A Rapid Bioassay for Predicting Toxicity of PHC-Contaminated Soil

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ABSTRACT

The quest for a rapid bioassay for oil-contaminated soil was prompted by the expense and turn-around time of tests prescribed by current regulations, which aim to quantify the toxicity of such materials towards organisms that represent various soil ecosystem levels.
In Alberta Environment's Eco-Contact Guideline, the Weight of Evidence approach requires, as well as tests with other species, earthworm reproduction bioassays which run for several months and can be costly, depending on the number of samples required per site.

The rapid bioassay developed in the course of this project requires only two working days, and will cost less than $1,000 per sample. It involves shaking soil with aqueous cyclodextrin (CD), which extracts bio-available petroleum hydrocarbons (PHC) by forming an association complex.
After centrifugation, the supernatant CD extract is pasteurized to suppress natural soil enzyme activity, then a portion is treated with amylase enzyme and incubated in darkness at room temperature for 20 h to uncouple CD from any associated PHC.
A standard Microtox bioassay using the test organism *Vibrio fischeri* is then done, to assess extract toxicity.

Eight PHC-contaminated soils were donated to the project by CAPP member companies and their contractors, and a control soil was spiked with various rates of weathered diesel fuel.
Soils with total PHC concentrations between 1,000 - 20,000 mg/kg gave CD-extracts with 20 - 400 mg/L. After amylase treatment these extracts, and CD solutions spiked with diesel in that same mg/L range, were toxic towards *V. fischeri* in proportion to the level of PHC contained.

Earthworm bioassays were also done on all soils but, unfortunately, no correlations could be drawn between these bioassay results and CD-extract toxicity to *V. fischeri*. The donated soils inhibited earthworm reproduction independent of PHC level; this effect may be related to soil texture.
Organic amendments to subsoils to eliminate this confounding factor will be investigated in follow-up work (Phase 2), in which we plan to do similar bioassays on a range of true Tier-2 candidate soils (see below).

INTRODUCTION

Decisions regarding clean-up and disposal of oil-contaminated soils are generally made on the basis of comparing levels of solvent-extractable petroleum hydrocarbons (PHC) with CCME threshold values (CCME 2008).

PHC concentrations thus measured can exceed Tier 1 thresholds, although much of the residual F2-F4 may have become biologically unavailable over time and thereby non-toxic to biota, through processes such as PHC ageing, partition and sequestration (Semple et al. 2000: Axiom 2005). In such cases there is the option of providing a sufficient "weight of evidence" approach that could result in allowable thresholds being increased to a level specific for the soil and site in question (Tier 2 Eco-Contact Guideline Derivation Protocol; Alberta Environment, 2007). The Tier 2 approach is distinct from using a less conservative Tier 1 threshold by eliminating pathways to a receptor organism.

It is expensive and time-consuming to perform Tier 2 terrestrial toxicity screening of soils in order to confirm the absence of toxicity from residual PHC. Environment Canada has developed biological test methods using species that represent major trophic levels in soils – including plants, earthworms, and springtails. A full suite of tests (two plant species, one earthworm and one springtail) on a soil as received, (tested at one concentration – no dilutions) compared against site and laboratory control soils, costs between $5-10 k, excluding analytical and reporting costs. Three to five samples are required from each site for analysis using the full-scale chronic bioassays for each test species.

The objective of this research project was to develop a fast and relatively inexpensive screening-level bioassay to indicate soil toxicity caused by bio-available PHC. This information would aid in deciding whether soils that exceeded Tier 1 guidelines might have a likely chance of satisfying the eco-contact requirements of the full battery of terrestrial toxicity tests.
Such a method, if it demonstrated accuracy and reliability, would also be suited to site reconnaissance in identifying areas of high bio-available PHC.

The approach used in this project involved using the Microtox® test to assess toxicity of cyclodextrin extracts of PHC-contaminated soil.

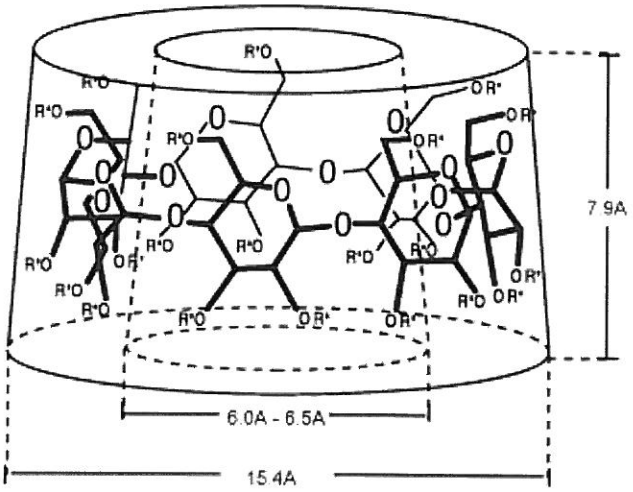
Cyclodextrin extraction

A milder analytical alternative to solvent extraction of oil-contaminated soil involves using an aqueous solution of cyclodextrin (CD), which can be any one of a family of sugary polymers each with a hydrophilic exterior and a relatively waxy, open centre (Fig. 1) that can either form an association complex with, or actually accommodate, PHC molecules.

Alpha, beta and gamma-forms of CD have 6, 7 and 8 glucosidic units respectively per molecule and the corresponding formula weights (FW, unsubstituted) are 972, 1135 and 1297 Daltons.

A hydroxypropyl-substituted β-cyclodextrin was used in research into degradability of PAH residues in soil (Semple et al. 2001).

Fig. 1. Diagrammatic representation of β-cyclodextrin



PHC in aqueous CD can be quantified by partition into dichloromethane, followed by gas chromatography. Good correlations observed between (a) amounts of PHC extracted by aqueous CD from oil-contaminated soils and (b) earthworm reproduction in those soils indicated that CD solution indeed extracts only bio-available PHC (Axiom 2005).

However, clean-up thresholds for CD-extractable PHC in soil have not yet been established (CCME 2008) and soil clean-up / disposal decisions continue to be made on the basis of solvent-extractable PHC.

Microtox® bioassay

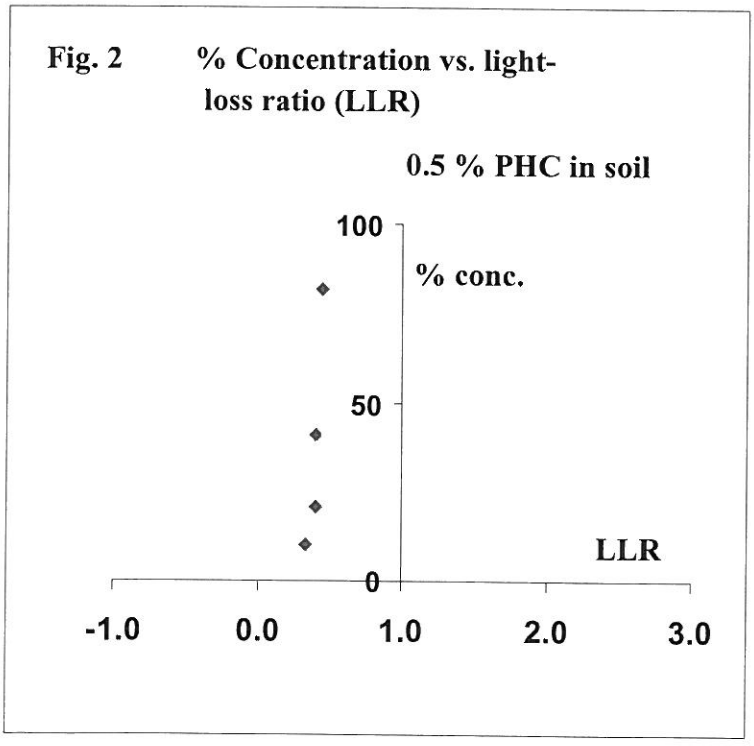
The Microtox bioassay is a rapid method for assessing toxicity of aqueous solutions containing PHC and other toxicants. It is currently used in many

environmental jurisdictions around the world and is employed in routine drilling waste testing in western Canada.
The method uses the luminescent microorganism *Vibrio fischeri*, and toxicity is assessed from measurements of light output of these bacteria, mixed with a series of 2-fold dilutions of the original test fluid, in 2 % NaCl. In typical plots of Microtox data for contaminated fluids, bacterial light loss increases with sample concentration. The EC50 value (a measure of sample toxicity) is the concentration of test fluid at which bacterial light output was halved (light loss ratio LLR = 1). The EC50 is obtained by fitting a line to data points, to obtain the intercept value at LLR = 1.

Toxicity of CD-extracts

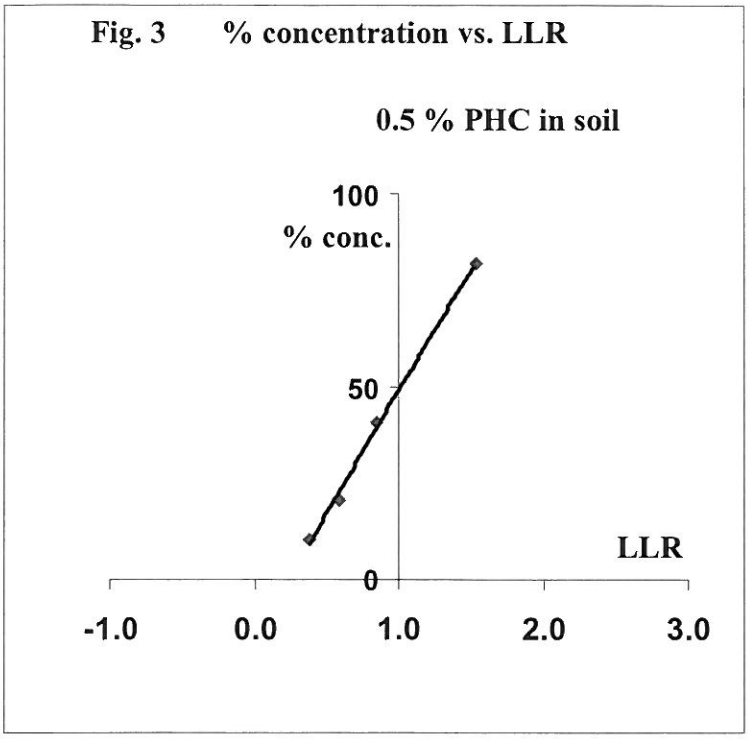
Untreated CD-extracts of oil-contaminated soil generally show little dependence of light output on concentration and LLR may always be < 1 (Fig. 2). This observed lack of toxicity of CD solutions known to contain PHC may be due to low levels of free PHC, due to occlusion of PHC molecules in the waxy CD pocket.

Fig. 2 **% Concentration vs. light-loss ratio (LLR)**



Removal of PHC from CD

The challenge undertaken by ALS was to find a way to weaken or break the CD-PHC association complex, with release of occluded PHC and possible concomitant effects on light loss in the Microtox bioassay, resulting in one or more LLR values > 1 and an intercept at LLR = 1, indicating a degree of toxicity, as in Fig. 3.



We considered four methods of opening up the PHC-CD occlusion complex:

1. Solvent extraction of PHC:

Removal of PHC from CD using solvents is done for analytical purposes but is not feasible as a precursor to Microtox testing, due to solvent toxicity to *V. fischeri*. This approach was not tried.

2. Surfactants:

Attempts to transfer PHC from its complex with CD into the aqueous phase, by shaking with a surfactant or wetting agent, are described below.

3. Acid hydrolysis of cyclodextrin:
There has been much pharmaceutical research into drug delivery by means of CD; the method relies on degradation of CD in the alimentary tract with release of its associated drug to the patient. The degradation process is analogous to the use of strong acids to hydrolyze starch.
In preliminary work, acidification and re-neutralization of CD extracts (beta form of CD) had apparently increased their toxicity (Ashworth and Oosterbroek 2010). Fig. 3 above, with LLR > 1 at the highest test concentration, was obtained in that work.

4. Enzyme-catalysed hydrolysis of CD:
Gamma-CD is reportedly far more susceptible to enzyme-catalyzed hydrolysis, e.g. by amylase, than the alpha or beta forms. Jodál et al. (1984) suggested that the size and flexibility of the gamma CD molecule may conform better to the helical chain of amylase.

Comparison with earthworm data
Whatever the chosen method of treating CD extracts, our plan was to compare Microtox bioassay results, for extracts thus treated, with results of acute and chronic earthworm bioassays using a series of PHC-contaminated soils, in order to quantify the correlation of toxicity of the soils to earthworms with that of CD soil extracts to *V. fischeri*.

MATERIALS

Soils

Eight soils were donated to the project by CAPP companies or their contractors. An uncontaminated loam from near Bassano, retained by ALS from a separate project, was also used. Analytical characteristics of the soils are in Table 1.

Table 1. Analytical data for project soils

Soil number	Texture	OM(%)	pH	EC (dS/m)	SAR	Elements (mg/kg)	Hydrocarbons		
							F2	F3	F4
1	Loam	6.5	6.9	3.6	1.3	Cu = 53 Zn = 130 Pb = 110	43	3740	2900
2	Loam	7.0	6.8	3.6	1.6	Cu = 49 Zn = 140 Pb = 100	49	3800	3020
3	Clay	3.7	7.2	4.5	3.9	OK	34	1270	800
4	Loam	1.7	7.8	4.7	16	OK	17	400	350
5	Loam	1.5	7.4	0.6	0.4	OK	200	410	96
6	Sandy Loam	8.0	7.2	2.9	0.4	OK	1400	5150	70
7	Loam	4.9	8.0	2.2	17	OK	3040	8770	2760
8	Loam	1.5	7.8	3.0	0.8	OK	n/d	n/d	n/d
9	SCL	1.8	7.4	0.8	0.2	OK	n/d	630	290

Notes: OM, organic matter; EC electrical conductivity; SAR, sodium adsorption ratio; Cu, copper; Zn, zinc; Pb, lead.

The term "OK" under the column heading Elements signifies that all trace elements were at typical background concentrations. Only soils 1 and 2 tested high for any potentially toxic element. Other results in red were also of concern in terms of earthworm viability.
In most cases there was little information about the provenance of the soils supplied. Judging from the F2-F4 PHC content, many were not true Tier 2 candidate soils.

HydroQual Laboratories Ltd. (HydroQual) generated additional soils for analysis by spiking Bassano loam (soil # 8) with nominal concentrations 0, 312, 1,250, 5,000 and 20,000 mg/kg of PHC, in the form of weathered diesel fuel (these are soils 10-14 in Table 4, below).

All soils were kept refrigerated in glass screw-cap jars with Teflon-lined lids, except for Bassano loam (plastic pail). All samples used for the earthworm bioassay were stored at room temperature in large plastic pails.

The possible effect of ageing on soil PHC toxicity was investigated in a set of Microtox tests on CD extracts of soils 10-14 stored at room temperature for an extra 4 months.

Cyclodextrins

Beta cyclodextrin (2-hydroxypropyl β-cyclodextrin; CAS 128446-35-5) was purchased from Aldrich Chemistry. It has a nominal FW = 1,380 and cavity diameter = 0.78 nm. Hydroxypropyl substituted beta CD was used by Semple et al. (2001) and Axiom (2005).
Gamma cyclodextrin (Hydroxypropyl γ-cyclodextrin; CAS 128446-34-4) was purchased from Fluka Analytical. The hydroxypropyl gamma form (8-membered ring) has FW = 1,760 and a cavity size of 0.95 nm.

Amylase

The amylase used in this study was 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 degrade γ-CD. Fungamyl liquid has 120 g NaCl per litre (ALS data); it was kept refrigerated.
Before adding Fungamyl to CD extracts, it was diluted 2:1 (v/v) with calcium chloride solution (2.75 % w/v, Ricca Chemical Co.). This solution is a ready-made standard, used in determining biological oxygen demand. Dilution with $CaCl_2$ was done for several reasons:

- The extra calcium thus added to soil CD-extracts would mask any variance in soluble soil calcium, which has the potential to affect amylase efficacy (Okita and Preiss 1980);
- The solution lowers the viscosity of Fungamyl, thus making the mixture easier to pipet accurately,
- The high salinity of Fungamyl was thus brought down to a level suited to *Vibrio fischeri*.

Weathered diesel

Diesel fuel from a commercial service station was placed in an evaporating dish under a strong air current entering a fume hood at ALS Edmonton. Weight loss data indicated that, after 48 h, vapour loss was extremely slow. The residue was kept in capped amber glass bottles at room temperature.

Analysis by GC-FID of solvent extracts of spiked Bassano soils indicated that the weathered fuel had approximately 55 % F2 and 45 % F3, with no F4.

METHODS

CD solutions and PHC-spiked CD solutions

Solutions of beta- or gamma-CD (8 %) were prepared by shaking *x* gm of solid CD with deionized water and making up to 12.5 *x* mL in an amber glass bottle. PHC-spiked CD solutions were prepared by first placing a droplet of weathered diesel on the inner wall of a tared glass BTEX vial, recording the weight in mg (*w)* to 0.1 mg. Then a stock 200 mg/L solution was prepared by adding 5 *w* mL of 8 % CD solution to the vial, which was capped and shaken mechanically for 30 min.

Portions of this stock solution were diluted 1:2 or 1:4 in 8 % CD to produce 100 mg/L and 50 mg/L standards, respectively.

The amount of PHC in a supposedly 50 mg/L standard solution was measured by dcm extraction and GC-FID of a sample, to confirm that the concentration was as intended. The amount found in 8 mL was 370 μg PHC (expected amount = 400 μg).

Soil extraction

The method of soil extraction with aqueous CD was similar to the one described in Axiom 2005. Field-moist soil (5.0 g) was treated with 40 mL of an 8 % CD solution in a glass BTEX vial and shaken for 30 min. The suspension was transferred to a plastic centrifuge tube and centrifuged for 20 min. at 5,000 rpm. The clear supernatant (soil CD extract) was transferred by pipet to a clean BTEX vial.

The additional de-ionized water wash step (Axiom 2005) was omitted, as this procedure can cause extract turbidity, which interferes with light output measurements in Microtox testing. Tests indicated that omitting this wash step had little effect on PHC recovery.

Due to the cost of CD and because only a small volume is needed for a Microtox bioassay, half-scale extractions were also done, ensuring in all cases that the 2.5 g sub-sample was made up of multiple small portions taken from a windrow of the entire jar of soil.

Microtox tests were done on all soil CD extracts, both before and after amylase treatment (see below). For comparison with the Microtox test result, portions of each extract were analyzed by partition into dichloromethane followed by GC-FID, in order to measure extract PHC concentration.

Surfactants

In attempts to transfer PHC into the aqueous phase without introducing toxicity from the solvating agent, CD extracts were mixed and shaken with various surfactants or wetting agents with a range of hydrophilic-lipophilic balance (HLB) values (Table 2),

Table 2. Hydrophilicity of surfactants used

Surfactant	Hydrophilicity (HLB value)
Brij 35	17.2
Witconate 60T	14 (estimated)
Span 20	8.6
Span 80	4.3 (lipophilic)

Acid hydrolysis of beta-CD

The Microtox test can be affected by pH values outside the range 5-9 (WCMUC 1994).

In order to obtain pH-neutral samples for testing, sodium bicarbonate was used to avoid over-adjusting the pH, when neutralizing acidified β-CD extracts. Various acid types, strengths and duration of acidification were tried.

The test requires 2 mL of solution; typically, 4 mL of CD-extract was treated with 0.2 mL of 1 *M* hydrochloric acid (HCl) and left for 2 hours before neutralizing with 0.3 mL of saturated $NaHCO_3$ solution (0.7 *M).*

Soil enzyme de-activation ("pasteurization").

Preliminary results suggested that natural enzymes in gamma-CD soil extracts were increasing extract toxicity and acidity. To suppress this effect, CD soil extracts were pasteurized by placing 10-30 mL in tightly capped glass BTEX vials in a 2 L plastic pail. The pail was partly immersed in hot water in a pressure cooker (Fresco, 5 L), and left to steam for 10 minutes with the lid closed. This brief exposure to boiling heat is enough to deactivate most enzymes (Pelczar & Reid 1972). Vials were cooled to room temperature before treating portions of the extract with amylase.

Enzyme-catalysed hydrolysis of γ-CD extracts

Gamma-CD extracts (4 mL) were treated with 0.4 mL of 2:1 Fungamyl-$CaCl_2$ mixture (see above) and left in a closed cupboard overnight (18-22 h) at room temperature (21 ± 2 °C) before a Microtox bioassay was performed.

Earthworm bioassays

Alberta Environment's Eco-Contact Guideline (2007) specifies earthworm tests in conjunction with other terrestrial bioassays to evaluate the potential biological effects of hydrocarbon contaminated soils. Both screening survival tests and full-scale chronic toxicity tests are outlined within this regulation. The acute toxicity test evaluates adult earthworm survival over a 14-day period. The chronic toxicity test evaluates adult survival and juvenile production and growth over a period of 56-63 days.

Prior to chronic test set up, acute (survival) earthworm tests were performed on soils 1-7 to determine if the soils were toxic to adult survival. Chronic earthworm tests were done on soil 8, treated with diesel fuel to give a range of PHC levels as described above, and also on soils 2 & 7 which, based on PHC content, were expected to affect earthworm reproduction differently.

RESULTS AND DISCUSSION

Soil extraction with CD

Amounts of PHC recovered from soils 1-9 by extracting with β- and γ-CD are shown below.

Table 3. PHC recovered by beta or gamma CD extraction

Soil no.	F2-F4 extracted by β-CD (mg/kg)	F2-F4 extracted by gamma-CD
1	404	245
2	450	264
3	224	101
4	134	95
5	256	144
6	879	412
7	1,490	890
8	n/d	n/d
9	300	138

Recovery by both forms of CD was less than 10 % of the amounts of F2-F4 found by solvent extraction (Table 1). Surprisingly, despite the bigger size of the molecule and its central cavity, gamma CD recovered less PHC than the beta form (on average, 55 %). Low recovery is of no great concern, as long as amounts recovered correlate with extract toxicity.

In a second set of extractions using gamma-CD, part of each extract was analyzed by solvent extraction/GC-FID and the rest underwent Microtox testing.
For the recently spiked soils 11-14, γ-CD extraction (Table 4) recovered approximately 30 % of the applied weathered diesel.
Some analytical variability in the amount of PHC recovered can be seen when results in Tables 3 & 4 are compared. The result for soil 7 (with the most total PHC of all donated soils) may be erroneously low.

Table 4. PHC recovered by γ-CD, based on concentration in the extract

Soil no.	F2-F4 extracted by gamma-CD (mg/kg)	F2-F4 concentration in CD extract (mg/L)
1	326	17
2	316	17
3	93	5
4	51	3
5	84	5
6	570	30
7	560	30
8	87	5
9	66	3
10	87	5
11	63	3
12	430	23
13	2,140	112
14	5,850	307

Pre-treatment of extracts before Microtox test

Partition into surfactants:
This approach was discontinued due to the toxicity of some of the agents to *Vibrio fischeri* and because, with non-toxic agents, resulting Microtox plots for PHC-contaminated soils still resembled Figure 2 above, showing little dependence of light loss on sample concentration.

Acid hydrolysis of beta-CD extracts:
Preliminary results had been encouraging enough to justify continuing to explore this method. However, after many attempts over an extended period, this approach was also discontinued, due to inconsistent results and small LLR values. In some instances, plots resembling Fig. 3 were obtained but, more often, the same procedures generated plots resembling Fig. 2.
As mentioned, to avoid pH over-correction, acid-treated CD solutions were neutralized with sodium bicarbonate rather than sodium hydroxide. Once acidified extracts are neutralized with $NaHCO_3$ solution, CO_2 evolution can continue for an extended period, especially in the confines of a Microtox test cuvette (5 cm high, 1 cm diameter). Speculating, the presence of nascent CO_2 bubbles on the rough membrane surface of *V. fischeri* may have induced some of the light loss observed, which on occasion matched the PHC content of the extracts being tested. However, these effects could not be reproduced well enough to justify persisting with this approach.

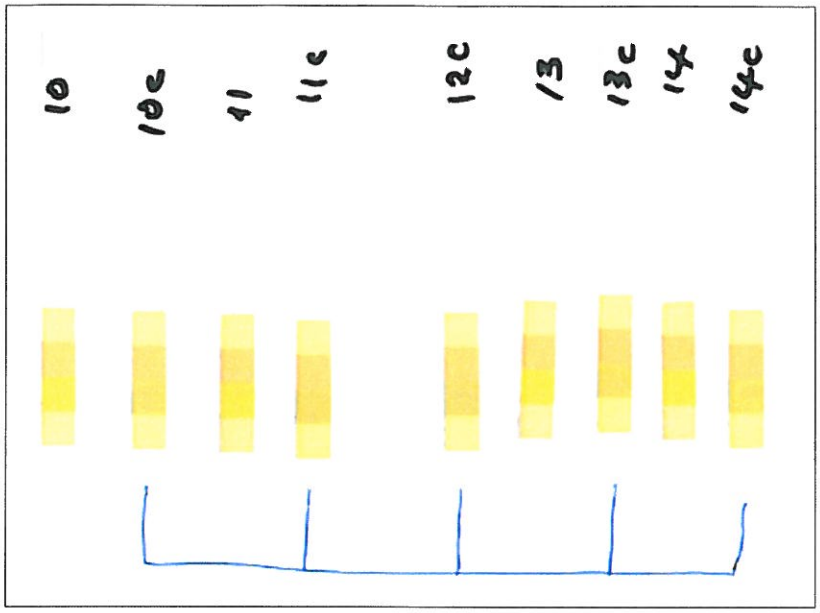
Enzyme-catalyzed hydrolysis of gamma-CD extracts:
Marked increases in toxicity (LLR > 2) were obtained following amylase treatment of gamma CD-extracts of PHC-contaminated soils and PHC-spiked CD solutions.
In contrast, the toxicity of beta-CD solutions spiked with PHC was essentially unaffected 20 h after amylase treatment. Plots of resulting Microtox data again resembled Fig. 2 above. These findings are consistent with the minimal hydrolysis of beta-CD by amylase, reported by Jodál et al (1984).

Effect of light:
Inconsistencies in the onset of amylase activity in treated gamma-CD extracts of soil were initially observed, but no delay was noticeable when treated extracts were kept in darkness. Inhibition of amylase activity by light (Van Onckelen et al. 1977) may be related to *in vivo* starch degradation in plants at night (Okita & Preiss 1980).

Pasteurization step:
Solutions of gamma-CD spiked with PHC remained clear and pH-neutral after amylase addition, but CD soil extracts became acidic and cloudy, soon developing an unpleasant, sour smell and eventually a white precipitate. No attempt was made to identify the precipitate.
These effects were delayed by "pasteurizing" soil extracts as described above, before adding amylase. Soil extracts thus "cooked" (designated by

the letter **c** in Fig. 4) still had neutral pH 20 h after amylase addition (as indicated by the green square on an inserted pH strip), whereas "uncooked" samples of the same extracts were acidic by then.

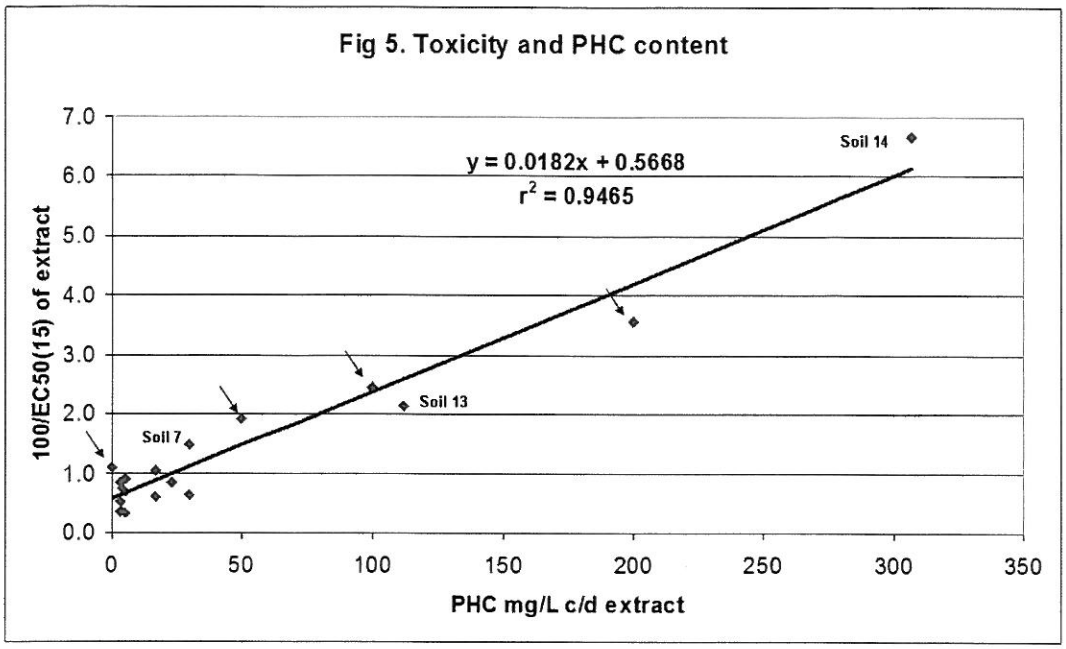
Fig. 4 pH strip tests of amylase-treated γ-CD soil extracts.



The letter **c** after the soil number, and the hand-drawn lines at the foot of the Figure indicate pasteurized samples. (There was insufficient residue of "uncooked" extract 12 for a pH test.)

Incubation temperature:
Incubating at 35 °C is near-optimum for amylase (Jodál et al. 1984) but, for practical reasons, a 20-h period at room temperature was adopted. Soil extraction and treatment followed by several hours' incubation at 35 °C make sample preparation too long for subsequent Microtox tests to be done conveniently on the same working day.

<u>Microtox testing of amylase-treated CD extracts and solutions</u>
Non-pasteurized gamma-CD extracts of PHC-contaminated soil required pH adjustment for acidity after amylase treatment, and were more toxic than CD solutions with similar levels of PHC.
However, when the above pasteurization step was done, toxicity of amylase-treated soil extracts (Fig. 5) fell into line with results for CD solutions with similar PHC levels; extracts were neutral, requiring no pH-adjustment.



The arrows in Fig. 5 indicate amylase-treated gamma-CD solutions with 0, 50, 100 and 200 mg/L of PHC (as weathered diesel).

Similar EC50(15) values were obtained in replicate toxicity tests on all 14 test soils. There was little or no effect of an extra 4 months storage of soils 10-14 at room temperature.

All y-axis values < 1 are imprecise, because EC50(15) results (> 100 %) used to calculate them were obtained (as is necessary) by extrapolating to LLR = 1.

The actual relationship in Fig. 5 may be curvilinear; a straight line has been fitted for the purpose of demonstration. A Phase 2 objective will be to test more soils with higher PHC levels and toxicity.

Most of the donated soils gave CD extracts with < 20 mg/L of PHC and displayed little toxicity to *V. fischeri*. Soil # 7 (with 30 mg/L of PHC in the CD extract) was the most toxic and was selected for earthworm reproduction testing, along with soil # 2 (17 mg/L) for comparison.

The sensitivity of the test can be adjusted as necessary, to bring it into line with earthworm bioassay results, by using a different incubation temperature and/or enzyme:substrate ratio.

Earthworm testing

Survival:

Data from 14-day acute earthworm bioassays indicated that none of the donated soils (# 1-7) affected adult earthworm survival.

Soil 8, freshly spiked with diesel hydrocarbons, significantly affected adult survival after 28 days. An LC50 = 825 (644-1057) mg/kg PHC was obtained (untrimmed Spearman-Karber model; CETIS v.1.7.0).

Reproduction:

Soils 2 and 7, as well as diesel-spiked soil 8, were used for chronic earthworm testing.

No earthworm reproduction was observed in soil 8 at any PHC level (including zero PHC) at test end. Therefore critical PHC concentrations for reproduction could not be calculated. Soil 8 was unfortunately not an appropriate standard for this purpose.

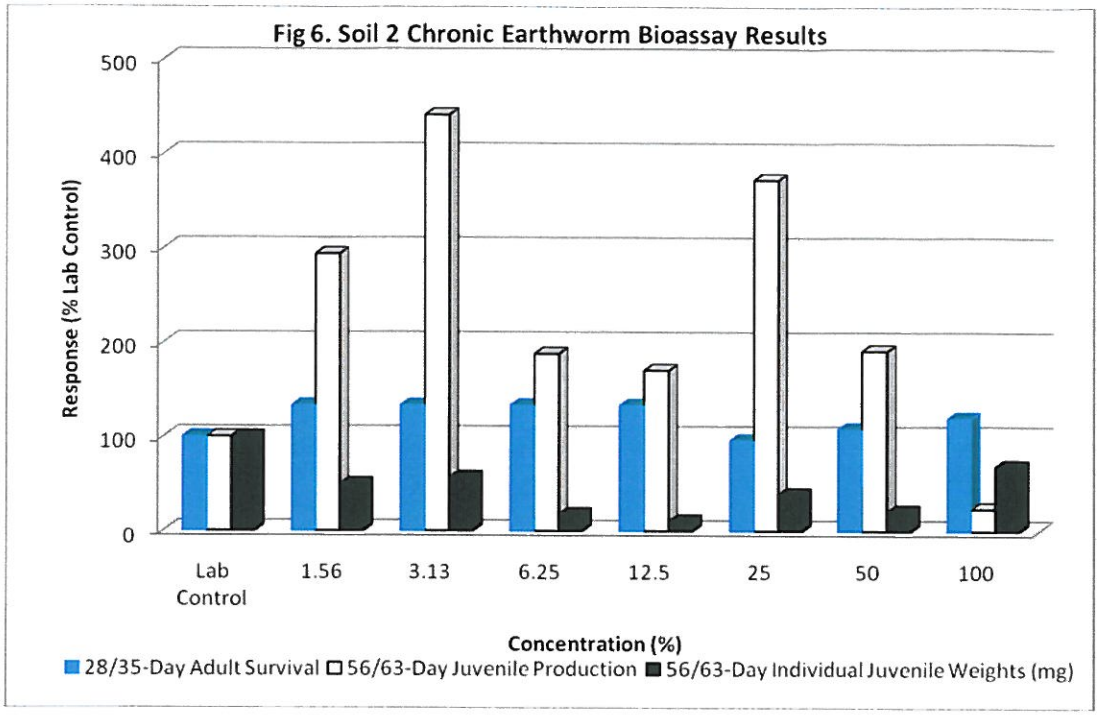
An unexpected research challenge was that texture and/or OM content of the donated soils was found to impact earthworm reproduction, such that no meaningful correlations between bio-available PHC levels and earthworm reproduction data could be made, due to this confounding factor.

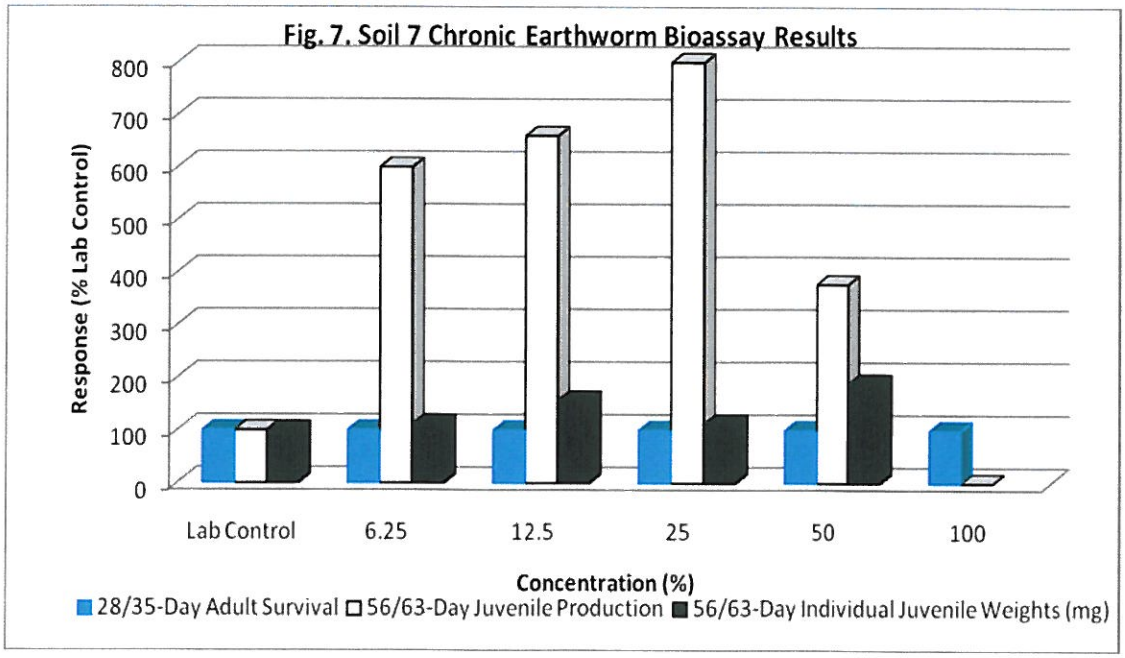
The 56-day chronic earthworm bioassay results for soils 2 and 7 are presented in Figs. 6 & 7, respectively. (These soils showed no effects on adult survival after 28-days, which was consistent with the corresponding 14-day acute test results.)

The x-axis in Figs. 6 & 7 indicates the relative proportion of test soil and the artificial soil with which it was mixed, expressed as a percentage.

The results observed displayed an unexpected response, with reproduction at low-end mix concentrations (6.25%-25%) being higher than in control artificial soil. Reproduction in 100 % undiluted test soil was less than in the control. This behaviour is considered a hormetic toxic dose response (stimulation at low concentrations, followed by inhibition at high test concentration.)

No significant trend was observed between individual juvenile weights and PHC concentrations in these chronic tests.





It is difficult to determine from these experiments whether the reproductive inhibition observed in the undiluted (100 %) soils 2 and 7 was due to soil quality or bio-available PHC. Further testing will be required to differentiate these effects. The higher reproduction observed for mixtures with artificial soil indicate that amending soils with peat (contained in

artificial soil) may help to differentiate soil-derived effects from PHC effects.
In order to determine critical PHC concentrations using a soil matrix appropriate for reproduction, a diesel-spike test using artificial soil will be conducted in Phase 2. An investigation into whether or not soils can be amended with peat to increase OM content and thus improve earthworm reproduction is also proposed. This should allow natural soils to be assessed un-amended and also amended for chronic earthworm bioassays, in order to eliminate confounding factors.

CONCLUSIONS

A rapid bioassay of oil-contaminated soil has been developed, involving soil extraction with aqueous gamma-cyclodextrin, extract pasteurization, treatment with amylase enzyme and overnight incubation, followed by a Microtox bioassay on day 2.
The resulting degree of toxicity using this test was well correlated with the concentration of PHC in gamma cyclodextrin extracts and solutions.
There was little or no toxicity of gamma CD extracts / solutions with PHC that had not been treated with amylase.
Beta-CD solutions with appreciable PHC remained non-toxic after treatment with amylase.

None of the soils donated to the project affected earthworm survival in acute 14-day bioassays.

In a chronic earthworm bioassay on an uncontaminated soil freshly spiked with diesel, no test concentrations (including the control) produced any juveniles, perhaps due to the low organic matter content of this soil.
Chronic earthworm tests were also done on two of the donated soils, at a range of dilutions with artificial soil. Reproductive effects were highly variable, and it was difficult to determine whether the inhibition observed in the undiluted soils was due to soil characteristics or to bio-available PHC.

A chronic earthworm bioassay using artificial soil spiked with increments of PHC will be done in future work, to help separate the effects of PHC on earthworm reproduction.
Further work will also be done to determine the most appropriate method to amend soils so that fine texture or low OM does not affect earthworm reproduction.

LITERATURE REFERENCES

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