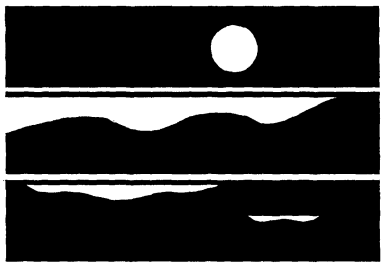


# Early Seedling Growth Protocol for Soil Toxicity Screening

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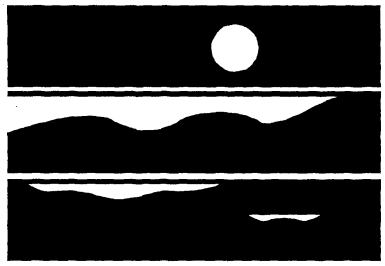


# Early Seedling Growth Protocol for Soil Toxicity Screening

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# Table of Contents

Appendices

# Summary of Test Conditions for Early Seedling Growth Bioassay

| | |
|---|---|
| Test type: | 14 Day |
| Temperature: | 20 - 30°C |
| Photoperiod: | 16-hours light/8-hours darkness, using a light intensity of 100 $u$Em$^{-2}$s$^{-1}$ (measured at a distance equal to the top of the test container from the light source) |
| Test container: | Any container inert to the test materials with a height to width ratio between 1:1 and 2:1, an approximate volume of 400 - 500ml, and at least four drainage holes (≥5mm ID) in the bottom. |
| Total amount (soil weight)/sample: | 1000g |
| Test amount/replicate: | 300g |
| Soil moisture content: | Field capacity |
| Test Organism: | Butter crunch lettuce (*Lactuca sativa*). Perennial ryegrass (*Lolium perenne)* is substituted if pH 8.5 - 10 |
| Number of seeds/replicate: | 20 |
| Number of replicates/sample: | 3 |
| Soil pH: | ≥5.0 to ≤10, do not adjust. If pH outside range, do not run test |
| Endpoint measured: | Survival, biomass |
| Positive control: | Boric acid |
| Negative control: | Artificial soil |

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- Andrea J. LaTier and Wayne G. Landis, Huxley College of Environmental Studies, Western Washington University, Bellingham, WA.

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- Greg Linder, Oregon State University, Corvallis, OR.

- Larry Kapustka, Joe Garrison, and Karen Stephenson, Ecological Planning and Toxicology, Inc., Corvallis, OR.

- Nigel Blakley and Joan LeTourneau, Washington State Department of Ecology, Olympia, WA.

# 1. Introduction

## 1.1 Application and Background

Plant bioassays that focus on early seedling survival and growth are widely used for phytotoxicity testing at hazardous waste sites. Krawczyk *et al.* (1991) describes an application of the plant growth test to assess the phytotoxicity of contaminated soils at a superfund site. Other applications of early seedling growth and survival testing to hazardous waste site soils have also been described by the following investigators (Nwosu *et al.*, 1991; Linder *et al.*, 1990; Greene *et al.*, 1988; Gorsuch, *et al.*, 1991; Miller *et al.*, 1985; Thomas and Cline, 1985; Thomas *et al.*, 1986).

The Early Seedling Growth Protocol described in this document is intended to be used in screening-level assessments of soil toxicity at hazardous waste sites being investigated under the Washington Model Toxics Control Act (MTCA) Cleanup Regulation. The protocol has been adapted from procedures developed by the United States Environmental Protection Agency (USEPA) for conducting the lettuce seed germination test (Greene *et al.*, 1988). Briefly, the procedure involves germinating and growing lettuce seeds in field-collected site soils. No dilution series is required and no nutrients are added during the test duration. After 14 days the number of seedlings surviving are counted and the above ground biomass is harvested, dried and weighed.

## 1.2 Health and Safety

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

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

# 2. Sample Requirements

## 2.1 Sample Size

Approximately 1000g of soil per sample is required to perform this test. Each sample is tested using three replicates (a replicate is defined as one 300g aliquot of the bulk sample), for a total of 900g per sample. The remainder of the bulk sample (approximately 100g) is available for ancillary determinations such as pH. If chemical characterization of the soil, beyond the parameters listed in this protocol, is to be performed in conjunction with the toxicity testing, additional soil must be collected. At the time of collection, sample containers should be filled completely to minimize headspace.

## 2.2 Sample Storage

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

# 3. Supplies

## 3.1 Equipment

The following equipment is required to perform the early seedling growth test:

- Wire mesh screens for sizing seeds (fractions of an inch): 1/6x1/28, 1/6x1/30, 1/6x1/32, and 1/6x1/3
- Forceps
- pH meter
- Weighing/drying vessels for seedlings (e.g., watch glasses, crystallizing dishes, etc)
- Wash bottles for rinsing glassware and probes
- Environmental chamber capable of maintaining temperature at 20 - 30°C and minimum of 100 micro-Einsteins ($u Em^{-2}s^{-1}$) of light (measured at a distance equal to the top of the test container from the light source) operating on a clock timer to control photoperiod (16-hours light/8-hours darkness)
- Photometer capable of measuring the visible spectrum
- Water purification system that produces Type II water
- Top loading balance, capable of weighing soil sample to 1.0g; and analytical balance, capable of weighing seedlings to the nearest 0.001g
- Reference weights, Class S, for checking performance of balance. Weights should bracket the expected weights of the weighing pans as well as the expected weights of the pans plus samples
- Test containers, any container inert to the test substances (glass, stainless steel, polyethylene, or equivalent) with a volume of approximately 400 - 500 ml, a height to width ratio between 1:1 and 2:1, and a minimum of four drainage holes (≥5mm ID) in the bottom
- Volumetric flasks and graduated cylinders, Class A, borosilicate glass or non-toxic plastic labware, 10 to 1000ml

- Volumetric pipettes, Class A, 1 to 100ml
- Serological pipettes, 1 to 10 ml. graduated
- Syringe or other measuring device for preparation of positive controls
- Sharp knife or blade for harvesting seedlings
- Pipette bulbs and fillers
- Bulb thermograph or electronic-chart type thermometers for continuous recording of temperature. Automated electronic data collection systems designed to monitor chamber conditions are also acceptable and in fact preferred
- Glass thermometer or thermal probe

## 3.2 Materials

The following materials are required to perform the early seedling growth test:

### Soil Hydration Water

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

### pH Buffers

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

### Reference Toxicant

The reference toxicant is boric acid.

### Artificial Soil

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

## 3.3 Test Organisms

### Lettuce

The early seedling growth test described in this protocol should be conducted using butter crunch lettuce (*Lactuca sativa*) (Wang, 1987; Miller *et al.*, 1985). Lettuce has been used extensively in phytotoxicity evaluations and, where comparisons have been made, is often among the most sensitive plants to toxic chemicals (Linder *et al.*, 1990; Gorsuch *et al.*, 1990; Miller *et al.*, 1985). However, the pH of the test soil may indicate the use of an alternative species.

### Ryegrass

When the pH of the test soil is in the range of 8.5 to 10.0, perennial ryegrass (*Lolium perenne*) should be substituted for lettuce.

No seedling test is recommended whenever the clay content of the soil is >30%. The primary reason being neither plant species would be expected to emerge at the acceptance level of ≥90% in the negative control.

Lettuce and perennial ryegrass seeds are available from commercial seed companies. Seeds should be purchased at least annually, preferably within one year from the date of determination of the seed germination response. A sufficient volume of seeds should be obtained to run an entire test with the same seed lot. Information on seed lot, year, or growing season in which they were collected, and germination percentage should be provided by the seed supplier. Only untreated (not treated with fungicide, repellents, etc.) seeds are acceptable for use in this test. If during any test the germination rate in the negative control is <90%, the test must be repeated using fresh seed.

After purchase, size-grading of seeds must be conducted on the entire seed lot. Samples are sized in small lots of 100-150 seeds. Additionally, the seed lot must be inspected to remove trash, empty hulls and damaged seed. To separate seeds by size, nest the four wire mesh screens, with the largest screen size on top and successively smaller screen sizes in sequence below. A blank or bottom pan is used to collect the fraction that passes through all screens.

Pour the seeds onto the top screen, then vigorously shake the entire set of nested screens until all the seeds remain on one screen or reach the bottom pan. The separated fractions

are set aside and retained. This procedure is repeated until all the seed in the lot has been sized. The size class containing the most seed is used for testing. The seeds from each size class are divided into small lots, placed in separate envelopes or bags which have been labeled with seed type, seed size, lot number, and year of growing season collected. The bags are then stored in airtight, waterproof containers in a refrigerator at 4°C.

# 4. Preparation of Artificial Soil

## 4.1 Ingredients

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

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

Once the materials are combined together, mix thoroughly, and add an amount of calcium carbonate ($CaCO_3$) equal to 0.40% of the combined total weight. This should adjust the pH of the mixture to 7.0 ± 0.5. Check the pH of an aliquot using the procedure given in Section 5.3. Additional calcium carbonate may be added to the mixture if needed to adjust the pH to 7.0± 0.5.

# 5. Reference Toxicant Testing (Boric Acid)

## 5.1 Preparation of Positive Controls

Reference toxicant testing can be conducted either separately or concurrently with each new batch of samples analyzed. However, it is recommended that acceptable seedling response be established prior to actually testing samples. Boric acid is the recommended reference toxicant. The following concentrations (concentration of boric acid in solution added) are recommended for the positive control series: 40ppm, 80ppm, 160ppm, 320ppm, and 640ppm (Kaputska, 1996). Artificial soil is used as the test media for the positive controls.

The following procedure is used to prepare 1 liter of a 640ppm boric acid stock solution. Sufficient volume of the boric acid solution should be prepared to treat all test containers in the positive control series.

1) Using a graduated cylinder, measure 750ml of deionized water into an Erlenmyer flask

2) Weigh 640mg of boric acid in a clean weighing vessel and add it to the Erlenmeyer flask

3) Add a magnetic stir bar and stir on a magnetic plate until all the boric acid has dissolved

4) Measure an additional 250ml of deionized water into the flask

5) Stir until mixed

6) Label container with contents and cover with parafilm until ready to use

Additional concentrations of the boric acid solution can be made by diluting the 640ppm stock solution. For example the 320ppm concentration would be prepared by adding equal amounts of the stock solution and deionized water.

After preparing the boric acid solutions, weigh the amount of artificial soil added to each test container. Place a watch glass under each container and add the boric acid solution to the watchglass, using a syringe or other measuring device to measure the amount added. Allow the container to wick up the solution from the base. Later, if desired, the concentration can be expressed as ppm boric acid in artificial soil. The following data are examples of results obtained using boric acid as a reference toxicant. The treatments are identified as ppm in the solution added, not as ppm in soil (Kapustka, 1996).

Examples of Boric Acid Positive Control Data using Butter Crunch Lettuce

| Endpoint | Treatment (ppm in solution) | Mean Response (%) | Standard Deviation (%) |
|---|---|---|---|
| Germination (mean) | 0 | 100 | 0 |
| | 160 | 88 | 12 |
| | 320 | 80 | 32 |
| | 640 | 71 | 37 |
| Wet Weight (mean) | 0 | 100 | 0 |
| | 160 | 89 | 19 |
| | 320 | 76 | 18 |
| | 640 | 53 | 14 |
| Dry Weight (mean) | 0 | 100 | 0 |
| | 160 | 89 | 24 |
| | 320 | 85 | 28 |
| | 640 | 58 | 11 |

## 5.2 Reference Toxicant Data Analysis

Report the concentrations of the positive controls on the laboratory data collection sheet. Calculate and report the LC50 and the 95% confidence limits for the positive control treatments and check the results graphically. If the Trimmed Spearman-Karber method is used, follow the methods described by Hamilton *et al.*, 1977. If the Probit technique is used, follow the methods described by Weber *et al.*, 1989. Any other statistical method(s) approved by EPA may be substituted for the trimmed Spearman-Karber or Probit techniques.

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# 6. Conducting the Test

## 6.1 Test Containers and Labeling

Any container that is inert to the test material (glass, stainless steel, polyethylene or equivalent) may be used for testing. The container selected should have a height to width ratio between 1:1 and 2:1, an approximate volume of 400 - 500ml, and a minimum of 4 drainage hole (≥5mm ID) in the bottom. Commercially produced, disposable test containers and covers may be used as received if pre-testing demonstrates no significant contamination is present that would impact the test species. If non-disposable containers are used, the containers and lids should be decontaminated with hot water/laboratory grade detergent, rinsed, and allowed to dry. If it is not possible to remove visible contamination the container should be discarded. All containers should be labeled on the side with at least the following information: sample #, replicate #, and date of test initiation.

## 6.2 Filling Test Containers

Physical sorting to remove non-soil debris (e.g., sticks, twigs, pea stone chunks) may be necessary in some situations. However, soil samples should not be screened or mixed.

Working under a fume hood, place the appropriate amount of soil (300g) into individual test containers using a spatula or scoop. Measure a single initial temperature (using the procedure described below) in one container. This procedure assumes that all soils have been held together long enough for temperature equilibration to have occurred. If a thermal probe is used to record soil temperatures (after seeds have been planted), be careful not to damage or disturb seedlings. The temperature of the test soils should be within the range of 20 - 30°C.

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

2) If the initial temperature is outside the range of 20 - 30°C, place the test soils in the environmental chamber for 1 hour to equilibrate to the test range.

3) Recheck the temperature

## 6.3 Measurement of Soil pH

To measure soil pH, make a slurry of dilution water and soil on a 1:1 ratio (1ml water = 1g). Combine 25g of soil and 25ml of hydration water in a 100ml beaker. For soils high in clay content, an additional 25ml of hydration water may need to be added so that a

mixable slurry will be produced. Mix the slurry with a magnetic stir bar on a magnetic stir plate for 5 min, then measure and record the pH. Allow the slurry to settle for 30 min and recheck and record the pH of the supernatant.

## 6.4 Positioning Seeds in Test Containers

To assure equal spacing of test plants, a template made of stainless steel or wood may be used to make seed planting holes. A carefully spaced, 5 x 4 grid pattern may also be developed utilizing a small wooden dowel or stainless steel instrument. Planting holes should be a maximum of 5mm in depth with one seed deposited in each of the 20 holes. All seeds should be at least 1cm from the edge of the pot to ensure they will be covered with the test soil. After the seeds have been placed in the holes, tap the test container lightly to cover the seeds, being careful not to cover the seeds with more than about 2.5mm of soil (Garrison, 1996). A rule of thumb is that the depth of planting should not exceed 1.5 - 2 times the seed diameter.

After planting the seeds and hydrating the samples, place a clear cover over the top of the test container to help prevent moisture loss until the seedlings emerge (usually 3-5 days). Randomize positions of the test containers in the environmental chamber. After emergence of plants to a height of 5 - 8mm, remove the clear covers.

The test is conducted at 20 - 30°C with a photoperiod of 16 hours light/8 hours darkness (light intensity 100 $u Em^{-2}s^{-1}$ measured at a distance equal to the top of the test container from the light source). Plant-grow lights are recommended as the lighting source to prevent spindly seedlings (Gorsuch, 1993). Do not allow direct sunlight to reach the seedlings for the duration of the test. Record the starting time of the test on the data collection sheet.

## 6.5 Hydration of Test Soils

Plants grow best in soil that is saturated to field capacity. Field capacity is achieved in each replicate by watering from the top with a low-flow nozzle until water emerges through the drain holes in the bottom of the container. It is recommended that hydration of the test soils be performed after the seeds have been planted (See section 7.4 for seed planting procedure).

## 6.6 Negative Control

Use artificial soil without the addition of any toxicant for the negative control. Three replicates of the negative control are required. Press twenty seeds into each replicate using the placement technique described in Section 7.4, then hydrate to field capacity. An acceptable test must have a minimum of $\geq$90% germination in the negative control.

# 7. Test Monitoring

## 7.1 Monitoring of Test Conditions

No plant observations are required during the test. However, the following measurements of test conditions should be recorded:

- The pH of the test soils should be measured at the beginning and end of the test. For the test termination pH measurements, determine the pH in one replicate of each sample tested including the positive and negative controls. After harvesting the seedlings, use the procedure described in section 7.3 to determine the pH of the soil.

- Minimums and maximums of ambient temperature are measured each 24 hours during the test

- Light intensity is measured each 7 days during the test. Measure light intensity at a distance equal to the top of the test container from the light source.

- Document the replacement of any light bulbs (type, number, frequency and location)

## 7.2 Watering Schedule

During the testing period the seedlings should be watered daily to field capacity. It is recommended that tempered (20 - 30°C) water be added from the top using a low-flow regulated nozzle until water emerges through the drain holes. While it is not necessary to determine the exact amount of water added to each container on a daily basis, a record of how often watering was conducted should be maintained.

## 7.3 Seedling Survival

At test termination, count the number of seedlings that survived the test in each replicate and record the number on the data collection sheet. Also record the presence of any sublethal effects such as chlorosis/discoloration, mottling, desiccation, or/and wilting on each seedling.

## 7.4 Biomass Determinations

At test termination, determine biomass by harvesting the above-ground portion of the seedlings from each replicate and determine the combined dry weight of all surviving seedlings per replicate. Seedling removal can be accomplished by cutting the above-ground portion of the plant at the soil interface with a sharp knife or other blade.

Place all the seedlings from each replicate in a previously tared and labeled vessel and weigh them to obtain an initial wet weight. To determine a moisture reduction factor for an entire days replicates to be weighed, randomly select 10% of the replicates for a dry weight determination. To determine dry weight, place the vessel containing the seedlings in a drying oven set at 70±5°C for 24 hours. Monitor the oven temperature to avoid scorching or charring the plants. After 24 hours, place the dried plants in a desiccator to cool, and then re-weigh them. Replace the sample in the drying oven for an additional 2 hours. Again, place the sample in the desiccator to cool and re-weigh the sample. If the weight is the same, record the weight as the final dry weight. If not, repeat the drying and re-weighing on a 2-hour interval until there is no longer a decrease in sample weight. Record both the total replicate and seedling/replicate (divide the total replicate biomass by the number of surviving seedlings) dried weight on the data sheet. The average percent moisture for the 10% of the replicates tested is used to calculate the final dry weight for the entire days seedlings harvested.

The biomass determination is only performed if lettuce seedlings are being used. No biomass determination is performed if perennial ryegrass is being used as the test species.

# 8. Data Analysis and Reporting

## 8.1 Cover Sheet

The following is a suggested format for recording relevant information about the test. Other formats are acceptable for reporting purposes, provided the same information is included.

The cover sheet should contain specific information regarding the test including, but not limited to, the following (see Appendix B for example):

- Name of site where the soil was collected and other identifying information
- Date and time the test is started
- Name of person performing the test
- pH value of site soil as received
- Any problems with or deviations from the protocol

## 8.2 Data Report

The final report should include all data collection, calculation, and observation sheets. The observation sheets should include all data obtained during the test that are suggestive of toxicity. If not provided elsewhere, also include the following information:

### Data Handling

Calculate the percent survival for each replicate of the test and control samples at the end of the 14-day period and enter the results on the data collection sheet. Also report the average survival for each test sample and each control across replicates. Record any morphological changes on seedlings that were observed. Report the wet and dry weight of the biomass, with standard deviation and sample size (number of plants weighed) for each replicate, as well as across replicates for each sample.

### Statistical Analysis

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

## Control Charts

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

## Test Organisms

Detailed information about the species of plant chosen should be recorded including seed lot, seed size, year or growing season in which they were collected, and germination percentage. The number of seeds used, as well as the number of seeds germinating in the negative control and experimental treatments, should also be recorded.

## Test Conditions

Describe the test conditions especially if there was any deviation from this document in soil preparation, addition of chemicals, lighting, temperature, replicates or the number of replicates per treatment. Record all chemical analyses of the test material if performed including methods, method validations and reagent blanks. Record the temperature and pH of the soil samples at the beginning and end of the test.

## Test Containers

Describe the test container used, its size, volume and weight of soil used in each container, number of test containers per concentration and preparation of the test containers.

## Reporting of Protocol Deviations and Problems

Provide a description about any deviations from the direction provided in this document or anything unusual encountered in the test (e.g. equipment failure, fluctuations in temperature or other environmental conditions).

# 9. Quality Assurance/Quality Control (QA/QC)

## 9.1 Requirements and Specifications

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

### Handling of Soil Sample

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

### Facilities, Equipment and Test Chambers

Laboratory temperature control equipment must be adequate to maintain the required temperature (20 - 30°C) throughout the test.

### Analytical Methods

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

### Calibration and Standardization

Instruments used for routine chemical and physical parameters, such as pH or temperature, must be calibrated and standardized according to the instrument manufacturer's procedures.

### Test Conditions

Soil and air temperatures must be maintained within the limits specified for the test. The pH of the soils must be checked at the beginning and end of the test in one replicate of each sample and one replicate of the controls. The ambient air temperature must, at a minimum, be measured every 24 hours throughout the test duration.

## Hydration Water

The hydration water used in this procedure must, at a minimum, meet the specifications for Type II water (see Appendix A).

## Quality of Test Species

Butter crunch lettuce (*Lactuca sativa)* is the test species used in this protocol. Perennial Ryegrass (*Lolium perenne*) should be substituted if the pH of the test soil is between 8.5 - 10. Since a laboratory will not have an ongoing culturing program for seeds and obtains them from an outside source, the sensitivity of each batch of seeds obtained from outside sources must be evaluated. Evaluations must be performed with a reference toxicant in a positive control series.

## Test Acceptability

For the test to be acceptable, mean germination in the negative control must be at least $\geq$90%. An individual test may be conditionally acceptable if temperature and other specified conditions fall outside specifications, depending on the degree of the variation and the objectives of the test. Deviations from test specifications must be noted when reporting data from the test.

## Precision

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

## Replication and Test Sensitivity

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

## Control Charts

Prepare a control chart for the reference toxicant by plotting successive toxicity values (LC50) and examining the results to determine whether they are within the prescribed limits. The mean and upper and lower control limits ($\pm$ 25%) are recalculated with each successive point until the statistics stabilize. Maintain a running plot for the toxicity values

of successive tests with the reference toxicant. For further details, see Weber *et al.* (1989) or Greene *et al.* (1988).

If the LC50 from a given test with the reference toxicant does not fall in the expected range for the plant species, then the sensitivity of the species and the overall credibility of the test system are suspect. In this case, the test procedure should be examined for defects and repeated with a different batch of seeds.

## Record Keeping

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

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# Appendices

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# Appendix A: Specifications* for Type II Water

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

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

# Appendix B: Laboratory Data Collection Cover Sheet

Name of Field Site _____

Date of Field Sample Collection _____

Chemical Analysis Results of Soil Sample:

Identified Contaminants _____

_____

_____

Suspected Contaminants _____

_____

_____

Laboratory Test Initiation Date _____ Time _____

Laboratory Test Termination Date _____ Time _____

Name of Laboratory Technician Conducting Test _____

pH of Sample Soil: Slurry start_____ end_____

Supernatant start_____ end_____

Deviations/Problems with Protocol (list and describe below):

_____

_____

_____

_____

_____

_____

# Appendix C: Sample Laboratory Test Monitoring Data Sheet

| Lab ID No. | Replicate No. | Soil Slurry pH start (s.u.) | Soil Supernatent pH start (s.u.) | Soil Slurry pH Day-14 (s.u.) | Soil Superantent pH Day-14 (s.u.) | No. of seeds planted start | No. of seedlings emerging | No. of live seedlings day-14 | Percent Survival | Wet Biomass (g) | Dry Biomass total replicate (g) | Sublethal effects | Total seedlings with sublethal effects (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
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| | | | | | | | | | | | | | |

Comments _____

_____

Light Intensity: Start= _____ Day 7= _____ Day 14= _____

# Appendix C: Sample Laboratory Test Monitoring Data Sheet (continued))

Daily Temperature (DT), Watering Performed (WP)

| Day | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| DT (EC) | | | | | | | | | | | | | | |
| WP (yes/no) | | | | | | | | | | | | | | |