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EARTHWORM CHRONIC TESTING

PHASE 2: IDENTIFYING EFFECTIVE SOIL CONDITIONERS AND AMENDMENT LEVELS IN CLAY AND SAND REFERENCE SOILS FOR USE IN TOXICITY TESTING

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IDENTIFYING EFFECTIVE SOIL CONDITIONERS AND AMENDMENT LEVELS IN CLAY AND SAND REFERENCE SOILS

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1.0 Introduction

Many of the soil samples received for toxicity assessments are difficult to work with and are considered to be 'suboptimal' soils for the various test organisms recommended by regulatory agencies for use in toxicity testing. The structure of the soils is often not acceptable for the biological organisms because it does not support survival, growth, and/or reproduction of the test organism.

Clay soils can be difficult to work with. When clay soils are wet, water drains slowly from the soil, so the clay can become waterlogged and compacted, resulting in anoxic conditions (Riofrio and Wittmeyer, 1992; Glattstein, 1989). When clay soils dry out, they often become hard, form crusts, and crack on the soil surface (Riofrio and Wittmeyer, 1992; Glattstein, 1989). Clay soils are made up of fine soil particles that are small and flat, which can pack together tightly. This results in soils low in air, organic matter and microbial activity, although they are high in mineral nutrients and have relatively high bulk densities (Reynolds et al., 2003; Glattstein, 1989).

Sand soils typically have low moisture retention and low organic matter content, and they are considered 'suboptimal' soils for use in toxicity testing with earthworms. Pore spaces between sand particles, especially with coarse sandy soils, tend to be large so that water leaches and moves quickly through the soil, causing these soils to dry out (Li et al., 2000; Riofrio and Wittmeyer, 1992).

From past experience, earthworms seem especially sensitive to the structure and condition of the test soils and, although earthworms can survive in soil with little structure, they rarely reproduce. Since earthworms inhabit soils, they need to be able to move through the soil to live, and they are sensitive to the physical properties of soil, including texture and structure, moisture content, pH, particle aggregation, and aeration of the test soil. In tests where earthworm survival, reproduction, and growth were inhibited, it was unclear whether the observed responses were due to the contaminants in the soil or to the structure and physico-chemical characteristics of the soil being tested.

In an earlier phase of this project, Stantec Consulting Ltd. (Stantec) conducted a literature review for Petroleum Technology Alliance Canada (PTAC) on the use of soil conditioners to amend 'suboptimal' soils to improve their ability to support earthworm reproduction. Based on the findings of the literature review, four soil conditioners were identified for use in Phase 2 of this project. Phase 2 was designed to evaluate the effectiveness of peat, coir, perlite and gypsum at five amendment levels (0, 2.5, 5, 10 and 15%) to improve the structure and physical characteristics of a clay and a sand reference soil. An experimental negative control soil was also included in the test design; it was an artificial soil (AS) recommended by the Environment Canada biological test method (EC, 2004). The AS was included in the assessment as an internal QA/QC measure of test organism health and performance, technician proficiency, and experimental conditions. Tests were conducted with the earthworm, *Eisenia andrei*. The goal

of the project was to identify potential soil conditioner/amendment level combinations that would improve the physical characteristics of test soils and be suitable and effective for use in toxicity testing. Acceptable soil conditioner/amendment combinations could then be used in future toxicity tests when working with difficult soils, to determine the effects of contaminants on earthworm survival, growth, and reproduction, while reducing the effect of suboptimal soil properties on the test organism.

1.1 TEST SOILS

1.1.1 Reference Soils

The assessment of soil conditioners was conducted at the Stantec Consulting, Ltd., Soils Laboratory (361 Southgate Drive, Guelph, ON, N1G 3M5) and the soils used for testing were soils that had been identified as suboptimal for earthworm reproduction and used for previous ecotoxicity assessments. A suitable clay and sand soil were selected for testing and retrieved from storage in the Soil Sample Storage Room (clay = June 25, 2007; sand = July 9, 2007). The three buckets of clay reference soil had been collected in July 2005 and the two buckets of sand reference soil had been collected in October 1999. Both the clay and sand soils were composite samples. Each soil sample was assigned a unique identification number and was entered into the Soils Laboratory Sample logbook (Table 1). Soil samples were collected from the clay and sand buckets for chemical analysis, to ensure samples were not contaminated, and for soil and nutrient analysis.

Table 1 Description of the Reference Soils at the Stantec Soils Laboratory.

Soil Sample Descriptor	Date Received / Out of Storage	Number of Buckets	Unique Sample ID	pH	Conductivity (µS/cm)
Clay Reference Soil	July 28, 2005 June 25, 2007	3 x 20-L	0750-KB	5.58	13.8
Sand Reference Soil	1999 July 9, 2007	2 x 20-L	0754-SR2&3	7.62	526

The clay soil required preparation before it could be used for testing. The clay was wet upon initial inspection, so it was broken up by hand and spread out on a piece of vapour barrier. The soil was left to dry for approximately 24 to 48 hours, until sufficiently dried. Once dried, the soil was ground using the soil grinder. The soil from the replicate buckets was homogenized together to make a composite sample for testing. The soil was stored in the original buckets until used in the test.

The sand soil had been previously sieved and dried; it only required homogenization of the replicate buckets to make a composite sample for testing. The soil was stored in the original buckets until tested.

Both the clay and sand samples were stored at room temperature prior to testing (21.5 ± 1.1 °C and 21.7 ± 1.1 °C, respectively). The pH, conductivity, and soil moisture content were determined for each soil prior to testing.

The test with clay was initiated with the addition of the earthworms to the test soils on August 22, 2007. The test was terminated on October 24, 2007. The sand test was initiated on August 30, 2007 and terminated on November 1, 2007.

1.1.2 Physical and Chemical Characterization of Test Soils

Prior to testing, soil samples were collected from both the clay and sand reference soils to determine physical and chemical properties of each soil. The pedologic characteristics of the soils were measured to satisfy the requirements of the Environment Canada test method (EC, 2004). A subsample of each reference soil was collected and submitted to the University of Guelph's Soil and Nutrient Laboratory (Guelph, ON) for physical and chemical characterization. Soil samples from the clay and sand reference soils were also collected and sent to ALS (ALS Laboratory Group, Waterloo, ON) to characterize the soils based on metals scan and petroleum hydrocarbon (F2-F4) analyses. This was to ensure that the reference soils being tested were not contaminated. All results are present in Appendices B and C (Tables B.4, B.5, C.4, and C.5).

The Environment Canada test method also requires that pH, moisture content and water-holding capacity be measured for all test soils; these parameters and electrical conductivity were measured at the Stantec Soils Laboratory and are reported in Appendices B and C (Tables B.3 and C.3). These parameters were determined at the time of test initiation and test termination. The water-holding capacity of each soil treatment was determined once testing had been initiated from extra soil collected from the test setups (August to September, 2007). Descriptions of the methods used to determine water-holding capacity, soil pH, moisture content and electrical conductivity are provided in Appendix A.

1.1.3 Negative Control Soil

The negative control soil used for the assessments was a formulated artificial soil (AS), recommended by Environment Canada for toxicity testing (EC, 2004). AS was formulated from natural ingredients of silica sand, kaolinite clay, and *Sphagnum* peat, and was buffered to a neutral pH range (6.0 – 7.5) with calcium carbonate ($CaCO_3$). This negative control soil served as an experimental QA/QC soil to check test organism health, technician proficiency, experimental conditions, and testing procedures. The formulation of this artificial soil (AS) is described in Appendix A, and the soil characteristics are described in Appendices B and C.

1.2 TEST SPECIES

The test species used to assess the effectiveness of the different soil conditioners and amendment levels was the earthworm (*Eisenia andrei*). This species is commonly referred to as the red wiggler or compost worm. This invertebrate species was selected because:

- it has a relatively short life cycle that makes it possible to conduct reproduction tests in the laboratory;
- it is sensitive to soil physical characteristics (often affects reproduction endpoints);
- it is a commonly used invertebrate toxicity test species;
- it is an important member of the soil fauna;
- performance criteria are available;
- reliable cultures are available;
- toxicity data generated from tests with this species are reproducible and sensitive; and
- standardized test methods exist for this test species (Environment Canada biological test method (EC, 2004)).

All of the earthworms used for testing with the reference soils were cultured in the Stantec Soils Laboratory and were removed from the in-house culture prior to testing.

1.3 SOIL CONDITIONERS

The soil conditioners included in the tests were selected based on the literature review completed in Phase 1 of this project. An ideal soil conditioner for the purpose of toxicity testing would have the ability to improve and correct any deficiencies in the physical properties of the soil for the duration of a test, making suboptimal soils more acceptable for earthworms. This would allow the effects of suboptimal soil properties to be separated from the effects of the contaminants in the test soil. The requirements for an ideal soil conditioner to be used for toxicity testing with earthworms include:

- inert to avoid interactions with contaminants;
- no negative effects on biological test organisms;
- sufficiently porous to provide good air/water balance, preventing soils from drying out or becoming waterlogged, compacted, and anaerobic;
- resistant to physical and microbial breakdown;
- low fertility/nutrient status to avoid affecting test results;
- decreases bulk density of heavy clay soils;
- increases formation of aggregates;
- maintains pH within acceptable range;
- free from pests, disease, and weeds;
- readily available, and guaranteed supplier/source;
- consistent quality for test results to be repeatable over time;
- easy to work with (calculations, preparation, applications, and mixing);

- low cost so conditioner is affordable for use in tests when needed; and
- effective for the duration of the toxicity test with little change over the test duration (63 days for earthworm chronic tests).

Based on these criteria and the findings of the literature review, four soil conditioners were selected for testing: peat, coir, perlite and gypsum. The reference soils were amended with the conditioners at 0, 2.5, 5, 10, and 15%.

The peat purchased for amending the test soils was PROMOSS Fine Grind Peat shipped as a 107-L bale (purchased from Canadian Hydrogardens, Ltd., Ancaster, ON, received on July 24, 2006). This peat has short-fibers and is suitable for use in toxicity testing without sieving. To prepare the peat for toxicity testing, a sufficient amount of peat was removed from the original package and clumps were broken up by hand to make homogenization of the test soil and the amendment easier. The peat was stored in a clean, labeled plastic bag until use.

A 25 kg bale of coconut coir was purchased for amending the test soils (purchased from Millenniumsoils Coir, St. Catharines, ON, received on June 27, 2007). Coir was removed from the original package and clumps were broken up by hand to make homogenization of the test soil and the amendment easier. A sufficient amount of coir was prepared for testing and stored in a clean, labeled plastic bag until use.

A coarse perlite (9.5 kg) was purchased for testing (Plant Products, Brampton, ON, received on June 21, 2007). No preparation was required to prepare the perlite for testing.

Agricultural gypsum (22.68 kg) was purchased for testing (Plant Products, Brampton, ON, received on June 21, 2007). No preparation was required to prepare the gypsum for testing.

The pH and conductivity of the soil conditioners were determined before testing to identify any of the amendments which might affect the pH and/or conductivity of the test soil. Results are presented in Table 2.

Table 2 Characteristics of the Soil Conditioners Used in the Assessments with Earthworms.

Soil Conditioner	pH	Conductivity (µS/cm)	Appearance
Peat	3.68	128	Fibrous material; brown; fluffy and lightweight
Coir	5.72	429	Fibrous material; brown; fluffy and lightweight
Perlite	8.75	60.4	White granules
Gypsum	7.84	1.20 mS/cm	White powder

1.4 REFERENCE TOXICITY TESTS

A reference toxicity test was conducted according to the recommendations in the Environment Canada test method (EC, 2004). This test is also a mandatory requirement for accreditation by the Canadian Association of Environmental Analytical Laboratories (CAEAL). The Stantec Soils Laboratory is CAEAL-accredited for the Environment Canada plant, earthworm and collembola

test methods. The reference toxicant used for conducting the earthworm reference toxicity test was boric acid and the reference test soil was the artificial experimental control soil described in Subsection 1.1.3. The purpose of conducting reference toxicity tests was to evaluate the health of the test organisms, precision and accuracy of laboratory techniques and technicians, and suitability of the experimental conditions. Organisms used in the reference toxicity tests were from the Stantec Soils Laboratory in-house culture. The results from the reference toxicity test are reported in Appendices B and C.

2.0 Chronic Testing of Soil Conditioners at Different Amendment Levels

The chronic earthworm test is a 63-day test. The measurement endpoints for the test include adult survival (Day 35), number of progeny produced (Day 63), and wet and dry mass of individual progeny (Day 63). Chronic earthworm tests were conducted with artificial soil (negative control) and a reference control soil amended with one of four soil conditioners, added to the soil at different amendment levels. Artificial soil was included in the experimental design for QA/QC purposes only, to assess test organism health, technician proficiency, experimental conditions, testing procedures, and test validity. Two chronic earthworm tests were conducted, one with a clay reference soil and the second with a sand reference soil. The soil conditioners were selected for testing based on the findings of the literature review conducted in Phase 1 of this project. The selection criteria for the soil conditioners are presented in Section 1.3. The soil conditioners included in this assessment included: peat, coir, perlite and gypsum. The amendment levels selected were 0, 2.5, 5, 10, and 15%. The purpose of conducting the earthworm tests was to identify effective soil conditioners and appropriate amendment levels that would improve the structure of 'suboptimal' soils. Therefore, the "performance" of test organisms in these amended soils was compared.

2.1 MATERIAL AND METHODS

The tests were conducted following the Environment Canada test method (EC, 2004) and details of the experimental procedures can be found in this document. The experimental design and test conditions for each test are summarized in Table A.1 (Appendix A) and in the test reports in Appendices B and C. The test reports also contain the results of the chronic tests and any modifications to, or deviations from, the procedures and conditions recommended in the test method.

Due to the nature of the selected soil conditioners, some of the soil treatments were prepared in advance of test initiation. The pH of each of the reference soils was compared to the pH of each soil conditioner. Based on the comparisons, decisions were made to adjust soil pH and allow for stabilization of soil pH prior to testing. Soil treatments in which the soil conditioner amendment was expected to lower the pH of the soil were prepared in advance. For the clay reference soil, peat was identified as a conditioner that might lower the pH of the soil. For the sand reference soil, which had a higher pH than the clay reference soil, both peat and coir were identified as conditioners that might lower soil pH. Soil treatments were considered acceptable for test initiation once the maximum pH difference between the 0% treatment and the amended treatments was ≤ 0.5 pH units.

2.1.1 Clay Reference Soil Test

The clay soil treatments amended with peat were prepared on August 9, 2007. Soils were mixed and stored in stainless steel mixing bowls and covered with aluminum foil for the duration of the buffering period. The pH of the soil decreased with the addition of peat. The decrease in pH was greater as the amendment level of peat increased, so the treatment with the highest peat amendment resulted in the lowest pH. The peat amended soils were buffered with $CaCO_3$ over a 2-week period. The pH and conductivity of each soil treatment were checked approximately 3 days after each buffering event; soil pH was measured and further buffering performed as required. Following the final buffering event, the maximum pH difference relative to the 0% treatment for the peat amended soils was 0.24 (0% peat = 6.31 and 15% peat = 6.55). Based on the acceptability criterion for soil pH, the soils were deemed ready for testing.

The clay soil treatments amended with coir, perlite and gypsum, along with the AS treatment, were prepared on August 21, 2007. These soil treatments were not buffered prior to testing. Based on the comparison of clay reference soil pH to the pH of each soil conditioner, no significant changes in pH were expected for these treatments.

The amount of soil added to each test unit was adjusted so that each test unit was ~ 2/3 full of test soil. Because of bulk-density differences, this equated to approximately 200 – 270 g of soil in each test unit. Testing was initiated with the addition of the earthworms to the test soils on August 22, 2007. Food and water were added to each test unit every 14 days over the test duration. The adult worms were removed on September 26, 2007 (Day 35) and the test was terminated on October 24, 2007 (Day 63).

The pH, conductivity, and soil moisture content of each of the treatments were determined at the time of test initiation and test termination. The water-holding capacity of each soil treatment was determined once testing had been initiated, from extra soil collected from the test setup (August to September, 2007).

2.1.2 Sand Reference Soil Test

The sand soil treatments amended with peat and coir were prepared on August 9, 2007. Soils were mixed and stored in stainless steel mixing bowls and covered with aluminum foil for the duration of the buffering period. The pH of the soil decreased with the addition of peat. The decrease in pH was greater as the amendment level of peat increased, so the treatment with the highest peat amendment resulted in the lowest pH. The peat soils were buffered with $CaCO_3$ over a period of approximately 3 weeks. The pH and conductivity of each soil treatment were checked approximately 3 days after each buffering event; soil pH was measured and buffered as required. Following the final buffering event, the maximum pH difference relative to the 0% treatment for the peat amended soils was 0.49 (0% peat = 8.22 and 15% peat = 7.73). Based on the acceptability criterion for soil pH, the soils were deemed ready for testing.

The coir amendment resulted in an increase in soil pH as the amendment level increased. The coir amended soils were left to sit for approximately 3 weeks without further amendment. The

maximum pH difference relative to the 0% treatment for the coir amended soils was 0.23 (0% coir = 8.22 and 5% coir = 7.99). Based on the acceptability criterion for soil pH, the soils were deemed ready for testing.

The sand soil treatments amended with perlite and gypsum were prepared on August 23, 2007. The treatments were prepared following the same procedures as those used to prepare the peat and coir amended soils. The pH of the soil decreased with the addition of gypsum. The pH decrease was similar among all of the treatments amended with gypsum. The gypsum soils were buffered with $CaCO_3$ over a period of approximately 1 week. The pH and conductivity of each soil treatment were checked approximately 2 days after each buffering event; soil pH was measured and buffered as required. Following the final buffering event, the maximum pH difference relative to the 0% treatment for the gypsum amended soils was 0.25 (0% gypsum = 7.95 and 2.5% gypsum = 7.70). Based on the acceptability criterion for soil pH, the soils were deemed ready for testing.

The perlite amendment resulted in an increase in soil pH as the amendment level increased. The perlite amended soils were left to sit for approximately 1 week without further amendment. The maximum pH difference relative to the 0% treatment for the perlite amended soils was 0.16 (0% perlite = 8.10 and 15% perlite = 8.26). Based on the acceptability criterion for soil pH, the soils were deemed ready for testing.

The AS treatment was prepared on August 29, 2007. Testing of all soil treatments was initiated with the addition of the earthworms to the test soils on August 30, 2007. The amount of soil added to each test unit was adjusted so that each test unit was ~ 2/3 full of test soil. Because of bulk-density differences, approximately 230 – 350 g of soil was added to each test unit. Food and water were added to each test unit every 14 days over the test duration. The adult worms were removed on October 4, 2007 (Day 35) and the test was terminated on November 1, 2007 (Day 63).

The pH, conductivity, and soil moisture content of each of the treatments were determined at the time of test initiation and test termination. The water-holding capacity of each soil treatment was determined once testing had been initiated, from extra soil collected from the test setup (September, 2007).

2.1.3 Statistical Analyses

Statistical analyses were performed using SYSTAT 12 (SSI, 2007). Statistical differences among treatments (soil conditioner and % amendment) were determined for both the clay and sand reference soils by applying analyses of variance procedures (ANOVAs) to the data for all endpoints (adult survival, progeny production, and progeny wet and dry mass) for each soil. Tests for ANOVA assumptions include the Shapiro-Wilk Test (normality) and the Levene's Test (homogeneity of variance). Nonparametric statistics were performed for any endpoint data for which at least one of the assumptions of normality and homogeneity was not met, after transforming the data and checking for outliers.

For the test results with the clay reference soil, at least one of the assumptions of normality or homogeneity of variance was not met. Therefore, the non-parametric equivalent procedure (e.g., Kruskal-Wallis One-way Analysis of Variance) was applied to the data, followed by the appropriate means comparison test (Mann-Whitney U-Test).

The test results for the sand reference soil were analyzed in a manner similar to that used for the clay reference soil data. For adult survival and wet and dry mass, at least one of the assumptions of normality and homogeneity was not met. Data were analyzed using the Kruskal-Wallis One-way Analysis of Variance followed by the Mann-Whitney U-Test. An ANOVA was performed to analyze the progeny production data because both assumptions of normality and homogeneity were met when one outlier was removed from the data set.

2.2 RESULTS

2.2.1 Clay Reference Soil Test

Detailed descriptions of the experimental design, conditions, and test results are provided in the test report for the earthworm test conducted in clay reference soil in Appendix B.

There was no significant difference for mean adult survival among soil treatments. The mean percent adult survival was 100% for all treatments tested (Figure B.1 and Table B.2; Appendix B). Organisms appeared to be healthy at the time of the Day 35 adult removal.

Significant differences were found among treatments for mean progeny production (Figure B.1 and Table B.2; Appendix B). At the 0, 2.5, 5, and 10% amendment levels, no significant differences among the soil conditioners were found. However, significant differences were found at the 15% level. Progeny production was highest in the peat amended soil. Peat, coir and gypsum were not significantly different from each other; perlite and gypsum were not significantly different. When comparing percent amendments grouped by soil conditioner, significant differences were found within the peat and coir groupings. In the peat comparison, the 0, 2.5, 5 and 10% amendments were not significantly different; the 10 and 15% amendment treatments were not significantly different. Progeny production was highest at the 15% amendment level. In the coir grouping, the 0, 2.5, 5 and 10% amendments were not significantly different; the 5, 10 and 15% amendments were not significantly different. Progeny production was highest at the 15% amendment level; production increased as the amendment level increased. Progeny produced during the test in all treatments appeared to be healthy at the end of the test.

There were no significant differences found for mean wet and dry mass of individual progeny (Figure B.2 and Table B.2; Appendix B).

The pH of each soil treatment was measured at the start and end of the test. The maximum difference in soil pH relative to the respective unamended control soil (0%) for each treatment at the beginning of the test (Day 0) was 0.39, 0.44, 0.28, and 0.79 pH units for peat, coir, perlite and gypsum, respectively. The maximum difference in soil pH relative to the respective

unamended control soil for each treatment at the end of the test (Day 63) was 0.28, 0.55, 0.78, and 0.72 pH units for peat, coir, perlite and gypsum, respectively (Table B.3; Appendix B). In general, the pH of the soil treatments dropped over the test duration. The potential influence of pH on *E. andrei* test results is discussed in Subsection 3.3.1.

Electrical conductivity was measured at the start and end of the test. The maximum difference in soil electrical conductivity relative to the respective unamended control soil (0%) for each treatment at the beginning of the test (Day 0) was 117, 163, 10, and 2033 µS/cm for peat, coir, perlite and gypsum, respectively. The maximum difference in electrical conductivity relative to the respective unamended control soil for each treatment at the end of the test (Day 63) was 190, 133, 231, and 2021 µS/cm for peat, coir, perlite and gypsum, respectively (Table B.3; Appendix B). In general, conductivity was found to increase over the duration of the test for peat and perlite and decrease for coir. For gypsum, conductivity both increased and decreased with the different amendment levels. The potential influence of the conductivity on *E. andrei* test results is discussed in Subsection 3.3.2.

The initial and final soil moisture contents (% WHC) for each of the soil treatments are summarized in Table B.3 (Appendix B). At the start of the test, the moisture content of each soil treatment was assessed by eye and an appropriate volume of water was added to the soil to create a crumbly texture. The volume of water was sufficient to ensure the soils were adequately hydrated, but not over-watered. For the peat and coir amended treatments, soil moisture content increased with an increase in amendment level, at both the start and end of the test. For perlite and gypsum amended treatments, soil moisture content remained around the same level for all amended treatments. Soil moisture contents generally increased over the duration of the test. Only two soil treatments had soil moisture contents above 100% of WHC, and these treatments were 15% peat (initial and final) and 15% coir (final).

All performance criteria for test acceptability were met for the artificial soil treatment (EC, 2004), indicating that the test procedures, conditions, organism health and technical proficiency were acceptable (Table B.1; Appendix B). Reference toxicity QA/QC data were also within the historical warning limits (Appendix B).

2.2.2 Sand Reference Soil Test

Detailed descriptions of the experimental design, conditions, and test results are provided in the test report for the earthworm test conducted in sand reference soil in Appendix C.

There was no significant difference for mean adult survival among soil treatments. The mean percent adult survival was 100% for all treatments tested, except for the 2.5% peat treatment, which had a mean percent adult survival of 80% (0 adult worms found in one test unit) (Figure C.1 and Table C.2; Appendix C). Organisms appeared to be healthy at the time of the Day 35 adult removal.

Significant differences were found among treatments for mean progeny production (Figure C.1 and Table C.2; Appendix C). At the 0% amendment level, no significant differences were found

among the soil conditioners tested. At the 2.5% amendment level, progeny production in the coir treatment was significantly higher than progeny production in soils amended with the other soil conditioners. Peat, coir and perlite treatments were not significantly different at the 5% amendment level, while perlite and gypsum treatments were not significantly different. Progeny production was highest in the coir amended soil. At the 10% amendment level, progeny production in peat, coir and perlite treatments was not significantly different, while coir, perlite and gypsum were not significantly different. Progeny production was highest in the peat treatment at this amendment level. Significant differences among the soil conditioners at the 15% amendment level were found. Progeny production was significantly higher in the peat treatment than in the treatments with the other soil conditioners. The coir and perlite treatments were not significantly different, and no significant difference was found between the perlite and gypsum treatments. When comparing percent amendments grouped by soil conditioner, significant differences were found with the peat and coir groupings. In the peat comparison, progeny production generally increased as the peat amendment level increased, with the highest progeny production at the 15% amendment level. No significant difference was found between 0 and 2.5% amendment levels; among 0, 5 and 10% amendment levels; or among 5, 10 and 15% amendment levels. In the coir grouping, the highest progeny production was observed at the 5% amendment level. Progeny production at the 2.5% amendment level was significantly different from production in 0, 10 and 15% amendment levels; the 5% amendment level was not significantly different from any of the other amendment levels. Progeny produced during the test for all treatments appeared to be healthy at the time of the test process.

There were no significant differences found for mean wet mass of individual progeny, based on soil treatment (Figure C.2 and Table C.2; Appendix C).

Significant differences were found for mean individual progeny dry mass, based on soil conditioner and on percent amendment. At the 0% amendment level, progeny dry mass from the peat and gypsum amended treatments were significantly different. Dry mass was highest in the peat amended treatment. The progeny dry mass from the gypsum treatment was significantly lower than the other conditioner treatments at the 2.5% amendment level. At the 5% amendment level, progeny dry mass from the perlite and gypsum amended soils were significantly different, with the highest progeny dry mass found in the perlite treatment. When comparing treatments based on soil conditioner, significant differences were found with the peat, coir and gypsum groupings. In both the peat and coir amended soils, the 2.5 and 15% amendment levels were significantly different. Also, the mean individual progeny dry mass decreased as the amendment level of each conditioner increased. Significant differences were found among the treatments amended with gypsum. The mean individual progeny dry mass increased from the 2.5 to 15% amendment level.

The pH of each soil treatment was measured at the start and end of testing. The maximum difference in soil pH relative to the respective unamended control soil (0%) for each treatment at the beginning of the test (Day 0) was 0.40, 0.11, 0.14 and 0.33 pH units for peat, coir, perlite and gypsum, respectively. The maximum difference in soil pH relative to the respective unamended control soil for each treatment at the end of the test (Day 63) was 0.25, 0.22, 0.11 and 0.53 pH units for peat, coir, perlite and gypsum, respectively (Table C.3; Appendix C). In general, the pH of the soil treatments dropped over the test duration. The potential influence of pH on *E. andrei* test results is discussed in Subsection 3.3.1.

Electrical conductivity was measured at the start and end of the earthworm test. The maximum difference in soil electrical conductivity relative to the respective unamended control soil (0%) for each treatment at the beginning of the test (Day 0) was 115, 109, 115 and 1774 µS/cm for peat, coir, perlite and gypsum, respectively. The maximum difference in electrical conductivity relative to the respective unamended control soil for each treatment at the end of the test (Day 63) was 36, 87, 24 and 1640 µS/cm for peat, coir, perlite and gypsum, respectively (Table C.3; Appendix C). In general, conductivity was found to decrease for the soil treatments over the duration of the test. The potential influence of the conductivity on *E. andrei* test results is discussed in Subsection 3.3.2.

The initial and final soil moisture contents (% WHC) for each of the soil treatments are summarized in Table C.3 (Appendix C). At the start of the test, the moisture content of each soil treatment was assessed by eye and an appropriate volume of water was added to the soil to create a crumbly texture. The volume of water was sufficient to ensure the soils were adequately hydrated, but not over-watered. For the peat, coir and perlite amended treatments, soil moisture content increased with an increase in amendment level, at both the start and end of the test. For the gypsum amended treatments, soil moisture content remained around the same level for all amended treatments. Soil moisture contents generally increased over the duration of the test. Only two treatments had soil moisture contents above 100 % of WHC, and these treatments were 10% coir (initial and final) and 15% coir (final).

All performance criteria for test acceptability were met for the artificial soil treatment (EC, 2004), indicating that the test procedures, conditions, organism health and technical proficiency were acceptable (Table C.1; Appendix C). Reference toxicity QA/QC data were also within the historical warning limits (Appendix C).

3.0 Summary and Discussion

3.1 CLAY - *Eisenia andrei*

The soil conditioners and amendment levels used in this test did not significantly affect earthworm adult survival, or individual progeny wet and dry mass in the clay reference soil. Progeny production was significantly affected by the treatments tested (refer to Subsection 2.2.1 for significant and not significant differences among treatments). At the 15% amendment level, progeny production was highest in the peat amended soils. The amendment level of peat and coir had a significant effect on progeny production. In general, as the amendment level increased, so did progeny production. Overall, the 15% peat treatment resulted in the highest number of progeny produced in this test. This result was significantly different from some, but not all other treatments.

The peat and coir amendments increased the organic matter content of the clay reference soil, and broke up the large clay aggregates into smaller soil aggregates. At the higher amendment levels, the amended clay soil was fluffier and not as sticky as the 0% amended soil. Soil amended with peat and coir was easier to pick through versus the 0% amended treatment, especially at the higher peat and coir amendment levels. The perlite broke up the clay soil, although the soil was still very clayey (i.e., sticky) for all amendments levels. Perlite was clearly visible in the test soils as white chunks. At the 15% amendment level, the soil was clayey but also had a grainy feel from the addition of perlite. The addition of gypsum did not visibly change the soil in any significant way. The gypsum amended treatments looked very similar to the 0% gypsum treatment and were still very sticky and compacted. Only the peat and coir amendments appeared to improve the structure of the clay soil.

3.2 SAND - *Eisenia andrei*

Earthworm adult survival and individual progeny wet mass were not significantly affected by the type of soil conditioner or amendment level in the sand reference soil. However, progeny production was significantly affected by the treatments tested (refer to Subsection 2.2.2 for significant and not significant differences among treatments). The coir treatment resulted in higher progeny production at the 2.5 and 5% amendment levels; these results were significantly different from some but not all other treatments. At the 10 and 15% amendment levels, the peat amendment resulted in an increase in progeny production. Again, these results were significantly different from some but not all other treatments. As the peat amendment level increased, progeny production was found to increase. For coir, the highest progeny production occurred at the 5% level. Overall, the 5% coir amendment resulted in the highest number of progeny produced in this test; however, depending on the amendment level of the soil conditioner, coir and peat were found to be equally effective in increasing progeny production.

Mean individual progeny dry mass was significantly affected by the treatments tested in the sand reference soil (refer to Subsection 2.2.2 for significant and not significant difference among treatments). The largest dry mass of individual progeny at the 2.5 and 5% amendment levels was found in the perlite amended treatment. The amendment level of peat, coir and gypsum had a significant effect on progeny dry mass. For both peat and coir, as the amendment level increased, the dry mass decreased. However, in general, as the amendment levels for peat and coir increased, the number of progeny produced also increased, suggesting that more worms were produced, of a smaller size. For gypsum, as the amendment level increased, so did the progeny dry mass. In general, fewer worms were produced in the gypsum treatments than in soils amended with the other soil conditioners, and the progeny produced were not as large as those produced in the soils amended with perlite.

The sand reference soil used for testing was a fine sandy loam, and when hydrated with the water and homogenized, the soil began to form aggregates and 'crumbs' without amendment. The peat and coir amendments increased the organic matter content of the sand reference soil. Both the peat and coir amendments were found to break up the soil and make it fluffier. The perlite amendment was clearly visible in the test soils as white chunks. The perlite broke up the soil; at the higher amendment levels, the perlite gave the soil a grainy feel. The addition of gypsum did not visibly change the soil in any significant way.

3.3 SOIL PHYSICAL CHARACTERISTICS

3.3.1 Test Soil pH

Soil pH affects the distribution, number and species composition of earthworms in a given soil (Edwards and Lofty, 1972), and can affect worm weight, number of juveniles per cocoon, and cocoon production and hatchability (Jänsch et al., 2005). *E. andrei* prefers neutral to slightly acid conditions, typically 5.0 to 7.4, but can tolerate soil pH values from 4.0 to 9.0 (Jänsch et al., 2005; Edwards and Lofty, 1972). Earthworms tend to be rare in soils with pH values below 4.5 and are usually absent from soils with pH values below 3.5 (Edwards and Lofty, 1972). Earthworms also tend to avoid alkaline soils.

For the clay reference soil, the pH of the peat, coir and perlite amended soils on Day 0 ranged from 5.15 to 6.60, which was within the preferred pH range for *E. andrei*. The pH of the gypsum amended soils ranged from 4.80 to 6.22, with 2 of the treatments at or below pH 5.00 (2.5 and 5% treatments). Our experience has shown that the threshold pH for *E. andrei* reproduction in soil ranges between pH 4.8 and 5.2 (Feisthauer et al., 2006); however, this pH range still falls within the pH range tolerated by *E. andrei*. At Day 63, the pH of the peat ranged from 5.66 to 6.04. For coir, perlite and gypsum, the pH ranged from 4.78 to 6.14, with 4 of the treatments at or below pH 5.00 (10 and 15% coir, 0% perlite and 2.5% gypsum). The results of the test indicate that earthworms reproduced in all of these treatments except for the 0% perlite and 5% gypsum treatments.

The pH values measured on Day 0 and Day 63 for the sand reference soil were at the top end of the acceptable range for earthworm reproduction, but were still within the pH range tolerated by *E. andrei*. On Day 0, the pH values ranged from 7.79 to 8.30 for peat, coir, perlite and gypsum. For the same treatments on Day 63, the pH values ranged from 7.56 to 8.24.

The pH values for the treatments varied due in part to the conditioner used as the soil amendment and in part, to the amount of calcium carbonate required to buffer the soils during test soil preparation.

3.3.2 Test Soil Conductivity

The conductivity of the clay treatments amended with peat, coir and perlite ranged from 16 to 190 µS/cm and 26 to 280 µS/cm on Day 0 and 63, respectively. The conductivity range measured for the gypsum amended soils (excluding 0%) was much higher than all other soil treatments. The conductivity values measured for the 0% gypsum treatment were 27 and 39 µS/cm for Day 0 and 63, respectively. However, the conductivity range for the gypsum amended treatments (2.5 to 15%) ranged from 2020 to 2060 µS/cm for both Day 0 and 63.

A similar difference between the gypsum and the other soil amendments was observed with the sand soil. The conductivity range for the treatments amended with peat, coir and perlite was 307 to 467 µS/cm and 208 to 313 µS/cm for Day 0 and 63, respectively. The conductivity range measured for the gypsum amended soils (excluding 0%) was much higher than all other soil treatments. The conductivity values measured for the 0% gypsum treatment were 466 and 210 µS/cm for Day 0 and 63, respectively. However, the conductivity range for the gypsum amended treatments (2.5 to 15%) ranged from 2140 to 2240 µS/cm and 1620 to 1850 µS/cm for Day 0 and 63, respectively.

Differences in the conductivity of the test soil treatments were due in part to the amount of buffering that occurred to prepare the treatments for testing, and to the conditioner used as the soil amendment. Amending the test soils with gypsum resulted in a substantial increase in soil conductivity. It was expected that the much higher conductivity in the gypsum amended soil treatments would negatively affect earthworm reproduction (i.e., cause a decrease in reproduction). Despite the increase in conductivity in clay soil amended with gypsum, earthworms did reproduce in all treatments except 0 and 5%. A similar increase in conductivity was observed for the sandy soil amended with gypsum; however, earthworms we produced in all treatments.

Gypsum is a hydrous calcium sulphate, consisting of finely powdered rocks. When added to soil, gypsum is a good source of calcium for both plants and soil invertebrates (Wallace and Wallace, 2006). In spite of the high conductivity measured in the gypsum amended treatments, it is possible that the earthworms were using the added calcium as a nutrient, allowing them to continue to reproduce.

3.3.3 Test Soil Moisture Content (% WHC)

The soil moisture contents for each soil treatment were measured at the start and end of the test. A calculation sheet based on the wet and dry masses of the reference soil and soil amendment was used as a guideline to adequately hydrate the test soils for the test setup; however, final judgment on adequate moisture content was made by eye.

Soil moisture content (% WHC) for the clay reference soil amended with peat and with coir at the start of the test was 38 to 126% and 40 to 77%, respectively. At the end of the test, the soil moisture contents were 51 to 143% and 62 to 109%, respectively. In general, for both the peat and coir amendments, the soil moisture content increased as the amendment level increased. For the soil amended with perlite and gypsum, the soil moisture contents at the start of the test were 41 to 47% and 33 to 46%, respectively. For the end of the test, the moisture contents were 57 to 73% and 41 to 59%, respectively. For these treatments, the soil moisture content did not increase or decrease as the amendment level increased; instead, the values stayed relatively the same.

For the sand reference soil, moisture content (% WHC) for the peat, coir and perlite amended treatments at the start of the test were 35 to 73%, 36 to 113%, and 39 to 71%, respectively. At the end of the test, the soil moisture contents for the same treatments were 40 to 90%, 40 to 108%, and 48 to 85%, respectively. In general, as the amendment level increased, the soil moisture increased. The gypsum amended treatments had moisture contents (% WHC) ranging from 38 to 53% at the start of the test and 42 to 53% at the end of the test. Soil moisture content did not increase or decrease as the amendment level increased; instead, the values stayed relatively the same.

The soil conditioners affected soil moisture content (% WHC) differently. In both the clay and sand reference soils amended with peat and coir, the soil moisture content increased as the amendment level increased. Peat and coir, both organic soil conditioners, have the ability to affect the physical and chemical properties of soils and the way the soils behave (Schulte and Kelling, 1998). Organic matter amendments tend to improve drainage of heavy soils and help dry soils retain moisture and nutrients. Perlite, an inorganic soil conditioner, tends to be used to reduce soil compaction, increase porosity, aeration and drainage, and trap moisture and nutrients from the soils (Crawley and Zabcik, 2005). Gypsum tends to be used to improve water penetration and aeration of soils, and it can be used to alleviate unsuitable soil chemical conditions as it is a very good source of calcium (Wallace and Wallace, 2006; Bathke et al., 1992; Glattstein, 1989).

3.3.4 Soil Organic Matter

One of the most important components of a soil is the organic matter content (Schulte and Kelling, 1998). Organic matter affects the physical and chemical properties, and the ‘behaviour’ of soil, by increasing the water-holding capacity, improving drainage and aeration of heavy soils, and increasing the retention of moisture and nutrients in dry soils (Riofrio and Wittmeyer, 1992; Schulte and Kelling, 1998). Microbial activity and earthworm populations have been observed

to increase with the amendment of soil with organic matter (Kukkonen et al., 2004). Earthworm reproduction is very sensitive to organic matter content level in soils, particularly for *Eisenia* species, and the threshold levels for optimal earthworm reproduction are generally between 3 and 4% (Jänsch et al., 2005).

The organic matter contents for the reference soils used in testing were 0.3% for the clay soil and 1.3% for the sand soil; both soils were well below the threshold levels for organic matter content presented by Jänsch et al. (2005). However, the sand soil had a higher organic matter content than the clay. The results of the tests indicate that the worms did better overall in the sand reference soil (progeny production, and individual progeny wet and dry mass) than in the clay reference soil. Also, progeny production in both soils increased with the addition of organic matter, in the form of peat and clay. In general, progeny production increased as the peat and coir amendment levels increased.

3.4 SUMMARY

Based on the results of the Phase 2 testing, the organic soil amendments seemed to perform better than the inorganic amendments when amending the clay and sand reference soils with low organic matter contents. Both the peat and coir improved progeny production.

Peat and coir are both lightweight, fluffy, fibrous materials. Both improve the balance of air and water in the soil while reducing soil compaction (Feiger, 2005; Riofrio and Wittmeyer, 1992). Clay soils amended with peat and coir are broken up so that aeration of the soil is improved. In sandy soils, peat and coir bind and aggregate the soil, and help to retain moisture. In general, the more organic matter present in the soil, the better the soil physical properties (Wallace and Wallace, 1986). For this assessment, both the peat and coir were effective in breaking up the clay soil and aggregating the sand soil, and in improving the organic matter content of the reference soils.

Perlite is often used to prevent soil compaction and is able to trap moisture and nutrients in the soil (Crawley and Zabcik, 2005). Perlite was successful in breaking up the clay and sand reference soils used in the tests; however, it most likely would have performed better in relation to the organic amendments in soils where the organic matter contents were not so low. The fact that the perlite was hard, abrasive and gave the soils a grainy texture might have affected the earthworms in the test.

Gypsum (hydrous calcium sulphate) is used to improve water penetration and aeration of soil and to prevent soil compaction (Wallace and Wallace, 2006; Glattstein, 1989). It can also be used to alleviate unsuitable soil chemical conditions (Bathke et al., 1992). The calcium is used by various soil invertebrates and plant species as a nutrient, and is important in creating water-stable soil aggregates by cementing together soil particles (Wallace and Wallace, 2006; Glattstein, 1989). Gypsum did not visibly change the physical characteristics of either reference soil, as the amended (2.5 to 15%) and the nonamended (0%) soil treatments looked very similar. However, soil conductivity was increased substantially with this amendment.

3.5 PHASE 3 TESTING

Phase 3 of this project will involve another set of earthworm tests. Instead of testing only clay and sand reference soils, this next round of testing will involve both reference and contaminated soils. The purpose of the testing will be to determine if the soil conditioners selected for further testing from Phase 2 (peat and coir) have any effect on contaminant bioavailability (e.g., bind contaminants) while improving conditions for earthworm reproduction. Both clay and sand soils will be tested; the reference and contaminated soils for each soil type will be matched as closely as possible to reduce the influence of differences in the soil physical characteristics on the results of the test. If the current contamination level in the contaminated soils is not appropriate, the contaminated soils might be amended by either spiking the soil with a toxicant or diluting the soil with clean reference soil.

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Appendix A

Test Design, Procedures and Conditions

Table A.1. Experimental Design and Conditions of Chronic Earthworm Tests.

Test	Earthworm
Test type	Chronic Screening
Test duration (d)	63 (35-d adult survival)
Test unit (chamber)	Glass 500-mL mason jar
Amount of soil	270 g wet weight
Temperature (day/night)	20 ± 2°C
Photoperiod (h)	16 light : 8 dark
Treatments	Artificial Soil (QA/QC for both tests) Clay Reference Soil • 0, 2.5, 5, 10 and 15% peat • 0, 2.5, 5, 10 and 15% coir • 0, 2.5, 5, 10 and 15% perlite • 0, 2.5, 5, 10 and 15% gypsum Sand Reference Soil • 0, 2.5, 5, 10 and 15% peat • 0, 2.5, 5, 10 and 15% coir • 0, 2.5, 5, 10 and 15% perlite • 0, 2.5, 5, 10 and 15% gypsum
Number of replicate test units per treatment	5
Number of organisms per test unit	2
Lighting (Type & Intensity)	Fluorescent 400-800 Lux
Physico-chemical measurements	Conductivity, pH, % moisture
Biological endpoint measurements	Adult survival (Day 35) Progeny production (Day 63) Progeny wet and dry masses (Day 63)
Statistical endpoints	Significant difference among soil treatments
Description of methods	EC, 2004

A.1. FORMULATION OF ARTIFICIAL SOIL

The artificial control soil (AS) was formulated in the laboratory by mixing ingredients in their dry form, then gradually hydrating with de-ionized water and mixing further until the soil was visibly uniform in colour and texture. The ingredients of AS were 70% silica

sand (No. 200, Barco 71; Optima Minerals, Waterdown, ON), 20% kaolinite clay (Tuckers Pottery Supplies, Richmond Hill, ON), 10% *Sphagnum* spp. peat (Canadian Hydrogardens Ltd., Ancaster, ON) and calcium carbonate (10-30 g per 1 kg peat). A 12 kg batch of AS was formulated on a dry weight basis by adding 7 kg of sand, 2 kg of kaolinite clay, 1 kg of peat, approximately 30 g of $CaCO_3$ (sieved), and 2 L of de-ionized water. The amount of calcium carbonate required adjusts the soil pH to 6.0-7.5, depended on the nature (i.e., acidity) of the *Sphagnum* peat and the silica sand. Each time a new batch of either of these ingredients was used, it might be necessary to adjust the amount of $CaCO_3$ used in each batch of formulated soil.

A.2. DETERMINATION OF SOIL MOISTURE CONTENT

Prior to the day of test soil formulation, a 3 to 5 g sample of control soil wet weight (wet wt.) was placed into a pre-weighed aluminum weigh boat (1 or 2.5 g) and the wet mass recorded. Each weigh boat was then placed into a drying oven at 105°C for a minimum of 24 hours. The dry weight of each soil was then determined. Percent moisture content was calculated by expressing the dry mass as a percentage of the wet mass:

$$\text{Percent Moisture} = \frac{\text{wet mass (g wet wt.)} - \text{dry mass (g dry wt.)}}{\text{wet mass (g wet wt.)}} \times 100$$

The initial moisture content of the soils was needed in order to standardize the moisture content in the test soils.

A.3. DETERMINATION OF WATER-HOLDING CAPACITY

The water-holding capacity of each soil was determined by placing ~130 g wet weight of soil sample into a large aluminum container and drying the sample at 105°C to a constant weight for at least 48 hours. Subsequent to drying, the sample was removed from the oven and cooled in a desiccator for at least 1 hour until the soil reached room temperature. 100 g of the dried soil sample were placed into a 250-mL glass container and 100 mL of de-ionized water were added to the sample and mixed thoroughly with a spoon to ensure that all of the sample was wetted and that a slurry of soil and water existed. A circle of filter paper was folded into quarters and placed into a glass funnel; the folded filter paper was level with the top of the funnel. 7 mL of de-ionized water were slowly added, using a pipette, to the filter paper to wet the entire surface. The combined weight of the funnel and hydrated filter paper was measured. The weight of the dried soil, funnel and hydrated filter paper was recorded as the initial weight. The funnel was placed into an appropriately sized Erlenmeyer flask and the slurry of soil and water was slowly poured onto the hydrated filter paper held in the funnel. Any soil remaining on the glass container and spoon was rinsed into the funnel with minimal amounts of de-ionized water to ensure that all of the solid material had been washed onto the filter. The funnel was covered tightly with aluminum foil and allowed to drain for 3 hours at room temperature. After 3 hours, the funnel, hydrated filter paper, and soil were weighed and recorded as the final weight. The water-holding capacity, expressed as mL water/100 g soil, was equal to the difference between the final and initial weights of the funnel, filter, and sample.

A.4. MEASURING SOIL PH AND CONDUCTIVITY (WATER SLURRY) (MODIFIED FROM THE SOIL ANALYSIS HANDBOOK, 1992).

Approximately 25 g (wet wt.) of test soil and 50 mL of deionized water were placed into a glass beaker and stirred with a glass rod for two minutes. The beakers sat at room temperature in the laboratory for a minimum of 20 minutes. Immediately prior to measuring pH and conductivity, the soil slurry was mixed again. Soil pH was measured with a pH and ATC probe submersed in the soil slurry that was gently agitated until the readings were constant. Conductivity was measured with a conductivity and ATC probe submersed in the freshly mixed slurry, and was recorded once the readings were constant. The slurry was not agitated while conductivity measurements were taken. The soil pH and conductivity were measured using an Accumet® Meter (Fisher Scientific Model 20) that had been calibrated before use with three (pH 4, 7 and 10) external pH buffers and an external conductivity standard. The probes were washed between samples with deionized water.

Appendix B

Test Conditions, Experimental Design, Data Summaries, and Results of the Earthworm Chronic Test Conducted in Clay Reference Soil

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Sample Identification

Client:	Petroleum Technology Alliance Canada
Sample(s) description:	Clay reference soil from Alberta
Sample(s) identification:	0750-KB
Date collected/formulated:	July 2005
Method of soil collection:	Composite samples
Date sample(s) received:	July 28 2005
Time sample(s) received:	10:00 am
Temperature on arrival:	Not available
Soil storage temperature:	Outdoor storage unit until 2007-06-25, then 21.5 ± 1.1°C
Date sample(s) tested:	August 22 – October 24, 2007
Technicians:	Ben Smith, Carolyn Brown, Kelly Olaveson, Natalie Feisthauer, Jessica Sosa, Yvonne Busby, Gladys Stephenson, Joe Keene
Analyst:	Kelly Olaveson
QA/QC:	Gladys Stephenson

Test Organism

Test Organism:	*Eisenia andrei*
Organism Source:	In house culture Ea 07-1, 07-2, 07-3, 07-4, 07-5, 07-6, 07-7
Initial mean adult wet weight (g):	0.449 ± 0.062

Test Conditions and Procedures

Test type:	Static, chronic
Test duration:	63 days
Number of treatments:	21, including 1 negative control (AS) Clay reference soil amended with: • 0, 2.5, 5, 10 and 15% peat • 0, 2.5, 5, 10 and 15% coir • 0, 2.5, 5, 10 and 15% perlite • 0, 2.5, 5, 10 and 15% gypsum
Temperature:	20.3 ± 0.3 °C
Light intensity:	552 ± 105 lux
Photoperiod:	16 h light; 8 h dark
Watering regime:	De-ionized water, misted at test initiation and every 14 days, as required
Feeding regime:	Cooked oatmeal (~ 5 mL), fed at test initiation and every 14 days, as required
Test unit description:	500-mL glass wide-mouthed mason jar
Soil volume/test unit:	200-270 g (depending on soil volume)
No. organisms per test unit:	2
No. replicate test units/treatment:	5 replicates
Measured soil chemistry parameters:	Initial and final soil pH, electrical conductivity, and percent moisture content
Measured endpoint(s):	Day 35 adult survival, number of progeny produced at Day 63, and wet and dry mass of individual progeny at Day 63
Test Protocol:	Biological Test Method: Tests for Toxicity of Contaminated Soil to Earthworms (*Eisenia andrei*,

Eisenia fetida, or *Lumbricus terrestris*). Report EPS 1/RM/43, June 2004. Method Development and Applications Section, Environmental Technology Centre, Environment Canada, Ottawa, Ontario.

Statistical Analyses: Mean, SD – Microsoft Excel (2002)
Kruskal-Wallis One-way Analysis of Variance; Mann-Whitney U-Test (SSI, 2007)
Nominal ☒ measured ☐ concentrations analysed

Test acceptability criteria met? See Table B.1

Table B.1. Performance of earthworms in negative control soil treatment relative to test method validity criteria.

Criterion in Negative Control Soil	Negative Control Soil	Criteria Met?	Positive Control Soil	Solvent Control Soil
28- or 35-d adult survival ≥ 90%	100	Yes	NA	NA
Mean # live progeny/adult ≥ 3	10.0	Yes	NA	NA
Mean dry wt of individual progeny ≥ 2.0 mg	9.27	Yes	NA	NA

Boric Acid Reference Toxicant Data for Artificial Soil

Type of Test: Acute lethality
Test Duration 7 days
Date Tested: 2007-09-06
Organism Lab Code: Laboratory Code No. Ea 07-2, 07-14, 07-19
LC50 Survival: 5754 mg/kg
95% CL: 5070 to 6310 mg/kg
Statistical Analyses: Probit (Stephen, 1977)
Historical Mean LC50: 4282 mg/kg
Warning Limits (± 2 SD): 2684 to 6031 mg/kg
Technician(s): Kelly Olaveson and Natalie Feisthauer
Analyst(s): Natalie Feisthauer

Results

Table B.2. Effect of amending clay reference soil with four different soil conditioners at five amendment levels on earthworm (*E. andrei*) survival (Day 35), growth (Day 63), and reproduction (Day 63). Results are reported as treatment means, and the values in brackets indicate one standard deviation of the mean.

Soil Treatment	%	Mean Percent 35-d Adult Survival (n = 2 adults)	Mean Number of Progeny Produced	Mean Individual Wet Mass of Progeny (mg)	Mean Individual Dry Mass of Progeny (mg)
AS		100 (0)	20.0 (9.6)	43.57 (24.18)	9.27(5.72)
Peat	0	100 (0)	0.0 (0)	-	-
	2.5	100 (0)	0.0 (0)	-	-
	5	100 (0)	1.0 (2.2)	9.18*	1.84*
	10	100 (0)	0.8 (1.1)	23.08 (3.08)	3.88 (0.04)
	15	100 (0)	13.6 (13.8)	15.63 (2.67)	2.74 (0.53)
Coir	0	100 (0)	0.0 (0)	-	-
	2.5	100 (0)	0.2 (0.4)	11.50*	0.80*
	5	100 (0)	1.0 (1.7)	29.08 (23.16)	5.45 (5.44)
	10	100 (0)	2.2 (2.2)	9.67 (1.26)	1.52 (0.27)
	15	100 (0)	6.4 (7.5)	16.05 (6.69)	2.53 (0.77)
Perlite	0	100 (0)	0.0 (0)	-	-
	2.5	100 (0)	0.0 (0)	-	-
	5	100 (0)	1.6 (3.0)	16.84 (10.69)	3.15 (1.77)
	10	100 (0)	0.0 (0)	-	-
	15	100 (0)	0.0 (0)	-	-
Gypsum	0	100 (0)	0.0 (0)	-	-
	2.5	100 (0)	1.2 (1.8)	11.76 (4.51)	2.19 (0.87)
	5	100 (0)	0.0 (0)	-	-
	10	100 (0)	2.2 (3.2)	4.70 (2.34)	0.97 (0.45)
	15	100 (0)	1.8 (4.0)	15.78*	3.07*

* These values have no standard deviation as there was only one data point per treatment.
- No data for these endpoints as there were no progeny produced in this treatment.

The results reported relate only to the sample(s) tested

Date: February 5, 2008 Approved by: Gladys I Stephenson
Laboratory Director

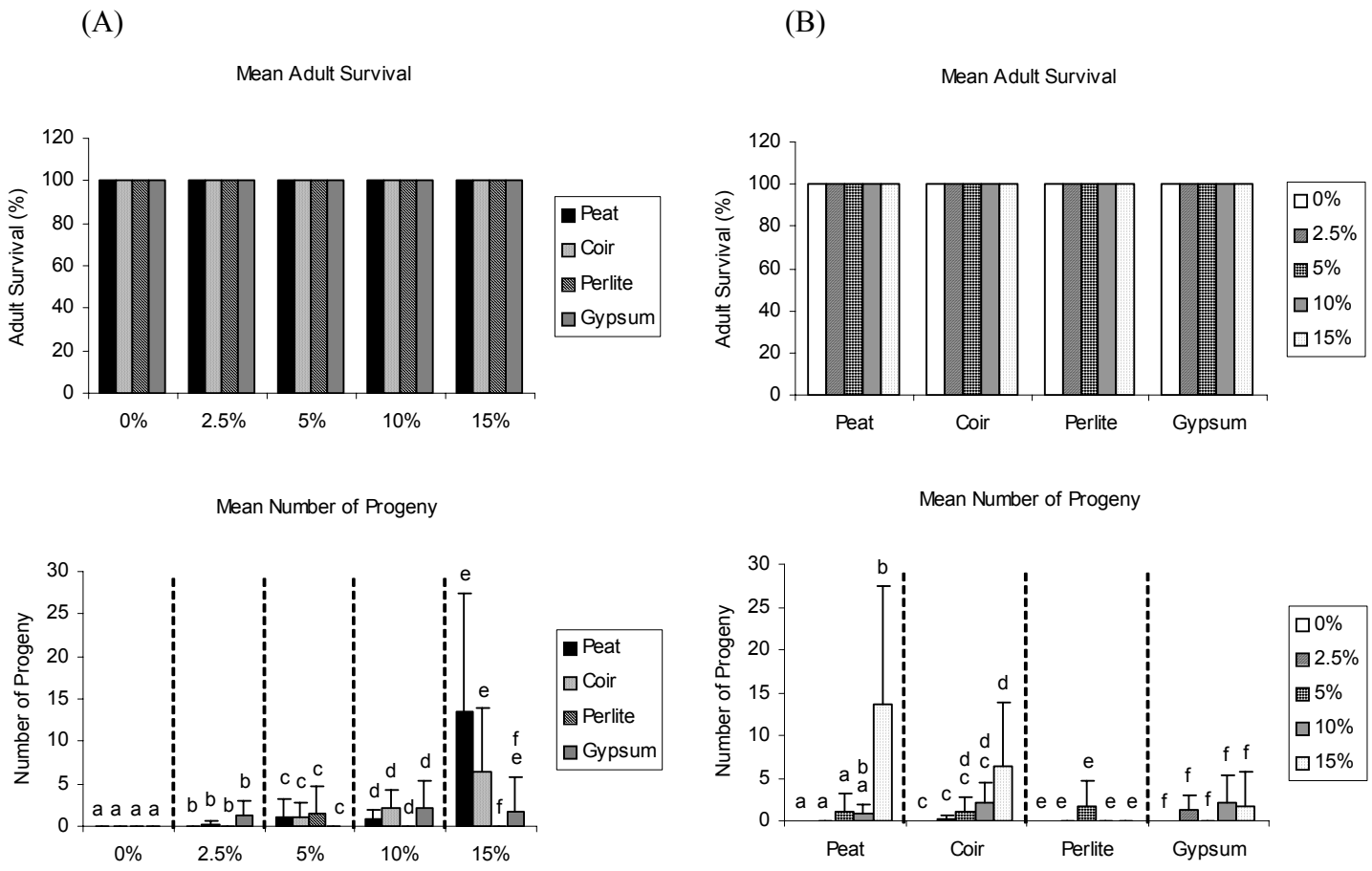

Figure B.1. Earthworm (*E. andrei*) mean adult survival (%) (Day 35) and mean progeny production (Day 63) following the amendment of clay reference soil with four different soil conditioners at five amendment levels. Columns indicate treatment means and bars above the columns represent one standard deviation of the mean. Results are grouped together by percent amendment (A) and by soil conditioner (B) in separate graphs. On each graph, results were compared only within the grouping, not across all treatments tested. Letters above the columns indicate significant difference(s) ($p < 0.05$) among means within a particular conditioner or amendment-level grouping, with different letters indicating where differences were found. There were no significant differences among treatments for adult survival.

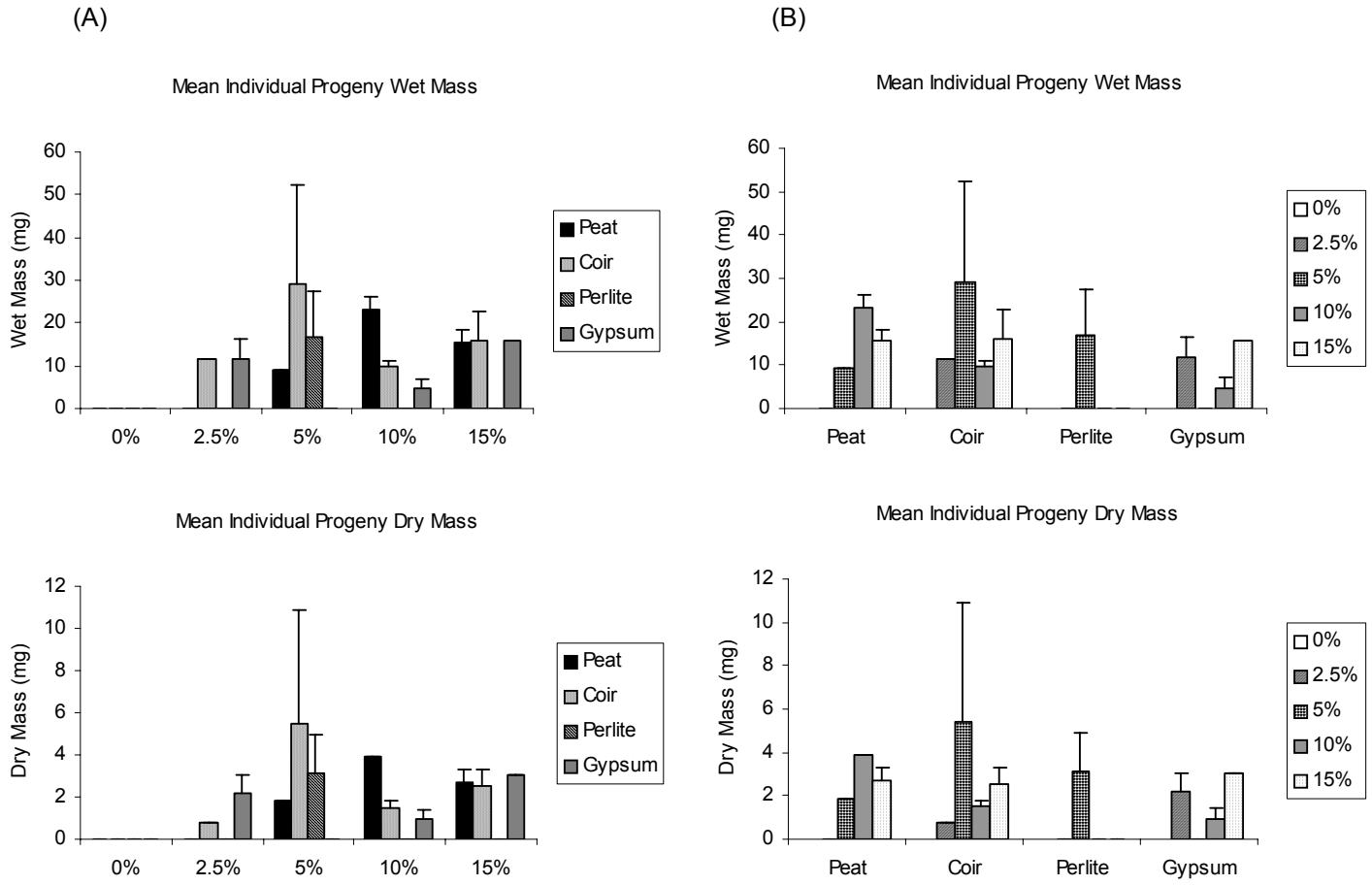

Figure B.2. Earthworm (*E. andrei*) mean individual progeny wet and dry mass (Day 63) following the amendment of clay reference soil with four different soil conditioners at five amendment levels. Columns indicate treatment means and bars above the columns represent one standard deviation of the mean. Results are grouped together by percent amendment (A) and by soil conditioner (B) in separate graphs. On each graph, results were compared only within the grouping, not across all treatments tested. Letters above the columns indicate significant difference(s) ($p < 0.05$) among means within a particular conditioner or amendment-level grouping, with different letters indicating where differences were found. There were no significant differences among treatments within the groupings.

Soil Characteristics

Table B.3. Moisture content, conductivity and pH of test soils at the beginning (Day 0) and end (Day 63) of the test.

Soil Treatment	%	Initial pH[1]	Final pH[1]	Initial Conductivity[1] (µS/cm)	Final Conductivity[1] (µS/cm)	Initial Soil Moisture[2] (% WHC)	Final Soil Moisture[2] (% WHC)
AS		7.40	6.92	164	186	84	90
Peat	0	6.21	5.76	16.2	39	38	51
	2.5	6.13	5.66	31.2	53	51	61
	5	6.60	6.04	94.6	123	87	70
	10	6.22	5.76	116	164	53	93
	15	6.55	5.89	133	229	126	143
Coir	0	5.59	5.54	27.1	26	46	62
	2.5	5.39	5.24	88.6	58	40	64
	5	5.26	5.10	139	103	47	77
	10	5.17	4.99	168	150	58	75
	15	5.15	5.01	190	159	77	109
Perlite	0	5.59	4.92	27.1	280	46	57
	2.5	5.81	5.27	20.2	77	44	61
	5	5.81	5.44	21.6	49	41	58
	10	5.74	5.31	23.7	91	43	73
	15	5.87	5.70	17.6	59	47	71
Gypsum	0	5.59	5.50	27.1	39	46	59
	2.5	4.80	4.78	2050	2020	41	50
	5	5.02	5.11	2020	2060	36	48
	10	5.61	5.77	2060	2060	33	41
	15	6.22	6.14	2040	2050	41	51

[1] pH and conductivity were measured using a 2:1 water:soil slurry.
[2] % WHC - percent of water-holding capacity of the soil.

Table B.4. Texture, organic matter content, carbon content and fertility of test soils (prior to testing).

Parameter	AS	Clay Reference Soil 0750-KB
Soil Texture	Fine Sandy Loam	Clay Loam
Sand (%)	78.9	23.6
Silt (%)	8.5	37.1
Clay (%)	12.7	39.3
Organic Matter (%)	7.9	0.3
Total Carbon (%)	3.85	0.19
Inorganic Carbon (%)	0.05	0.00
Organic Carbon (%)	3.80	0.19
Total Nitrogen (%)	0.11	0.02
Plant Available Phosphorus (mg/kg)	14	18

AS = Artificial Soil

Table B.5. Analytical Results for Clay Reference Soil.

Sample Details/Parameters	Result
L530908-1 0750-KB	
Sampled By: K. Olaveson on 16-JUL-07 @15:30	
Matrix: SOIL	
F2-F4 (O.Reg.153/04)	
CCME Total Hydrocarbons	
F2 (C10-C16) (mg/kg)	<10
F3 (C16-C34) (mg/kg)	<50
F4 (C34-C50) (mg/kg)	<50
Chromatogram to baseline at nC50	YES
F2-F4 (O.Reg.153/04)	
Prep/Analysis Dates	19-JUL-07
Octacosane (%)	100
Regulation 153 Metals, Hg, Cr6+, Avail B	
Boron (B), Available	
Boron (B), Available (ug/g)	<0.1
Hexavalent Chromium in Soil	
Chromium, Hexavalent (mg/kg)	<2
Mercury by CVAA	
Mercury (Hg) (ug/g)	0.07
Standard Metal Scan (ICP)	
Antimony (Sb) (mg/kg)	5
Arsenic (As) (mg/kg)	9
Barium (Ba) (mg/kg)	325
Beryllium (Be) (mg/kg)	0.7
Cadmium (Cd) (mg/kg)	<1
Chromium (Cr) (mg/kg)	33
Cobalt (Co) (mg/kg)	13
Copper (Cu) (mg/kg)	34
Lead (Pb) (mg/kg)	12
Molybdenum (Mo) (mg/kg)	<1
Nickel (Ni) (mg/kg)	34
Selenium (Se) (mg/kg)	<1
Silver (Ag) (mg/kg)	<0.2
Thallium (Tl) (mg/kg)	<1
Vanadium (V) (mg/kg)	44
Zinc (Zn) (mg/kg)	74
% Moisture	6.7

Comments

No organisms exhibiting unusual appearance, behaviour or undergoing unusual treatment were used in this test.

Test Method Modifications

1. Soil pH was measured using a soil-water slurry, which represents our normal practices and is a method modified from the Soil Analysis Handbook (1992), instead of using a $CaCl_2$ slurry, as recommended by the method for pH. This had no impact on the results of the test. The method of using $CaCl_2$ was developed for soil scientists who were comparing the pH of different soils, and wished to minimize the variability of the different pHs (McKeague, 1978). As a result, the $CaCl_2$ method will, by design, minimize the variability of the soil pH among soil samples, and will be less sensitive to differences in pH. In addition, soil pH measured in water is considered to be the pH closest to the pH of soil solution in the field (Hendershot et al., 1993).

2. Test soils were prepared on different days, depending on the conditioner being used to amend the clay reference soil. Peat had a lower pH than the other soil conditioners, so treatments amended with peat were prepared ~ 2 weeks before the other treatments. This allowed time for buffering the peat amended soils with $CaCO_3$ and for the pH of these treatments to stabilize prior to testing.

Test Method Deviations

1. Soil pH, conductivity and moisture content of the test soils were measured on Day -1 of the test rather than on Day 0 as required by the Environment Canada method. The treatments amended with peat were prepared on August 9 2007, to allow for pH adjustment and stabilization. All other treatments were prepared on August 21 2007. Due to the fact that the soils were non-contaminated reference soil amended with different conditioners at different amendment levels, as well as the fact that the soil was undisturbed after dispensed to test units until the earthworms were added to the test units on Day 0 (August 22 2007), this deviation is not expected to affect the pH, conductivity and moisture content results, and cannot affect test results.

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Hendershot W.H., H. Lalonde, and M. Duquette. 1993. Soil reaction and exchangeable acidity. P 141-145 in: Soil Sampling and Methods of Analysis, M.R. Carter, ed., Canadian Society of Soil Science, Lewis Publishers, Boca Raton, Florida.

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Appendix C

Test Conditions, Experimental Design, Data Summaries, and Results of the Earthworm Chronic Test Conducted in Sand Reference Soil

Stantec Consulting Ltd.
361 Southgate Drive
Guelph, ON N1G 3M5
Tel: (519) 836-6050 Fax: (519) 836-2493
stantec.com

Sample Identification

Client:	Petroleum Technology Alliance Canada
Sample(s) description:	Sand reference soil
Sample(s) identification:	0754-SR2&3
Date collected/formulated:	October 28, 1999
Method of soil collection:	Composite samples
Date sample(s) received:	1999
Time sample(s) received:	10:00 am
Temperature on arrival:	Not available
Soil storage temperature:	Outdoor storage until 2007-07-09, then 21.7 ± 1.1°C
Date sample(s) tested:	August 30 – November 1, 2007
Technicians:	Ben Smith, Carolyn Brown, Kelly Olaveson, Natalie Feisthauer, Yvonne Busby, Jessica Sosa, Joe Keene
Analyst:	Kelly Olaveson
QA/QC:	Gladys Stephenson

Test Organism

Test Organism:	*Eisenia andrei*
Organism Source:	In house culture Ea 07-13, 07-14, 07-15, 07-16, 07-17, 07-18, 07-19, 07-20
Initial mean adult wet weight (g):	0.435 ± 0.065

Test Conditions and Procedures

Test type:	Static, chronic
Test duration:	63 days
Number of treatments:	21, including 1 negative control (AS) Sand reference soil amended with: • 0, 2.5, 5, 10 and 15% peat • 0, 2.5, 5, 10 and 15% coir • 0, 2.5, 5, 10 and 15% perlite • 0, 2.5, 5, 10 and 15% gypsum
Temperature:	20.2 ± 0.3 °C
Light intensity:	552 ± 105 lux
Photoperiod:	16 h light; 8 h dark
Watering regime:	De-ionized water, misted at test initiation and every 14 days, as required
Feeding regime:	Cooked oatmeal (~ 5 mL), fed at test initiation and every 14 days, as required
Test unit description:	500-mL glass wide-mouthed mason jar
Soil volume/test unit:	230-350g (depending on soil volume)
No. organisms per test unit:	2
No. replicate test units/treatment:	5 replicates
Measured soil chemistry parameters:	Initial and final soil pH, electrical conductivity, and percent moisture content
Measured endpoint(s):	Day 35 adult survival, number of progeny produced at Day 63, and wet and dry mass of individual progeny at Day 63
Test Protocol:	Biological Test Method: Tests for Toxicity of Contaminated Soil to Earthworms (*Eisenia andrei*, *Eisenia fetida*, or *Lumbricus terrestris*). Report EPS

1/RM/43, June 2004. Method Development and Applications Section, Environmental Technology Centre, Environment Canada, Ottawa, Ontario.

Statistical Analyses: Mean, SD – Microsoft Excel (2002)
ANOVA; Kruskal-Wallis One-way Analysis of Variance; Mann-Whitney U-Test (SSI, 2007)
Nominal ☒ measured ☐ concentrations analysed

Test acceptability criteria met? See Table C.1

Table C.1. Performance of earthworms in negative control soil treatment relative to test method validity criteria.

Criterion in Negative Control Soil	Negative Control Soil	Criteria Met?	Positive Control Soil	Solvent Control Soil
28- or 35-d adult survival ≥ 90%	100	Yes	NA	NA
Mean # live progeny/adult ≥ 3	9.4	Yes	NA	NA
Mean dry wt of individual progeny ≥ 2.0 mg	6.30	Yes	NA	NA

Boric Acid Reference Toxicant Data for Artificial Soil

Type of Test: Acute lethality
Test Duration 7 days
Date Tested: 2007-09-06
Organism Lab Code: Laboratory Code No. Ea 07-2, 07-14, 07-19
LC50 Survival: 5754 mg/kg
95% CL: 5070 to 6310 mg/kg
Statistical Analyses: Probit (Stephen, 1977)
Historical Mean LC50: 4282 mg/kg
Warning Limits (± 2 SD): 2684 to 6031 mg/kg
Technician(s): Kelly Olaveson and Natalie Feisthauer
Analyst(s): Natalie Feisthauer

Results

Table C.2. Effect of amending sand reference soil with four different soil conditioners at five amendment levels on earthworm (*E. andrei*) survival (Day 35), growth (Day 63), and reproduction (Day 63). Results are reported as treatment means, and the values in brackets indicate one standard deviation of the mean.

Soil Treatment	%	Mean Percent 35-d Adult Survival (n = 2 adults)	Mean Number of Progeny Produced	Mean Individual Wet Mass of Progeny (mg)	Mean Individual Dry Mass of Progeny (mg)
AS		100 (0)	18.8 (12.8)	32.62 (16.20)	6.30 (2.83)
Peat	0	100 (0)	16.6 (5.0)	64.91 (24.75)	16.05 (6.42)
	2.5	80 (45)	11.2 (7.0)	63.80 (12.93)	15.76 (3.45)
	5	100 (0)	19.6 (9.5)	51.21 (23.66)	10.96 (5.13)
	10	100 (0)	21.2 (11.8)	63.69 (23.97)	12.84 (5.33)
	15	100 (0)	25.0 (5.5)	52.07 (12.03)	9.89 (2.39)
Coir	0	100 (0)	12.4 (6.8)	52.32 (26.48)	13.97 (7.58)
	2.5	100 (0)	26.4 (2.8)	53.29 (6.56)	12.47 (1.84)
	5	100 (0)	27.0 (19.2)	45.69 (18.25)	10.57 (4.57)
	10	100 (0)	17.2 (5.9)	51.61 (7.03)	10.45 (1.82)
	15	100 (0)	16.4 (7.9)	54.81 (9.45)	9.92 (1.61)
Perlite	0	100 (0)	13.4 (5.0)	57.02 (17.65)	14.35 (5.40)
	2.5	100 (0)	17.0 (5.8)	62.10 (21.29)	16.43 (5.97)
	5	100 (0)	14.0 (8.9)	86.54 (43.62)	21.83 (11.15)
	10	100 (0)	17.8 (2.6)	49.64 (8.38)	12.49 (2.52)
	15	100 (0)	12.6 (2.9)	60.28 (24.35)	14.52 (5.95)
Gypsum	0	100 (0)	14.0 (6.0)	34.30 (17.38)	8.45 (4.56)
	2.5	100 (0)	10.4 (3.2)	16.05 (3.03)	2.81 (0.55)
	5	100 (0)	7.8 (4.9)	26.27 (12.71)	6.07 (2.78)
	10	100 (0)	10.2 (6.5)	44.81 (37.50)	10.39 (7.98)
	15	100 (0)	6.4 (4.0)	54.73 (21.43)	14.07 (6.36)

The results reported relate only to the sample(s) tested

Date: February 5, 2008 Approved by: Gladys L Stephenson
Laboratory Director

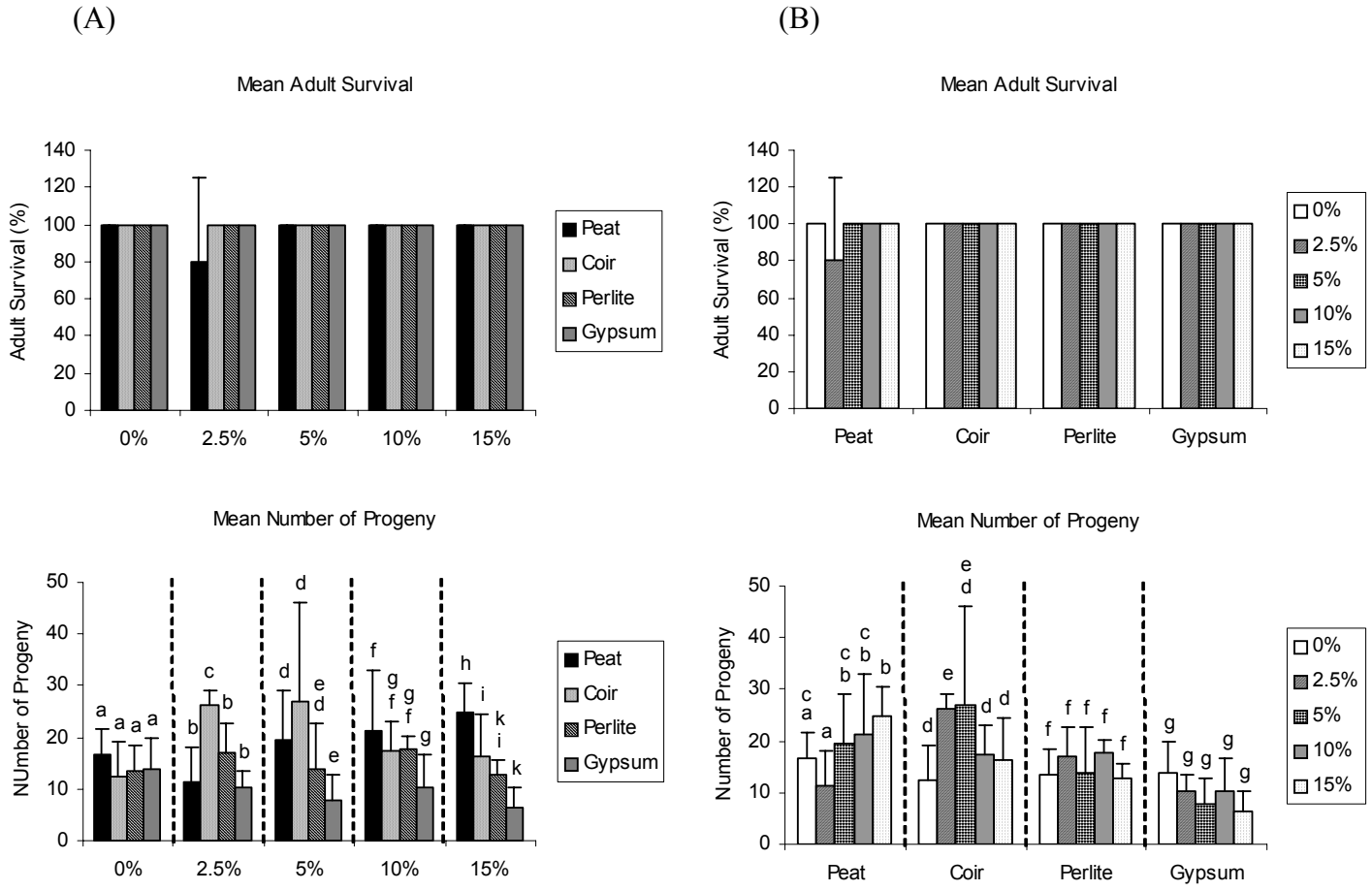

Figure C.1. Earthworm (*E. andrei*) mean adult survival (%) (Day 35) and mean progeny production (Day 63) following the amendment of sand reference soil with four different soil conditioners at five amendment levels. Columns indicate treatment means and bars above the columns represent one standard deviation of the mean. Results are grouped together by percent amendment (A) and by soil conditioner (B) in separate graphs. On each graph, results were compared only within the grouping, not across all treatments tested. Letters above the columns indicate significant difference(s) ($p < 0.05$) among means within a particular conditioner or amendment-level grouping, with different letters indicating where differences were found. There were no significant differences among treatments for adult survival.

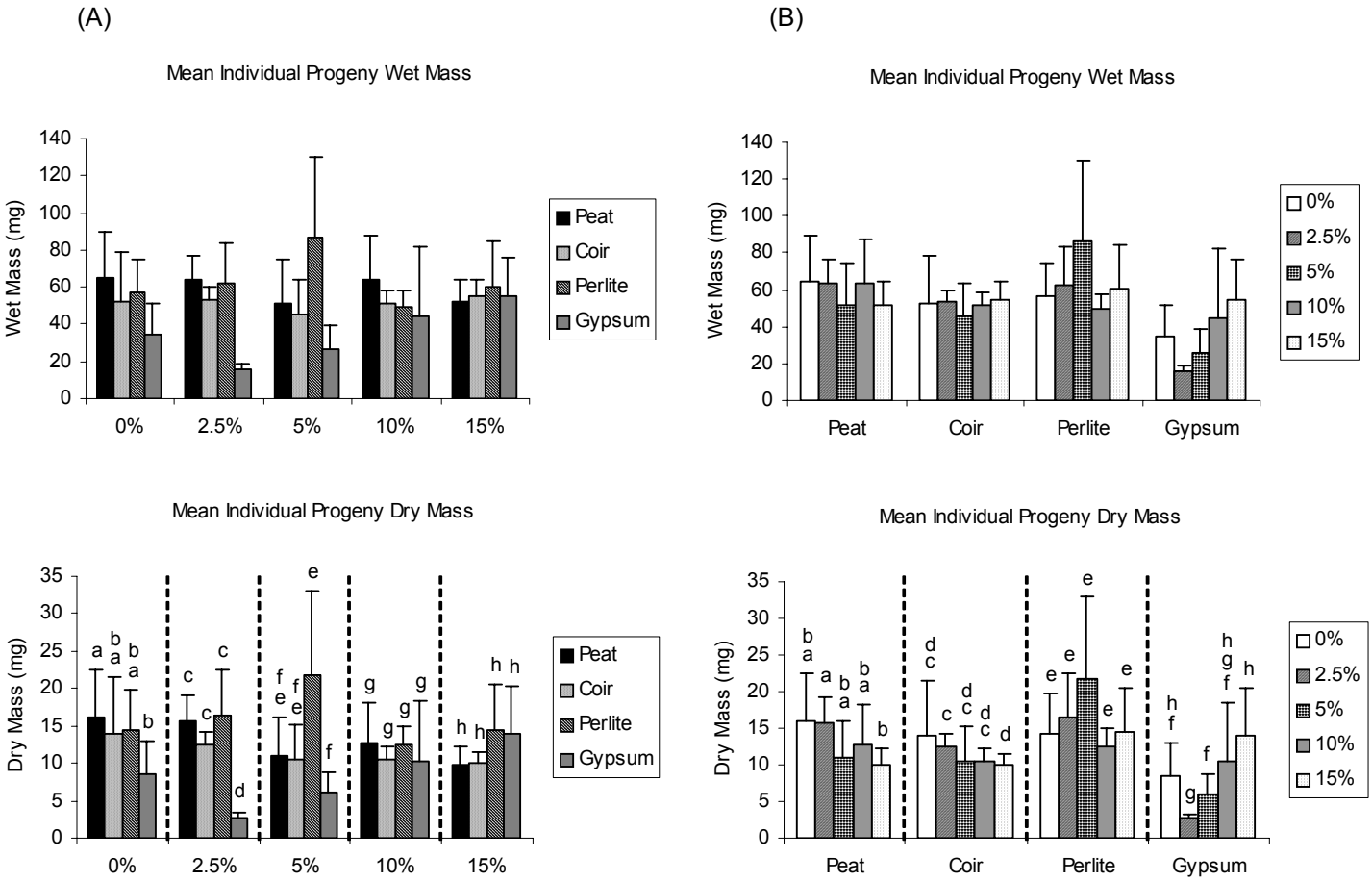

Figure C.2. Earthworm (*E. andrei*) mean individual progeny wet and dry mass (Day 63) following the amendment of sand reference soil with four different soil conditioners at five amendment levels. Columns indicate treatment means and bars above the columns represent one standard deviation of the mean. Results are grouped together by percent amendment (A) and by soil conditioner (B) in separate graphs. On each graph, results were compared only within the grouping, not across all treatments tested. Letters above the columns indicate significant difference(s) ($p < 0.05$) among means within a particular conditioner or amendment-level grouping, with different letters indicating where differences were found. There were no significant differences among treatments within groupings for progeny wet mass.

Soil Characteristics

Table C.3. Moisture content, conductivity and pH of test soils at the beginning (Day 0) and end (Day 63) of the test.

Soil Treatment	%	Initial pH[1]	Final pH[1]	Initial Conductivity[1] (µS/cm)	Final Conductivity[1] (µS/cm)	Initial Soil Moisture[2] (% WHC)	Final Soil Moisture[2] (% WHC)
AS		7.24	6.76	159	268	78	98
Peat	0	8.19	7.95	467	231	35	40
	2.5	8.04	7.87	420	224	48	61
	5	7.97	7.79	415	255	57	66
	10	7.90	7.70	390	267	67	78
	15	7.79	7.70	352	208	73	90
Coir	0	8.19	8.02	467	226	36	40
	2.5	8.12	8.10	398	226	61	69
	5	8.09	8.13	374	241	87	92
	10	8.29	8.24	358	282	113	109
	15	8.30	8.13	365	313	91	108
Perlite	0	8.16	7.92	422	244	39	48
	2.5	8.22	8.03	383	222	47	64
	5	8.17	7.93	385	268	47	54
	10	8.25	7.97	331	256	62	85
	15	8.30	7.90	307	266	71	76
Gypsum	0	8.16	8.09	466	210	44	47
	2.5	7.83	7.58	2170	1700	42	49
	5	7.84	7.62	2200	1850	39	42
	10	7.83	7.56	2240	1620	38	46
	15	7.85	7.58	2140	1710	53	53

[1] pH and conductivity were measured using a 2:1 water:soil slurry.
[2] % WHC - percent of water-holding capacity of the soil.

Table C.4. Texture, organic matter content, carbon content and fertility of test soils (prior to testing).

Parameter	AS	Sand Reference Soil 0754-SR2&3
Soil Texture	Fine Sandy Loam	Fine Sandy Loam
Sand (%)	78.9	75.1
Silt (%)	8.5	15.2
Clay (%)	12.7	9.8
Organic Matter (%)	7.9	1.3
Total Carbon (%)	3.85	1.38
Inorganic Carbon (%)	0.05	0.58
Organic Carbon (%)	3.80	0.80
Total Nitrogen (%)	0.11	0.08
Plant Available Phosphorus (mg/kg)	14	10

AS = Artificial Soil

Table C.5. Analytical Results for Sand Reference Soil.

Sample Details/Parameters	Result
L527489-2 0747-SR-2 and L527489-3 0748-SR-3	
Sampled By: K. Olaveson on 09-JUL-07 @14:00	
Matrix: SOIL	
F2-F4 (O.Reg.153/04)	
CCME Total Hydrocarbons	
F2 (C10-C16) (mg/kg)	<10
F3 (C16-C34) (mg/kg)	<50
F4 (C34-C50) (mg/kg)	<50
Chromatogram to baseline at nC50	YES
F2-F4 (O.Reg.153/04)	
Prep/Analysis Dates	19-JUL-07
Octacosane (%)	102
% Moisture	8.6
Standard Metal Scan (ICP)	
Aluminum (Al) (mg/kg)	5400
Antimony (Sb) (mg/kg)	<1
Arsenic (As) (mg/kg)	4
Barium (Ba) (mg/kg)	75
Beryllium (Be) (mg/kg)	<0.5
Bismuth (Bi) (mg/kg)	<1
Boron (B) (mg/kg)	<5
Cadmium (Cd) (mg/kg)	<0.5
Calcium (Ca) (mg/kg)	20150
Chromium (Cr) (mg/kg)	9
Cobalt (Co) (mg/kg)	5
Copper (Cu) (mg/kg)	8
Iron (Fe) (mg/kg)	8885
Lead (Pb) (mg/kg)	5
Magnesium (Mg) (mg/kg)	3770
Manganese (Mn) (mg/kg)	234
Molybdenum (Mo) (mg/kg)	<1
Nickel (Ni) (mg/kg)	11
Phosphorus (P) (mg/kg)	415
Potassium (K) (mg/kg)	775
Selenium (Se) (mg/kg)	<1
Silver (Ag) (mg/kg)	<0.2
Sodium (Na) (mg/kg)	220
Strontium (Sr) (mg/kg)	17
Thallium (Tl) (mg/kg)	<1
Tin (Sn) (mg/kg)	4
Titanium (Ti) (mg/kg)	134
Uranium (U) (mg/kg)	<1
Vanadium (V) (mg/kg)	18
Zinc (Zn) (mg/kg)	26
Zirconium (Zr) (mg/kg)	7

Comments

No organisms exhibiting unusual appearance, behaviour or undergoing unusual treatment were used in this test.

Test Method Modifications

1. Soil pH was measured using a soil-water slurry, which represents our normal practices and is a method modified from the Soil Analysis Handbook (1992), instead of using a $CaCl_2$ slurry, as recommended by the method for pH. This had no impact on the results of the test. The method of using $CaCl_2$ was developed for soil scientists who were comparing the pH of different soils, and wished to minimize the variability of the different pHs (McKeague, 1978). As a result, the $CaCl_2$ method will, by design, minimize the variability of the soil pH among soil samples, and will be less sensitive to differences in pH. In addition, soil pH measured in water is considered to be the pH closest to the pH of soil solution in the field (Hendershot et al., 1993).

2. Test soils were prepared on different days, depending on the conditioner being used to amend the sand reference soil. Peat and coir amended soils were prepared ~ 3 weeks prior to the earthworms being added to the test units, due to the effect these conditioners had on the pH of the sand reference soil. Gypsum and perlite amended soils were prepared ~ 1 week prior to test initiation. Preparing the test soils ahead of time allowed for buffering treatments in which soil pH had been lowered and for the pH of these treatments to stabilize prior to testing. The artificial soil treatment was prepared the day before the earthworms were added to the test units.

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