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Final Report: AUPRF Project # 09-9159-50

Title: A rapid bioassay for predicting toxicity of PHC-contaminated soil, Phase 2

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Abstract.

The rapid (2-h) bioassay developed during this project is intended for soils that are contaminated with petroleum hydrocarbons (PHC) above Tier 1 remediation criteria, but which may be sufficiently weathered to make them candidates for Tier 2 site-specific assessment. The aim is not to replace any of Environment Canada's battery of Tier 2 tests, but to screen out soils with toxic levels of bio-available PHC, thus avoiding the fruitless expense of subjecting such soils to those time-consuming tests.

A representative soil sample is shaken with aqueous cyclodextrin (CD) to recover bio-available PHC; the centrifuged extract is treated with amylase before being subjected to a standard Microtox test, employing luminescent bacteria. Extracts with > 100 mg/L of PHC exhibit marked toxicity (bacterial light reduction), believed to be due to enzyme-catalyzed hydrolysis of the CD-PHC inclusion complex.

An uncontaminated sandy loam, and an artificial soil, each freshly spiked in the lab with a range of levels of diesel oil (F2 and F3 PHC), yielded toxic CD extracts at spike levels above 1,000 mg/kg PHC. Six real-world soils from PHC-contaminated sites in Alberta were donated to the project by CAPP/PTAC member companies or their contractors. CD extracts of these soils were non-toxic (toxicity index < 1) except in one case (soil 11-2094, with an index of 2.5).

Results of 35-day earthworm survival bioassays reflected the CD-Microtox bioassay results. Earthworm survival was similar to that in controls with no PHC, except for soil 11-2094 and the lab-spiked soils with > 1,000 mg/kg PHC. The rapid bioassay seems therefore to be a reliable indicator of earthworm survival.

However, in 63-day reproduction bioassays, juvenile production and juvenile weights were lower in all six real-world soils, as well as in the two lab-spiked soils, than in controls. Improved reproduction observed in three of the real world soils, after they were amended with peat, suggests that low soil porosity may exacerbate the effects of PHC on earthworm reproduction.

Introduction

Decisions regarding remediation or disposal of PHC-contaminated soil are based on solvent-extractable PHC concentrations, which can exceed established guidelines (CCME 2008) even though residual PHC may have become biologically unavailable with time and thereby non-toxic to biota (Reid et al. 2000; Axiom 2005). Our aim was to develop a fast and relatively inexpensive screening-level bioassay to predict soil toxicity caused by bio-available PHC. Provided that a correlation could be demonstrated between the screening test and one or more terrestrial bioassays, such information would aid in deciding whether soils exceeding CCME thresholds might

satisfy the eco-contact requirements of the full Tier 2 battery of terrestrial toxicity tests, thus justifying the expense of this further testing.
A milder analytical alternative to solvent extraction of PHC employs aqueous cyclodextrin. CD molecules have toroidal structures, with a hydrophilic exterior and an open, relatively lipophilic centre. They are known to form water-soluble host-guest complexes, with inclusion compounds that in isolation are relatively insoluble. Water solubility of the complex is further improved when the CD molecule also carries a hydroxypropyl substituent group.
Beta (β) and gamma (γ) forms of CD have 7 and 8 glucopyranose units per molecule, respectively. Recent reports suggest that CD solution extracts mainly bio-available PHC from contaminated soil. Aqueous hydroxypropyl-β-CD was used to assess bio-availability of PAH residues in soil (Reid et al. 2000). A good correlation was seen between amounts of PHC recovered by aqueous hydroxypropyl-β-CD from a range of PHC-contaminated field soils, and earthworm reproduction in them (Axiom 2005).
Although the potential of CD extraction to indicate bio-available PHC has been recognized (CCME 2006), clean-up thresholds for CD-extractable PHC in soil have not yet been established; also, the need for GC analysis to quantify PHC in CD extracts precludes fast, inexpensive site assessment.

Microtox® bioassay. **This rapid test is used world-wide to assess toxicity of aqueous solutions containing PHC and other contaminants. It employs the luminescent marine organism *Vibrio fischeri*, sample toxicity being assessed from light output measurements, after aliquots of these bacteria are mixed with a series of dilutions of the test fluid, in 2 % NaCl. The EC_{50} value (indicating sample toxicity) is the fluid concentration at which initial bacterial light output is halved (usually after 15 min. contact). The EC_{50} is the intercept of a line fitted to the light loss data points, where the light-loss ratio LLR (also called Gamma) = 1.**

Toxicity of CD-extracts. **CD-extracts of soils with as much as 1 % PHC show little dependence of light output on extract concentration and have LLR < 1 (Ashworth & Oosterbroek 2010). This low toxicity of CD solutions known to contain PHC may be due to occlusion of PHC by CD molecules. The reported susceptibility of γ-CD to enzyme-catalyzed hydrolysis by amylase (Jodál et al. 1984) offered a way to break apart the CD structure, with possible release of occluded PHC and concomitant light loss effects in the Microtox bioassay.**

<u>Materials and Methods</u>:
Cyclodextrins. **Beta cyclodextrin (2-hydroxypropyl β-cyclodextrin; CAS 128446-35-5) was purchased from Aldrich Chemistry. Gamma cyclodextrin (Hydroxypropyl γ-cyclodextrin; CAS 128446-34-4) was purchased from Sigma Life Sciences. Weighed amounts of β- or γ-CD were magnetically stirred into de-ionized water then made up to the volume required for 8 % solutions (8 g per 100 mL).**

Amylase. **We used Sigma Life Sciences' liquid formulation Fungamyl® 800L, containing α-amylase from the fungus *Aspergillus oryzae*, which was the source of the amylase used by Jodál et al. (1984) to hydrolyze γ-CD. It was kept at 4±2 °C. Analysis of a sample indicated that Fungamyl has 120 g NaCl per litre. Before adding Fungamyl to aqueous CD, therefore, it was diluted (2 parts by volume) with de-ionized water (1 part), in order both to lower its viscosity (thus making it easier to pipet reproducibly), and to bring the salinity of Fungamyl down to a level suited to routine Microtox testing.**

Hydrocarbons. **Diesel fuel was employed as a convenient PHC source. GC analysis indicated that, after 48-h weathering in a current of air, the fuel had approximately 55 % fraction-2 (F2) hydrocarbons (C10-C16), 45 % F3 (C16-C34), and little or no F4 (> C34). The absence of F4 compounds was not considered a disadvantage since they are generally less toxic than F2-F3 compounds (CCME 2008).**

Soils. **An uncontaminated loam subsoil from near Bassano, Alberta (Orthic Brown Chernozem, with 18 % clay, 42 % sand, and 1.4 % OM) was spiked with weathered diesel fuel at 20,000 mg/kg, and successively diluted with clean soil 4-fold to obtain first 5,000 then 1,250 and 312 mg/kg. One screw-cap glass jar (250 mL) of each spiked soil was stored at 4±2 °C. Solvent extraction and GC-FID analysis recovered 90-95 % of the added spike, indicating that the target rates had been achieved.**
An artificial soil was prepared at HydroQual according to Environment Canada (2004) guidelines and similarly spiked with a range of PHC concentrations, using the same weathered diesel fuel.
Six real-world soils were donated to the project. They were analyzed for various inorganic and organic parameters and were found to range widely in PHC content (see Table). Two soils had elevated levels of Ba and/or Zn and another was low in OM, characteristics that may be of concern for earthworm reproduction; all soils were subjected to earthworm testing (see below).

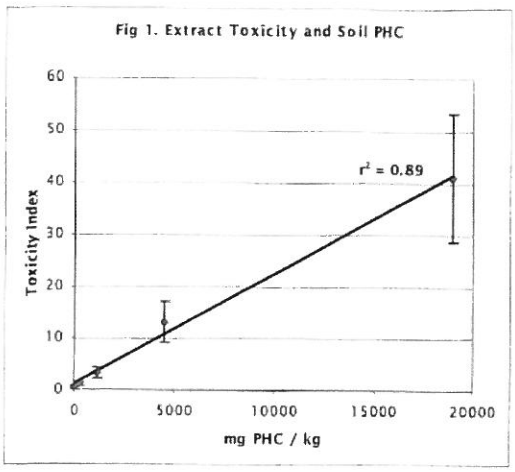
Soil no.	Name	Texture	Org. Matter	pH	EC	SAR	Elem-ents mg/kg	Toxicity Index = 100/EC50	Hydrocarbons F2	F3	F4
1	11-1945	Loam	2.3 %	7.7	1.9 dS/m	0.4	OK	0.5	11	339	71
2	11-2094	Sandy Loam	17.5	6.7	1.3	0.3	OK	2.5	159	2800	670
3	Matrix Nov	Sandy Clay Loam	1.7	7.9	0.6	0.5	OK	0.3	35	298	96
4	NorAlta 06-26	Loam	3.7	7.6	0.7	0.2	Ba = 1320	0.4	12	171	n/d
5	NorAlta 14-08	Loam	8.9	7.5	1.1	0.1	Ba = 860 Zn = 102	0.4	61	629	28
6	Pembina Clark Lake	Loam	2.1	7.8	1.1	0.4	OK	0.8	n/d	28	n/d

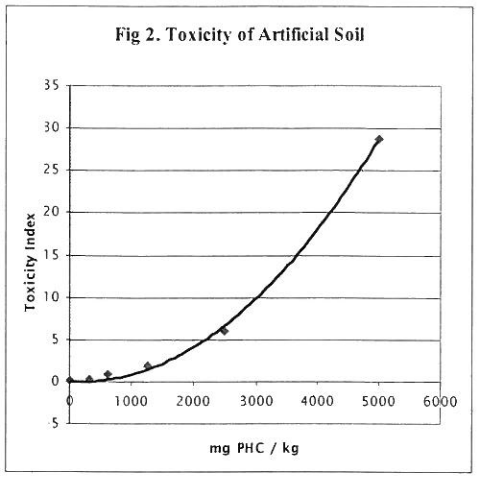
Soil extraction. Moist soil subsamples (= 5.0 g dry soil) were treated with 40 mL of 8 % CD solution. This combination of soil:solution ratio and CD concentration (Axiom 2005) provided a large stoichiometric excess of CD over total PHC in the soils tested. Extraction was done in plastic centrifuge tubes shaken end to end at 120 excursions per min. for 30 min., the time that gave the best recovery of added PHC in previous work (Axiom 2005). The resulting suspension was centrifuged for 15 min. at 3,000 x g and the clear supernatant (CD extract) transferred to a glass vial.

Enzyme-catalysed hydrolysis and Microtox testing. CD soil extracts thus obtained (4 mL) were treated with 0.4 mL of 2:1 Fungamyl-water mixture at room temperature (21 ± 2 °C) and a Microtox test done within 1 h, using the 4-serial dilutions ISA procedure (10.2, 20.5, 40.9 and 81.8 %), with aqueous phenol as the reference standard (WCMUC 1994). No pH-adjustment or colour-correction was required.

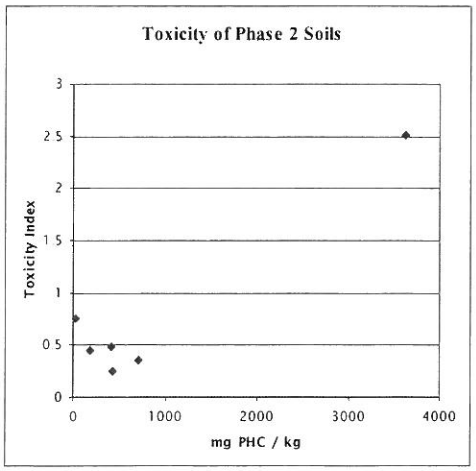
Earthworm bioassays. Chronic earthworm bioassays were conducted at HydroQual using modified Environment Canada (2004) methods. Modifications applied to the numbers of concentrations tested for each soil, and not to other test parameters. After homogenization, the raw soils were successively diluted with artificial soil so as to obtain 90.9, 45.5, 22.7 and 11.4 % concentrations of each original soil. Lab controls (100 % artificial soil) were also prepared. The available quantities of soils 11-2088, -2089 and -2094 were sufficient to allow additional replicates of the 90.9 and 11.4 % concentrations to be amended with 10 % peat by volume to qualitatively assess amendment effects.
Following soils preparation, testing was initiated according to the Environment Canada method (2004). Adult survival was assessed after 35 days, and juvenile production and growth were assessed after 63 days. For each soil, adult survival, juvenile production (number of juveniles), and juvenile growth (dry mass per juvenile) were assessed for each concentration relative to controls (i.e., controls set to 100%), and EC_{50} and EC_{20} values calculated for each parameter.

Results and Discussion.
EC_{50} values are inversely related to toxicity, which is therefore often expressed as an index = 100 / EC_{50}. Using the above rapid bioassay, the Bassano loam (Fig. 1) and the artificial soil (Fig. 2) showed increasing toxicity index values at PHC spike levels above 1,000 mg/kg. This rate of soil contamination is in line with current CCME clean-up thresholds for F2 & F3. The curvilinear response with the artificial soil was largely due to its high toxicity at the highest PHC spike rate, suggesting that it had a smaller sorption capacity for PHC than the real-world Bassano loam.

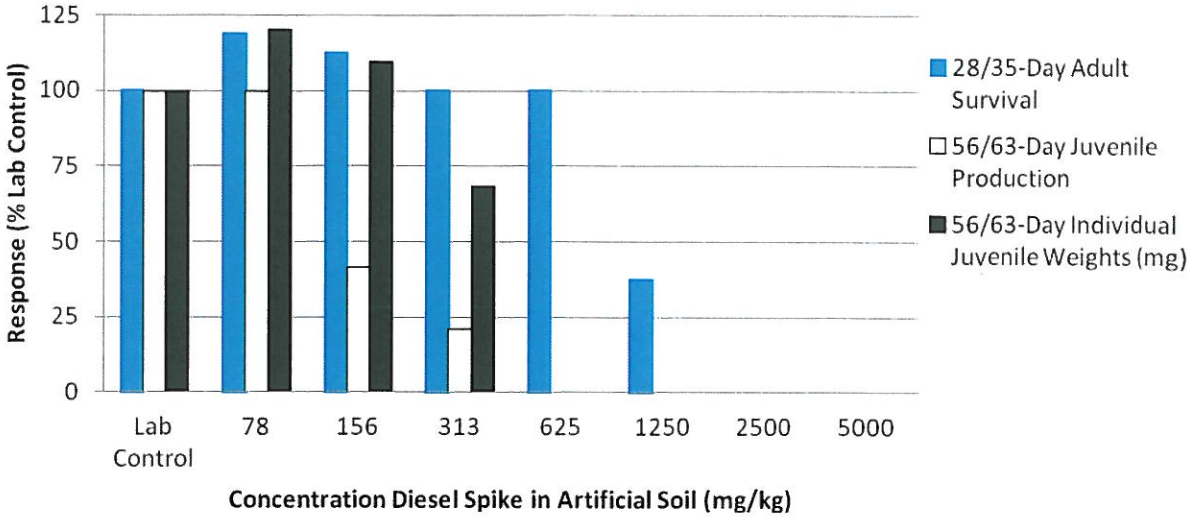




Of the six soils from contaminated field sites donated in 2011, only one (11-2094) had solvent-extractable PHC in excess of Tier 1 criteria (3,600 ppm F2-F4). The rapid CD-Microtox bioassay picked out soil 11-2094 as the only one with significant toxicity (see Fig. 3). Judging from the results of testing the lab-spiked soils (Figs. 1 & 2), the mean toxicity index of 2.5 for soil 11-2094 is approx. equivalent to 1,500 ppm of freshly applied F2+F3.



For the spiked artificial soil (Fig. 2), the CD toxicity index was > 1 only for PHC rates above 1,000 mg/kg, reflecting the 35-d earthworm survival results for this soil (below). However, as also shown below, 63-d earthworm juvenile production was affected at lower PHC rates, in the range 100 - 500 mg/kg (Appendix 1; 12-0147), at which the CD toxicity index was < 1 [EC50(15) > 100 %].



Earthworm bioassays.

The results of earthworm tests at HydroQual on the six real-world soils are contained in Appendix 1. With these donated soils, as with the lab-spiked soils, earthworm reproduction was more sensitive to increasing contaminant concentration than earthworm survival. While only soil 11-2094 was toxic enough to produce an adult survival EC_{50} value, juvenile production EC_{50}s for all six donated soils fell within the range of concentrations tested (i.e., equivalent to toxicity index values > 1). Juvenile weights were more varied, with EC_{50} values for 3 of the 6 soils (including 11-2094) falling within the range of concentrations tested.

As observed above, the CD-Microtox test gave toxicity index values < 1 in all cases except 11-2094, which had the most PHC contamination. The rapid CD-Microtox bioassay therefore would at first sight seem to be capable of indicating problems of earthworm survival in oil-contaminated soils, but not of predicting the likelihood of earthworm reproduction in them.

However, adding extra peat to the mixtures of soils 11-2088, -2089 and -2094 resulted in improved weights and rates of juvenile production (Appendix 1), suggesting that earthworm reproduction may depend on physical soil characteristics such as porosity, as well as on concentrations of PHC contaminants. Since the earthworm bioassay integrates all adverse effects experienced by the organisms, and we had insufficient soil to run full, side-by-side peat amended trials, it is unclear to what extent peat amendment mitigated the effects of non-PHC factors.

Conclusions

In outline, the rapid bioassay (Ashworth & Bullecer 2012) consists of:

1. **Shake the oil-contaminated soil with gamma CD solution (0.5 h)**
2. **Centrifuge the mixture (0.2 h)**
3. **Treat a portion of the centrifugate with amylase enzyme (0.8 – 1.0 h) to hydrolytically weaken the CD-PHC association complex**
4. **Perform a Microtox bioassay on the treated CD extract (0.3 h) and calculate a toxicity index = 100 / EC50(15)**

No effects on 35-d earthworm survival were observed in soils with CD-Microtox toxicity index values < 1. However, soil 11-2094 gave a toxicity index = 2.5, and in this one case, earthworm survival was drastically reduced at the 90.9 % concentration.

At soil mix concentrations below 90.9 %, juvenile production was significantly reduced in all six donated soils, and weights were significantly reduced in three of them, findings which do not correlate with the CD-Microtox results. The rapid CD-Microtox bioassay would therefore seem to be a good indicator of earthworm survival but not of reproduction.

However, adding peat to three of the PHC-spiked soils improved juvenile production and weights, indicating that soil porosity may also be a factor affecting earthworm reproduction. It is hoped to be able to investigate this possibility further in later work, proposed for Phase 3 of this project.

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