ECOTOXICITY OF WEATHERED PHC F2 – PHASE 2

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1.0 Introduction

Stantec Consulting Ltd. (Stantec) has been instrumental in the process of developing Canada-wide Standards (CWS) for Petroleum Hydrocarbons (PHCs) in soil. Stantec developed the terrestrial toxicity test methods used to generate the data from which, in part, the standards were derived. The fraction-specific testing was completed by Stantec using these draft standard methods (Stantec, 2003). The Tier 1 standards (first issued June of 2001 and revised March 2007 and re-issued January 2008; CCME 2008a) serve as soil quality standards to assist with the management of site soils contaminated with petroleum hydrocarbons. The concentrations of the CCME fractions (F1 to F4) are measured in soil from a site and screened against these Tier 1 standards. If the concentrations measured in the site soils are higher than the Tier 1 fraction-specific values for the soil contact exposure pathway, there is an option to proceed to Tier 2. At this time, there is no provision at Tier 2 for adjustment of the Tier 1 standards associated with this soil contact exposure pathway. There is, however, a provision for the potential elimination of the exposure pathway (CCME, 2008b).

In the course of the re-evaluation of the Tier 1 standards, the F2 values were lowered significantly from 900 mg/kg to 150 mg/kg and from 1,500 mg/kg to 260 mg/kg soil dry weight for soils associated with agricultural/residential and commercial/industrial land use classes, respectively. These values apply equally to both fine and coarse soils. The basis for the lowering of the values cited in the CCME (2008a) Supporting Technical Document was: 1) the test soils, that were used to generate the data from which the initial Tier 1 standards were derived, were fine textured and not coarse textured so the application of an adjustment factor of 2 was an error; 2) the effects endpoints used in the revised derivation were EC/IC/LC25s as opposed to EC/IC/LC50s; and, 3) the revised derivation included effects data for plant and soil invertebrate species as opposed to plant species only. The result was a significant lowering of the Tier 1 F2 standards.

It is generally acknowledged that weathering and aging of petroleum hydrocarbons in soil decreases the toxicity of these soils to ecological receptors. It is hypothesized that the decrease in toxicity could be attributed to a decrease in the bioavailability of the constituents due to sequestration and/or a preferential degradation of the more toxic constituents. However, the magnitude of this decrease depends on a number of factors including the concentration and composition of the hydrocarbons in soil, the nature of the soil, and the chemical, physical, and biological conditions under which the weathering and aging occurs.

Weathering by definition refers to the relative change in the composition of contamination due to the preferential loss of constituents with time. Note that weathering has been used in various contexts within the literature. Aging refers to the time-dependent change in bioavailability of a compound(s) in soil (Stantec, 2004). In this definition, aging includes all sorption and sequestration processes, including the formation of non-extractable residues. Sequestration describes the time-dependent movement of contaminant molecules into remote, inaccessible

areas of soil particles and/or organic matter. Sequestration does not involve the formation of covalent bonds. Non-extractable residues result from the time-dependent formation of residues that cannot be solvent extracted from soils, and which can only be removed upon hydrolysis with a strong alkali or acid (Alexander, 1999). These residues might involve the formation of covalent bonds between the parent compound or a metabolite with the organic matter. Residual fraction is the contaminant(s) remaining in the soil following weathering and aging (Stantec, 2004). This fraction includes those compounds that are resistant to degradation and other loss mechanisms, as well as those that are unavailable to organisms for degradation. Therefore, it is the fraction that will remain in the soil with relatively little change in both composition and concentration over an indefinite period of time.

Phase 1 testing of fine-grained soil spiked with F2 and "weathered" in the laboratory was completed by Stantec Consulting Ltd. in 2009 (Angell et al., 2012; Stantec, 2009). The species sensitivity distribution constructed from the Phase 1 data indicates that weathered F2 is half as toxic as fresh F2 to ecological receptors in soil (Angell et al., 2012; Stantec, 2009). A "Request for Proposals" was issued by PTAC for a toxicity assessment of coarse-grained soils spiked with F2 and "weathered" in the laboratory to a stable endpoint where the F2 concentration is no longer decreasing significantly. The objective of Phase 2 ecotoxicity testing was to generate LC/EC/IC25s and LC/EC/IC50s for multiple endpoints and for a test species battery exposed to weathered PHC F2 in a coarse-grained soil.

1.1 SCOPE OF REPORT

This report contains the test reports and analytical reports and summaries for Phase 2 of a project facilitated by PTAC and in collaboration with Steve Kullman (Husky Energy) and Miles Tindal (Axiom Environmental Consulting Ltd.) whereby a toxicity assessment was conducted on coarse-grained artificial soils spiked with F2 and "weathered" in the laboratory to a stable endpoint. A stable endpoint is achieved when the F2 concentrations are no longer decreasing significantly. Weathering cannot occur realistically under laboratory conditions but the soils can be spiked with F2 and aged and a simulation of weathering can take place. The aim of the testing in this project was to generate LC/EC/IC25s and LC/EC/IC50s for multiple endpoints and for a test species battery using coarse-grained soils that were contaminated with "weathered and aged" F2. Specific objectives were to:

1. Amend a coarse-grained soil with a range of F2 concentrations and "age" and "weather" these soils under laboratory conditions until F2 residuals are chemically stable.

2. Expose a battery of test species (plant and soil invertebrate species) to these soils with a gradient of stable residuals to quantify the exposure concentration-response relationships for each endpoint and each species.

The test species are representative of two major groups of soil organisms, plants and soil invertebrates. The monocotyledonous plant species were northern wheatgrass (*Elymus lanceolatus*) and barley (*Hordeum vulgare*), and the dicotyledonous plant species was alfalfa (*Medicago sativa*). The earthworm species is commonly referred to as the red wiggler or

compost worm (*Eisenia andrei*) and soil arthropods were represented by the springtail (Collembola – *Folsomia candida*). The test species used are consistent with the earlier work completed in support of the development of Tier 1 PHC standards and Phase 1 of the present PTAC project; however, *Folsomia candida* replaced *Orthonychiurus folsomi* because, although the sensitivities to petroleum hydrocarbons are comparable, the former has less variability associated with test results. The test methods and procedures were those of Environment Canada (EC, 2007, 2005a, 2004).

Reference toxicity tests with boric acid and each test species were also conducted concurrently to comply with the test protocols of Environment Canada; they are also a mandatory requirement for QA/QC purposes for CALA-accredited laboratories. The results of the reference testing have been included in each test report.

2.0 Materials and Methods

2.1 TEST SOILS AND PRODUCT (FRACTION 2)

2.1.1 Reference Soils

Artificial soils (hereafter referred to as F2 artificial soil) free of petroleum hydrocarbons were formulated for the present project by Stantec Consulting Ltd. from March 29 to April 6, 2011. F2 artificial soil formulation followed the same procedure that Stantec Consulting Ltd. uses to formulate the reference control artificial soil (Section 2.1.2) with the following exception: the amount of peat added to the mixture was decreased slightly from the current Environment Canada recommended formulation, from 2.0 kg down to 1.8 kg, in order to ensure consistency with the artificial soil used in the testing for the PHC CWS. Three batches of F2 artificial soil were selected using a random number generator. A sample was collected from each of these three batches on April 6, 2011, and submitted to the University of Guelph Soil and Nutrient Laboratory (Guelph, ON) the same day for characterization. The analytical report from the University of Guelph was received on April 18, 2011. The University of Guelph analysis confirmed that the soils texturally classified as “coarse-grained” using the CCME definition.

Soil storage temperature was monitored and the water-holding capacity was determined for the artificial soils prior to testing.

Table 1: Description of the F2 Artificial Soil formulated at the Stantec Consulting Ltd. Soils Laboratory (Guelph)

Artificial Soil Batch #	Date of Formulation
F2 AS 2011-03-1	March 29, 2011
F2 AS 2011-03-2	March 29, 2011
F2 AS 2011-03-3	March 30, 2011
F2 AS 2011-03-4	March 30, 2011
F2 AS 2011-03-5	March 30, 2011
F2 AS 2011-03-6	March 31, 2011
F2 AS 2011-03-7	March 31, 2011
F2 AS 2011-03-8	March 31, 2011
F2 AS 2011-04-1	April 1, 2011
F2 AS 2011-04-2	April 1, 2011
F2 AS 2011-04-3	April 1, 2011
F2 AS 2011-04-4	April 4, 2011
F2 AS 2011-04-5	April 4, 2011
F2 AS 2011-04-6	April 4, 2011
F2 AS 2011-04-7	April 5, 2011
F2 AS 2011-04-8	April 5, 2011
F2 AS 2011-04-9	April 6, 2011
F2 AS 2011-04-10	April 6, 2011

Table 2: Description of the F2 Artificial Soil homogenized at the Stantec Consulting Ltd. Soils Laboratory (Guelph)		
New Artificial Soil Batch #	**Artificial Soil Batches Mixed**	**Date of Mixing**
F2 AS mixed 2011-04-26-1	F2 AS 2011-03-2 F2 AS 2011-04-8 F2 AS 2011-04-9 F2 AS 2011-04-10 F2 AS 2011-03-7 F2 AS 2011-03-4	April 26, 2011
F2 AS mixed 2011-04-26-2	F2 AS 2011-04-4 F2 AS 2011-04-7 F2 AS 2011-04-5 F2 AS 2011-03-6 F2 AS 2011-03-5 F2 AS 2011-04-6	April 26, 2011
F2 AS mixed 2011-04-26-3	F2 AS 2011-04-2 F2 AS 2011-04-3 F2 AS 2011-04-1 F2 AS 2011-03-3 F2 AS 2011-03-1 F2 AS 2011-03-8	April 26, 2011

2.1.2 Negative Control Soil

In addition to the artificial negative control soil treatment (e.g. F2 artificial soil free of PHC contamination); an artificial negative control soil (AS) was included in the experimental design of each toxicity test. The artificial negative control soil treatment and artificial negative control soil (AS) are slightly different. The artificial negative control soil (AS) is the artificial soil included for Quality Assurance/Quality Control (QA/QC) purposes only. The artificial negative control soil treatment refers to the "0 mg/kg" artificial soil, formulated for the present project as detailed in Section 2.1.1, and were slightly coarser-grained than the QA/QC AS. Additionally, the artificial negative control soil treatment was artificially weathered along with the spiked exposure concentrations for ~6 months prior to testing.

The QA/QC AS was formulated in the laboratory by mixing the 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 (Barco 71; Opta Minerals, Inc., Waterdown, ON), 20% kaolinite clay (EPK Pulverized Kaolin Clay; Tucker's Pottery Supplies, Inc., Richmond Hill, ON), 10% *Sphagnum* spp. fine grind peat (Premier Pro-Moss Fine Grind Peat; Canadian HydroGardens Ltd., Ancaster, ON), and calcium carbonate ($CaCO_3$). 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 (dry weight basis) of fine grind peat (approximately 2 mm), approximately 160 mL of $CaCO_3$ (sieved), and 2 L of de-ionized water. The amount of $CaCO_3$ required to adjust the soil pH to 6.0-7.5, depends on the nature (i.e., acidity) of the *Sphagnum* peat and the silica sand. When a new batch of either of these ingredients is used, it is often necessary to adjust the amount of $CaCO_3$ used in each batch of formulated soil. The AS was allowed to stabilize for at least three days. The pH was checked, and the AS was buffered if necessary with $CaCO_3$ to

adjust the soil pH to 6.0-7.5. Once the pH stabilized within the acceptable range, it was ready for use in testing.

The AS is characterized as a sandy-loam soil and served as an experimental control soil to evaluate the health of the test organisms, the influence of the experimental conditions on test organism performance (e.g., survival and/or reproduction), technical proficiency, and the acceptability of the test (i.e., performance is measured and compared to the validity criteria outline in the test methods).

2.1.3 Fraction 2 (>nC10 to C16)

Three containers of F2 were available in-house for testing (received October 23, 2000, December 10, 2002, and February 16, 2011). Sub-samples from each container were sent for characterization by ALS Laboratories Inc. (ALS, Waterloo ON) on April 12, 2011. Data were received from ALS on April 19, 2011. Two of the containers (received December 10, 2002 and February 16, 2011) had very similar materials and exhibited cleaner cuts than the third so these two containers were combined and used for spiking the soils (Analytical report in Appendix G). The F2 used for this project was distilled in 2002 by Imperial Oil Ltd. (IOL), Sarnia, ON from Federated Crude Oil; distillates of Federated Crude Oil were used to generate the toxicity data for the tests comprising Janet Cermak's (McCann et al., 2006) Ph.D. thesis and some of these data were used by the technical subcommittee in the re-evaluation of the Tier 1 Canada-wide Standards for PHCs in soil.

The IOL F2 was characterized and exhibited minimal overlap with either F1 or F3 (Appendix G). Phase 1 of the present study found that soils spiked with lower concentrations of F2 experienced a greater loss (i.e., percent volatilization) than soils spiked with higher concentrations of F2, so this non-linear decrease was used to determine the initial spiking concentrations (Angell et al., 2012; Stantec, 2009).

Stantec had approximately 3 L of F2 available for testing. Results of characterization of the F2 are provided in Appendix G. The volume allowed for a minimum of ten batches of soil to be spiked at sufficiently high concentrations to result in the desired range of residual concentrations of F2 (n=10). The desired range of exposure concentrations was selected to span a range of residual F2 concentrations between 10 and 20,000 mg/kg soil dry weight after aging and weathering. This range represented exposure levels below the existing Tier 1 standards (e.g., no-effect concentrations), close to the existing standards (e.g., 150 and 260 mg/kg soil dry wt., and those just above) and well above the existing standards.

There were a number of assumptions implicit in this approach. The first assumption was that stable residuals would be reached within 4-6 months. The second assumption was that, over this period of time, percent reduction of F2 would follow a similar pattern to that observed in Phase 1 of the present study (Angell et al., 2012; Stantec, 2009). The third assumption was that the time to reach stability was concentration independent (i.e., stability will occur within the same time frame, regardless of the initial spiking concentration). The fourth assumption was

that stable residuals would be reached in the different batches of spiked soil (e.g., treatments) such that a gradient of exposure concentrations would be produced.

The initial spiking concentrations are summarized in Appendix G. The estimated required amounts of F2 (mL) and soil (kg dry wt.) and the detailed calculations with assumptions are provided in Table G.1 (Appendix G; Table G.1)

2.2 SOIL PREPARATION

The soil amendments occurred by spiking the F2 into the soil and mixing the soils in a metal bowl with an electric mixer. Addition of F2 to the soils was done to minimize the potential for product loss. To do this, the batch of soil was placed into a metal mixing bowl and holes were uniformly placed into the soils. The amount of F2 required by weight to achieve the desired concentration was added using a calibrated pipette by adding equal aliquots to each hole and immediately covering the hole with soil. Once the F2 had been added to the batch of soil, the soil was well mixed with an electric mixer for 5-10 minutes; a homogeneous mixture can be achieved with this standardized duration (Stantec, 2009). Sub-samples of soil were collected and submitted for analyses. The spiked soils were placed into a metal bucket (e.g., microcosm) and allowed to equilibrate for 14 days with the lids on the buckets. On day 14, the lids were removed, and the contents of each bucket were placed into a large metal mixing bowl. The contents of each bucket were mixed vigorously with an electric mixer for 5-10 minutes after hydrating the soils to 35% of the water holding capacity and then returned to the appropriate bucket. The buckets were then covered and placed into storage at room temperature. Thereafter, the contents in each bucket were subjected to a mixing regime identical to that used for Phase 1 (bi-weekly for four weeks, and weekly thereafter) (Angell et al., 2012; Stantec, 2009). Sub-samples of selected test soils with low, medium, and high F2 concentrations were collected for chemical analyses following the schedule outlined in Section 2.3 below, and triplicate samples of all test soils were collected immediately at test setup. The analytical results are provided in Appendix H. Extra soil was built into the calculations for archival of duplicate samples. Concentrations were considered to have reached residual levels when there was less than a 10% decrease between two subsequent sampling events.

2.3 WEATHERING OF F2-SPIKED SOILS

On day 14, the lids were removed and the contents of each bucket were placed into a large metal mixing bowl. The contents of each bucket were mixed vigorously with an electric mixer for 5-10 minutes; the test soil was then returned to the appropriate bucket. The buckets were then covered and placed into storage at room temperature. Thereafter, the contents in each bucket were subjected to the same mixing regime and hydrated to maintain the same moisture level. Selected low, medium, and high exposure concentrations were sub-sampled over the course of weathering to monitor weathering progress. Sub-samples of all exposure concentrations were collected in triplicate for chemical analyses at test setup. Residual levels were considered stable when there was less than a 10% decrease between two subsequent sampling events.

The preparation of the F2 spiked soils occurred on April 28, 2011 and mixing occurred bi-weekly from May 12 to June 9, 2011 and weekly from June 23 to October 6, 2011; mixing occurred on the following dates May 12 and 26, 2011; June 9, 16, 23, and 30 2011; July 7, 14, 21, and 29, 2011; August 4, 11, 19, and 25, 2011; September 1, 8, 15, 22, and 29, 2011; and October 6, 2011. Samples were collected and shipped to ALS for analysis at the time of spiking on April 28, 2011 and on mixing dates May 26, June 23, July 21, August 19, and September 15, 2011, and at test setup on October 13, 2011.

Initial exposure concentrations were consistent with the desired concentrations (see attached analytical reports in Appendix H).

2.4 TEST SET-UP

Soils were prepared on day 0 for the plant tests and day -1 for the soil invertebrate tests.

The soil moisture content and water-holding capacity were determined for the test soil prior to test set-up. Water-holding capacity was measured on May 19, 2011. Three randomly selected sub-samples of the F2 artificial soil were sent to the University of Guelph's Soil and Nutrient Laboratory for characterization on April 6, 2011, in accordance with the Environment Canada biological test methods. Results were received April 18, 2011. All characterization results from the University of Guelph's Laboratory Services are presented in Appendix F.

Tests were set up on October 13, 2011 for plants and October 14, 2011 for invertebrates. At the time of each test setup, moisture content, soil pH, electrical conductivity were measured and triplicate sub-samples of soil from each exposure concentration were submitted for the contamination profile (e.g., PHC F2 concentration). Soils were stored in the main laboratory in their original buckets until used for testing.

Soils were prepared for testing (hydrated and mixed) on October 13, 2011. Seeds were added to the test soil the day the soils were prepared for testing; the invertebrates were added to the test units the next day. The Barley test was terminated on October 27, 2011. The Northern Wheatgrass and Alfalfa tests were terminated on November 3, 2011. The collembola test was processed on November 11, 2011. The earthworm test was processed on December 16 and 17, 2011.

2.5 PHYSICAL AND CHEMICAL CHARACTERIZATION OF TEST SOILS

The pedological characteristics of the artificial and site soils were measured to satisfy the requirements of the Environment Canada biological test methods (EC, 2004, 2005 and 2007). Subsamples of the F2 artificial soils were collected and submitted to Laboratory Services at the University of Guelph (Soils and Nutrient Laboratory, Guelph, ON) for physical and chemical characterization (Tables A.7, B.7, C.7, D.5, E.5, Appendices A to E, respectively). The analytical reports for soil characterization are provided in Appendix F. The Environment Canada biological test methods also require that soil pH, electrical conductivity, moisture content and water-holding capacity be measured for all test soils; these parameters were measured at the

Stantec Soils Laboratory and are reported in the test reports (Tables A.6, B.6, C.6, D.4, E.4, Appendices A to E, respectively).

2.6 TOXICITY TESTS

The test battery consisted of three plant species, one earthworm species and one collembolan species. The test species were consistent with the work completed earlier in support of the development of Tier 1 PHC standards and they include Northern Wheatgrass, Alfalfa, and Barley, *Eisenia andrei*, and *Folsomia candida* in place of *Orthonychiurus folsomi,* because, although the sensitivities to petroleum hydrocarbons are comparable, the former has less variability associated with test results. The test methods and procedures were those of Environment Canada (EC 2005a, 2004, 2007, respectively).

The design of the tests supported the use of regression analyses to determine the toxicity endpoints. The exposure concentrations were selected to bracket the Tier 1 standards themselves and with the expectation that, upon weathering and aging, the estimates for toxicity would be higher than those for freshly-spiked petroleum product.

At the beginning of testing, sub-samples of test soils were collected in triplicate from all exposure concentrations and submitted for chemical analyses in triplicate.

The F2 artificial negative control soil (treatment without PHCs; e.g., coarse-grained soil free of F2) served as the experimental control treatment. The artificial soil (AS) is characterized as a sandy-loam soil and will serve as the QA/QC negative control to evaluate the health of the test organisms, the influence of the experimental conditions on test organisms health and/or reproduction, and the acceptability of the test (measured against the “validity” criteria outlined in the test methods).

The Environment Canada test methods require that, as a minimum, the following soil properties be measured and reported for each test soil. Therefore, a sample of the coarse-grained reference soil will be submitted to Laboratory Services, University of Guelph, Guelph, ON, for analysis.

- Particle size distribution (% sand, % silt and % clay);
- Total organic carbon content (%);
- Organic matter content (%);
- Moisture content (%);
- Water-holding capacity (%);
- Total nitrogen;
- Total phosphorus;

- pH; and,
- Conductivity.

The soil pH, conductivity, moisture content, and water-holding capacity were measured in-house.

The test organisms, including plant seeds purchased from reliable suppliers and earthworms and collembola from in-house cultures were provided by Stantec; this ensured that the test organisms were as similar as possible to those used for Phase 1.

Chronic (earthworm and collembola) and definitive (plant) screening tests were conducted with artificial negative control soil, the F2 artificial negative reference control soil, and the 10 exposure concentration soils. The test methods and species were those recommended by Environment Canada (2004, 2005a, and 2007). The purpose of the longer-term plant and chronic invertebrate tests was to examine the effects of prolonged exposure to the weathered, F2-spiked soils on the survival, growth, and reproduction of earthworms and collembola and the emergence and growth of plants.

The measurement endpoints for the 63-day earthworm test included 35-day adult survival, 63-day mean number of progeny produced, and 63-day wet and dry mass of individual progeny. The measurement endpoints for the 28-day collembolan test were adult survival and mean number of progeny produced. The measurement endpoints for each plant test included seedling emergence, shoot and root length, and shoot and root dry mass. Plant test durations were 14 days for Barley, and 21 days for Alfalfa and Northern Wheatgrass.

2.6.1 Test Species Selection

The test species are representative of two major groups of soil organisms, plants and soil invertebrates. The monocotyledonous plant species were northern wheatgrass (*E. lanceolatus*), and barley (*H. vulgare*), and the dicotyledonous plant species was alfalfa (*M. sativa*). The earthworm species is commonly referred to as the red wiggler or compost worm (*Eisenia andrei*) and soil arthropods were represented by a parthenogenic species of springtail (Collembola – *Folsomia candida*).

The plant species were selected because:

- they are consistent with the earlier work completed in support of the development of Tier 1 PHC standards and Phase 1 of the present PTAC project;
- they include di- and monocotyledonous species;
- they include annual and perennial species;
- they include a nitrogen-fixing species;
- reliable seed sources are available;

- performance criteria are available;
- they are considered to be relatively sensitive to PHCs in soil; and,
- they are species recommended for ecotoxicity assessments by Environment Canada.

The invertebrate species were selected because:

- they have a relatively short life cycle that make it possible to conduct reproduction tests in the laboratory;
- they are easily cultured in the laboratory;
- they are commonly used invertebrate toxicity test species;
- they are considered to be relatively sensitive to PHCs in soil;
- performance criteria are available for both species;
- reliable cultures are available for both species;
- toxicity data generated from tests with these species are reproducible and sensitive;
- the earthworm species is the same as that used to generate data for the CCME Tier 1 CWS for PHCs in soil and for Phase 1 of the present project; and,
- standardized test methods exist for both test species (EC, 2004 and 2007).

All tests were conducted following the Environment Canada biological test methods (EC, 2004, 2005a, and 2007). The experimental design and test conditions for each test species are summarized in Table 3 (below), and in the test reports comprising Appendices A, B, C, D, and E. The test reports summarize the results of the definitive and chronic tests and any modifications to, or deviations from, the procedures and conditions recommended in the test methods.

Table 3: Experimental design and conditions of definitive plant and chronic invertebrate toxicity tests.

Test	Plant	Earthworm	Collembola
Test type	Definitive Screening	Chronic Screening	Chronic Screening
Test duration (d)	14 or 21	63 (35-d adult survival)	28
Test unit (chamber)	1-L polypropylene container	Glass 500-mL mason jar	Glass 125-mL mason jar
Amount of soil	500 g wet wt.	270 g wet wt.	30 g wet wt.
Temperature (day/night)	24/15 ± 3°C	20 ± 2°C	20 ± 2°C
Photoperiod (h)	16 light : 8 dark	16 light : 8 dark	16 light : 8 dark
Treatments	Artificial soil (AS); F2 artificial negative reference control soil; 10 exposure concentrations	Artificial soil (AS); F2 artificial negative reference control soil; 10 exposure concentrations	Artificial soil (AS); F2 artificial negative reference control soil; 10 exposure concentrations
Number of replicate test units per treatment	4	10	5 for AS and F2 artificial negative control soil; 3 for exposure concentrations
Number of organisms per test unit	5 – Barley 5 – Northern Wheatgrass 10 – Alfalfa	2	10
Lighting (Type & Intensity)	Full spectrum Durotest or Vita Lights 200-400 μmoles/(m^2·s)	Fluorescent 400-800 Lux	Fluorescent 400-800 Lux
Physicochemical	Conductivity, pH, % moisture	Conductivity, pH, %	Conductivity, pH, %

Table 3: Experimental design and conditions of definitive plant and chronic invertebrate toxicity tests.

Test	Plant	Earthworm	Collembola
measurements		moisture	moisture
Biological endpoint measurements	Emergence, shoot and root length and shoot and root dry mass	Adult survival, number of progeny produced, progeny wet and dry mass	Adult survival, number of progeny produced
Statistical endpoints	L/E/IC25s; L/E/IC50s	L/E/IC25s; L/E/IC50s	L/E/IC25s; L/E/IC50s
Description of methods	EC 2005a	EC 2004	EC 2007

2.6.2 Reference Toxicity Tests

Reference toxicity tests were conducted as required by the Environment Canada test methods (EC, 2004, 2005a, and 2007). They are also a mandatory requirement for accreditation by the Canadian Association for Laboratory Accreditation (CALA). The Stantec Southgate Laboratory is CALA-accredited for the Environment Canada plant, earthworm and collembolan test methods. The reference toxicant used was boric acid and the reference toxicity test soil was the artificial negative control soil described in Subsection 2.1.2. The purpose of conducting reference toxicity tests is to evaluate the health of the test organisms, precision and accuracy of laboratory techniques and technicians, and suitability of the experimental conditions. Organisms used for the reference toxicity tests were from the same batch as those used in the ecotoxicity assessment. The results from the reference toxicity tests are reported in Appendices A to E.

2.6.3 Statistical Analyses

Data analyses were conducted according to the Statistical Guidance recommended by Environment Canada (EC, 2005b). Data for each quantal endpoint were analyzed using logit procedures to determine E/LC25s (West, 1995; R Development Core Team, 2010). Research by Hubert indicates that for data with fewer than 30 organisms per treatment, χ^2 is not "statistically justified" (Hubert, 1984). Therefore, models for quantal endpoints were chosen based on approximate χ^2 and closeness to E/LC50 estimation via hand graphed probit regression. The emergence data for the barley and northern wheatgrass tests were not amenable to logit analysis due to lack of partial-effects data which is typical for longer-term tests.

Data for each sub-lethal toxicity endpoint were described by a non-linear or linear regression model and IC25s (25% inhibitory concentration) were determined (Systat Software Inc., 2007). Goodness-of-fit for quantitative endpoint models was assessed by line fit to scatter plot, r^2, and closeness of confidence intervals (Table 5). Data for quantitative endpoints were assessed for normality (Shapiro-Wilk normality test; $p>0.05$) and homogeneity of variances (ANOVA; $p>0.05$). For quantitative endpoints which did not fit a model using non-linear regression, ICPIN was used to estimate the IC25 (Norberg-King, 1993).

2.7 ANALYTICAL CHEMISTRY

2.7.1 PHC Analyses

The CCME reference method (CCMEa, 2000) for measuring CCME PHC fractions (BTEX/F1-F4) in soil requires solvent extraction. The soils were soxhlet extracted with a 1:1 ratio of hexane:acetone with an *in situ* silica gel cleanup; quantification was by GC/FID.

The test soils were analyzed for fractions 2 to 4 (e.g., F2-F4) prior to toxicity testing. Samples were submitted by Stantec to ALS (Waterloo, ON). Appendix H contains the ALS analytical reports. Samples were tightly packed (zero headspace) into Teflon lined, 120-mL glass sample jars provided by ALS. Samples were stored in one of the Stantec Southgate Laboratory refrigerators before being picked up by ALS and placed into a cooler containing ice. The ALS Chain of Custody and Analytical Results for the test soils are presented in Appendix H. Results are discussed in more detail in section 3.1.1.

3.0 Results

The calculations used for the test soil preparation are summarized in Appendix G. The test reports for the tests with barley, northern wheatgrass, alfalfa, collembola, and earthworms are presented in Appendices A, B, C, D, and E, respectively. The results of the soil physico-chemical characterization from the University of Guelph Soil Analytical Laboratory are presented in Appendix F. The analytical reports for the PHC analyses from ALS are contained in Appendix H. The toxicity test results are summarized in the following tables with the toxicity estimates derived using the measured exposure concentrations in soil at the start of each test.

3.1 CHEMICAL ANALYSES OF TEST SOILS

3.1.1 Petroleum Hydrocarbons

The analytical results for F2 are presented in Figures 1 to 3. The ALS Chains of Custody and Analytical Results for test soils are presented in Appendix H.

The solvent extraction data for the present study were compared to the data for F2 spiked into fine-grained soils (Phase 1), both at time of spiking (Figure 1) and after the completion of the artificial weathering process (Figure 3). Concentrations of F2 decreased over the course of weathering (Figures 2). Overall, the percentage decrease in F2 over time was greatest in the lower exposure concentrations and least in the higher exposure concentrations. This pattern was observed in Phase 1 of this project, and in other studies with petroleum spiked, artificially weathered soils (Hanna and Weaver, 2002; Salanitro et al., 1997). Table 4 details the measured F2 at each exposure concentration immediately after spiking (Day 0), and at completion of artificial weathering (Day 168).

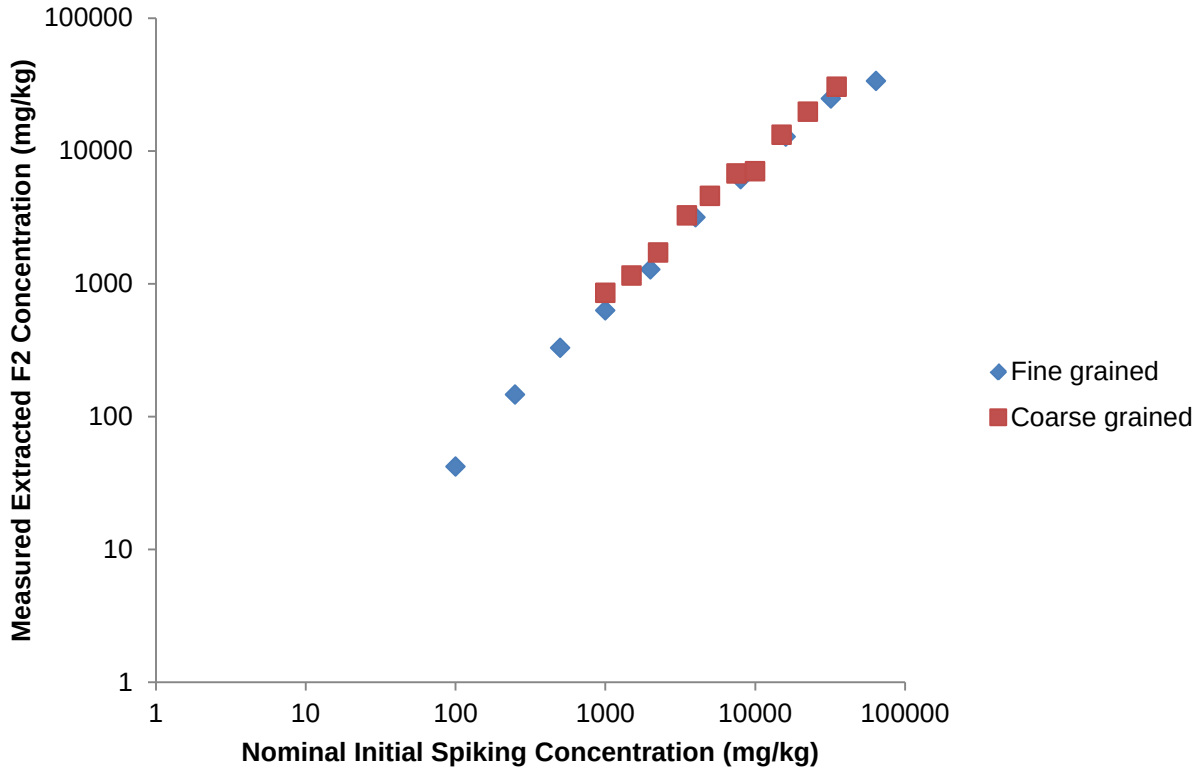

Figure 1: Measured concentrations of F2 (mg/kg) in test soils immediately after spiking (Day 0) for Phase 1 (fine-grained) and Phase 2 (coarse-grained) soils.

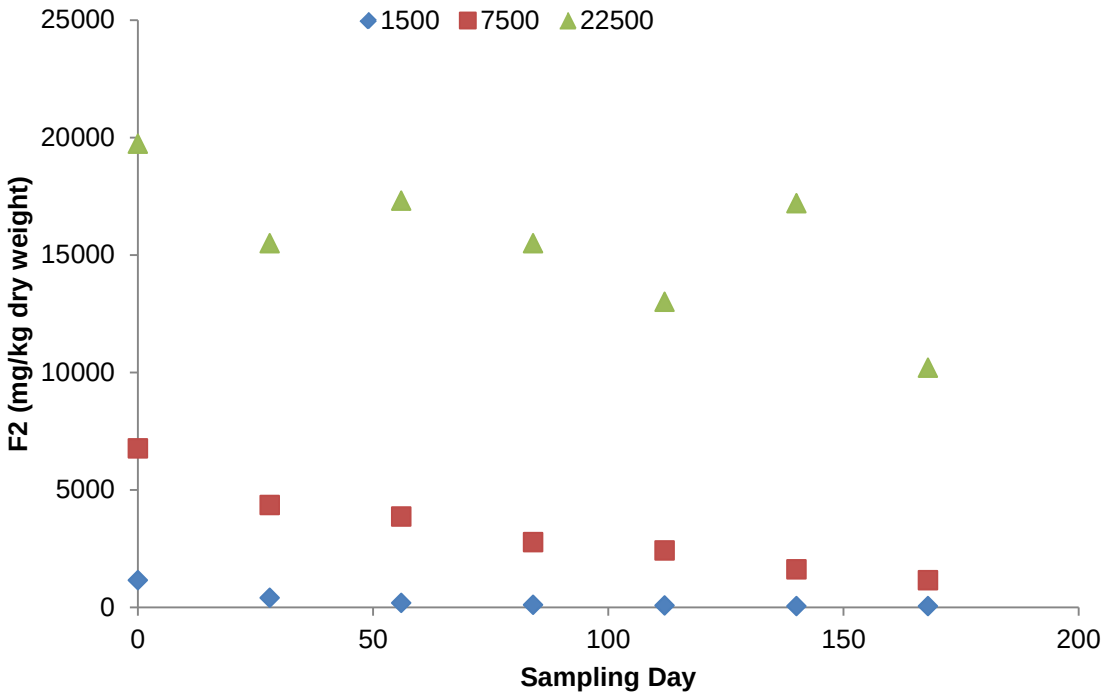

Figure 2: F2 degradation (mg/kg) over the course of weathering for selected low, medium, and high exposure concentrations.

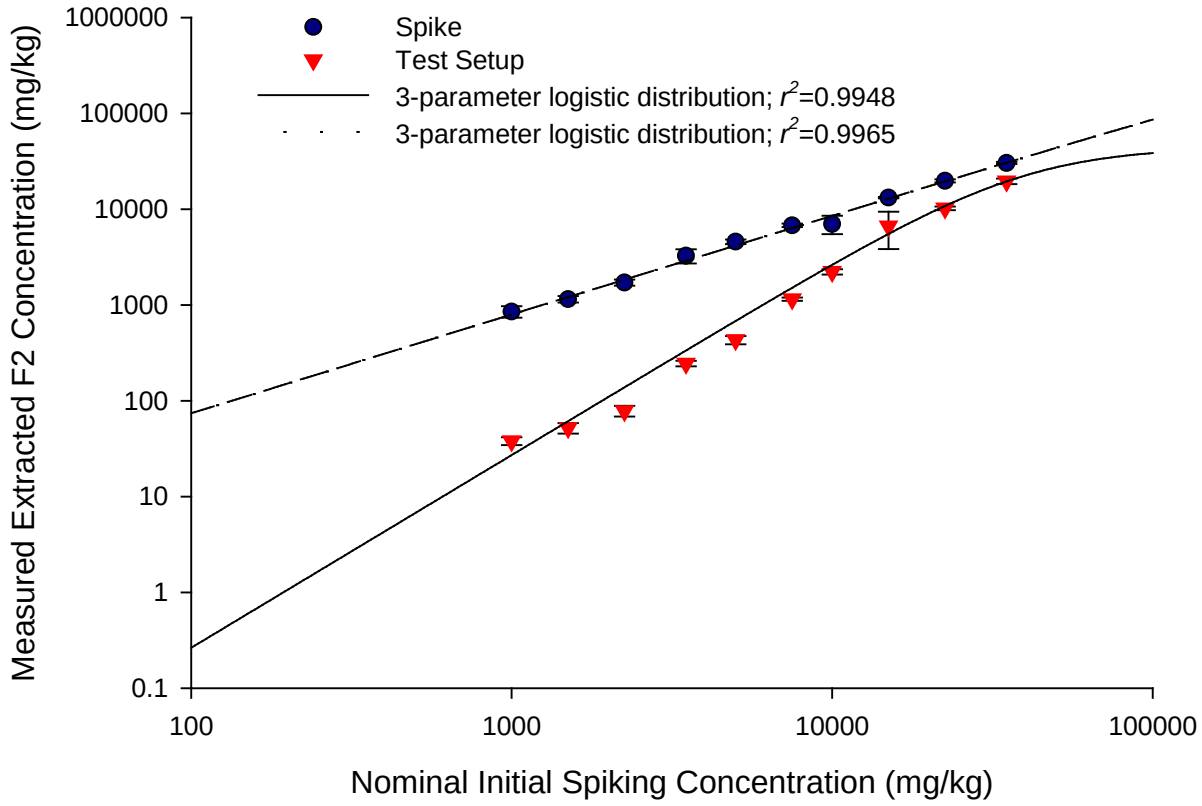

Figure 3: F2 concentration (mg/kg) measured in the test soils for all exposure concentrations immediately after spiking (Day 0) and after the completion of the artificial weathering (Day 168).

Table 4: PHC concentrations in soil immediately after spiking and upon completion of weathering. Concentrations were measured following the CCME reference method (hexane:acetone extraction).

F2 Nominal Spiking Concentration (mg/kg)	F2 Concentration Immediately after Spiking (mg/kg)	F2 Concentration Upon Completion of Weathering (at Test Set-up) (mg/kg)
0	<10	<10
1000	855 ± 117	38 ± 4
1500	1150 ± 92	52 ± 7
2250	1713 ± 127	78 ± 10
3500	3260 ± 547	245 ± 16
5000	4573 ± 253	431 ± 42
7500	6770 ± 284	1150 ± 46
10000	7013 ± 1527	2220 ± 144
15000	13200 ± 200	6613 ± 2786
22500	19733 ± 764	10193 ± 440
35000	30300 ± 755	19533 ± 1274

3.2 TOXICITY TESTS

3.2.1 Barley

Detailed descriptions of the experimental design, conditions, and test results are provided in the test report for Barley in Appendix A.

The soil pH for all exposure concentrations including the reference control soil ranged from 7.82 to 7.94 at the start of the test and from 7.76 to 8.15 at the end of the test. The change in soil pH from the start to the end of the test was acceptable. Initial soil conductivity[1] ranged from 205 to 230 µS/cm. At the end of the test, soil conductivity[1] ranged from 223 to 361 µS/cm (Table A.6, Appendix A). The changes in soil pH and conductivity from the start to the end of the test were acceptable. The changes in soil pH (0.04 pH units) and electrical conductivity (287 µS/cm) of the artificial control soil over the duration of the test were acceptable. The initial soil moisture contents were similar and ranged from 85 to 91 (%WHC). The initial moisture content for the artificial soil was 89% (Table A.6, Appendix A).

All performance criteria for test acceptability were met for the artificial soil treatment (EC, 2005a), indicating that the test procedures, conditions, seed quality and technical proficiency were acceptable (Table A.1, Appendix A). Reference toxicity QA/QC data were also within the limits of the historical mean.

3.2.1 Northern Wheatgrass

Detailed descriptions of the experimental design, conditions, and test results are provided in the test report for Northern Wheatgrass in Appendix B.

The soil pH for all exposure concentrations including the reference control soil ranged from 7.82 to 7.94 at the start of the test and from 7.82 to 8.19 at the end of the test. The change in soil pH from the start to the end of the test was acceptable. Initial soil conductivity[1] ranged from 205 to 230 µS/cm. At the end of the test, soil conductivity[1] ranged from 223 to 689 µS/cm (Table B.3, Appendix B). The changes in soil pH and conductivity from the start to the end of the test were acceptable. The changes in soil pH (0.11 pH units) and electrical conductivity (489 µS/cm) of the artificial control soil over the duration of the test were acceptable. The initial soil moisture contents were similar and ranged from 85 to 91 (%WHC). The initial moisture content for the artificial soil was 89% (Table B.6, Appendix B).

All performance criteria for test acceptability were met for the artificial soil treatment (EC, 2005a), indicating that the test procedures, conditions, seed quality and technical proficiency were acceptable (Table B.1, Appendix B). Reference toxicity QA/QC data were also within the historical warning limits (Appendix B).

3.2.1 Alfalfa

Detailed descriptions of the experimental design, conditions, and test results are provided in the test report for Alfalfa in Appendix C.

The soil pH for all exposure concentrations including the reference control soil ranged from 7.82 to 7.94 at the start of the test and from 7.94 to 8.14 at the end of the test. The change in soil pH from the start to the end of the test was acceptable. Initial soil conductivity[1] ranged from 205 to 230 µS/cm. At the end of the test, soil conductivity[1] ranged from 222 to 385 µS/cm (Table C.3, Appendix C). The changes in soil pH and conductivity from the start to the end of the test were acceptable. The changes in soil pH (0.08 pH units) and electrical conductivity (320 µS/cm) of the artificial control soil over the duration of the test were acceptable. The initial soil moisture contents were similar and ranged from 85 to 91 (%WHC). The initial moisture content for the artificial soil was 89% (Table C.6, Appendix C).

There was a non-conformance associated with this test. The validity criterion for root length (≥ 120 mm) was not met in the artificial soil for this test. Root length was 83 mm. The results of the test were scrutinized, the test methods and conditions reviewed. Four of the five validity criteria were met for artificial soil in this test. The four criteria that were met were percent seedling emergence, percent survival of emerged seedlings, percent of emerged control seedlings exhibiting phytotoxicity or developmental anomalies and seedling shoot length. Seedlings that emerged in the negative control soil were healthy; however, they did not meet the validity criteria for root length. Seedling emergence was excellent and plants appeared vigorous and healthy with few signs of stress and it is unclear why the root length validity criterion was not met in this test. We reviewed the test procedures and concluded that the experimental conditions were acceptable. All validity criteria for performance tests in the past with this batch of seed were met and the validity criteria for the reference toxicant with the batch of seed used in this test were met as well. All other performance criteria for test acceptability were met for the artificial soil treatment (EC, 2005a), indicating that the test procedures, conditions, seed quality

and technical proficiency were acceptable (Table C.1, Appendix C). Reference toxicity QA/QC data were also within the limits of the historical mean.

3.2.2 *Folsomia candida*

Detailed descriptions of the experimental design, conditions, and test results are provided in Table 3 (see subsection 2.6.1) and in the test report for collembola (Appendix D).

The soil pH for all exposure concentrations including the reference control soil ranged from 7.94 to 8.07 at the start of the test and from 7.83 to 7.96 at the end of the test. The change in soil pH from the start to the end of the test was acceptable. Initial soil conductivity[1] ranged from 178 to 216 µS/cm. At the end of the test, soil conductivity[1] ranged from 189 to 229 µS/cm (Table D.4, Appendix D). The change in soil conductivity from the start to the end of the test was acceptable. Soil pH and electrical conductivity of the artificial control soil was stable over the duration of the test. The initial soil moisture contents were similar and ranged from 85 to 100 (%WHC). The initial moisture content for the artificial soil was 92%. The final soil moisture (% WHC) ranged from 42 to 113 % for the test soils. The moisture content of the artificial soil at the end of testing was 108 % (Table D.4, Appendix D).

Both of the performance criteria for test acceptability were met for the artificial soil treatment (EC, 2007), indicating that the test procedures, conditions, organism health and technical proficiency were acceptable (Table D.1, Appendix D). Reference toxicity QA/QC data were also within the historical warning limits (Appendix D).

3.2.3 *Eisenia andrei*

Detailed descriptions of the experimental design, conditions, and test results are provided in Table 3 (see subsection 2.6.1) and in the test report (Appendix E).

The soil pH[1] for all exposure concentrations ranged from 7.94 to 8.07 at the start of the test and from 7.32 to 7.69 at the end of the test. The change in soil pH from the start to the end of the test was acceptable. Initial soil conductivity[1] ranged from 178 to 216 µS/cm. At the end of the test, soil conductivity[1] ranged from 186 to 236 µS/cm (Table E.4, Appendix E). The change in soil conductivity from the start to the end of the test was acceptable. Soil pH and electrical conductivity of the artificial control soil were stable over the duration of the test. The initial soil moisture contents were similar and ranged from 85 to 100 (%WHC). The initial moisture content for the artificial soil was 92%. The final soil moisture (% WHC) ranged from 85 to 101 % for the F2 artificial soils. The moisture content of the artificial soil at the end of testing was 111 % (Table E.4, Appendix E). The organic matter content for the artificial soil spiked to create the test soils was 7.3 ± 0.1% dry soil; the OM content for the artificial soil was 6.9 % dry soil which is typical (Table E.5, Appendix E).

[1] Soil pH and electrical conductivity were measured at the beginning and end of the tests by Stantec using the standard procedures for the water slurry method.

The performance criteria for test acceptability for progeny production and mass of individual progeny were met for the artificial soil treatment (EC, 2004), indicating that the test procedures, conditions, organism health and technical proficiency were acceptable (Table E.1, Appendix E); adult survival in the negative control treatment was 100% (n=20). Reference toxicity QA/QC data were also within the historical warning limits (Appendix E).

4.0 Discussion

4.1 TOXICITY TESTS

Toxic effects were observed for all test species and the E/I/LC50s and E/I/LC25s for all test species are presented in Table 5. Northern wheatgrass and barley EC50/EC25 for emergence could not be calculated using the approved EC statistical methods due to lack of partial effects. Endpoint E/I/LC25s ranged from 58 mg/kg F2 (alfalfa root length) to 18,197 mg/kg F2 (alfalfa emergence). There was no pattern in the sensitivity of test organisms. Invertebrates were not more sensitive to weathered F2 than plants. Plant emergence was the least sensitive endpoint relative to weathered-F2 contamination. EC25s for plant emergence were not-calculable for two of the three plant species, and alfalfa emergence was the least sensitive endpoint with the highest E/I/LC25 of those calculated (Table 5).

Table 5: Summary of E/L/ICxs calculated using the measured exposure concentrations at the start of each test (Day 0).

Parameter	Model	E/IC50 (mg/kg)	LCL[a] (mg/kg)	UCL[b] (mg/kg)	E/IC25 (mg/kg)	LCL (mg/kg)	UCL (mg/kg)	r^{2c}	χ^2 (df, p value)[d]	W[e]
NORTHERN WHEATGRASS										
Emergence	NC[g]	NC	NC	NC	NC	NC	NC	NA	NA	NA[h]
Shoot Length	Logistic	5321	3365	8414	1455	733	2884	0.970	NA	N
Root Length	Logistic	1991	1297	3055	533	292	975	0.974	NA	N
Shoot Dry Mass	ICPIN	961	905	1042	644	620	673	NA	NA	NA
Root Dry Mass	Logistic	1074	675	1714	508	269	962	0.927	NA	N
ALFALFA										
Emergence	Spontaneous Probit	20578	NC	NC	18197	NC	NC	NA	15.89 (7,0.03)	NA
Shoot Length	Hormesis	2032	1067	3864	298	162	548	0.984	NA	N
Root Length	ICPIN	4472	162	7490	58	17	121	NA	NA	NA
Shoot Dry Mass	ICPIN	803	400	1000	96	72	188	NA	NA	NA
Root Dry Mass	Logistic	796	461	1374	278	144	537	0.915	NA	Y
BARLEY										
Emergence	NC	NC	NC	NC	NC	NC	NC	NA	NA	NA
Shoot Length	ICPIN	17791	16569	18387	5618	3422	7096	NA	NA	NA
Root Length	ICPIN	876	708	974	474	185	597	NA	NA	NA
Shoot Dry Mass	ICPIN	14911	13286	16535	2552	989	3364	NA	NA	NA
Root Dry Mass	Logistic	2630	1271	5433	312	115	845	0.973	NA	N
F. candida										
Adult Survival	Logit	3418	2538	4605	1508	1087	2092	NA	67.38 (8,1.63×10^{-11})	NA
Progeny Production	ICPIN	3726	3463	3848	2784	2492	2904	NA	NA	NA
E. andrei										
Adult Survival	Logit using R	6159	NC	72645517	6324	NC	91060	NA	5.17 (108,1.0)	NA
Progeny Production	ICPIN	363	290	486	77	61	310	NA	NA	NA
Progeny Wet Mass	ICPIN	845	710	972	579	155	649	NA	NA	NA
Progeny Dry Mass	ICPIN	805	620	984	569	88	627	NA	NA	NA

[a]Lower 95% confidence limit
[b]Upper 95% confidence limit
[c]Coefficient of determination for regression analysis
[d]Chi-square lack of fit (degrees of freedom, *p* value)
[e]Indicates if data have been weighted by the inverse of the variance (N=No, Y=Yes)
[g]Not calculated (NC)
[h]Not applicable (NA)

E/IC25s for the various species (Table 5) were used to generate a species-sensitivity distribution (SSD), from which the direct soil contact values for ecological receptors were derived for the land-use classifications (Systat Software Inc., 2008). The derivation process followed the precedent set by the 2008 Canadian Council of Ministers of the Environment (CCME) protocol which utilized rank species sensitivity analysis. The geometric mean was calculated and used to combine redundant endpoints (single endpoint wet and dry weights). Regression procedures were applied to the ranks, and the 25th percentile was used to derive soil contact values for agricultural/residential land-use areas; the 50th percentile was used for commercial/industrial land-use areas. This data set meets all requirements for the Weight of Evidence method outlined by the CCME (≥10 data points; ≥2 plant + 2 invertebrate taxa) except for number of studies (≥3). The potential soil contact standards for weathered F2 in coarse-grained soil study were determined using species sensitivity distribution (SSD) regression with the 3-parameter logistic distribution (Figure 4; r^2= 0.9771).

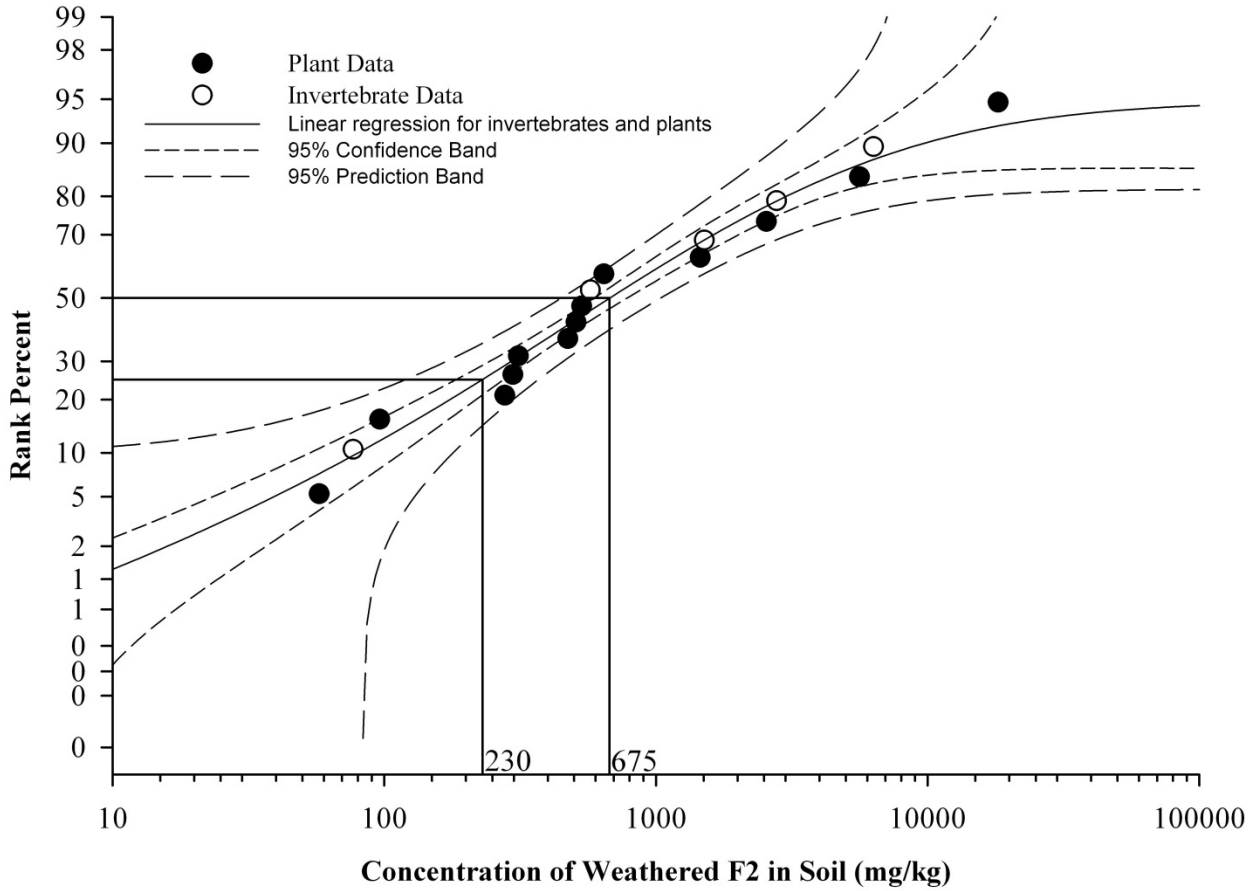

Figure 4: Species sensitivity distribution of rank values for weathered F2 using E/IC25s calculated using measured concentrations at the beginning of the tests. Threshold effect concentrations for 25th (agricultural and residential land-use classes) and 50th percentile (industrial and commercial land-use classes) were 230 and 675 mg/kg soil dry weight, respectively.

The potential soil contact standards for weathered F2 in coarse-grained soil are 230 mg/kg for agricultural and residential areas and 675 mg/kg in soil for commercial and industrial areas. These soil standards are less restrictive than current Tier 1 CWS for PHC fractions in soil based on the ecotoxicological data for soil receptors exposed to fresh F2 in soil (Table 6). Additionally, while the soil contact standard for coarse-grained soil for agricultural and residential areas is marginally more restrictive than that derived for fine-grained soils (Phase 1), the soil contact standard for commercial and industrial areas was almost two-fold greater for coarse-grained soils than fine-grained soils.

Table 6 Summary of Tier 1 Soil Standards for F2 in Surface Soil (mg/kg).

	Agricultural/Residential (mg/kg)	Commercial/Industrial (mg/kg)
Current values based on fresh product (CCME, 2008a)	150	260 (230- groundwater)
Proposed values for fine-grained soils based on weathered product (Phase 1; Angell et al., 2012; Stantec, 2009)	262	338
Proposed values for coarse-grained soils based on weathered product (Phase 2)	230	675

4.2 CONCLUSIONS

The present study determined that an artificially weathered, PHC Fraction 2-spiked, coarse-grained soil was toxic to the earthworm, collembola, and plant species exposed during testing. L/E/IC25s ranged from 58 to 18,197 mg/kg F2. When these L/E/IC25s were ranked and used to create a species sensitivity distribution, the distribution was described best by a Logistic regression model. Using CCME methodology, the proposed agricultural/residential and commercial/industrial standards for weathered F2 in a coarse-grained soil would be 230 and 675 mg/kg, respectively. These soil standards are less restrictive than current Tier 1 CWS for PHC fractions in soil based on the ecotoxicological data for soil receptors exposed to fresh F2 in soil. Additionally, the commercial/industrial standard based on the present testing with an artificially weathered, F2-spiked, coarse-grained soil is twice as high as the standard derived from testing with fine-grained soil (Phase 1).

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