpha = 0.01) = 13.277

ıta PASS normality test. Continue analysis.

ıRTHWORM SURVIVAL
ıle: WORM14 Transform: ARC SINE(SQUARE ROOT(Y))

ıapiro Wilks test for normality

= 0.285

= 0.943

cit al W (P = 0.05) (n = 18) = 0.897
ritical W (P = 0.01) (n = 18) = 0.858

ata PASS normality test at P=0.01 level. Continue analysis.

ARTHWORM SURVIVAL
ile: WORM14 Transform: ARC SINE(SQUARE ROOT(Y))

artley test for homogeneity of variance
artletts test for homogeneity of variance

hese two tests can not be performed because at least one group has
ero variance.

ata FAIL to meet homogeneity of variance assumption.
dditional transformations are useless.

TITLE: EARTHWORM SURVIVAL
FILE: WORM14
TRANSFORM: ARC SINE(SQUARE ROOT(Y)) NUMBER OF GROUPS: 6

GRP	IDENTIFICATION	REP	VALUE	TRANS VALUE
1	CONTROL	1	1.0000	1.4120
1	CONTROL	2	1.0000	1.4120
1	CONTROL	3	0.9000	1.2490
2	5 MG/L	1	1.0000	1.4120
2	5 MG/L	2	1.0000	1.4120
2	5 MG/L	3	0.8000	1.1071
3	10 MG/L	1	1.0000	1.4120
3	10 MG/L	2	1.0000	1.4120
3	10 MG/L	3	1.0000	1.4120
4	20 MG/L	1	0.9000	1.2490
4	20 MG/L	2	0.5000	0.7854
4	20 MG/L	3	0.9000	1.2490
5	40 MG/L	1	0.8000	1.1071
5	40 MG/L	2	0.8000	1.1071
5	40 MG/L	3	1.0000	1.4120
6	80 MG/L	1	0.0000	0.1588
6	80 MG/L	2	0.0000	0.1588
6	80 MG/L	3	0.0000	0.1588

EARTHWORM SURVIVAL
File: WORM14 Transform: ARC SINE(SQUARE ROOT(Y))

SUMMARY STATISTICS ON TRANSFORMED DATA TABLE 1 of 2

GRP	IDENTIFICATION	N	MIN	MAX	MEAN
1	CONTROL	3	1.249	1.412	1.358
2	5 MG/L	3	1.107	1.412	1.310
3	10 MG/L	3	1.412	1.412	1.412
4	20 MG/L	3	0.785	1.249	1.094
5	40 MG/L	3	1.107	1.412	1.209
6	80 MG/L	3	0.159	0.159	0.159

EARTHWORM SURVIVAL
File: WORM14 Transform: ARC SINE(SQUARE ROOT(Y))

SUMMARY STATISTICS ON TRANSFORMED DATA TABLE 2 of 2

GRP	IDENTIFICATION	VARIANCE	SD	SEM
1	CONTROL	0.009	0.094	0.054
2	5 MG/L	0.031	0.176	0.102
3	10 MG/L	0.000	0.000	0.000
4	20 MG/L	0.072	0.268	0.155
5	40 MG/L	0.031	0.176	0.102
6	80 MG/L	0.000	0.000	0.000

EARTHWORM SURVIVAL
File: WORM14 Transform: ARC SINE(SQUARE ROOT(Y))

ANOVA TABLE

SOURCE	DF	SS	MS	F
Between	5	3.316	0.663	27.927
Within (Error)	12	0.285	0.024	
Total	17	3.601		

Critical F value = 3.11 (0.05,5,12)
Since F > Critical F REJECT Ho:All groups equal

EARTHWORM SURVIVAL
File: WORM14 Transform: ARC SINE(SQUARE ROOT(Y))

DUNNETTS TEST - TABLE 1 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	T STAT	SIG
1	CONTROL	1.358	0.967		
2	5 MG/L	1.310	0.933	0.376	
3	10 MG/L	1.412	1.000	-0.432	
4	20 MG/L	1.094	0.767	2.092	
5	40 MG/L	1.209	0.867	1.184	
6	80 MG/L	0.159	0.000	9.529	*

Dunnett table value = 2.50 (1 Tailed Value, P=0.05, df=12,5)

EARTHWORM SURVIVAL
File: WORM14 Transform: ARC SINE(SQUARE ROOT(Y))

DUNNETTS TEST - TABLE 2 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	NUM OF REPS	Minimum Sig Diff (IN ORIG. UNITS)	% of CONTROL	DIFFERENCE FROM CONTROL
1	CONTROL	3			
2	5 MG/L	3	0.209	21.6	0.033
3	10 MG/L	3	0.209	21.6	-0.033
4	20 MG/L	3	0.209	21.6	0.200
5	40 MG/L	3	0.209	21.6	0.100
6	80 MG/L	3	0.209	21.6	0.967

FEED LOG

BATCH ID: _____

Alfalfa wt: _____ g Water volume: _____ mls

Fermentation Start Date: _____

Total Start Volume: _____ mls

Date	Volume Used (mls)	Volume Remaining (mls)	Culture Tray ID:

EARTHWORM SURVIVAL CONTROL CHART

EC50=	Mean =	X-bar=	55.28	U.L.=
	Trials =	n=	1	#DIV/0!
	Standard Deviation =	SD=	#DIV/0!	L.L.=
	Coefficient of Variation =	CV%=	#DIV/0!	#DIV/0!

Trimmed-Spearman 7-Day

Dates	Trial	L.L.	U.L.	EC50	NOEC	LOEC	IC25	IC50	Probit
4/29/98	A	52.82	57.85	55.28	40	80	48.96	59.31	

EARTHWORM SURVIVAL CONTROL CHART

EC50=	Mean =	X-bar=	44.74	U.L.=
	Trials =	n=	1	#DIV/0!
	Standard Deviation =	SD=	#DIV/0!	L.L.=
	Coefficient of Variation =	CV%=	#DIV/0!	#DIV/0!

Trimmed-Spearman 14-Day

Dates	Trial	L.L.	U.L.	EC50	NOEC	LOEC	IC25	IC50	Probit
4/29/98	A	38.49	51.99	44.74	40	80	44.49	56.33	42.42

ESAT REGION V STANDARD OPERATING PROCEDURES FOR BIOLOGICAL SUPPORT ACTIVITIES

TITLE:

STANDARD OPERATING PROCEDURE FOR ACUTE SOLID WASTE TOXICITY TESTING, USING THE COMMON EARTHWORM EISENIA FOETIDA

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SOP FOR ACUTE SOLID WASTE TOXICITY TESTING USING THE COMMON EARTHWORM, EISENIA FOETIDA

1. SCOPE AND APPLICATION

This method has been modified from the methods described by U.S. EPA Region IV, Corvallis Environmental Research Laboratory. It estimates the acute toxicity of solid hazardous wastes to the earthworm *Eisenia foetida* in a 14-day static test. The responses measured include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components that adversely affect the physiological and biochemical functions of the organisms. The method could also be used to test aqueous samples by using 100% artificial soil and by using the aqueous samples in place of distilled or deionized water to hydrate the test soils. This method should be performed under the supervision of professionals experienced in environmental toxicity testing.

Detection limits of the toxicity of a hazardous waste solution or a pure substance are organism-dependent.

2. PURPOSE

To estimate the potential biological impact that may result from exposure to any and all contaminants associated with soils and sediments.

3. SAFETY AND WASTE HANDLING

3.1 General Precautions

Collection and use of solid samples in toxicity tests may involve significant risks to personal safety and health. Personnel collecting solid samples and conducting toxicity tests should take all safety precautions necessary for the prevention of bodily injury and illness which might result from ingestion or invasion of infectious agents, inhalation or absorption of corrosive or toxic substances through skin contact, and asphyxiation due to lack of oxygen or presence of noxious gases.

Prior to sample collection and laboratory work, personnel should determine that all necessary safety equipment and materials have been obtained and are in good working condition.

3.2 Safety Equipment

Personnel should use safety equipment, as required, such as rubber aprons, laboratory coats, respirators, gloves, and safety glasses.

3.3 General Laboratory Operations

Most soil, sediment, and water samples collected from hazardous waste sites have little chemical or biological background information. Therefore, these samples are considered hazardous to the health of persons handling them until demonstrated otherwise. Safety protocol should be based on two worst case assumptions:

3.3.1 Incoming samples have unpredictable, extremely high, acute toxicity. Inadvertent or overt exposure to these samples may cause immediate short- or long-term adverse health effects or even, in rare instances, death.

3.3.2 Incoming samples have unpredictable, extremely mutagenic, teratogenic or carcinogenic properties. Inadvertent or overt exposure to these samples may cause short- or long-term adverse health effects.

3.3.3 These assumptions require that extreme care be exercised in the collection, shipping, and receiving of hazardous samples. The following statements should be applied when working with hazardous waste site samples for toxicity tests:

3.3.3.1 Work should be performed in compliance with accepted rules pertaining to the handling of hazardous materials. It is recommended that personnel carrying out toxicity tests not work alone in the laboratory.

3.3.3.2 Because the chemical composition and the concentration of the constituents in waste samples is usually only poorly known, every sample should be considered as a potential health hazard, and exposure should be minimized by using appropriate chemical resistant laboratory gloves and protective attire.

3.3.3.3 It is advisable to cleanse exposed parts of the body with soap and water immediately after contact with test samples.

3.3.3.4 Further guidance on safe practices for handling test samples can be obtained from "Prudent Practices for Handling Hazardous Chemicals in Laboratories" by the National Research Council.

4. SUMMARY OF METHOD

Earthworms (*Eisenia foetida*) are exposed, in an environmental chamber set at 20 ± 2 °C, to various concentrations (in triplicate) of site soil (SS) mixed with artificial soil (AS) or site specific dilution soil (SSDS) for a period of 14 days. Positive and negative controls are also used for each test. Sediments are not evaluated with this test. Mortality, behavioral and pathological observations are checked at seven and fourteen days. Each replicate consists of 200 g (dry weight) of test soil (TS) with 10 worms. The test soil (TS) is hydrated with Milli-Q® water to create a moist environment for the worms. If the pH of the site soil is outside of the range of 5.00 - 9.00, adjust within range before testing.

5. SAMPLE HANDLING AND PRESERVATION

Soil and sediment samples from hazardous waste sites are heterogeneous mixtures of natural chemicals in the substrate matrix (e.g., clays and silts and sands in varying proportions) (Bohn et al. 1979; Brady 1974), along with anthropogenic chemicals that may be present as contaminants (Morrill et al. 1982).

5.1 At each sample location, soil from the top three inches will be homogenized *in situ* using a stainless steel trowel. Large rocks and organic debris will be removed by shifting the soil through a ¼" soil sieve. Enough soil will be collected to fill a 3.5-gallon High Density Polyethylene-HDPE plastic pail (or the sample container recommended for the amount of sample required to conduct the bioassays and analyses for the specific waste site). Samples collected which are not submitted for analysis will remain on-site. Residual sample wastes from toxicity tests and other analyses will be returned to the site.

5.2 Line the HDPE plastic pail with two 4-mm plastic bags and fill the inner bag with sample material to a level about

three inches from the top of the pail. Twist and seal the inner bag with PVC (electrician's) tape. Each sample is identified with a sample tag, i.e. a sequentially numbered and accountable document that identifies where, when, how and who collected the sample. The sample tag is placed inside the second bag, which is then twisted and sealed with PVC tape. All collected samples are recorded directly into a bound logbook and on the Chain-of-Custody (COC) record along with other identifying information while the samples are still in the custody of the sampling team.

5.3 Secure the lid on the pail, insert the sealed samples upright into an ice chest and fit frozen cold packs amongst the samples. An equal volume of cold packs to sample volume is optimal to keep the samples cool. Fill any empty space in the ice chest with an absorbent (vermiculite, floor-dry, etc.). The absorbent acts both as insulation and to stabilize the sample containers. Copies of the Chain-of-Custody records for all samples in each chest should be placed in a waterproof document protector (such as a Glad-Lock® bag) and enclosed in the ice chest with the samples. Close and latch the lid, then wrap duct tape around the ice chest to secure the lid.

Aeration during collection and transfer of solid wastes should be minimized to reduce the loss of volatile chemicals. The time elapsed from collection of a sample to the initiation of the toxicity tests should not exceed 72 hours. Sample toxicity may be effected when held for longer than 72 hours. Samples must be chilled after collection and maintained at 4 °C until used for testing, unless toxicity tests are initiated within 24 hours of sample collection (Greene, et al. 1988).

6. APPARATUS

6.1 Instrumentation

6.1.1 Environmental chamber capable of maintaining a uniform temperature of 20 ± 2 °C and 4300 ± 430 lux of light operating on a clock timer to control diurnal cycling.

6.1.2 Water purification system -- Millipore Milli-Q® or equivalent.

6.1.3 Balance, analytical, Sartorius® Research -- capable of accurately weighing earthworms to 0.0001 g.

6.1.4 Balance, top-loading, Sartorius® Basic -- capable of weighing soil samples to 1000.0 g.

6.1.5 Reference weights, Class S -- for checking performance of balance. Weights should bracket the expected weights of the weighing pans and the expected weights of the pans plus worms or soil.

6.1.6 Bulb-thermograph, electronic-chart type thermometers or temperature chart recorder, or Temperature Indicator strips -- for continuous recording of temperature.

6.1.7 Drying oven.

6.1.8 pH meter -- Orion Research® digital pH/millivolt meter or equivalent.

6.1.9 Motorized Pipet-Aid® or equivalent.

6.2 Glassware/Plasticware

6.2.1 Sample containers -- High Density Polyethylene (HDPE) plastic pails with covers. Consolidated Plastics Company, Part #33400AJ (1.0 Gal), #33404AJ (3.5 Gal) or equivalent.

6.2.2 10" x 11", 4-ml polyethylene or equivalent resealable bags (e.g., Glad-Lock®, Yellow and Blue Make Green® Seal, gallon-size freezer bags).

6.2.3 Test chambers -- standard 1-pint Kerr® mason jars with bands and lids, or equivalent.

6.2.4 Volumetric flasks and graduated cylinders - Class A, borosilicate glass or non-toxic plastic labware, 10- to 1000-ml.

6.2.5 Volumetric pipets -- Class A, 10- to 100-ml.

6.2.6 Serological pipets -- 1- to 10-ml and 1000-µl, graduated.

6.2.7 National Bureau of Standards certified thermometer.

6.2.8 Earthworm sorting/counting tray.

6.2.9 Desiccator.

6.2.10 100-mm (top inside diameter) glass funnel.

6.2.11 Glass stirring rod.

6.2.12 Plastic Trash bags, 1' x 2', VWR Scientific #11215-392, or equivalent.

6.2.13 100 to 500-ml glass beakers.

6.3 Other Supplies

6.3.1 Disposable rubber gloves with approved protection factor (NIOSH).

6.3.2 Sieve -- 2.36-mm (#8 equivalent).

6.3.3 ¼" soil sieve.

6.3.4 185-mm diameter VWR 617 Filter paper.

6.3.5 Disposable petri dishes.

6.3.6 Indelible ink marking pens.

6.3.7 PVC (electrician's) tape.

6.3.8 Stainless steel trowel.

6.3.9 Mortar and pestle.

6.3.10 Pipet bulbs and fillers -- Propipet® or equivalent.

6.3.11 Aluminum foil.

6.3.12 Aluminum weigh boats.

6.3.13 Magnetic stir plate with magnetic stir bars.

6.3.14 Magnetic stir bar retriever.

7. REAGENTS AND CONSUMABLES

7.1 Reagent water -- defined as activated carbon-filtered, distilled or deionized water that does not contain substances toxic to the test organisms. A water purification system may be used to generate reagent water.

7.2 Solid hazardous waste sample -- approximately 1500 g dry weight.

7.3 Artificial Soil (AS) consisting, by weight, of:

7.3.1 10% sphagnum peat (carbonized vegetable tissue), passed though a 2.36-mm screen; (The peat moss used in this test is baled peat that can be purchased at garden supply centers. The following types have been used by other EPA laboratories: Rose, Lakeland and Sunshine brands of Canadian sphagnum peat.)

7.3.2 20% colloidal kaolinite clay (aluminum silicate used in ceramics and refractories), less than 40 μ; (The clay currently used is produced by US Silica.)

7.3.3 70% silica sand, 53 - 300 μ particle size; (The sand is also a US Silica product and is described as grade F95 silica sand.)

7.3.4 0.42% calcium carbonate, agricultural lime. (The calcium carbonate can be Lawn and Garden Agricultural Lime or any chemical purity grade with a calcium carbonate content of not less than 93%. CAUTION: Do not use slaked or hydrated lime. Use the kind that can be mixed with feed, or that is used to line athletic fields.)

7.4 pH buffers 4.00 ± 0.01 @ 25 °C, 7.00 ± 0.01 @ 25 °C, and 10.00 ± 0.02 @ 25 °C (or as per instructions of instrument manufacturer) for standards and calibration check.

7.5 Reference toxicant (99% or greater purity 2-chloroacetamide) -- positive control.

7.6 Test organisms -- earthworms (Eisenia foetida). The species taxonomy should be verified using appropriate systematic keys. Adult Eisenia foetida used for each test should: be at least 2 months old with a fully developed clitellum; weigh 300 to 600 mg; and be from the same culture.

8. PROCEDURE

8.1 Test Preparation.

8.1.1 Earthworm Culture.

The culturing of earthworms involves the set up of growing (culture) containers, feeding of worms and general maintenance of the cultures. These procedures are explained in detail in the SOP: BIO-WCUL-01 SOP for the Laboratory Culture of the Common Earthworm, Eisenia foetida.

8.1.2 Artificial Soil.

The artificial soil (AS) was developed with the advice of pedologists to overcome the variability between different soil types and has an adsorptive capacity resembling typical loam soils (i.e., a mixture of clay, silt, and sand). The following constituents are mixed together on a dry weight basis to produce the artificial soil (AS): 10% sphagnum peat moss (that portion passing through a 2.36-mm screen); 20% kaolinite clay (very fine powder, $\leq 40\ \mu$); and 70% silica sand. After these materials are mixed together, an amount of calcium carbonate equal to 0.42% of the total weight of artificial soil (AS) is added to the mixture to adjust the pH to the range of 6.00 - 7.00. For example:

$$5{,}000\ g \times 0.0042 = 21\ g$$

5,000 grams of artificial soil (AS) would require 21 grams of calcium carbonate to neutralize the acidity of the mix.

8.1.3 Site Specific Dilution Soil (ONLY if required by the sampling plan).

Site specific dilution soil (SSDS) is used as an alternative to Artificial Soil (AS). Since many site soils and sediments differ in porosity and adsorptive capacity, when possible one should utilize dilution soil which has nearly the identical characteristics as the site soil (SS). That is, if the site soil (SS) were 90% fine sand and 10% organic matter, the dilution soil would be composed of 90% fine sand and 10% pH-adjusted sphagnum peat. The site soil characteristics can be obtained from the latest edition of the soil survey (USDA SCS) from the county where the site is located and may be confirmed by soil analysis.

8.1.4 Site Soil.

Site soil (SS) samples should be screened through a ¼" soil sieve prior to testing. The samples are mixed with artificial soil (AS) to produce a series of test soil (TS) concentrations.

8.1.5 Soil pH.

Initial pH values of the high and low concentrations, as well as the positive and negative controls are recorded on the Earthworm Survival Data Sheet (ESAT-5-046.1). A pH value within the range of 5.00 - 9.00 is required for initiation of the test. Kaplan, et. al. (1980) and others have documented that earthworms die within 7 days when exposed to pH values <5 or >9.

The method for measuring pH was taken from "SW 846 Method 9045, Soil pH", (11/86) an electrometric procedure which has been approved for measuring pH in noncalcareous soils.

8.1.5.1 Place 20 g of soil in a 50-ml beaker, add 20 ml of deionized water and stir the suspension for 30 minutes.

8.1.5.2 Let the soil suspension stand for about 1 hour to allow most of the suspended clay to settle out from the suspension.

8.1.5.3 Adjust the electrodes in the clamps of the electrode holder so that, upon lowering the electrodes into the beaker, the glass electrode will be immersed just deep enough into the supernatant solution to establish a good electrical contact through the ground-glass joint or the fiber-capillary hole. For combination electrodes, immerse just below the suspension.

8.1.5.4 Measure the temperature of the soil solution. If the sample temperature differs by more than 2 °C from the buffer solutions, the measured pH value must be corrected as described in the pH meter instruction manual.

8.1.5.5 Report the results as "soil pH measured in water".

8.1.6 Soil pH Adjustment

If the site soil (SS) needs to be pH adjusted, the 40 gram slurry (20 g soil plus 20 ml deionized water) of site soil (SS) and deionized water used for pH measurement is utilized. Site soil (SS) pHs that fall outside of the 5.00 - 9.00 range are adjusted to the closest pH necessary for the survival of the test organisms. pH adjustment is done with 5N HCl (hydrochloric acid) or 5N NaOH (sodium hydroxide) solutions depending on which direction that the pH is to be adjusted. Also a different Normality: 12N HCl and 10N NaOH solutions can be used to reduce the amount of pH adjusting solutions added to the site soil (SS). If the site soil (SS) pH is less than 5.00, then the pH should be adjusted to pH ≥ 5.00. Also, if the pH is above 10.00, it should be adjusted to pH ≤ 10.00. The amounts of NaOH and HCl and the Normality used to adjust the pH of the slurry to an acceptable range, should be recorded and then used in calculating the amounts of NaOH and HCl needed for adjusting the pH of the calculated wet weights of site soil (SS) needed for the test. Site soil (SS) pH adjustment is normally performed during the hydration stage; when moisture is being added to test soils (TS) in the Glad-Lock® bags.

An example of a site soil (SS) with a pH of 4.00 is used for demonstration. Five normal NaOH is slowly added to the slurry using a serological pipette and a Pro-pipet® bulb or a finely calibrated burette (25 ml,

for example). It is difficult to ascertain the amount of base to add to a particular soil because soils have varying buffering capacities. Small amounts of NaOH such as 500 µl are used initially. The slurry is allowed to mix. The immediate reaction to an input of NaOH is to increase the pH, but it slowly decreases thereafter. Stabilization of the slurry is achieved, when the pH of the slurry does not change over the course of one hour. This procedure is repeated, slowly and carefully, until the slurry becomes stabilized at an acceptable pH level. This process requires a minimum of 4 hours and often portions of two separate days. The soil slurry is checked periodically and the pH is recorded. A record of the pH adjustment process is kept. Note the amount of acid or base used to adjust the 40 ml slurry and extrapolate the amount to the total volume of soil being mixed. The amounts will be recorded on the Earthworm Bioassay Calculation Worksheet-2 (ESAT-5-108.0).

Note: Site soils are not pH adjusted unless this procedure is specifically requested by the client.

8.1.7 Test Soil.

Test soil (TS) is composed of site soil (SS) that has been adjusted for acceptable pH and moisture levels. The site soil (SS) may be used at 100% or diluted with artificial soil (AS) or site specific dilution soil (SSDS) (see below). One of two dilution factors, 0.3 or 0.5, is commonly used. A dilution factor of approximately 0.3 allows testing between 100% and 1%, using only five concentrations (100%, 30%, 10%, 3%, and 1%). This series of dilutions minimizes the level of effort, but because of the wide interval between test concentrations, provides poor test precision (± 300%). A dilution factor of 0.5 provides greater precision (± 100%), but requires several additional dilutions to span the same range of concentrations. Improvements in precision decline rapidly as the dilution factor is increased beyond 0.5. A dilution factor of 0.5 is generally preferred.

If the hazardous waste is known or suspected to be highly toxic, a lower range of concentrations should be used (such as 10%, 5%, 2.5%, 1.25%, and 0.63%). If a high rate of mortality is observed during the first 1

to 2 hours of the test, additional dilutions at the lower range of concentrations should be added.

The volume of hazardous waste required for three replicates per concentration, each containing 200 g of test soil (TS), is approximately 1300 g. Prepare enough test soil (TS) at each concentration to provide sufficient soil for chemical analyses.

To dilute the site soil (SS), homogenized site soil (SS) is mixed with artificial soil (AS) or site-specific dilution soil (SSDS) to prepare geometric test soil (TS) concentrations (i.e. 100, 50, 25, 12.5, 6.25, and 0 percent dry wt/dry wt.). For each concentration, 660 g of test soil (TS) is prepared. Test soil (TS) mixtures are based on the ratio of site soil (SS) to artificial/dilution soil used for each level of exposure. For example:

If an 80% concentration with 3 replicates of 200 g each is desired, then we have

*660 g Site Soil (SS) x 0.80	=	528 g SS
660 g Artificial Soil (AS) x 0.20	=	132 g AS
	Total =	660 g Test Soil (TS)

*The additional 60 g of test soil (TS) prepared for each concentration is to allow for soil lost during preparation and dispensing (container walls, etc.), and for soil chemistry measurements. The pH of the site soil (SS) should be measured prior to the preparation of test soil (TS). The site soil (SS) pH should fall within the range of 5.00 - 9.00. If the pH lies outside this range, adjust the pH to the closest allowable pH with either 5.0 N NaOH or 5.0 N HCl as outlined above in 8.1.6.

Once the test soils (TS) are mixed, they need to be hydrated with distilled or deionized water to create a moist, but non-saturated testing environment. The test soil (TS) is thus hydrated to approximately 75% of its water-holding-capacity. To ensure even distribution of the test soil mixture, the total amount for each concentration is mixed together before dividing into replicates.

8.1.8 Soil Moisture content (m) and Water-Holding-Capacity (WHC).

Soils and sediments need to be at a proper moisture content for worm growth and survival. However, a water content that ranges from 25% to 42% of the dry weight of the basic substrate is sufficient to run the test with reproducible results. The key criterion is that the test soil (TS) be wetted only to a point where there is no standing water.

Moisture content (m) can be determined by the following process. First weigh an empty aluminum weigh boat or crystallizing dish (A). Next, a representative 125 grams sample of the soil or sediment is removed and placed in an aluminum weigh boat. The combined weight of the boat and sample equals the initial wet weight and is recorded to 0.1 g (B). The wet wt. (C) is calculated by subtracting the wt of the empty weigh boat (A) from the combined (boat + soil) wt. (B). The weigh boat and subsample are placed in the drying oven at 100 ± 5 °C for 24 hours. Remove and immediately cool in a desiccator. The weight of the boat and dried sample are recorded as the final dry weight (D). The moisture content of the sample is determined according to the following formula:

$$m = \frac{\text{init. wet wt. (B)} - \text{final dry wt. (D)}}{\text{wet wt. of soil (C)}} \times 100$$

where: m = % moisture fraction.

Save the dried soil in an airtight container for use in determining the water-holding-capacity (WHC) of the sample. Ideally, 100.0 g of dry soil is used, but because sometimes the moisture content may be so high as to leave less than 100.0 g after drying, a 125.0 g sample is more commonly utilized. If this situation occurs, whatever amount of dried sample soil that remains is used for the procedure and calculations are made on a 100.0 g basis. After drying, a soil may be in the form of a hard cake. If it is, it is placed in a mortar and pulverized with a pestle until it becomes a powder or until all of the large particles are broken up and a uniform particle size exists. If the sample is not in the form of a hard cake, it is placed in a resealable plastic bag and pulverized by hand until a uniform particle size exists. If a soil is low in

density that 100 g is too much volume for the holding funnel, then a lesser amount is used to determine WHC and the results are extrapolated to a 100.0 g sample size.

A 500-ml glass beaker is tared on a top loading balance, the soil added and its weight recorded to 0.1 g (G) on the Earthworm Bioassay Calculation Worksheet-1 (ESAT-5-045.1). One hundred mls of Milli-Q water is added to the sample and mixed thoroughly with a glass stir rod to ensure that all particles are wetted and a slurry of soil and water exists. A circle of 185-mm diameter, coarse porosity, qualitative, crepe filter paper (VWR grade 617) is folded into eighths and placed in a 100-mm glass funnel. The folded filter paper should be level with the top of the glass funnel. Next, an empty 500-ml Erlenmeyer flask is tared on a top-loading balance (H). The funnel with filter paper is placed in this 500-ml Erlenmeyer flask for support. Using a serological pipette and a Pro-pipet® bulb, 9 mls of Milli-Q water are slowly added to the filter paper so as to uniformly wet the entire surface. The flask with funnel and wetted filter paper is weighed and this weight is recorded (I). In the following example this weight is 481.2 g. Next, the slurry of soil and water is slowly poured onto the wetted filter paper held in the funnel. All remaining soil in the beaker and on the stirring rod is rinsed into the funnel with Milli-Q water. Use the minimum volume of water necessary to ensure that all of the solid material has been washed onto the filter. The weight of the funnel and hydrated filter paper (J) is calculated by subtracting the weight of the empty flask (H) from the weight of the flask plus funnel plus hydrated filter paper (I). Add the weight of dried soil (F) to the weight of the funnel and hydrated filter (J) paper to obtain the initial weight and record on the Calculation Worksheet 1 (ESAT-5-045.1) The funnel is covered tightly with aluminum foil and allowed to drain for a minimum of three (3) hours and a maximum of twenty-four (24) hours at room temperature.

After the drying period, a second 500-ml Erlenmeyer flask (L) is tared on a top loading balance and the funnel with drained soil is weighed to find the final weight (M). Remove foil cover prior to weighing. In the example, the final weight is 477.3 g. The final

weight minus the initial weight is the number of mls of water held by the soil (1 ml of water = 1 g).

grams of dry wt. utilized = 100.1 g
wt. funnel & wet filter paper = 316.4 g +
initial weight (sample, funnel and wet filter paper) = 416.5 g

final wt. (sample, funnel and drained filter paper) = 477.3 g

water held = final wt. - initial wt. = 60.8 g

$$WHC = \frac{60.8 \text{ g (ml) water}}{100.1 \text{ g (dry wt.) soil}} \times 100$$

= 60.7%

In this example, this amount is 60.7 g. The water held, when related to the amount of soil, is the water-holding-capacity (WHC). In the example, the WHC is 60.7 g water/ 100 g (dry weight) soil.

When testing sediments and/or saturated soils, moisture will be adjusted as follows:

Soil that is too wet is to be placed in a class I ventilated cabinet and vacuum filtered. Pore water is withdrawn from the soil and retained. The filtered soil is then brought up to a sufficient water content with the pore water (between 25-42%) to run the tests. Dry weight of soil will be taken after the test is run to reconfirm the moisture of the test soils and controls.

Water holding capacities of both the artificial soil (AS) and site soil (SS) must be known so appropriate moisture adjustments can be made.

8.1.9 Reference Toxicant Stock Solution Preparation.

The reference toxicant of choice for the terrestrial toxicity test is anhydrous 2-Chloroacetamide (C_2H_4ClNO, cas# 79-07-2). A 5000 mg/L 2-chloroacetamide stock solution is prepared and the stock solution is diluted to obtain the right amount needed for the positive control. Weigh out 5 g (5000 mg) 2-chloroacetamide

into a one liter (1L) volumetric flask. Add enough deionized water to fill the flask up to one half of the 1-liter mark. Mix the solution on a magnetic stir plate with a stir bar until all crystals are dissolved. Heat the solution at the low heat setting to enable the crystals to dissolve faster. If a clear solution is not achieved, apply more heat until all the crystals are dissolved. When a clear solution is obtained, retrieve the stir bar with a stir bar retriever and add enough deionized water to fill to the 1-liter mark. Place the stir bar back into the flask and mix solution on a stir plate for about 5 minutes. Seal the flask with a glass stopper and store at room temperature until needed.

Accuracy of the concentration is dependent on the accuracy of the balance and on the calibration markings on the glassware. For example, if the 1-L flask actually held only 995 mLs instead of 1000 mLs, the dissolution of 5.0 g of 2-chloroacetamide would actually produce a concentration of 5025 mg/L.

$$\frac{5.0\ g\ (5000\ mg)}{995\ mL} = 5025\ mg/L$$

Since the stock solution would be more concentrated than desired, less stock solution would be needed for making the desired positive control concentration. For instance, we generally add 4.7 mls of 5000 mg/L stock solution to 123.5 mls of deionized water to produce a concentration of 25 mg/L. If we started with a 5025 mg/L stock solution we would only require 4.67 mls of stock solution to be added to the deionized water to make the desired concentration:

x = unknown amount of stock solution

$$(x\ mls)\ (5025\ mg/L) = (4.7\ mls)\ (5000\ mg/L)$$

Solve for x: $$x = \frac{(4.7\ mls)\ (5000\ mg/L)}{(5025\ mg/L)}$$

$$= 4.67$$

For our applications, the nominal concentration is adequate.

8.1.10 Positive Controls.

The positive control is the treatment that duplicates all the conditions of the exposure treatments, but contains an effective concentration of a known toxicant that is added to the diluted medium. The positive control is used as a routine, continuing check for such factors as feeding, source of worms, culturing changes, etc. Refer to section 8.2.3 for the procedure on how to make up the positive controls.

8.1.11 Negative Controls.

The negative control is the treatment that duplicates all the conditions of the exposure treatments, but contains no test material. One hundred percent artificial soil (AS) or site-specific dilution soil (SSDS) is used as the negative control in this test.

8.1.12 Earthworm Purgation (OPTIONAL).

Earthworms may be harvested and purged of their gut contents for 24 hours prior to testing. Purging is done by placing 30-40 worms on a circle of 617 grade filter paper wetted with 10 mls of deionized water in a 150-mm plastic petri dish. The lid is placed on the petri dish and the worms are incubated at 20 ± 2 °C under continuous light (400-800 lux). The average weight of the worms should be 300 - 600 mg prior to the purging.

8.2 Procedure.

8.2.1 Test Containers and Labeling.

Standard one-pint canning jars with lids and screw rings are used as test containers. The lids should have a small (one-sixteenth inch) hole drilled or punched in their center to allow air exchange. All test concentrations are made in triplicate. The sides of each jar are labeled with the concentration and the replicate number, e.g. 25% #1; 25% #2, etc. Controls are labeled "pos. C #1, pos. C #2, pos. C#3; for the positive controls and "neg. C #1, neg. C #2, and neg. C #3 for the negative controls.

8.2.2 Set-up of Test Containers.

Artificial soil (AS) is weighed for each concentration and the controls and placed in 10 inch by 11 inch Glad-Lock® plastic bags. Bag and soil are tared on a top loading balance. Dust is kept to a minimum if the artificial soil (AS) is weighed under a hood. Using the information in column (5) of the Test Soil Preparation Worksheet (ESAT-5-112.0) the proper amount of artificial soil (AS) for each concentration and the controls is weighed and placed into Glad-Lock® bags. Each bag is marked with the appropriate concentration using a permanent marking pen. Using the 25% concentration as an example, 495 g of artificial soil (AS) are placed into a Glad-Lock® bag.

660 g x 0.25 =	165 g	dry Site Soil (SS)
660 g x 0.75 =	495 g	dry Artificial Soil (AS)
	660 g	total Test Soil (TS)

For the negative control 660 g of artificial soil (AS) are needed. The appropriate amounts of wet site soil (SS) are added to each Glad-Lock® bag. A stainless steel scoop or spatula is used to dispense the site soil (SS). For example to make a 25% concentration when the moisture content of the site soil is 0.558 and the moisture content of the artificial soil is negligible, 373.3 g of wet site soil (SS) would be added to 495 g of artificial soil (AS). See Sample Test Soil Preparation Calculations in Attachment. Each bag of site soil (SS) and artificial soil (AS) are mixed by hand to ensure that the two constituents are homogenous. After mixing, the material is known as Test Soil (TS).

8.2.3 Hydration of Test Soil.

The values needed for the hydration of Test Soil (TS) are in column (9) of the Earthworm Test Soil Preparation Worksheet (ESAT-5-112.0). The corresponding amounts of water are added to the test soils (TS) using a 100-ml graduated cylinder. The positive controls, however, need to be measured with volumetric flasks and volumetric pipettes. The hydrated test soil (TS) is mixed by kneading the plastic bag by hand to ensure a uniform distribution of moisture.

The calculations needed to determine the amount of 2-chloroacetamide solution to add to 660 g of artificial soil (AS) for the positive controls are as follows, using 25 mg/kg as an example:

Starting with a stock solution of 5000 mg/L 2-chloroacetamide

Desired conc. for positive control
= 25 mg/kg wet artificial soil

For this example, assume:
water-holding-capacity (WHC) of earthworm artificial soil = 60.7 mls/100 g

The desired % saturation of the artificial soil is 32%. (This provides enough moisture to wet the mixture, without leaving standing water in the bag.)

32% WHC = (0.32) (60.7 mls)/100 g = 19.4 mls/100 g

$$\frac{\text{test soil (TS) per concentration}}{\text{test soil (TS) per replicate}} = \frac{660\text{ g}}{200\text{ g}} = 3.3$$

Total ml water in control = (3.3) (32% WHC)
= (3.3) (19.4 mls/100 g)
= 64.1 mls/100 g

But since 200 g of soil are needed per replicate, the total amt of water is doubled;

(64.1 mls/100 g) (2) = 128.2 mls/200 g

Total wet wt of artificial soil
= 788.2 g (660 g artificial soil + 128.2 mls of water) (Based on 1 ml water = 1g)

Number of mg 5000 mg/l 2-chloroacetamide needed in 25 mg/kg
= 25 mg/kg x 0.7882 kg (788.2 g) = 19.7 mg

19.7 mg/5000 mg/l = 0.0039 liters = 3.9 mls

Total solution amt - stock conc. amt = Milli-Q® water amt

128.2 mls - 3.9 mls = 124.3 mls

Therefore, 3.9 mls of 5000 mg/L stock solution plus 124.3 mls of deionized water will provide the necessary amount of 25 mg/L 2-chloroacetamide solution to wet 660 g of artificial soil to 32% of the artificial soils's water-holding-capacity (WHC).

Pipet 3.9 mls of 2-chloracetamide stock solution into a beaker with 124.3 mls of deionized water (Neuhauser, et al., 1986a). The solution is mixed with a glass stir rod. The mixture is poured into the Glad-Lock® bag containing the artificial soil (AS) for the positive control and mixed by hand through the polyethylene bag. All of the mixed and hydrated test soils (TS) should be mixed 24 hrs prior to test initiation to facilitate equilibration.

The test soils (TS) are placed in the test containers using a spatula or scoop. The values from column (10) of the Earthworm Test Soil Preparation Worksheet (ESAT-5-112.0) are used to determine the appropriate amount in each jar. The test soil (TS) is consolidated into one mass in a Glad-Lock® bag. This test soil (TS) is separated with the aid of a spatula or scoop and placed in the individual mason jars.

A standard test has five concentrations of site soil (SS) and positive and negative controls. A dilution factor of 0.5 (e.g., 100%, 50%, 25%, 12.5%, and 6.25%) and 10 worms per replicate are used. Site soils (SS) are tested as percent of site soil (dry weight) per concentration.

Each concentration of test soil (TS) and control soils are divided equally among replicate test containers. If large interstitial spaces occur after placing the soil into the jars, the soil is pressed down to remove these spaces. The soil is not pressed unless these spaces occur.

An initial temperature is taken from only one test container. A glass thermometer is placed in the selected jar (at mid-depth in the test soil) and allowed to equilibrate for 10 minutes before the temperature is read. If the initial temperature is outside the range of 20 ± 2 °C, the test soils (TS) are placed in the testing chamber for one hour to become equilibrated.

8.2.4 Placement of Worms into Test Soil.

The worms are weighed and placed into the test jars. It is important in this process to transfer the worms without causing any damage. Worms are removed and not used in the toxicity tests if they appear to be injured. Each replicate is to have a biomass of 3.0 to 6.0 grams.

The worms used in testing are adult, clitellate worms with a wet weight range of 300-600 mg each (Edwards, 1983). Test jars with test soil (TS) are placed in an exhaust cabinet -- hood. The test jars are inoculated with worms in any sequence. The bottom half of a plastic, disposable petri dish or disposable weighing dish is tared on a top loading balance. Ten worms are removed from the culture tray and weighed in the dish. The total weight is then recorded in grams on the Earthworm Survival Data sheet (ESAT-5-046.1) for the corresponding replicate. It may not be possible to remove all of the bedding from each individual worm, but as little bedding as possible is weighed. The petri dish with worms is gently emptied onto the surface of the test soil in the selected replicate. The worms are allowed to burrow into the test soil (TS).

The jars are now taken to the testing chamber. The tests are run at 20 ± 2 °C. The worms are exposed to continuous lighting which promotes burrowing. The lighting is to be at a minimum intensity of 7 microeinsteins. The jars are randomly placed on the shelves. The starting time and date for the test is recorded on the Earthworm Bioassay Cover Sheet (ESAT-5-044.1).

8.2.5 24-Hr Observations. (Optional)

Observations are made after 24 hours of exposure to determine if the worms are burrowing and if any mortality has occurred on the surface. Lack of burrowing in the presence of continuous light is considered a behavioral response to the toxicants. Each test container is opened and the number of dead and living worms on the surface is recorded on an Observation Sheet (ESAT-5- .0). It may not be possible to determine if a worm is dead at this time,

since the test is not to be disturbed other than by removing the lid. Dead worms are not removed.

8.2.6 Seven Day Count and Observations.

The worms are counted and observed for behavioral and pathological changes at 7 and 14 days. The test jars are emptied one at a time on a counting tray. As the count is made, the following observations are made: lack of burrowing, ulceration, coiling, "balling" together, contraction, rigidity, elongation, mid-segmental swelling, segmental constriction and segmental loss.

Each replicate is counted and the number of mortalities is entered on the Earthworm Survival Data Sheet (ESAT-5-046.1) and the Earthworm Bioassay Observation sheet (ESAT-5-107.0). A worm is considered dead if it does not respond to a gentle mechanical stimulus to its anterior end. If no mortality occurs, a zero is placed on the data sheet. The soil is gently sorted with gloved fingers or with the aid of a flat, stainless steel spatula. As the worms are located, they are placed in the lid. Ten worms are accounted for in each replicate. Mortality seldom occurs in the negative control. After the worms are accounted for, the soil is pushed back into the test jar. The worms are placed back into the jar on the surface and allowed to burrow into the soil again. The other replicates of the negative control are counted and the jars returned. The tray is wiped off with paper towels. The positive controls are counted next. Some mortality is to be expected in the positive controls. Dead worms are removed from the soil and discarded. If any replicate of a concentration has 100% mortality, the test soil (TS) and the dead worms are placed into double plastic bags and labeled as waste. Worms decay rapidly in the moist testing environment and if 10 worms are not accounted for, they are presumed dead and completely decomposed. In addition, worms can lose a number of segments and still be able to move. This "half" of a worm is still considered alive. The sorting tray is washed with soap and water and then dried with paper towels after the last replicate of the positive controls are counted.

Next, the lowest test concentration is counted. If the test jars are counted from the lowest to the highest

concentration, the tray is not washed between concentrations and is just wiped off with paper towels. If 100% mortality, i.e., 10 dead worms, occurs in the high or low concentrations or controls on the 7-day count, the final pH for that concentration is taken. The final step for the 7-day count is the calculation of the percent effect (percent mortality) for each concentration. The calculations are made by dividing the total number of dead worms in a concentration by 30 and converting this fraction to a percent value.

$$\text{Percent effect (E\%)} = \frac{\text{\# dead worms}}{30} \times 100$$

8.2.7 Fourteen Day Count and Test Termination.

The procedure is similar to the 7-day count. If the survival rate does not change between 7 and 14 days, the mortality counts and the percent effect values are carried forward from the 7-day count. As this is the final count the sorting tray is not cleaned after the positive control is counted. It is cleaned at the end of all the counts. The test soil (TS) along with the dead and living worms are placed in double plastic bags and disposed of as potentially hazardous waste. The final pH values are taken from the concentrations remaining, the negative controls and the positive controls.

8.2.8 Completing the Earthworm Bioassay Cover Sheet.

Each data package should contain the name of the Analyst, the Site and sample number. The time at which the test is initiated as well as the water-holding-capacity (WHC) value of the site soil (SS) is noted. The batch number of the artificial soil (AS) and its water-holding-capacity (WHC) are noted. Hydration of the site soil (SS) is expressed in terms of percent water-holding-capacity (WHC). The site soil (SS) portion of the test soil (TS) is hydrated at 75% of its water-holding-capacity (WHC) value. The pH values of the site soil (SS) (after it is screened and mixed) and the artificial soil (AS) are recorded. The amount of mixing time associated with each pH value determination is also noted. The artificial soil (AS) portion of the test soil (TS) is hydrated at 45% of the dry weight of its components. Moisture content of the site soil (SS)

and the artificial soil (AS) are reported as percentages. The test uses three replicates per concentration, 10 worms per replicate for a total of 30 worms per concentration.

Initial pH values of the high and low concentrations as well as the positive and negative controls are measured and recorded on the Earthworm Survival Data sheets (ESAT-5-046.1).

8.2.9 Sample Disposition.

Samples are disposed of after analysis of all results are documented on the data sheets for each test. Disposal of samples is determined on a case by case basis. Determinations should be made if the samples are to be considered hazardous or not.

8.2.9.1 If the sample is low in toxicity (i.e. considered nonhazardous), it may be disposed of by dumping into a dumpster.

8.2.9.2 If the sample is highly toxic and or considered hazardous, it should be disposed of as follows: Place all possibly contaminated labware such as paper towels, aprons, gloves, pipets, disposable glassware, and respirator cartridges into plastic bags. Label the bag as to its contents, the start accumulation date, and the name of the analyst who initiates the waste stream. Notify Safety Officer for proper storage and disposal.

8.3 Preparation and Testing Timetable

Prior to the arrival of samples:

a. Prepare Artificial Soil - 70% silica sand, 20% colloidal kaolinite clay, and 10% sphagnum peat.
b. Measure pH of Artificial Soil. (4 hr)
c. Perform moisture content measurement of Artificial Soil. (24 hr)
d. Perform Water-Holding-Capacity measurement of Artificial Soil. (4 hr min.)
e. Prepare 2-chloroacetamide stock solution.

Day 1 a. Begin moisture content measurement of Site Soil. (24 hr min.)

b. Measure pH of Site Soil.
c. Perform Soil Characterization of Site Soil (optional).

Day 2
a. Conclude moisture content measurement of Site Soil.
b. Perform Water-Holding-Capacity measurement of Site Soil. (4 hr min.)
c. Label test jars.
d. Prepare Test Soil Preparation Worksheet.

Day 3
a. Mix artificial soil and site soil to produce test soil.
b. Hydrate test soil.
c. Store in test chamber overnight to equilibrate soil temperature.

Day 4
a. Obtain initial pH values of test soil concentrations.
b. Place test soil into test containers.
c. Obtain initial temperature values.
d. Introduce worms to test soil.
e. Place test containers into the testing chamber.

Day 5
a. Make 24-hr observations.

Day 11
a. Perform 7-day counts and observations.

Day 18
a. Perform 14-day counts and observations.
b. Take final pH values.
c. Calculate median LC50.

9. CALCULATIONS

The percent effect values are calculated at the end of the 7 and 14 day counts and entered on the data sheet. For the test results to be acceptable, survival in the negative controls must be at least 90%. After the control chart has been established, the 14-day results must fall within this range.

9.1 Calculations/Data Analysis for Single Concentration Tests.

9.1.1 Express survival as the proportion surviving (e.g. if 7 of 10 worms survive, the proportion surviving is 0.70).

9.1.2 Transform the data using arcsin square root transformation.

9.1.3 Calculate the mean (X-bar) and standard deviation (S-squared) of the replicates for the control and test concentrations.

9.1.4 Calculate the F-statistic.

If the calculated F is less than 199.0 (the critical value of F for this data), use the T-test to analyze the data.

If the calculated F is greater than 199.0, analyze the data using the modified T-test.

If the T-test indicates a significant difference in survival between the test concentration and the control. The test sample is considered "toxic".

9.2 Calculations/Data analysis for Multi-Concentration Tests.

9.2.1 Determining the NOEC*/LOEC**.

a. Express worm mortality as a proportion (i.e. if 8 of 10 worms die, the proportion dying is 0.80).

b. Transform the data using arcsin square root transformation.

c. Perform a Shapiro-Wilk's test on the transformed data to test for normality. If the data is normal, proceed to Bartlett's Test.

If the data fails the test for normality, no further statistical analysis is recommended. This situation, however, applies only to tests conducted with 3 or fewer replicates per test concentration. If four or more replicates per test concentration are used, the data can be analyzed using Steel's Many-One Rank Test (used when the control and test concentrations have equal numbers of replicates) or using the Wilcoxon Rank Test (when the control and test

concentrations have unequal numbers of replicates).

d. Perform Bartlett's Test on transformed, normal data to test for homogeneity of variance.

If the requirement for equality of variance is met, the data is analyzed using Dunnett's Procedure (when the control and test concentrations have equal numbers of replicates) or using Bonferroni's T-test (when the control and test concentrations have unequal numbers of replicates).

If the data fails the test for equality of variance, no further statistical analysis is recommended when the test was conducted with 3 or fewer replicates per test concentration. If however, the test was conducted with 4 or more replicates, Steel's Many-One Rank test or Wilcoxon'S Rank Sum Test can be used to analyze the data as explained above.

Note that if the data fails the test for normality and/or the test for homogeneity of variance, and the test was conducted with 3 replicates per test concentration, (1) a parametric test such as Dunnett's or Bonferroni's T-test can be performed but the results <u>must be used with extreme caution</u>, or (2) the results of the test can simply be recorded as the percent mortality of the test concentrations compared to the percent mortality of the control.

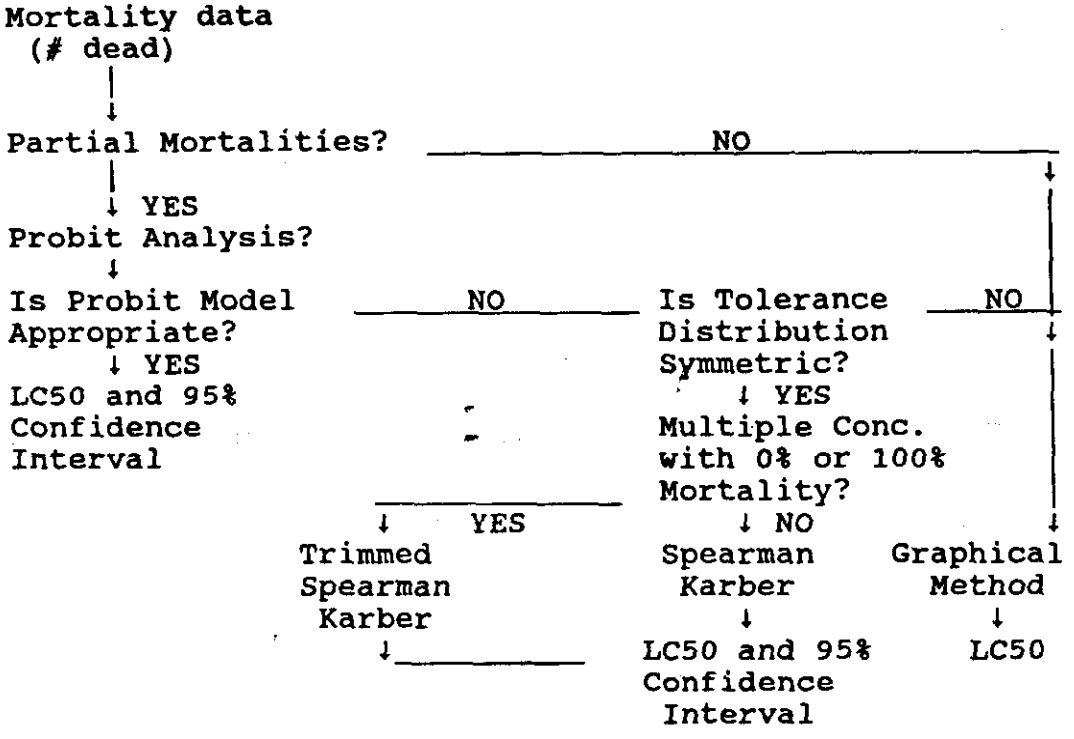
e. When Dunnett's Procedure (or Bonferroni's T-test, or Steel's Many-One Rank Sum Test, or Wilcoxon Rank Sum Test) indicates a significant difference in mortality between the control and a test group, the sample at that test concentration is considered "toxic". Then, by definition:

* The NOEC (No Observed Effect Concentration) is the highest concentration of test sample to which the worms were exposed that caused no observable adverse effect.

** The LOEC (Lowest Observed Effect Concentration) is the lowest concentration of test sample to which the worms were exposed that cause the observed effect.

9.2.2 Determining the LC50.

The LC50 is an estimate of the median lethal concentration. To calculate or estimate the LC50 for a chronic multi-concentration test, follow the flow chart below. The first test of choice is the Probit Analysis, followed by the Spearman-Karber and finally the Graphical Method. The analyses are best performed using computer programs.



10. QUALITY CONTROL

For the test results to be acceptable, mean survival in the negative controls must be at least 90%.

11. INTERFERENCES

11.1 Toxic substances may be introduced by contaminants in water, glassware, sample hardware, artificial soil, and testing equipment. Low dissolved oxygen (DO) concentration or saturation of soils with water may mask the presence of toxic substances.

11.2 Pathogenic organisms in test materials may also affect test organisms survival, and confound test results.

11.3 Improper hazardous waste sampling and handling may adversely affect test results.

EARTHWORM BIOASSAY COVER SHEET

Site Soil:_____ Analyst: _____

Sample Number:_____ Test Start Date: _____ Time:_____

AS Batch #:_____

AS pH:_____ (Time:_____) SS pH: _____ (Time:_____)

WHC of AS: _____ WHC of Dry SS:_____

Moisture content AS: _____ Moisture content SS: _____

Hydration of AS: _____ Hydration of SS:_____

No. Repl./Conc._____ No. Worms/Repl._____ No. Worms/Conc. _____

Positive Controls = _____ Negative Controls = 0% SS = 100%

EARTHWORM TEST SOIL PREPARATION WORKSHEET

Site Soil: _____ Analyst: _____

Sample Number: _____ Test Dates: _____

(1) % Site Soil (SS)	(2) wgt. dry Site Soil (SS)	(3) wgt. wet Site Soil (SS)	(4) water needed for dry Site Soil (SS)	(5) wgt. dry Arti-ficial (AS)	(6) water needed for dry Arti-ficial Soil (AS)	(7) total water needed for Test Soil (TS)	(8) water in wet Site Soil (SS)	(9) total water added to Test Soil (TS)	(10) wgt. wet Test Soil (TS) per jar
Pos. C									
Neg C									

WHC =

EARTHWORM SURVIVAL DATA

Site Soil:_____ Analyst:_____

Sample Number:_____ Test Dates:_____

Repl. No.	Conc.	Init. Temp.	Init. pH	Biomass per Repl.	7 days		14 days		Final pH
					Mort	%E	Mort	%E	
1									
2									
3									
1									
2									
3									
1									
2									
3									
1									
2									
3									
1									
2									
3									
1	Pos. C								
2									
3									
1	Neg. C								
2									
3									

Mort = Mortality
%E = Effective concentration

EARTHWORM BIOASSAY OBSERVATION SHEETS

Site Soil: _____ Analyst: _____

Sample Number: _____ Date: _____

Observation Time: 24-hr Day 7 Day 14 Time: _____

Conc.	Rep No.	Observations
	1	
	2	
	3	
	1	
	2	
	3	
	1	
	2	
	3	
	1	
	2	
	3	
	1	
	2	
	3	
Pos C	1	
	2	
	3	
Neg C	1	
	2	
	3	

EARTHWORM BIOASSAY CALCULATION WORKSHEET - 1

Dataset:_____ **Analyst:**_____
Soil I.D.: Sample or Diluent (Circle One)
MOISTURE CONTENT (m) CALCULATION:
Weight of empty aluminum boat = _____ (g) **A**

Initial weight of boat + wet soil = _____ (g) **B**

Grams Wet Weight utilized = _____ (g) **B-A=C**
Date/Time In: _____ Drying Temperature (°C):_____
Date/Time Out:_____ Drying Time (min. 24 hrs):_____

Final Weight of boat + dried soil = _____ (g) **D**

Weight of water lost = _____ (g) **B-D=E**

Moisture content = _____% **(E/C) x 100**
WATER-HOLDING-CAPACITY (WHC) CALCULATION:
Grams Dry Weight Utilized = _____ (g) **F**

Empty 500-ml glass beaker = _____ (g) **G**

500-ml beaker + soil = _____ (g) **F+G**

Empty 500-ml Erlenmeyer flask = _____ (g) **H**

Flask + funnel + hydrated filter paper = _____ (g) **I**

Funnel + hydrated filter paper = _____ (g) **I-H=J**

Initial weight (funnel, soil, wet filter paper) = _____ (g) **F+J=K**
Drainage Date/Time started: _____
Drainage Date/Time completed: _____
Total Draining Time (3-24 hrs):_____

Weight of second 500-ml flask = _____ (g) **L**
Weight of flask + wet soil + funnel
+ hydrated filter paper = _____ (g) **M**

Final weight (soil, funnel, hydrated filter paper) = _____ (g) **M-L=N**

Water held = Final weight - Initial weight = _____ (ml) **N-K**

$$\text{WHC} = \frac{[\text{amt of water held (N-K)}]\ [\text{ideal wt utilized, 100.0 g soil}]}{[\text{amt of wt utilized (F)}]}$$

WHC = _____ ml water/100 g dry weight soil

EARTHWORM BIOASSAY CALCULATION WORKSHEET - 2

Dataset:_____ **Analyst:**_____
Soil I.D.: Sample or Diluent (Circle One)

SOIL pH MEASUREMENTS:

Weight of soil (g):_____ Volume of water (ml):_____
pH reading (10 minutes):_____ Concentration:_____

Weight of soil (g):_____ Volume of water (ml):_____
pH reading (10 minutes):_____ Concentration:_____

Weight of soil (g):_____ Volume of water (ml):_____
pH reading (10 minutes):_____ Concentration:_____

Weight of soil (g):_____ Volume of water (ml):_____
pH reading (10 minutes):_____ Concentration:_____

Weight of soil (g):_____ Volume of water (ml):_____
pH reading (10 minutes):_____ Concentration:_____

Weight of soil (g):_____ Volume of water (ml):_____
pH reading (10 minutes):_____ Concentration:_____

SOIL pH ADJUSTMENT CALCULATIONS:

To 40 gram slurry added _____ ml of _____ N NaOH or _____ N HCL

pH reading after 1 hour _____

pH reading after 2 hours _____

pH reading after 3 hours _____

pH reading after 4 hours _____

$$\frac{x}{40 \text{ g slurry}} = \frac{X}{(1200 - 20)} = \frac{X}{1180 \text{ g SS}}$$

x = amt of acid/base added to 40 gram slurry
X = amt of acid/base required to neutralize the remainder of SS sample

SUMMARY OF TEST CONDITIONS FOR EARTHWORM (E. FOETIDA) SURVIVAL TEST

1.	Test type:	Static
2.	Test duration:	14 days
3.	Soil temperature (°C):	20 ± 2°C
4.	Light quality:	Ambient laboratory light
5.	Light intensity:	540-1080 lux
6.	Photoperiod:	Continuous illumination
7.	Test vessel type and size:	1-pint glass canning jars with bands and lids. A 1/16 inch air hole in lid.
8.	Test soil mass:	200 g
9.	Test soil pH: *	≥ 5.00 but ≤ 9.00
10.	Artificial soil(% wt.):	10% 2.36-mm screened sphagnum peat, 20% colloidal kaolinite clay, and 70% grade 70 silica sand -- pH adjusted with 0.42% Calcium Carbonate.
11.	Test soil moisture content:	Approximately 75% of water holding capacity.
12.	Renewal of test soils:	None
13.	Age of test organisms:	> 60 days
14.	Number of test organisms per test chamber:	10
15.	Number of replicate test chambers per test concentration:	3
16.	Feeding regime:	Do not feed
17.	Dilution factor:	0.5
18.	Test Acceptability:	90% Survival of Negative Controls
19.	Effect measured:	Death

* If pH is outside this range, results may reflect pH toxicity. Adjustments of pH to 5.00 or 9.00 may result in altered toxicity of other constituents.

REFERENCES CITED

1. Greene, Joseph C., Cathy L. Bartels, William J. Warren-Hicks, Benjamin R. Parkhurst, Gregory L. Linder, Spencer A. Peterson, and William E. Miller. 1989. Protocols for Short Term Toxicity Screening of Hazardous Waste sites.

2. Harris, Todd, Dr. Jay Glover, and Dr. Jim Maudsley. Region IV; SOP for Toxicity Testing Hazardous Waste Assessments. Environmental Services Assistance Team, NSI Technology Services Corporation, U.S. EPA, ESD, College Station Road, Athens, Georgia. pp. XVII. 1-13.

3. Inglesfield, Christopher. 1984. Toxicity of the Pyrethroid Insecticides Cypermethrin and WL85871 to the Earthworm, Eisenia foetida Savigny. Bull. Environ. Contam. Toxicol. 33:568-570.

4. Kaplan, David L., Roy Hartenstein, Edward F. Neuhauser and Michael R. Malecki, 1980. Physicochemical Requirements in the Environment of the Earthworm, Eisenia foetida. Soil Biol. Biochem. 12:347-352.

5. Neuhauser, E.F., P.R. Durkin, M.R Malecke, and M. Anatra. 1986. Comparative toxicity of ten organic chemicals to four earthworm species. Comp. Biochem. Physiol. 83C(1): 197-200.

6. Warren-Hicks, William, Benjamin R. Parkhurst, and Samuel S. Baker, Jr. 1989. Ecological Assessments of Hazardous Waste Sites: A Field and Laboratory Reference Document.

ATTACHMENTS

Sample Test Soil (TS) Preparation Calculations:

A 25% concentration is a mixture of 25% Site Soil (SS) and 75% Artificial Soil (AS) mixed on a dry weight/dry weight basis.

$$660 \text{ g} \times 0.25 = 165 \text{ g SS}$$

Since a certain amount of water (1 ml of water = 1 g) is contained in the Site Soil (SS), this pre-existing moisture must be accounted for. The amount of wet Site Soil (SS) needed to yield the proper amount of dry Site Soil (SS) is calculated by the following equation:

$$\text{wet wt. SS} = \frac{\text{dry wt. SS}}{1 - \%\text{ moisture content (m)}}$$

For this example assume moisture content, m = 0.558

$$\text{wet wt. SS} = \frac{165 \text{ g}}{1-m} = \frac{165 \text{ g}}{1-0.558} = \frac{165}{0.442} = 373.3\text{g}$$

The amount of hydration water needed for the Site Soil (SS) is entered in column (4). The Site Soil (SS) fraction of the Test Soil (TS) is hydrated to 75% of the Site Soil's Water-Holding-Capacity (WHC). Assume the Water-Holding Capacity (WHC) of the Site Soil (SS) is 68.5 mls/100 g dry soil.

$$\begin{aligned}\text{water for dry SS} &= (\text{dry wt. SS})\ (75\%\ \text{WHC}) \\ &= (165\text{ g})\ (0.75)\ (68.5\text{ mls}/100\text{g}) \\ &= (165\text{ g})\ (51.4\text{ mls}/100\text{ g}) \\ &= 84.8\text{ mls}\end{aligned}$$

The amount of dry Artificial Soil (AS) that is mixed with the Site Soil (SS) to produce the desired concentration is entered in column (5).

$$\begin{aligned}\text{wt. dry AS} &= 660\text{ g} - (\text{wt. dry SS}) \\ &= 660\text{ g} - 165\text{ g} \\ &= 495\text{ g}\end{aligned}$$

Artificial Soil (AS) is hydrated to 45%. The Water-Holding-Capacity (WHC) for the artificial soil (AS) is 60.7 mls/100 g. The amount of hydration water is entered in column (6).

water for dry AS = (wt. dry AS) (45% WHC)
= (495 g) (0.45) (60.7 mls/100 g)
= (495 g) (27.3 mls/100 g)
= 135.1 mls

Total volume of water needed for the Test Soil (TS) is entered in column (7).

water for TS = (water for dry SS) + (water for dry AS)
= 84.8 ml + 135.1 ml
= 219.9 mls

Amount of water in wet Site Soil (SS) is entered in column (8). This amount is dependent on the moisture content of the Site Soil (SS). For this example, the moisture content (m) is 55.8 mls/100 g.

water content of wet SS = (wt. of wet SS) (moisture content)
= (373.3 g)(55.8 mls/100 g)
= 208.3 mls

Total amount of water actually added to the Test Soil (TS) is entered in column (9).

water added to TS = (water for TS) - (water in wet SS)
= 219.9 ml - 208.3 ml
= 11.6 mls

Wet wt. of Test Soil (TS) added to each replicate is entered in column (10).

X = wt. of TS per replicate

$$\frac{\text{dry wt./concentration}}{\text{dry wt/conc. + water for TS}} = \frac{\text{dry wt./replicate}}{X}$$

$$\frac{660\ g}{660\ g + 219.9\ g} = \frac{200\ g}{X}$$

$$\frac{660\ g}{879.9\ g} = \frac{200\ g}{X}$$

$$X = 266.6\ g$$

100% SS

wgt dry SS 660 g SS

wet wt. SS = $\frac{660\ g}{1-m} = \frac{660\ g}{1-0.558} = \frac{660}{0.442} = 1493.2\ g$

water for dry SS = (660 g) (51.4 mls/100 g) = 339.2 mls

wt. dry AS = 0 g

water for dry AS = 0 mls

water for TS = 339.2 ml + 0 ml = 339.2 mls

water content of wet SS = (1493.2 g)(55.8 mls/100 g)
= 833.2 mls

water added to TS = 339.2 ml - 833.2 ml = -494.0 mls
(i.e., no additional water is added to the mix)

X = wt. of TS per replicate = (200) (999.2)/660
= 302.8 g

50% SS: 50% AS

wgt dry SS 660 g x 0.50 = 330 g SS

wet wt. SS = $\frac{330\ g}{1-m} = \frac{330\ g}{1-0.558} = \frac{330}{0.442} = 746.6\ g$

water for dry SS = (330 g) (51.4 mls/100 g) = 169.6 mls

wt. dry AS = 660 g - (wt. dry SS) = 660 g - 330 g = 330 g

water for dry AS = (330 g) (27.3 mls/100 g) = 90.1 mls

water for TS = 169.6 ml + 90.1 ml = 259.7 mls

water content of wet SS = (746.6 g)(55.8 mls/100 g)
= 416.6 mls

water added to TS = 259.7 ml - 416.6 ml = -156.9 mls
(i.e., no additional water is added to the mix)

X = wt. of TS per replicate = (200) (919.7)/660
= 278.7 g

12.5% SS: 87.5% AS

wgt dry SS 660 g x 0.1250 = 82.5 g SS

wet wt. SS = $\frac{82.5\ g}{1-m} = \frac{82.5\ g}{1-0.558} = \frac{82.5}{0.442} = 186.7\ g$

water for dry SS = (82.5 g) (51.4 mls/100 g) = 42.4 mls

wt. dry AS = 660 g - (wt. dry SS) = 660 g - 82.5 g = 577.5 g

water for dry AS = (577.5 g) (27.3 mls/100 g) = 157.7 mls

water for TS = 42.4 ml + 157.7 ml = 200.1 mls

water content of wet SS = (186.7 g)(55.8 mls/100 g)
= 104.2 mls

water added to TS = 200.1 ml - 104.2 ml = 95.9 mls

X = wt. of TS per replicate = (200) (860.1)/660
= 260.6 g

6.25% SS: 93.75% AS

wgt dry SS 660 g x 0.0625 = 41.3 g SS

wet wt. SS = $\frac{41.3\ g}{1-m} = \frac{41.3\ g}{1-0.558} = \frac{41.3}{0.442} = 93.4\ g$

water for dry SS = (41.3 g) (51.4 mls/100 g) = 21.2 mls

wt. dry AS = 660 g - (wt. dry SS) = 660 g - 41.3 g = 618.7 g

water for dry AS = (618.7 g) (27.3 mls/100 g) = 168.9 mls

water for TS = 21.2 ml + 168.9 ml = 190.1 mls

water content of wet SS = (93.4 g)(55.8 mls/100 g)
= 52.1 mls

water added to TS = 190.1 ml - 52.1 ml = 138.0 mls

X = wt. of TS per replicate = (200) (850.1)/660
= 257.6 g

Positive Control:

wgt dry SS = 0 g

wet wt. SS = 0 g

water for dry SS = 0 mls

wt. dry AS = 660 g

water for dry AS = (660.0 g) (32% WHC)
= (660.0 g) (0.32) (60.7 mls/100 g)
= 128.2 mls

water for TS = 128.2 mls

water content of wet SS = 0 mls

water added to TS = 128.2 ml - 0 ml = 128.2 mls

X = wt. of TS per replicate = (200) (788.2)/660
= 238.8 g

Negative Control:

wgt dry SS = 0 g

wet wt. SS = 0 g

water for dry SS = 0 mls

wt. dry AS = 660 g

water for dry AS = (660.0 g) (45% WHC)
= (660.0 g) (0.45) (60.7 mls/100 g)
= 180.3 mls

water for TS = 180.3 mls

water content of wet SS = 0 mls

water added to TS = 180.3 ml - 0 ml = 180.3 mls

X = wt. of TS per replicate = (200) (840.3)/660
= 254.6 g

EARTHWORM TEST SOIL PREPARATION WORKSHEET

Site Soil: Commercial potting soil Analyst: A. C. Harvey

Sample Number: Example Test Dates: _____

(1) % Site Soil (SS)	(2) wgt. dry Site Soil (SS)	(3) wgt. wet Site Soil (SS)	(4) water needed for dry Site Soil (SS)	(5) wgt. dry Arti-ficial (AS)	(6) water needed for dry Arti-ficial Soil (AS)	(7) total water needed for Test Soil (TS)	(8) water in wet Site Soil (SS)	(9) total water added to Test Soil (TS)	(10) wgt. wet Test Soil (TS) per jar
100	660	1493.2	339.2	0	0	339.2	833.2	-494.0	302.8
50	330	746.6	169.6	330	90.1	259.7	416.6	-156.9	278.7
25	165	373.3	84.8	495	135.1	219.9	208.3	11.6	266.6
12.5	82.5	186.7	42.4	577.5	157.7	200.1	104.2	95.9	260.6
6.25	41.3	93.4	21.2	618.7	168.9	190.1	52.1	138.0	257.6
Pos. C	0	0	0	660	128.2	128.2	0	128.2	238.8
Neg C	0	0	0	660	180.3	180.3	0	180.3	254.6

WHC (SS) = 68.5 mls/100 g
75% WHC (SS) = 51.4 mls/100 g
m (SS) = 55.8 mls/100 g = 0.558

WHC (AS) = 60.7 mls/100 g
45% WHC (AS) = 27.3 mls/100 g
Pos Ctrl: 32% WHC (AS) = 19.4 mls

EARTHWORM BIOASSAY CALCULATION WORKSHEET - 1

Dataset: Test Soil **Analyst:** A. C. Harvey
Soil I.D.: Sample or Diluent (Circle One) = peat/clay/sand

MOISTURE CONTENT (m) CALCULATION:

Weight of empty aluminum boat = _____ (g) **A**

Initial weight of boat + wet soil = _____ (g) **B**

Grams Wet Weight utilized = _____ (g) **B-A=C**
Date/Time In: _____ Drying Temperature (°C): _____
Date/Time Out: _____ Drying Time (min. 24 hrs): _____

Final Weight of boat + dried soil = _____ (g) **D**

Weight of water lost = _____ (g) **B-D=E**

Moisture content = _____ % **(E/C) x 100**

WATER-HOLDING-CAPACITY (WHC) CALCULATION:

Grams Dry Weight Utilized = 100.17 (g) **F**

Empty 500-ml glass beaker = 170.04 (g) **G**

500-ml beaker + soil = 270.21 (g) **F+G**

Empty 500-ml Erlenmeyer flask = 164.79 (g) **H**

Flask + funnel + hydrated filter paper = 481.15 (g) **I**

Funnel + hydrated filter paper = 316.36 (g) **I-H=J**

Initial weight (funnel, soil, wet filter paper) = 416.53 (g) **F+J=K**
Drainage Date/Time started: 10-19-94/09:10 am
Drainage Date/Time completed: 10-19-94/01:10 pm
Total Draining Time (3-24 hrs): 4 hrs

Weight of second 500-ml flask = 163.82 (g) **L**

Weight of flask + wet soil + funnel
+ hydrated filter paper = 641.15 (g) **M**

Final weight (soil, funnel, hydrated filter paper) = 477.33 (g) **M-L=N**

Water held = Final weight - Initial weight = 60.80 (ml) **N-K**

WHC = [amt of water held (N-K)] [ideal wt utilized, 100.0 g soil]
[amt of wt utilized (F)]

WHC = 60.7 ml water/100 g dry weight soil