TOXICITY ASSESSMENTS OF ANTIMONY, BARIUM, BERYLLIUM, AND MANGANESE FOR DEVELOPMENT OF ECOLOGICAL SOIL SCREENING LEVELS (ECO-SSL) USING EARTHWORM (EISENIA FETIDA) BENCHMARK VALUES

Michael Simini
Ronald T. Checkai
Roman G. Kuperman
Carlton T. Phillips

RESEARCH AND TECHNOLOGY DIRECTORATE

Jason A. Speicher
David J. Barcliff

NAVAL FACILITIES ENGINEERING COMMAND
Lester, PA 19113-2090

November 2002

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Aberdeen Proving Ground, MD 21010-5424
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### 1. REPORT DATE (DD-MM-YYYY)
XX-11-2002

### 2. REPORT TYPE
Final

### 3. DATES COVERED (From - To)
Feb 2000-Sep 2002

### 4. TITLE AND SUBTITLE
Toxicity Assessments of Antimony, Barium, Beryllium, and Manganese for Development of Ecological Soil Screening Levels (Eco-SSL) Using Earthworm (*Eisenia fetida*) Benchmark Values

### 6. AUTHOR(S)
Simini, Michael; Checkai, Ronald T.; Kuperman, Roman G.; Phillips, Carlton, T. (ECBC); Speicher, Jason A.; and Barclift, David J. (EFANE)

### 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) AND ADDRESS(ES)
DIR, ECBC, ATTN: AMSRD-ECB-RT-TE, APG, MD 21010-5424
CO, NAVAFC, EFANE, 10 Industrial Highway, MS #82, Lester, PA 19113-2090

### 9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
CO, NAVAFC, EFANE, 10 Industrial Highway, MS #82, Lester, PA 19113-2090

### 12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

### 14. ABSTRACT
The U.S. Environmental Protection Agency (USEPA), in a collaborative effort with other Federal agencies, states, and private industry, is developing Ecological Soil Screening Levels (Eco-SSLs) for ecological risk assessment of contaminants at Superfund sites. Earthworm (*Eisenia fetida*) cocoon production and survival tests were conducted in a Sassafras sandy loam soil that supports relatively high bioavailability of barium (Ba), beryllium (Be), manganese (Mn), and antimony (Sb). For the metals tested, cocoon production was a more sensitive endpoint than was survival. Bounded Lowest Observed Effect Concentrations (LOECs) (mg kg\(^{-1}\)) for cocoon production, as determined by analysis of variance (ANOVA), were 83, 86, 433, and 1236 for Be, Sb, Ba, and Mn, as compared to LOECs for survival of 110, 697, 1585, and 2222, respectively. Bounded No Observed Effect Concentrations (NOECs) (mg kg\(^{-1}\)) for cocoon production, as determined by ANOVA, were 57, 60, 258, and 1111 for Be, Sb, Ba, and Mn, as compared to NOECs for survival of 83, 617, 1348, and 1444, respectively. Non-linear regression analysis of cocoon production data showed that the relative toxicity (EC\(_{20}\) mg kg\(^{-1}\)) of the four metals was in the order of Sb (30) > Be (52) > Ba (370) > Mn (629). These results will be submitted to the Eco-SSL Workgroup for review and inclusion in their database.

### 19a. NAME OF RESPONSIBLE PERSON
Sandra J. Johnson

### 19b. TELEPHONE NUMBER (include area code)
(410) 436-2914
PREFACE

The work described in this report was authorized under Sales Order No. 9KNM22. The work was started in February 2000 and completed in September 2002.

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Acknowledgments

This project was completed in cooperation with and from funding provided by the Engineering Field Activity Northeast (EFANE), Naval Facilities Engineering Command, Lester, PA.

The authors thank Stephen J. Ells for support and assistance, and acknowledge the Ecological Soil Screening Level National Program, administered under the auspices of the Office of Solid Waste and Emergency Response (OSWER), U.S. Environmental Protection Agency (USEPA), Washington, D.C.
# CONTENTS

1. INTRODUCTION ........................................................................................................... 7

2. MATERIALS AND METHODS ..................................................................................... 8
   2.1 Soil Collection and Characterization ................................................................... 8
   2.2 Earthworm Culture ............................................................................................... 8
   2.3 Chemicals and Reagents ...................................................................................... 9
   2.4 Soil Amendment Procedure ................................................................................ 9
   2.5 Aging/Weathering of Amended Soil ................................................................... 9
   2.6 Chemical Extraction and Analyses ...................................................................... 10
   2.7 Toxicity Assessment ............................................................................................ 10
   2.7.1 Earthworm Survival Test ................................................................................. 10
   2.7.1.1 Principle of the Test .................................................................................. 10
   2.7.1.2 Validity of the Test ................................................................................... 11
   2.7.1.3 Test Conditions ....................................................................................... 11
   2.7.1.4 Endpoint Determination .......................................................................... 11
   2.7.2 Earthworm Reproduction Test ....................................................................... 11
   2.7.2.1 Endpoint Determination .......................................................................... 12
   2.7.2.2 Validity of Test ....................................................................................... 12
   2.8 Selection of Metal Concentrations in the Soil ...................................................... 12
   2.9 Data Analysis ...................................................................................................... 13

3. RESULTS ...................................................................................................................... 13
   3.1 Soil Analysis ....................................................................................................... 13
   3.2 Earthworm Toxicity Tests .................................................................................. 14
   3.2.1 Barium .......................................................................................................... 14
   3.2.2 Beryllium ...................................................................................................... 17
   3.2.3 Manganese .................................................................................................... 17
   3.2.4 Antimony ....................................................................................................... 21

4. DISCUSSION ............................................................................................................... 21

5. CONCLUSIONS ......................................................................................................... 24

LITERATURE CITED ................................................................................................... 27
APPENDIXES

A - RANGE-FINDING TEST DATA ..................................................31

B - DEFINITIVE TEST DATA..........................................................35

C - STATISTICAL ANALYSIS OF TOXICITY TEST DATA..............43

FIGURES

1. Concentration-response curves for four metals vs. cocoon production in *Eisenia fetida* reproduction tests using Gompertz logistical model
   \[ Y = a \times e^{(\log(1-p)) \times (C/ECP) - b} \] ..................................................19

2. *Eisenia fetida* adult survival vs. soil concentration of barium, beryllium, manganese, and antimony ........................................20

TABLES

1. Physical and chemical characteristics of Sassafras sandy loam soil.........................8

2. Nominal Ba, Be, Mn, and Sb concentrations selected for definitive toxicity studies with *E. fetida*.................................................................12

3. Nominal and measured concentrations of metals in soil ....................................15

4. Initial total manganese concentrations and exchangeable manganese fractions during 18-week aging/weathering study using SSL soil amended with manganese sulfate ..............................................16

5. Summary of soil pH data ........................................................................16

6. Ecotoxicological parameters associated with exposure of *E. fetida*
to barium (Ba), beryllium (Be), manganese (Mn), and antimony (Sb)........18
TOXICITY ASSESSMENTS OF ANTIMONY, BARIUM, BERYLLIUM, AND MANGANESE FOR DEVELOPMENT OF ECOLOGICAL SOIL SCREENING LEVELS (ECO-SSL) USING EARTHWORM (EISENIA FETIDA) BENCHMARK VALUES

1. INTRODUCTION

The U.S. Environmental Protection Agency (USEPA) is developing Ecological Soil Screening Levels (Eco-SSLs) for ecological risk assessment of contaminants at Superfund sites. Eco-SSLs are soil concentrations of chemicals which, when not exceeded, will theoretically protect terrestrial ecosystems from unacceptable harmful effects. They are derived using data generated from laboratory toxicity tests with different test organisms, which represent the vast array of ecological receptors. Whenever sufficient quantity and quality of information existed, Eco-SSLs for soil invertebrates were developed from studies reported in literature. However, insufficient information to generate Eco-SSLs for barium (Ba), beryllium (Be), Manganese, (Mn), and antimony (Sb) necessitated standardized toxicity testing to fill the data gaps.

This study was designed to produce benchmark data for the development of Eco-SSLs for Ba, Be, Mn and Sb for soil invertebrates, and meet specific criteria (USEPA, 2000), including: (1) tests were conducted in soil having physico-chemical characteristics that support relatively high bioavailability of metals; (2) experimental designs for laboratory studies were documented and appropriate; (3) both nominal and analytically determined concentrations of chemicals of interest were reported; (4) tests included both negative and positive controls; (5) chronic or life cycle tests were used; (6) appropriate chemical dosing procedures were reported; (7) concentration-response relationships were reported; (8) statistical tests used to calculate the benchmark and level of significance were described; and (9) the origin of test species were specified and appropriate.

Several soil invertebrate toxicity tests, for which standardized protocols have been developed, can effectively be used to assess the toxicity and to derive protective benchmark values for metals (Stephenson et al., 2001; Lekke and Van Gestel, 1998). We used the Earthworm Survival Test (Greene, et al., 1989) and the Earthworm Reproduction Test (ISO 1998; Van Gestel et al., 1989) in this study. These tests were selected on the basis of their ability to measure chemical toxicity to ecologically relevant species. The reproduction test was used to meet the Eco-SSL requirement of inclusion of at least one reproductive component among the measurement endpoints. The survival test was included to compare lethal and non-lethal toxicity.

Special consideration in assessing chemical toxicity for Eco-SSL development was given to the effects of aging/weathering of soil contaminants on the exposure of relevant ecological receptors, as commonly occurs at Superfund sites. During chemical aging/weathering in soil, reduction in the exposure to the chemical may occur due to volatilization, microbial degradation and immobilization, or other fate processes (e.g., photodecomposition, hydrolysis, and hysteresis, etc.). This can result in a dramatic reduction in the amount of chemical that is
bioavailable, compared to tests conducted with freshly-amended chemicals or those tested following a short equilibration period (e.g., 24 h). Standardized methods for aging/weathering of chemicals in soil are not available. We used the approach developed to simulate at least partially, the aging and weathering process that included exposing soils amended with chemicals to periodic alternating wetting and air-drying cycles for 3 weeks, in a green house.

2. MATERIALS AND METHODS

2.1 Soil Collection and Characterization.

The soil used in these studies was Sassafras sandy loam [Fine-loamy, siliceous, mesic Typic Hapludult] (SSL) collected from a grassy field (M-Field) at Aberdeen Proving Ground, MD. Vegetation and the organic matter horizon were removed and the top six inches of the A horizon were then collected. The soil was sieved through a 5mm² mesh screen, air-dried for at least 72 h and mixed periodically to ensure uniform drying, passed through a 2-mm sieve, then stored at room temperature before use in testing. Soil was then analyzed for physical and chemical characteristics by the Cooperative Extension Service, University of Maryland Soil Testing Laboratory, College Park, MD. Results of these analyses are presented in Table 1.

<table>
<thead>
<tr>
<th>Soil Parameter</th>
<th>Sassafras Sandy Loam</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sand %</td>
<td>71</td>
</tr>
<tr>
<td>Silt %</td>
<td>18</td>
</tr>
<tr>
<td>Clay %</td>
<td>11</td>
</tr>
<tr>
<td>Texture %</td>
<td>Sandy loam</td>
</tr>
<tr>
<td>CEC cmol kg⁻¹</td>
<td>4.27</td>
</tr>
<tr>
<td>Organic matter %</td>
<td>1.2</td>
</tr>
<tr>
<td>pH</td>
<td>5.0</td>
</tr>
</tbody>
</table>

2.2 Earthworm Culture.

Earthworms (E. fetida) were bred in plastic containers filled with approximately 14 kg of a 1:1 mixture of sphagnum PRO-GRO peat moss (Gulf Island Peat Moss Co., PEL, Canada) and BACCTO® potting soil (Michigan Peat Co., Houston, TX, USA). The pH was adjusted to 6.26 ± 0.07 by adding calcium carbonate (pulverized lime). The culture was kept moist at 21±2°C with continuous light. Earthworm colonies were fed biweekly with dehydrated alfalfa pellets (27% fiber, 17% protein, 1.5% fat; OB of PA, York, PA) that were fermented, dried, and ground to a course powder. Cultures were synchronized so that all worms used in each test were approximately the same age. Adult worms, 0.3g to 0.6g, with fully developed clitella were used for testing.
2.3 Chemicals and Reagents.

Assessments were performed using sulfate salts, including BaSO₄ (CAS #7727-43-7, 97%; stock #13928; lot #11020, Alfa Aesar), BeSO₄·4H₂O (CAS #7787-56-6, 99.99%; stock #16104; lot #H09107, Alfa Aesar), MnSO₄·H₂O (CAS #10034-96-5, ACS, 98.0-101.0%; stock #33341; lot #118129, Alfa Aesar), and Sb₂(SO₄)₃ (CAS #7446-32-4, 97%, stock #33492; lot #L21128, Alfa Aesar). Additional tests were done for Ba and Sb to determine how carrier salts and their solubilities affect the toxicity to E. fetida. For Ba, these compounds included BaO (CAS #1304-28-5, 97%, lot #121011, Aldrich Chemical Company), Ba(NO₃)₂ (CAS #10022-31-8, ACS, lot #000420, Fisher Scientific Co.), and Ba(C₂H₃O₂)₂ (CAS #543-80-6, ACS, lot #995963, Fisher Scientific Co.). For Sb, we used Sb D-tartrate Sb₂(C₄H₆O₆)₃·6H₂O (CAS #126506-93-2, lot #111004-2, Pfaltz & Bauer). The positive control used in this study was 4-Nitrophenol (CAS #100-02-7, 98%, lot #6623HE, Aldrich). The main carrier salt control was sulfate as CaSO₄·2H₂O (CAS #10101-41-4, ACS, lot #C01704, J.T. Baker). All reagents used in extraction of chemicals from soils were either reagent or trace metal grade and purchased from commercial suppliers. Purified water (ASTM type I; American Society of Testing and Materials, http://www.astm.org) obtained using Milli-RO® 10 Plus followed by Milli-Q® PF Plus systems (Millipore®, Bedford, MA) was used throughout the studies. Glassware was washed with phosphate-free detergent followed by rinses with tap water, Milli-RO® water, nitric acid 1% (v/v) and finally with Milli-Q® water.

2.4 Soil Amendment Procedure.

Treatment concentrations for toxicity tests with all sulfate salts and Ba oxide were prepared by adding test chemicals directly to SSL soil in appropriate proportions to achieve nominal target concentrations. Soil was mixed for 3 h on a three-dimensional rotary mixer. After mixing, soil was hydrated with purified water to 100% of the soil water holding capacity (WHC; 18% water, on the basis of the dry soil mass) for toxicity testing, or 60% of the WHC for the aging/weathering procedure (see Paragraph 2.5). Soil prepared for toxicity tests was allowed to equilibrate for 24 h before exposing earthworms. The exception was soil amended with Ba acetate, which was incubated for 5 days to allow for acetate degradation by soil microbes. Treatment concentrations of Ba(C₂H₃O₂)₂, Ba(NO₃)₂ and Sb₂(C₄H₆O₆)₃ were prepared by dissolving appropriate amounts of chemical in purified water, then hydrating pre-weighted amounts of SSL soil to achieve target treatment concentrations in soil for each chemical, respectively, at the required moisture level.

2.5 Aging/Weathering of Amended Soil.

All soil treatment concentrations and negative controls were subjected to simulated aging/weathering procedure, which included alternating wetting/air-drying cycles for 3 weeks prior to commencement of definitive tests. Aging/weathering of test soils was conducted in open plastic bags in the green house. Soil treatments were initially hydrated to 60% of water holding capacity (WHC), and then allowed to begin drying. All soil treatments were weighed and adjusted to 60% of WHC twice each week, and afterward brought to 100% of WHC (18% water, on the basis of the dry soil mass) for initiation of bioassays. A separate study was conducted using Mn as a model chemical to determine if the 3-week duration of
aging/weathering procedure was adequate. The duration of this study was 18 weeks. Nominal Mn treatment concentrations included 0, 10, 18, 31, 54, 94, 164, 287, and 503 mg kg\(^{-1}\). Samples from each treatment concentration were analyzed for exchangeable Mn concentrations at 3-week intervals to determine if increase in duration of aging/weathering procedure beyond 3 weeks affects exchangeable Mn concentrations (directly related to bioavailable Mn).

2.6 Chemical Extraction and Analyses.

Soil was analyzed for total metal concentrations following USEPA Method 200.8 (USEPA, 1994) using inductively coupled plasma mass spectrometry (ICP-MS). Additional analysis was done to determine exchangeable Mn fraction. Exchangeable Mn was extracted from soil using 0.05\(M\) CaCl\(_2\) with agitation on a reciprocating shaker for 24 h. All reagents used in extraction of chemicals from soils were either reagent or trace metal grade, and purified water was used throughout the analytical studies. Glassware was washed with phosphate-free detergent followed by rinses with tap water, purified water, nitric acid 1% (v/v), and finally again with purified water. Analyses of exchangeable Mn concentrations were conducted using a Perkin-Elmer 5100 PC Atomic Absorption Spectrophotometer equipped with an AS-90 autosampler.

2.7 Toxicity Assessment.

Two earthworm toxicity tests were used to determine either acute or chronic endpoints. The acute test used was a 14-day survival test adapted from Greene et al. (1989). The endpoint of this test is number of adult survivors. The chronic test used in this study was a 21-day reproduction test (ISO 1998; adapted from Van Gestel et al., 1989). The endpoint of this test is number of cocoons produced. Guidelines for these assays were originally developed for use with artificial soil (USEPA Standard Artificial Soil), however our research showed that these tests could also be successfully conducted using natural soils (Kuperman and Simini, 2004).

2.7.1 Earthworm Survival Test

2.7.1.1 Principle of the Test

Adult *E. fetida* are exposed to a range of concentrations of the test chemical added to soil. The test consists of two steps: first, a range-finding test in which adult survival is assessed using few treatment concentrations (five) and replicates (two); and second, a definitive test in which survival, live weight, and dry weight are assessed using a greater number of concentrations and replicates. The duration of each test is 2 weeks. The number of adult survivors in treated soils is compared to the number in the control(s) to quantify ecotoxicological parameters. These parameters include the No Observed Effect Concentration (NOEC), the Lowest Observed Effect Concentration (LOEC) and the effective concentration that causes an x percent reduction in adults, i.e., EC\(x\) (e.g. EC\(_{20}\), EC\(_{50}\)).
2.7.1.2 Validity of the Test

The validity criteria are included in the test as part of the Quality Control procedures. They include the following performance parameters for the negative controls:

1. The mean mortality does not exceed 10% at the end of the range-finding and definitive tests;

2. The coefficient of variation for the mean number of survivors is $\leq$30% at the end of the test.

2.7.1.3 Test Conditions

1. Earthworms were acclimated for 48 h in the test soil. Worms with fully developed clitella were selected for uniformity and purged overnight on moist filter paper.

2. Worms were rinsed twice with de-ionized water, blotted on paper towels, weighed on an analytical balance, and placed on the surface of soil in each of four 400-ml (9 cm diam), glass containers with screw caps. The worms were selected randomly for placement across treatments.

3. Plastic film was stretched over the top of the containers and secured with the screw caps. Three small holes were made in the wrap with a push-pin to allow for air exchange.

4. Worms were incubated under continuous light at $22\pm2^\circ\text{C}$ for 14 days.

2.7.1.4 Endpoint Determination

At the end of the study, the number of surviving earthworms in each beaker were counted and recorded. These procedures are summarized below:

1. Soil was emptied into a clean dry pan. The number of earthworm survivors per beaker was counted and recorded.

2. Treatment means were calculated and the data were analyzed by Analysis of Variance (ANOVA), and mean separation was performed using Fischer’s Least Significant Difference (LSD) test using $p<0.05$ criterion.

2.7.2 Earthworm Reproduction Test

At the start of the 21-day chronic reproduction assay, earthworms, soil, and beakers were prepared as described above, except that a 2 g bolus of alfalfa food (see Culturing Conditions) was added to each beaker. The soil was then hydrated to 95% of SSL water-holding capacity.
2.7.2.1 Endpoint Determination

After 21 days, surviving earthworms were counted and weighed as described above. Cocoons were recovered by gently agitating the soil on a 1-mm sieve with water until only the cocoons remained on the surface of the sieve. The number of cocoons per container (from five earthworms per treatment replicate) was recorded.

2.7.2.2 Validity of Test

(1) The mean mortality does not exceed 10% at the end of the range-finding and definitive tests;

(2) The coefficient of variation for the mean number of cocoons is ≤30% at the end of the test.

2.8 Selection of Metal Concentrations in the Soil.

Preliminary lethal (survival) and sublethal (cocoons production) toxicity tests (range-finding tests) were performed with each metal to determine the range of concentrations to use in the definitive toxicity tests. Five concentrations were used for each of the metals for both survival and cocoons production assays. The range of concentrations varied according to the type of test (adult survival or reproduction).

Data from the range finding tests were used to select the chemical compound containing each metal that was appropriate to use for definitive toxicity tests with *E. fetida*, and to determine treatment concentrations for definitive tests. Additional consideration in chemical form selection was given to chemical solubility in water and the effect that each chemical form had on soil pH. Concentrations selected and increment factors used to determine treatment concentrations for definitive tests are shown in Table 2.

Table 2. Nominal Ba, Be, Mn, and Sb concentrations selected for definitive toxicity studies with *E. fetida*.

<table>
<thead>
<tr>
<th>Chemical Toxicity Test</th>
<th>Ba Survival</th>
<th>Reproduction</th>
<th>Be Survival</th>
<th>Reproduction</th>
<th>Mn Survival</th>
<th>Reproduction</th>
<th>Sb Survival</th>
<th>Reproduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nominal Concentration (mg kg⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>800</td>
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<td>20</td>
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<td>245</td>
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<tr>
<td>944</td>
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<td>333.2</td>
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<tr>
<td>1830</td>
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<td>269</td>
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<td>1278</td>
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<td>2584</td>
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<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

12
Controls included positive (30 mg kg\(^{-1}\) 4-Nitrophenol), negative (no chemical added) and sulfate (CaSO\(_4\)). Sulfate controls were based on estimated sulfate amounts in highest treatment concentrations, and were 7,000 and 35,000 mg kg\(^{-1}\) SO\(_4^{2-}\). Four replicates were used for each treatment concentration and controls.

2.9 Data Analysis.

Nonlinear regression procedures were applied to the cocoon production data. Nonlinear models included EC\(_x\) as a parameter to determine the metal concentration producing a specified percentage reduction in juvenile production. These “x” parameters included EC\(_{20}\) and EC\(_{50}\) levels. The first parameter (EC\(_{20}\)) is the benchmark value preferred for Eco-SSL development for soil invertebrates, and the second (EC\(_{50}\)), more commonly used in the past, was included to enable comparisons of the results produced in this study with results reported by other researches. The asymptotic standard error (a.s.e.) and 95% confidence intervals (C.I.) associated with the point estimates were determined.

Reproduction data were analyzed using the Gompertz model \(Y = a \times e^{\left(\log(1-p) \cdot \left(\frac{C}{EC_{p}}\right)^{ab}\right)}\) described in Stephenson et al., 2001. Histograms of the residuals and stem-and-leaf graphs were examined to ensure that normality assumptions were met, although normality is not particularly important for these analyses as long as the data are approximately symmetrically distributed (Stephenson et al., 2001). Variances of the residuals were examined to decide whether or not to weight the data, and to select potential models. The Gompertz model had the best fit for all metal data. The fit of the line was closest to the data points, the variances were the smallest and the residuals had the best appearance (i.e., most random scattering).

Analysis of variance (ANOVA) was used to determine the bounded NOEC and LOEC values in both range finding tests and definitive tests. These analyses were applied to adult survival data, and to cocoon production data. Mean separations were done using Fisher’s LSD (Least Significant Difference) pairwise comparison tests. Significance level of \(P \leq 0.05\) was accepted for determining the NOEC and LOEC values. The appropriate tests for assumptions of the models were applied as described above before using either ANOVA or regression procedures. All analyses were performed using SYSTAT 7.0.1 (SPSS, Inc., 1997).

Raw data for range-finding and definitive tests were tabulated and are listed in Appendixes A and B, respectively. Detailed results of statistical analysis of toxicity test data are listed in Appendix C.

3. RESULTS

3.1 Soil Analysis.

Analysis of negative control soil showed that Be concentration in natural SSL soil used in this study was below the method the detection limit (MDL) of 2.5 mg kg\(^{-1}\). Total Be concentrations in the experimental treatments ranged from 95 to 124% and averaged 107% of nominal (Table 3).
The natural background Mn concentration determined in the negative control treatment was 94 mg kg\(^{-1}\). Total extractable Mn concentrations (in excess of background) in the experimental treatments ranged from 50 to 117% and averaged 94% of nominal (Table 3). Exchangeable Mn fraction expressed as percent of total concentration increased with increasing soil Mn loads (Table 4). There were no trends within any treatment concentration in the amount of exchangeable Mn fraction beyond 3 weeks during the 18-week aging/weathering study. These results confirmed that the 3-week duration for simulated aging/weathering procedure used in to the definitive study design was adequate for the Eco-SSL benchmark development.

Analytical procedures for Sb determination did not confirm agreement with the nominal treatment concentrations. Total Sb treatment concentrations determined using USEPA Method 200.8 ranged from 4 to 21% and averaged 8% of nominal concentration. These results showed that this standard method was not sufficient for total Sb analysis in SSL soil. Additional effort was made to improve the analytical procedure. Soils were digested using procedures described in SW-846 Method 3050B (USEPA, 1996). This improved the efficiency of Sb extraction, however it remained relatively low and averaged 58% of nominal concentration. For this reason, nominal Sb concentrations were used in determining ecotoxicological parameters for Sb; however because ERA relies on the determination of soil concentrations extracted from soil, toxicity parameters determined from nominal concentrations may have to be adjusted to 58% of their values before determining an Sb Eco-SSL to best conservatively-correspond to the level of Sb extracted from soil at specific levels of Sb toxicity in soil.

The SSL soil pH value of 5.29 was within the range of Eco-SSL’s soil matrix of properties that support high bioavailability of cationic metals in natural soils. Soil pH decreased consistently with increasing chemical loads but the decrease did not exceed one pH unit for Ba, Mn, and Sb treatments (Table 5). The decrease in the highest Be treatment was 1.46 pH units compared with untreated SSL soil (negative control). In the sulfate control, soil pH decreased by less than 1.0 pH unit in both 7000 and 35000 mg kg\(^{-1}\) SO\(_4^{2-}\) treatments compared with negative control.

3.2 Earthworm Toxicity Tests.

3.2.1 Barium

Range-finding tests for barium (Ba) were performed using Ba nitrate (Ba\(_2\)O\(_4\)). Nominal concentrations of Ba (w/w) in soil were 0, 100, 500, 1000, 5000, and 10000 mg kg\(^{-1}\). Results showed cocoon production was 67% of control at 100 mg kg\(^{-1}\), and 22% of control at 500 mg kg\(^{-1}\). No cocoons were produced above 500 mg kg\(^{-1}\).

ANOVA results of definitive cocoon production tests using actual measured concentrations of Ba (Table 3), produced a bounded NOEC of 258 mg kg\(^{-1}\) and a bounded LOEC of 433 mg kg\(^{-1}\) at P ≤ 0.05 (Table 6). Cocoon production was analyzed by nonlinear regression. The Gompertz nonlinear model produced best fit (Figure 1). The EC\(_{20}\) derived from this model for toxicity of Ba to E. fetida cocoon production was 370 mg kg\(^{-1}\) and the EC\(_{50}\) was 664 mg kg\(^{-1}\) (Table 6).
Results of the definitive adult survival tests, using actual measured concentrations of Ba (Table 3), showed a bounded NOEC of 1348 mg kg\(^{-1}\) and a bounded LOEC of 1585 mg kg\(^{-1}\) (Table 6).

Table 3. Nominal and measured concentrations of metals in soil.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Ba Concentration (mg kg(^{-1}))</th>
<th>Be Concentration (mg kg(^{-1}))</th>
<th>Mn Concentration (mg kg(^{-1}))</th>
<th>Sb Concentration (mg kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inherent</td>
<td>measured</td>
<td>measured</td>
<td>measured</td>
<td>measured</td>
</tr>
<tr>
<td>inherent</td>
<td>34</td>
<td>34</td>
<td>inherent</td>
<td>&lt;2.5</td>
</tr>
<tr>
<td>800</td>
<td>1000</td>
<td>112.5</td>
<td>153</td>
<td>50</td>
</tr>
<tr>
<td>944</td>
<td>1124</td>
<td>225</td>
<td>258</td>
<td>70</td>
</tr>
<tr>
<td>1114</td>
<td>1222</td>
<td>451</td>
<td>433</td>
<td>98</td>
</tr>
<tr>
<td>1314</td>
<td>1348</td>
<td>519</td>
<td>578</td>
<td>137</td>
</tr>
<tr>
<td>1551</td>
<td>1585</td>
<td>597</td>
<td>689</td>
<td>192</td>
</tr>
<tr>
<td>1830</td>
<td>2000</td>
<td>686</td>
<td>744</td>
<td>269</td>
</tr>
<tr>
<td>2160</td>
<td>2194</td>
<td>789</td>
<td>791</td>
<td>376</td>
</tr>
<tr>
<td>2584</td>
<td>2697</td>
<td>907</td>
<td>1000</td>
<td>1043</td>
</tr>
<tr>
<td>Mean % nominal</td>
<td>109</td>
<td>112</td>
<td>116</td>
<td>108</td>
</tr>
</tbody>
</table>

Several extraction methods were attempted to measure Sb levels in the soil. The best extraction efficiency was 58% of the nominal Sb concentration. Nominal Sb concentrations were used for statistical analyses.
Table 4. Initial total manganese concentrations and exchangeable manganese fractions during 18-week aging/weathering study using SSL soil amended with manganese sulfate.

<table>
<thead>
<tr>
<th>Nominal Mn treatment (mg kg⁻¹)</th>
<th>Exchangeable Mn fraction (% of total)</th>
<th>Treatment mean (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 3</td>
<td>Week 6</td>
</tr>
<tr>
<td>0</td>
<td>5.4</td>
<td>4.9</td>
</tr>
<tr>
<td>10</td>
<td>18.0</td>
<td>16.3</td>
</tr>
<tr>
<td>18</td>
<td>27.1</td>
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<td>37.3</td>
</tr>
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<td>54</td>
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<tr>
<td>94</td>
<td>85.8</td>
<td>75.9</td>
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<td>164</td>
<td>75.2</td>
<td>63.9</td>
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<td>287</td>
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<tr>
<td>503</td>
<td>127.3</td>
<td>99.8</td>
</tr>
</tbody>
</table>

Table 5. Summary of soil pH data. Values were determined following a 3-week aging/weathering procedure in studies of nominal beryllium, manganese, antimony, and Ba concentrations amended individually in SSL soil. Data include levels used in both survival and reproduction tests with *E. fetida*.

<table>
<thead>
<tr>
<th>Ba</th>
<th>pH</th>
<th>Be pH</th>
<th>pH</th>
<th>Mn pH</th>
<th>pH</th>
<th>pH</th>
<th>Sb pH</th>
</tr>
</thead>
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<tr>
<td>0</td>
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<tr>
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<td>4.89</td>
<td>100</td>
<td>5.11</td>
<td>60</td>
<td>5.14</td>
</tr>
<tr>
<td>225</td>
<td>4.88</td>
<td>28</td>
<td>4.75</td>
<td>238</td>
<td>4.92</td>
<td>86</td>
<td>5.09</td>
</tr>
<tr>
<td>451</td>
<td>4.72</td>
<td>39</td>
<td>4.66</td>
<td>274</td>
<td>4.88</td>
<td>104</td>
<td>5.08</td>
</tr>
<tr>
<td>519</td>
<td>4.66</td>
<td>50</td>
<td>4.55</td>
<td>332</td>
<td>4.85</td>
<td>124</td>
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<td>4.37</td>
<td>537</td>
<td>4.81</td>
<td>215</td>
<td>4.82</td>
</tr>
<tr>
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<td>4.54</td>
<td>98</td>
<td>4.26</td>
<td>653</td>
<td>4.76</td>
<td>245</td>
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<td>137</td>
<td>4.13</td>
<td>903</td>
<td>4.72</td>
<td>412</td>
<td>4.66</td>
</tr>
<tr>
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<td>4.48</td>
<td>151</td>
<td>4.13</td>
<td>914</td>
<td>4.71</td>
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<td>4.61</td>
</tr>
<tr>
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<td>4.45</td>
<td>192</td>
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<td>1054</td>
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<td>617</td>
<td>4.50</td>
</tr>
<tr>
<td>1314</td>
<td>4.44</td>
<td>269</td>
<td>3.94</td>
<td>1278</td>
<td>4.68</td>
<td>697</td>
<td>4.39</td>
</tr>
<tr>
<td>1551</td>
<td>4.38</td>
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<td>3.83</td>
<td>1792</td>
<td>4.58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1830</td>
<td>4.36</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2160</td>
<td>4.29</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>4.26</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.2.2 Beryllium

Range-finding tests for beryllium (Be) were performed using Be sulfate (BeSO$_4$$\cdot$4H$_2$O). Nominal concentrations of Be (w/w) in soil were 0, 1, 10, 100, 500, and 1000 mg kg$^{-1}$. Results showed 100% survival rate up to 100 mg kg$^{-1}$. All adults were dead at 500 mg kg$^{-1}$ and 1000 mg kg$^{-1}$.

The results of the cocoon tests at 39 mg kg$^{-1}$ were invalid because the earthworms received no food and no cocoons were produced. These data were therefore not included in the statistical analyses. ANOVA results of definitive cocoon production tests using actual measured concentrations of Be (Table 3) produced a bounded NOEC of 57 mg kg$^{-1}$ and a bounded LOEC of 83 mg kg$^{-1}$ at P $\leq$ 0.05 (Table 6). Cocoon production was analyzed by nonlinear regression. The Gompertz nonlinear model produced best fit (Figure 1). The EC$_{20}$ derived from this model for toxicity of Be to *E. fetida* cocoon production was 52 mg kg$^{-1}$ and the EC$_{50}$ was 63 mg kg$^{-1}$ (Table 6).

Results of the definitive adult survival tests using actual measured concentrations of Be (Table 3) showed a bounded NOEC of 83 mg kg$^{-1}$ and a bounded LOEC of 110 mg kg$^{-1}$ at P $\leq$ 0.05 (Table 6; Figure 2).

3.2.3 Manganese

Range-finding tests for manganese (Mn) were performed using Mn sulfate (MnSO$_4$$\cdot$H$_2$O). Nominal concentrations of Mn (w/w) in soil were 0, 100, 500, 1000, 5000, and 10000 mg kg$^{-1}$. Results showed cocoon production was 69% of control at 500 mg kg$^{-1}$ and 4% of control at 1000 mg kg$^{-1}$. No cocoons were produced in soil containing mg more than 1000 mg kg$^{-1}$.

ANOVA results of definitive cocoon production tests using actual measured concentrations of Mn (Table 3) produced a bounded NOEC of 1111 mg kg$^{-1}$ and a bounded LOEC of 1236 mg kg$^{-1}$ at P $\leq$ 0.05 (Table 6). Cocoon production was analyzed by nonlinear regression. The Gompertz nonlinear model produced best fit (Figure 1). The EC$_{20}$ derived from this model for toxicity of Mn to *E. fetida* cocoon production was 629 mg kg$^{-1}$ (Table 6). The EC$_{50}$ was 927 mg kg$^{-1}$ (Table 6).

Results of the definitive adult survival tests using actual measured concentrations of Mn (Table 3), showed a bounded NOEC of 1444 mg kg$^{-1}$ and a bounded LOEC of 2222 mg kg$^{-1}$ (Table 6). Adult EC$_{20}$ and EC$_{50}$ values were 1718 mg kg$^{-1}$ and 1920 mg kg$^{-1}$, respectively (Table 6).
Table 6. Ecotoxicological parameters associated with exposure of *E. fetida* to barium (Ba), beryllium (Be), manganese (Mn), and antimony (Sb).*

<table>
<thead>
<tr>
<th>ENDPOINT</th>
<th>COCOONS</th>
<th>ADULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg kg⁻¹</td>
<td>95% CI</td>
</tr>
<tr>
<td><strong>Ba</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOEC</td>
<td>258</td>
<td>--</td>
</tr>
<tr>
<td>LOEC</td>
<td>433</td>
<td>--</td>
</tr>
<tr>
<td>EC₂₀</td>
<td>370</td>
<td>230-510</td>
</tr>
<tr>
<td>EC₅₀</td>
<td>664</td>
<td>558-770</td>
</tr>
<tr>
<td><strong>Be</strong></td>
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<td></td>
</tr>
<tr>
<td>NOEC</td>
<td>57</td>
<td>--</td>
</tr>
<tr>
<td>LOEC</td>
<td>83</td>
<td>--</td>
</tr>
<tr>
<td>EC₂₀</td>
<td>52</td>
<td>36-67</td>
</tr>
<tr>
<td>EC₅₀</td>
<td>63</td>
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<tr>
<td><strong>Mn</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOEC</td>
<td>1111</td>
<td>--</td>
</tr>
<tr>
<td>LOEC</td>
<td>1236</td>
<td>--</td>
</tr>
<tr>
<td>EC₂₀</td>
<td>629</td>
<td>102-1155</td>
</tr>
<tr>
<td>EC₅₀</td>
<td>927</td>
<td>587-1266</td>
</tr>
<tr>
<td><strong>Sb</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOEC</td>
<td>60</td>
<td>--</td>
</tr>
<tr>
<td>LOEC</td>
<td>86</td>
<td>--</td>
</tr>
<tr>
<td>EC₂₀</td>
<td>30</td>
<td>11-50</td>
</tr>
<tr>
<td>EC₅₀</td>
<td>70</td>
<td>49-90</td>
</tr>
</tbody>
</table>

* NOEC = no observed effects concentration. LOEC = lowest observed effect concentration. NOEC and LOEC were derived from Analysis of Variance and comparison of mean metal concentrations by Fisher's LSD test at P < 0.05. EC₂₀ = effective concentration at which 20% reduction occurs. EC₅₀ = effective concentration at which 50% reduction occurs. Cocoon EC₂₀ and EC₅₀ were derived from nonlinear regression using the Gompertz logistical model \( Y = a \times e^{(\log_{10} y)(\log(C/EC_{50})-A)} \). Ecotoxicological parameters for Sb are based on nominal Sb concentrations, used in statistical analyses.
Figure 1. Concentration-response curves for four metals vs. cocoon production in *Eisenia fetida* reproduction tests using Gompertz logistical model $Y = a \times e^{-(t-b)/c}$.
Figure 2. *Eisenia fetida* adult survival vs. soil concentration of barium, beryllium, manganese, and antimony.
3.2.4 Antimony

Range-finding tests for antimony (Sb) were performed using Sb sulfate (SbSO₄). Nominal concentrations of Sb (w/w) in soil were 0, 1, 10, 100, 500, and 1000 mg kg⁻¹. Results showed 100% survival rate up to 100 mg kg⁻¹. All adults were dead at 500 mg kg⁻¹ and 1000 mg kg⁻¹.

ANOVA results of definitive cocoon production tests using actual measured concentrations of Sb (Table 3), produced a bounded NOEC of 60 mg kg⁻¹ and a bounded LOEC of 86 mg kg⁻¹ @ p ≤ 0.05 (Table 6). Cocoon production was analyzed by nonlinear regression, based on nominal concentration values of Sb. The Gompertz nonlinear model produced best fit (Figure 1). The EC₂₀ derived from this model for toxicity of Sb to E. fetida cocoon production was 30 mg kg⁻¹ and the EC₅₀ was 70 mg kg⁻¹ (Table 6).

Results of the definitive adult survival tests using actual measured concentrations of Sb (Table 3), showed a bounded NOEC of 617 mg kg⁻¹ and a bounded LOEC of 697 mg kg⁻¹ (Table 6).

4. DISCUSSION

Development of screening level benchmarks for Ecological Risk Assessment (ERA) of contaminated soils has become a critical need in recent years (USEPA, 2000). To address this problem, the USEPA in conjunction with stakeholders is developing Eco-SSLs to identify concentrations of chemicals in soil that, when not exceeded, theoretically protective of terrestrial ecosystems within specific soil boundary conditions from unacceptable harmful effects. An extensive review of literature (USEPA, 2000) determined that there was insufficient information for Be, Mn, Sb, and Ba to generate Eco-SSL benchmarks for soil invertebrates. Our toxicity studies were designed to specifically fill this knowledge gap.

The majority of soil toxicity tests reported in the literature used standard artificial soil with high organic matter content (10%) and near neutral pH. In contrast, we selected SSL soil to meet the criteria for Eco-SSL development, in large part because it has characteristics supporting relatively high bioavailability of cationic metals. In addition, our aging/weathering procedure of the soils loaded with the range of metal concentrations allowed us to more realistically assess the toxicity under conditions more closely resembling the potential toxic effects of Be, Mn, Sb, and Ba in the field. This study was designed to produce benchmark data for use in developing Eco-SSLs for soil invertebrates for Ba, Be, Mn, and Sb. Thus results from our study may not directly compare to those of other studies in the literature, since none of them were designed to specifically quantify metal toxicity to soil invertebrates using the Eco-SSL requirements for toxicity testing of metals.

The present study has determined soil toxicity threshold parameters NOEC, LOEC, EC₂₀, and EC₅₀ for earthworms in response to soil contamination by Ba, Be, Mn, or Sb, respectively. Earthworm is considered to be an important component of the ecosystem for metabolizing organic matter, aerating the soil, and serving as food for higher organisms in food
Several species of earthworms have been used by investigators to assess toxicity caused by a number of metal contaminants in soils. Ecotoxicological parameters for earthworms have been well characterized for cadmium, copper, lead, and zinc (Kula and Larink, 1998; Spurgeon, et al., 1994, Van Gestel, et al., 1989). However, little work has been done to characterize earthworm soil toxicity thresholds for Ba, Be, Mn, and Sb in either artificial or natural soils.

Our results indicate that Sb and Be are the most toxic to E. fetida of the four metals tested in this study. Cocoon production EC$_{20}$ for Sb (nominal) and Be were 30 mg kg$^{-1}$ and 52 mg kg$^{-1}$, respectively (Table 6). Comparatively, toxicity of Ba and Mn to E. fetida was much less. Cocoon production EC$_{20}$ for Ba and Mn were 370 mg kg$^{-1}$ and 629 mg kg$^{-1}$, respectively (Table 6). Reproductive endpoints in all tests were more sensitive compared with adult survival (Tables 6). Spurgeon et al. (1994) found a similar response of E. fetida to cadmium, copper, lead, and zinc. Cocoon production EC$_{50}$ values were 46.3 mg kg$^{-1}$, 53.3 mg kg$^{-1}$, 1,940 mg kg$^{-1}$, and 276 mg kg$^{-1}$ for Cd, Cu, Pb, and Zn, respectively. Adult survival EC$_{50}$ in the Spurgeon et al. (1994) study ranged from 2 to 13 times greater than cocoon production EC$_{50}$. We found similar results. Adult survival LOEC in our study ranged from 2.5 to 8 times greater than cocoon production LOEC. These results support the Eco-SSL requirement of the use of reproductive endpoints for benchmark development.

Beryllium is one of the least studied metals regarding its effects on soil invertebrates, although it is considered one of the problem metals of the future (Newland, 1982). It is a component of various fossil fuel types and is increasingly used in aircraft industry, space research, nuclear energy development (Ireland, 1986), X-ray tube windows manufacturing and in production of non-sparking tools composed of copper-beryllium alloy (Thorat et al., 2001). Beryllium concentrations in Aberdeen Proving Ground soil (including contaminated sites) in the areas adjacent to soil collection ranged from 0.3 to 1.4 mg kg$^{-1}$ (Hlohowskyj et al., 1999).

Extensive toxicological studies of exposure effects in humans and experimental animals have established that Be can cause pulmonary and systemic granulomatous disease known as chronic Be disease (Prince and Kazami, 1980), necrosis and tumors in animals (Witschi, 1971), can inhibit certain enzymes, including alkaline phosphatase (Reinner, 1971), and can inhibit plant and animal growth (Newland, 1982). Ireland (1986) reported increased mortality and growth suppression in a terrestrial snail Achatina fulica (Pulmonata) fed 10 µg ml$^{-1}$ Be in the diet containing the sub-optimal calcium concentrations. Beryllium, along with Sb, was the most toxic metal among the four chemicals tested in our study, and the estimated ecotoxicological parameters for E. fetida are the first in the available literature for a soil invertebrate species.

Few studies have investigated Sb concentrations in soil (Cal-Prieto et al., 2001; Crecelius et al., 1974; Kabata-Pendias and Pendias, 1992; van der Voet and de Wolff, 1996). Reported concentrations ranged from 0.17 mg kg$^{-1}$ in organic soils in Norway to 1489 mg kg$^{-1}$ in vicinity of Sb smelter in northeast England (Ainsworth and Cooke, 1991), and corresponded with treatment concentrations used in our study. Antimony concentrations in soil (including contaminated sites) at the Aberdeen Proving Ground in the areas adjacent to the location where the SSL soil was collected ranged from 0.1 to 501 mg kg$^{-1}$ (Hlohowskyj et al., 1999). No information could be found in the available literature on ecotoxicological effects of Sb to soil invertebrates. Developing such information is especially important since input to the soil ecosystems was estimated at 26000 t y$^{-1}$ of Sb (Cal-Prieto et al., 2001). This anthropogenic
contribution of Sb is 10-fold higher compared with the Sb emissions from natural sources (ca. 2600 t y⁻¹) reported by Nriagu (1990). Limited data for soil biota were reported by Rafel and Popov (1988) as part of validation efforts for developing the USSR maximum allowable concentrations of Sb in soil. These authors reported 23-52% reduction in seed germination and 26-62% reduction in root growth at 1002 mg kg⁻¹ Sb in tests with barley, wheat, radish, peas, and onion. Decrease in ammonia mineralization and nitrate accumulation was observed at Sb concentrations of 52 and 102 mg kg⁻¹ in their study. Other measures of soil biological activity were also affected, including decrease in soil enzyme catalase activity and stimulation of soil respiration at 102 mg Sb kg⁻¹ (Rafel and Popov, 1988).

Difficulties encountered with the efficiency of extraction of Sb that is aged/weathered in soil prior to analytical determination, using natural SSL amended with Sb, may be symptomatic of a larger problem regarding chemical characterization data during ERA activities at contaminated sites. Low Sb recovery rates using standard USEPA methods suggest that true concentrations of this metal will be underestimated during site characterization efforts. The recovery rates of 8 and 58% determined for Sb aged/weathered in soil in our study, using USEPA methods 200.8 and 3050B respectively, were below recovery rates of 70 and 88% previously reported for freshly-spiked soils. This clearly indicates that USEPA method 3050B appears better suited to extract aged/weathered Sb from soil, such as that which typically occurs at Superfund and other contaminated sites, and this potential discrepancy in extractability should be corrected for at the time of compilation of a list of contaminants of potential ecological concern (COPEC) in the screening phase of ERA. To use the ecotoxicological parameters from this study, which are based on nominal Sb values, it is recommended that these nominal Sb values be adjusted to 58% of nominal to account for the aging/weathering of Sb in soil (i.e., adjusted to 58% of nominal prior to determining the Eco-SSL). Aging/weathering of Sb in soils typically occurs even more extensively in the field, but simulated aging/weathering provides a conservative estimate of what might otherwise be extractable from field soils. This is especially important given the steep slope of the concentration-response curve for reproductive endpoint determined from the Earthworm Reproduction Test in our study (Figure 1.), which establishes a narrow toxicity threshold range from 30 to 70 mg kg⁻¹ based on EC₂₀ and EC₅₀ estimates, respectively (Table 6). The 43% difference between these two estimates is within the potential recovery error rate of analytical methods used. Disregarding this potential error, especially without adjustment of the Eco-SSL for aging/weathering, can otherwise lead to a removal of Sb from the COPEC list while its extracted concentrations represent field concentrations toxic to relevant ecological receptors. Adjustment of the values of the ecotoxicological parameters determined from nominal concentrations, prior to determination of the Eco-SSL, is properly left to those evaluating benchmarks for Eco-SSL development; however, in these studies an adjustment to 58% of nominal corresponds to the mean recovery rate following 3 weeks of aging/weathering of Sb in soil.

Natural Mn concentration in SSL soil of 94 mg kg⁻¹ was within the range of Mn concentrations reported for soils (including contaminated sites) at the Aberdeen Proving Ground, which ranged from 4.9 to 1140 mg kg⁻¹ (Hlohowskyj et al., 1999). Manganese is a required nutrient essential for plants and animals. Manganese was the most previously investigated of the four metals in this study, however none of the previous studies involved invertebrate exposures in natural soils. Reinecke and Reinecke (1996) reported reduction in growth and development
(measured as time needed for clitellum development) of *E. fetida* fed with cattle manure spiked with Mn at 151.7 mg kg\(^{-1}\). This value falls well below the EC\(_{20}\) of 629 mg kg\(^{-1}\) determined in our study. In a later study, Reinecke and Reinecke (1997) reported damage to spermatozoan structure from treatments containing food spiked with Mn at 61.57 mg kg\(^{-1}\). Nottrot *et al.* (1987) reported no effect on feeding activity and growth of collembolan *Orchesella cincta* fed with green algae spiked with up to 25 :mol Mn g\(^{-1}\) dry mass, however that study was conducted on dental plaster. Joosse and van Vliet (1984) reported no effect on respiration of woodlice fed with litter containing Mn at 1000 mg kg\(^{-1}\) on a porous tile. There was no soil exposure incorporated in that study.

Natural Ba concentration in SSL soil of 34 mg kg\(^{-1}\) was within the Ba concentrations found in soils (including contaminated sites) at the Aberdeen Proving Ground, which ranged from 9.8 to 1580 mg kg\(^{-1}\) (Hlohowskyj *et al.*, 1999). Limited Ba ecotoxicological information for soil invertebrates is available from literature. Grace (1990) investigated oral toxicity of Ba metabolate to the Eastern Subterranean Termite *Reticulitermes flavipes* (Kollar) in no-choice assays by feeding termite workers for 15 days on filter papers treated with concentrations of Ba. Results of this study are similar to results of our 14-day adult survival definitive test. Grace (1990) reported 19% mortality in 1780 mg Ba kg\(^{-1}\) treatment, which was comparable with 24% adult mortality at 1551 mg Ba kg\(^{-1}\) treatment observed in our investigation. However, direct comparisons of feeding assay results with soil exposure studies using different species should be treated with caution.

5. CONCLUSIONS

This study has produced ecotoxicological benchmark data for barium (Ba), beryllium (Be), manganese (Mn), and antimony (Sb), based on soil toxicity to the earthworm *E. fetida*, for use in the developing Eco-SSLs. The relative toxicity of the 4 metals tested in this study was Sb (nominal) \(\geq\) Be \(>\) Ba \(>\) Mn. When the ecotoxicological parameters for Sb are adjusted by 58% to account for reduced extractability of Sb after 3 weeks of aging/weathering in soil, the relative toxicity becomes Sb \(>\) Be \(>\) Ba \(>\) Mn. It is strongly recommended that the nominal Sb benchmark values from this study be adjusted to 58% of nominal, to account for the aging/weathering of Sb in soil (i.e., adjusted to 58% of nominal prior to determining the Eco-SSL). Be and Sb (nominal) were eight to ten times more toxic to cocoon production in *E. fetida* than were Ba and Mn. Adult survival was 2.5 to 8 times greater than cocoon production indicating that reproduction tests provide a more sensitive evaluation of effect than survival and therefore should be used to set screening criteria. These tests were performed using a natural soil, Sassafras sandy loam. Sassafras sandy loam has relatively low pH, low organic matter, low cation exchange capacity, and high sand content. Such characteristics support relatively high bioavailability of cationic metals in soil. Furthermore, aging and weathering of the soil produced a soil microenvironment more similar to field conditions than previous studies where soil invertebrates were exposed immediately following spiking of soil. These study results will be provided to the Ecological Soil Screening Level (Eco-SSL) workgroup for review. Results will
undergo quality control review by the Eco-SSL task group before inclusion in the Eco-SSL database, and before being used for developing Ecological Soil Screening Levels (Eco-SSLs) for Be, Mn, Sb, and Ba.
LITERATURE CITED


APPENDIX A

RANGE-FINDING TEST DATA

Range-finding invertebrate assays
Fresh SSL soil

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Range-finding invertebrate assays
Fresh SSL soil

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### Range-finding invertebrate assays

**Fresh SSL soil**

**Compound:**  Ba [Ba(NO₃)₂]

**Start Date:**  27-Sep--00

**Invertebrate:**  *E. fetida*

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### Range-finding invertebrate assays

**Fresh SSL soil**

**Compound:**  Ba [Ba(C₂H₃O₂)₂]

**Start Date:**  27-Sep--00

**Invertebrate:**  *E. fetida*

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Range-finding invertebrate assays
Fresh SSL soil

**Compound:** Be [BeSO₄]
**Start Date:** 17-Apr-00
**Invertebrate:** *E. fetida*

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Range-finding invertebrate assays
Fresh SSL soil

**Compound:** Mn [MnSO₄]
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**Invertebrate:** *E. fetida*

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APPENDIX A 33
Range-finding invertebrate assays
Fresh SSL soil

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**Invertebrate:** *E. fetida*

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APPENDIX B

DEFINITIVE TEST DATA

Definitive invertebrate assays
Aged SSL soil
Compound: Ba [Ba(NO₃)₂]
Start Date: 28-Nov-00
Invertebrate: E. fetida
Survival

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Invertebrate: E. fetida Reproduction

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Definitive invertebrate assays
Aged SSL soil

Compound: Be [BeSO₄]
Start Date: 24-Aug-00
Invertebrate: E. fetida
Survival

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Definitive invertebrate assays
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Start Date: 22-Aug-00
Invertebrate: E. fetida
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Start Date: 24-Aug-00
Invertebrate: E. fetida
Survival

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### Definitive invertebrate assays

**Aged SSL soil**

**Compound:** Mn [MnSO₄]

**Start Date:** 22-Aug-00

**Invertebrate:** *E. fetida*

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Definitive invertebrate assays
Aged SSL soil
Compound: Sb [Sb₂(SO₄)₃]
Start Date: 24-Aug-00
Invertebrate: E. fetida
Survival

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APPENDIX B 41
Definitive invertebrate assays  
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Start Date: 22-Aug-00  
Invertebrate: *E. fetida*  
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APPENDIX C

STATISTICAL ANALYSIS OF TOXICITY TEST DATA
E. fetida BARIUM NONLINEAR REGRESSION Gompertz MODEL

TUE 4/09/02 10:32:15 AM

SYSTAT VERSION 7.0.1
COPYRIGHT (C) 1997, SPSS INC.

Welcome to SYSTAT!

***************EW BA EC50************

Iteration
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Dependent variable is COCOONS

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Raw R-square (1-Residual/Total) = 0.9
Mean corrected R-square (1-Residual/Corrected) = 0.8
R(Observer vs predicted) square = 0.8

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APPENDIX C 44
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Asymptotic Correlation Matrix of Parameters

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G & 1.0 & \\
X & -0.8 & 1.0 \\
B & -0.6 & 0.7 & 1.0
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\]

Data, estimates and residuals have been saved.

************** BARIUM EW COCOON EC20 **************

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Dependent variable is COCOONS

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Total 7904.0  40
Mean corrected 1999.1  39

APPENDIX C
Raw R-square (1-Residual/Total) = 0.9
Mean corrected R-square (1-Residual/Corrected) = 0.8
R( observed vs predicted) square = 0.8

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Asymptotic Correlation Matrix of Parameters

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APPENDIX C
Data, estimates and residuals have been saved.

GRAPH MODEL FOR COCOONS:

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graph
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JUVENILES',
    xmax=150, xmin=0, ymax=30, ymin=0
fplot y=17.732*exp((log(.5))*(concentr/2.389)^0.56); xmin=0, xmax=150, xlab=''
ymin=0, ylab='',
ymax=30
end
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SYSTAT Rectangular file H:\SIMINI.SYD,
created Tue Apr 09, 2002 at 10:40:18, contains variables:

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***************STEM AND LEAF PLOT***************

Stem and Leaf Plot of variable: RESIDUAL, N = 40

Minimum: -7.9
Lower hinge: -2.0
Median: -0.9
Upper hinge: 1.6
Maximum: 8.1

-7 8
** * * Outside Values ** **
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-3 0
-2 H 998554000
-1 H 54111000
-0 M 82
0 14448
1 H 01477
2 157
3 4
4
5 9
6 1
** * * Outside Values ** **
7 5
8 1

ANOVA for Ba effect on E. fetida Cocoons

TUE 4/09/02 2:48:26 PM

SYSTAT VERSION 7.0.1
COPYRIGHT (C) 1997, SPSS INC.

Welcome to SYSTAT!
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APPENDIX C 47
Effects coding used for categorical variables in model.

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BA (10 levels)
34, 153, 258, 433, 578, 689, 744,
791, 1000, 1222

Dep Var: COCOONS  N: 40  Multiple R: 0.9  Squared multiple R: 0.9

Estimates of effects B = (X'X)^{-1} X'Y

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Analysis of Variance

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Least squares means.

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APPENDIX C 48
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Durbin-Watson D Statistic 2.127
First Order Autocorrelation -0.127

ROW BA
1 34
2 153
3 258
4 433
5 578
6 689
7 744
8 791
9 1000
10 1222
Using least squares means.
Post Hoc test of COCOONS

Using model MSE of 9.617 with 30 df.
Matrix of pairwise mean differences:

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Fisher's Least-Significant-Difference Test.
Matrix of pairwise comparison probabilities:

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</table>
40 cases have been saved into a SYSTAT file

ANOVA for Ba effect on *E. fetida* Adults

WED 4/10/02 12:19:54 PM

SYSTAT VERSION 7.0.1
COPYRIGHT (C) 1997, SPSS INC.

Welcome to SYSTAT!
SYSTAT Rectangular file H:\SIMINI-1\ECOSSL\BABEMNSB\BA\BANAC.SYD,
created Thu Aug 16, 2001 at 15:39:54, contains variables:
CONC   SURVIVORS

Effects coding used for categorical variables in model.

Categorical values encountered during processing are:
CONC (9 levels)
   34,  1000,  1124,  1222,  1348,  1585,  2000,
   2194,  2697

Dep Var: SURVIVORS  N: 36  Multiple R: 1.0  Squared multiple R: 0.9

Estimates of effects $B = (X'X)^{-1}X'Y$

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<td>CONC 1124</td>
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APPENDIX C  50
## Analysis of Variance

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<th>Mean-Square</th>
<th>F-ratio</th>
<th>P</th>
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### Least squares means.

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### Durbin-Watson D Statistic

2.167

First Order Autocorrelation: -0.083

ROW CONC

| 1 | 34 |
| 2 | 1000 |
| 3 | 1124 |
| 4 | 1222 |
| 5 | 1348 |
| 6 | 1585 |
| 7 | 2000 |
| 8 | 2194 |
| 9 | 2697 |

Using least squares means.

Post Hoc test of SURVIVORS

---
Using model MSE of 0.694 with 27 df.
Matrix of pairwise mean differences:

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Fisher's Least-Significant-Difference Test.
Matrix of pairwise comparison probabilities:

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<td>1.0</td>
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---

E. fetida BERYLLIUM NONLINEAR REGRESSION WITH COCOONS

TUE 4/09/02 1:59:40 PM

SYSTAT VERSION 7.0.1
COPYRIGHT (C) 1997, SPSS INC.

************EC50 COCOONS************

Welcome to SYSTAT!
SYSTAT Rectangular file H:\SIMINI-1\ECOSSL\BABEMNSB\BE\BECH2.SYD,
created Thu Sep 21, 2000 at 16:08:20, contains variables:
TRT SURVIVAL BIOMASS

APPENDIX C 52
### Iteration

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<th>X</th>
<th>B</th>
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Dependent variable is COCOONS

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<th>Mean-Square</th>
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</table>

Raw R-square (1-Residual/Total) = 0.9
Mean corrected R-square (1-Residual/Corrected) = 0.7
R(observable vs predicted) square = 0.7

### Table

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<th>Param/A.E.</th>
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### Case Table

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<th>Residual</th>
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APPENDIX C 53
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28  0.0  0.0  0.0  0.0

Asymptotic Correlation Matrix of Parameters

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Data, estimates and residuals have been saved.

**************************EC20 COCONOUS**************************

Iteration

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Dependent variable is COCONOUS

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Total 859.0 28
Mean corrected 403.0 27

Raw R-square (1-Residual/Total) = 0.9
Mean corrected R-square (1-Residual/Corrected) = 0.7
R( observed vs predicted) square = 0.7

Wald Confidence Interval

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<th>Parameter</th>
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<th>A.S.E.</th>
<th>Param/AE</th>
<th>Lower &lt; 95%</th>
<th>Upper</th>
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Case | COCONOUS Observed | COCONOUS Predicted | Residual |
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APPENDIX C 54
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Asymptotic Correlation Matrix of Parameters

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<th>B</th>
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</tbody>
</table>

Data, estimates and residuals have been saved.
SYSTAT Rectangular file H:\simini.SYD,
created Tue Apr 09, 2002 at 14:08:42, contains variables:
BE COCOONS ESTIMATE RESIDUAL

SYSTAT Rectangular file H:\simini.SYD,
created Tue Apr 09, 2002 at 14:08:42, contains variables:
BE COCOONS ESTIMATE RESIDUAL

***************STEM AND LEAF PLOT***************

Stem and Leaf Plot of variable: RESIDUAL, N = 28
Minimum: -4.4
Lower hinge: -0.8
Median: 0.0
Upper hinge: 0.7
Maximum: 5.7

-4 3
* * * Outside Values * * *
-2 93
-1 3333
-0 M 33222000000
0 M 00679
1
2 6
* * * Outside Values * * *
2 9
3 06
5 6

APPENDIX C 55
ANOVA for Be effect on E. fetida Cocoons

TUE 4/09/02 3:00:10 PM

SYSTAT VERSION 7.0.1
COPYRIGHT (C) 1997, SPSS INC.

Welcome to SYSTAT!
SYSTAT Rectangular file H:\SIMINI-1\METANOVA\BA\BADAT.SYD,
created Tue Apr 09, 2002 at 14:50:00, contains variables:
BA COCOONS
40 cases have been saved into a SYSTAT file

Effects coding used for categorical variables in model.

Categorical values encountered during processing are:
BE (7 levels)
   2.5,  24,  28,  57,  83, 121, 154

Dep Var: COCOONS  N: 28  Multiple R: 0.9  Squared multiple R: 0.7

-1
Estimates of effects  B = (X'X) X'Y

COCOONS

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<td>BE</td>
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Analysis of Variance

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APPENDIX C  56
Least squares means.

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*** WARNING ***
Case 8 is an outlier (Studentized Residual = 3.0)

Durbin-Watson D Statistic 2.262
First Order Autocorrelation -0.134

Using least squares means.
Post Hoc test of COCOONS

Using model MSE of 5.060 with 21 df.
Matrix of pairwise mean differences:

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Fisher's Least-Significant-Difference Test.
Matrix of pairwise comparison probabilities:

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APPENDIX C

57
ANOVA for Be effect on E. fetida Adults

WED 4/24/02 3:17:22 PM

SYSTAT VERSION 7.0.1
COPYRIGHT (C) 1997, SPSS INC.

Welcome to SYSTAT!
SYSTAT Rectangular file C:\SIMINI-1\ECOSSL\BABEMNSB\BE\BEAC2.SYD,
created Tue Apr 09, 2002 at 13:52:48, contains variables:
TRT   SURVIVAL   BIOMASS
32 cases have been saved into a SYSTAT file
32 cases have been saved into a SYSTAT file

Effects coding used for categorical variables in model.

Categorical values encountered during processing are:
TRT (8 levels)
   2.5, 79, 83, 110, 144, 191, 308, 380

Dep Var: SURVIVAL   N: 32   Multiple R: 1.0   Squared multiple R: 1.0

-1
Estimates of effects  B = (X'X) X'Y

SURVIVAL

   CONSTANT   3.0
   TRT    2.5   2.0
   TRT    79   2.0
   TRT   83   1.8
   TRT  110   0.8
   TRT  144   1.5
   TRT  191  -2.0
   TRT  308  -3.0

APPENDIX C  58
### Analysis of Variance

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### Least squares means.

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*** WARNING ***

Case 13 is an outlier (Studentized Residual = 3.3)

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</table>

COL/
ROW TRT

1  2.5  2  79  3  83  4  110  5  144  6  191  7  308  8  380

Using least squares means.
Post Hoc test of SURVIVAL

---

Using model MSE of 0.271 with 24 df.
Matrix of pairwise mean differences:

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APPENDIX C 59
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8  -5.0  -5.0  -4.8  -3.8  -4.5
6    7    8
6   0.0
7  -1.0  0.0
8  -1.0  0.0

Fisher's Least-Significant-Difference Test.
Matrix of pairwise comparison probabilities:

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**E. fetida** MANGANESE NONLINEAR REGRESSION WITH COCOONS

***********EC50***********

Iteration

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Dependent variable is COCOONS

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Raw R-square (1-Residual/Total) = 0.8
Mean corrected R-square (1-Residual/Corrected) = 0.4
R(observed vs predicted) square = 0.4
### Wald Confidence Interval

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<th>Param/ASE</th>
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### Case

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### Asymptotic Correlation Matrix of Parameters

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Data, estimates and residuals have been saved.

### Iteration

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Dependent variable is COCOONS

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Raw R-square (1-Residual/Total) $= 0.8$
Mean corrected R-square (1-Residual/Corrected) $= 0.4$
R(observed vs predicted) square $= 0.4$

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Asymptotic Correlation Matrix of Parameters

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</table>
Data, estimates and residuals have been saved. created Wed Apr 17, 2002 at 10:18:34, contains variables: 
MN COCOONS ESTIMATE RESIDUAL

Stem and Leaf Plot of variable: RESIDUAL, N = 27
Minimum: -5.6
Lower hinge: -1.8
Median: -0.7
Upper hinge: 1.7
Maximum: 6.6

-5 51
-4
-3 633
-2 10
-1 H 65
-0 M 7776630
 0 39
 1 H 249
 2 3
 3 38
 4 4
 5 8
 6 6

ANOVA for Mn effect on E. fetida Cocoons

Effects coding used for categorical variables in model.

Categorical values encountered during processing are: 
MN (7 levels) 94, 386, 528, 697, 1067, 1111, 1236

Dep Var: COCOONS N: 27 Multiple R: 0.7 Squared multiple R: 0.4

Estimates of effects B = (X'X) x'y

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APPENDIX C 63
## Analysis of Variance

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Least squares means.

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Durbin-Watson D Statistic 2.421
First Order Autocorrelation -0.225

Using least squares means.
Post Hoc test of COCOONS

Using model MSE of 12.271 with 20 df.
Matrix of pairwise mean differences:

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APPENDIX C 64
Fisher's Least-Significant-Difference Test.
Matrix of pairwise comparison probabilities:

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   1  2  3  4  5
1  1.0
2  0.9 1.0
3  0.7 0.6 1.0
4  0.4 0.3 0.7 1.0
5  0.1 0.0 0.1 0.2 1.0
6  0.0 0.0 0.1 0.2 0.8
7  0.0 0.0 0.0 0.1 0.5
   6  7
   6  1.0
   7  0.6 1.0
```

27 cases have been saved into a SYSTAT file
***WARNING***
The file C:\SIMINI-1\METANOVA\MN\MNCOOC5.SYD was read for processing, and its contents have been replaced by saving the processed data into it.
27 cases have been saved into a SYSTAT file

**ANOVA for Mn effect on E. fetida Adults**

WED 4/17/02 11:03:19 AM

SYSTAT VERSION 7.0.1
COPYRIGHT (C) 1997, SPSS INC.

Welcome to SYSTAT!
SYSTAT Rectangular file C:\SIMINI-1\METPSSNL\MN\MNCOOC5.SYD,
created Wed Apr 17, 2002 at 10:27:36, contains variables:
MN COCOONS
27 cases have been saved into a SYSTAT file
32 cases have been saved into a SYSTAT file

Effects coding used for categorical variables in model.

Categorical values encountered during processing are:
MN (8 levels)
94, 326, 449, 611, 767, 1111, 1444, 2222

Dep Var: ADULTS  N: 32  Multiple R: 1.0  Squared multiple R: 1.0

-1
Estimates of effects  B = (X'X) X'Y

APPENDIX C  65
### ADULTS

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<tr>
<td align="center">MN 326</td>
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<tr>
<td align="center">MN 449</td>
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</tr>
<tr>
<td align="center">MN 611</td>
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<tr>
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#### Analysis of Variance

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<th>F-ratio</th>
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#### Least squares means.

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<th>N</th>
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<td>4</td>
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<td>=2222</td>
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#### *** WARNING ***

Case 22 is an outlier (Studentized Residual = -3.4)
Case 25 is an outlier (Studentized Residual = -3.4)
Case 32 is an outlier (Studentized Residual = -3.4)

Durbin-Watson D Statistic 2.250
First Order Autocorrelation -0.250

COL/ROW MN

1 94
2 326
Using least squares means.
Post Hoc test of ADULTS

Using model MSE of 0.094 with 24 df.
Matrix of pairwise mean differences:

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Fisher's Least-Significant-Difference Test.
Matrix of pairwise comparison probabilities:

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***WARNING***
The file C:\SIMINI-1\METANOVA\MN\MNADULT5.SYD was read for processing, and its contents have been replaced by saving the processed data into it. 32 cases have been saved into a SYSTAT file.

APPENDIX C  67
E. fetida ANTIMONY NONLINEAR REGRESSION WITH COCOONS

THU 8/16/01 9:51:32 AM

SYSTAT VERSION 7.0.1
COPYRIGHT (C) 1997, SPSS INC.

Welcome to SYSTAT!

SYSTAT VERSION 7.0.1
COPYRIGHT (C) 1997, SPSS INC.

nonlin
print=long
model cocoons=g*exp((log(1-.5))*(conc/x)^b)
save c:\imini data\ecoss1\babemnsb\sbresidsb4 / resid
estimate/ start = 9, 75, 2 iter=200

Iteration
No.   Loss     G       X       B
0   .577175D+02   .900000D+01   .750000D+02   .200000D+01
1   .569808D+02   .882524D+01   .683194D+02   .101910D+01
2   .490480D+02   .874241D+01   .704113D+02   .135438D+01
3   .489823D+02   .874258D+01   .697020D+02   .136696D+01
4   .489819D+02   .874420D+01   .696049D+02   .136163D+01
5   .489818D+02   .874404D+01   .696221D+02   .136265D+01
6   .489818D+02   .874407D+01   .696187D+02   .136245D+01
7   .489818D+02   .874406D+01   .696194D+02   .136249D+01

Dependent variable is COCOONS

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<tr>
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<th>Sum-of-Squares</th>
<th>df</th>
<th>Mean-Square</th>
</tr>
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<tr>
<td>Regression</td>
<td>479.018</td>
<td>3</td>
<td>159.673</td>
</tr>
<tr>
<td>Residual</td>
<td>48.982</td>
<td>28</td>
<td>1.749</td>
</tr>
<tr>
<td>Total</td>
<td>528.000</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>Mean corrected</td>
<td>266.710</td>
<td>30</td>
<td></td>
</tr>
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</table>

Raw R-square (1-Residual/Total) = 0.907
Mean corrected R-square (1-Residual/Corrected) = 0.816
R( observed vs predicted) square = 0.817

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<th>Estimate</th>
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<th>Param/ASE</th>
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<th>Upper</th>
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APPENDIX C 68
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Asymptotic Correlation Matrix of Parameters

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Data, estimates and residuals have been saved.

Iteration:

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Dependent variable is COCOONS

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<th>df</th>
<th>Mean-Square</th>
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<tbody>
<tr>
<td>Regression</td>
<td>479.018</td>
<td>3</td>
<td>159.673</td>
</tr>
<tr>
<td>Residual</td>
<td>48.982</td>
<td>28</td>
<td>1.749</td>
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<tr>
<td>Total</td>
<td>528.000</td>
<td>31</td>
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APPENDIX C

69
Mean corrected  266.710  30

Raw R-square (1-Residual/Total) = 0.907
Mean corrected R-square (1-Residual/Corrected) = 0.816
R(observed vs predicted) square = 0.817

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Wald Confidence Interval

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Asymptotic Correlation Matrix of Parameters

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Data, estimates and residuals have been saved.

> APPENDIX C  70
> graph

> begin

> plot cocoons*conc / title='', xlab='Sb SO4 concentration', ylab='NUMBER OF COCOONS',

> xmax=250, xmin=0, ymax=12, ymin=0

>

> fplot y=8.74*exp((log(.5))*(conc/69.62)^1.36); xmin=0, xmax=250, xlab=''

> ymin=0, ylab='', ymax=12

> end

> graph

> begin

> plot cocoons*conc / title='', xlab='Sb concentration', ylab='NUMBER OF COCOONS',

> xmax=250, xmin=0, ymax=12, ymin=0

>

> graph

> use d:\nonlin\residsb3 / resid

SYSTAT Rectangular file d:\nonlin\residsb3.SYD,
created Thu Aug 16, 2001 at 10:06:06, contains variables:
COCOONS CONC ESTIMATE RESIDUAL

> plot residual*conc

> plot residual*estimate

>

**ANOVA for Sb effect on E. fetida Cocoons**

MON 9/18/00 2:34:10 PM

SYSTAT VERSION 7.0.1

> ANOVA

> CATEGORY TRT/MISS

> COVAR

> DEPEND COCOONS / BONF

> ESTIMATE

Effects coding used for categorical variables in model.

APPENDIX C
Categorical values encountered during processing are:
TRT (8 levels) 0, 60, 86, 104, 124, 149, 179, 215

Dep Var: COCOONS N: 35 Multiple R: 0.83 Squared multiple R: 0.69

Analysis of Variance

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Durbin-Watson D Statistic 1.853
First Order Autocorrelation 0.000

ROW TRT
1 0
2 60
3 86
4 104
5 124
6 149
7 179
8 215

Using least squares means.
Post Hoc test of COCOONS

Using model MSE of 3.807 with 27 df.
Matrix of pairwise mean differences:

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APPENDIX C
Bonferroni Adjustment.
Matrix of pairwise comparison probabilities:

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ANOVA for Sb effect on E. fetida Adults

WED 9/20/00 3:04:44 PM

SYSTAT VERSION 7.0.1
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>ANOVA
>CATEGORY SBCONC
>COVAR
>DEPEND SURVIVORS / BONF
>ESTIMATE

Effects coding used for categorical variables in model.
Categorical values encountered during processing are:
SBCONC (7 levels)
0, 245, 318, 412, 537, 617, 697

Dep Var: SURVIVORS N: 32 Multiple R: 0.68 Squared multiple R: 0.46

Analysis of Variance

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APPENDIX C 73
*** WARNING ***
Case 30 is an outlier (Studentized Residual = 3.10)
Case 32 is an outlier (Studentized Residual = -3.10)

Durbin-Watson D Statistic  2.036  
First Order Autocorrelation  -0.125  

ROW SBCONC
  1 0
  2 245
  3 318
  4 412
  5 537
  6 617
  7 697

Using least squares means.
Post Hoc test of SURVIVORS

Using model MSE of 0.420 with 25 df.
Matrix of pairwise mean differences:

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Bonferroni Adjustment.
Matrix of pairwise comparison probabilities:

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