SHORT COMMUNICATION

A rapid bioassay to screen soils for toxicity of residual petroleum hydrocarbons

John Ashworth and Ian Bullecer

ALS Environmental Group, 9936-67 Ave., Edmonton, Alberta, Canada T6E 0P5.
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Ashworth, J. and Bullecer, I. 2012. **A rapid bioassay to screen soils for toxicity of residual petroleum hydrocarbons**. Can. J. Soil. Sci. **92**: 901–904. The following 2-h procedure is proposed as a screen to indicate how soils contaminated with petroleum hydrocarbons (PHC) might perform in bioassays required for site remediation in Canada. Bio-available PHC in soil is extracted by aqueous cyclodextrin; a centrifuged extract portion is treated with amylase, then subjected to a standard Microtox® bioassay. Extract toxicity is correlated to available PHC content.

Key words: Cyclodextrin, amylase, Microtox, remediation, oil

Ashworth, J. et Bullecer, I. 2012. **Dosage rapide pour les sols pollués avec des résidus d'hydrocarbures**. Can. J. Soil. Sci. **92**: 901–904. La procédure de deux heures décrite dans cet article sert à déterminer la mesure dans laquelle les sols contaminés avec des hydrocarbures peuvent réagir avec les tests requis dans le cadre de la restauration des sites au Canada. On extrait les hydrocarbures biodisponibles du sol avec de la cyclodextrine aqueuse, puis traite une partie centrifugée de l'échantillon avec de l'amylase. On applique ensuite au produit résultant l'essai normalisé Microtox®. La toxicité de l'extrait est corrélée à la teneur en hydrocarbures disponibles.

Mots clés: Cyclodextrine, amylase, Microtox, restauration, pétrole

Decisions regarding remediation or disposal of petroleum hydrocarbon (PHC) contaminated soil are based on solvent-extractable PHC concentrations, which can exceed established Canadian guidelines [Canadian Council of Ministers of the Environment (CCME) 2008] even though residual PHC may become biologically unavailable with time and thereby non-toxic to biota (Reid et al. 2000; Axiom 2005). It is expensive and time-consuming to confirm toxicity to biota in such cases. Biological test methods specified by Environment Canada involve using species that represent major trophic levels. A full suite of tests (two plant species, one earthworm and one springtail) costs more than $10 000 and takes months.

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 Canadian Council of Ministers of the Environment thresholds might satisfy the eco-contact requirements of the full battery of terrestrial toxicity tests, thus justifying the expense of this further testing.

A milder analytical alternative to solvent extraction employs aqueous cyclodextrin (CD). Numerous on-line reference sources indicate that 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 would otherwise be relatively insoluble. Water solubility is further increased by adding hydroxypropyl substituent groups to the CD molecule.

Beta (β) and gamma (γ) forms of CD have seven and eight glucopyranose units per molecule, respectively, resulting in central cavities of differing size, and host-guest interactions of differing stability. The thermodynamics of CD-PHC inclusion complexes are beyond the scope of our empirical work, which is concerned with their toxicity in the Microtox bioassay (see below).

Recent reports suggest that CD solution extracts mainly bio-available PHC from oil-contaminated soil. Aqueous hydroxypropyl-β-CD was used to assess bioavailability of polyaromatic hydrocarbon 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 gas

Abbreviations: **CD**, cyclodextrin; **EC**, effective concentration; **LLR**, light loss ratio; **PHC**, petroleum hydrocarbon

chromatography 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. A detailed description of the version of the test used to regulate drilling waste disposal in western Canada is available on-line [Western Canada Microtox Users Committee (WCMUC) 1994].

The test uses the luminescent marine organism *Vibrio fischeri*, sample toxicity being assessed from light output measurements after mixing aliquots of these bacteria with a series of dilutions of the test fluid, in 2% NaCl. Fluid dilution reduces light loss; the effective concentration (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 Cyclodextrin Extracts

Cyclodextrin extracts of soils with as much as 1% PHC show little dependence of light output on extract concentration and have LLR < 1 (Ashworth and 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.

MATERIALS AND METHODS

Diesel fuel was employed as a convenient PHC source. Approximately 200 mL was placed in a porcelain dish under a strong air current entering a fume hood; after 48 h weight loss was < 0.2 g h^{-1}, allowing accurate weighing of sub-samples, with negligible volatile loss. The residue was kept at room temperature in a capped amber glass bottle. Gas chromatography analysis indicated that the fuel thus weathered 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 180 g kg^{-1} clay, 420 g kg^{-1} sand, organic carbon = 7 g kg^{-1} and pH = 7.5) was spiked with weathered diesel fuel at 20 000 mg kg^{-1}, and successively diluted with clean soil fourfold to obtain first 5 000 then 1250 and 312 mg kg^{-1}. One screw-cap glass jar (250 mL) of each spiked soil was stored at $4 \pm 2^{\circ}C$. Solvent extraction and gas chromatography-flame ionization detector analysis recovered 90–95% of the added spike, indicating that the target rates had been achieved.

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 100 mL^{-1}).

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 $4 \pm 2^{\circ}C$.

Analysis of a sample indicated that Fungamyl has 120 g NaCl L^{-1}. Before adding Fungamyl to aqueous CD, therefore, it was diluted (two parts by volume) with de-ionized water (one 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.

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 $3000 \times g$ and the clear supernatant (CD extract) transferred to a glass vial.

Enzyme-catalysed Hydrolysis and Microtox Testing

Cyclodextrin soil extracts thus obtained (4 mL) were treated with 0.4 mL of 2:1 Fungamyl-water mixture at room temperature ($21 \pm 2^{\circ}C$) and a Microtox test done within 1 h, using the four-serial dilutions increased sensitivity assay 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.

RESULTS AND DISCUSSION

Soil Extraction with Cyclodextrin

Judging from the difference between PHC in soil before and after γ-CD extraction, approximately 30% of each PHC spike was recovered by CD. Relative proportions of residual F2 and F3 remained in the range 55–60% and 40–45%, respectively.

Microtox Testing of Amylase-treated Cyclodextrin Extracts

In the absence of both PHC and amylase treatment, soil CD extracts were non-toxic in Microtox tests. Amylase-treated CD solution (with no added PHC) showed some initial light loss, but light levels largely recovered within 15 min. (all LLR values <1). However, marked increases in toxicity (LLR ≥ 2 at the highest test concentration) were obtained following amylase treatment of γ-CD extracts of soil with 1250 mg kg^{-1} PHC or more (Fig. 1). In contrast, β-CD extracts of soil with >1250 mg kg^{-1} PHC remained essentially non-toxic (LLR <1, data not shown) after amylase treatment, a finding consistent with the minimal hydrolysis of β-CD by amylase reported by Jodál et al (1984).

Toxicity Index Values

EC_{50} values are inversely related to toxicity, which is therefore often expressed as an index $= 100/EC_{50}$ (e.g., Ikehata and Nicell 2000). Toxicity index values calculated from $EC_{50}(15)$ data for amylase-treated soil extracts (Fig. 2) were linearly correlated with the known amount of PHC spike (mg kg^{-1}) added to the soil.

Factors Affecting Cyclodextrin-extract Toxicity

Incubating at 35°C is reportedly near-optimum for amylase (Jodál et al. 1984), but results of ruggedness tests (Youden and Steiner 1975) on our amylase treatment procedure suggested that the enzyme-catalyzed reaction goes quite rapidly to completion at lower temperatures. Allowing amylase-treated soil CD extracts to stand at room temperature for >1 h before running the Microtox test in fact slightly lessened toxicity; doing the amylase treatment at 5°C had little effect on results, as did using a stronger 3:1 amylase:water mixture (instead of 2:1).

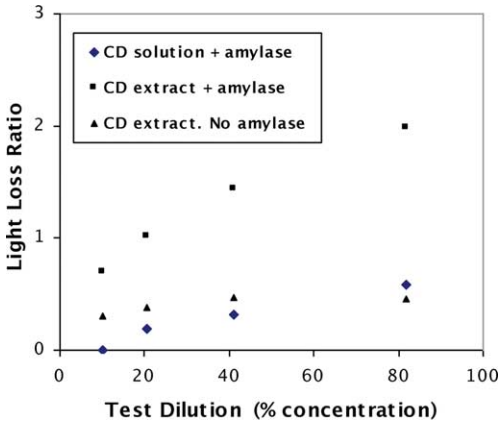

Fig. 1. Light loss in Microtox bioassays of pure γ-CD solution + amylase, and of γ-CD extracts of soil with 1250 mg kg^{-1} of PHC, with and without amylase.

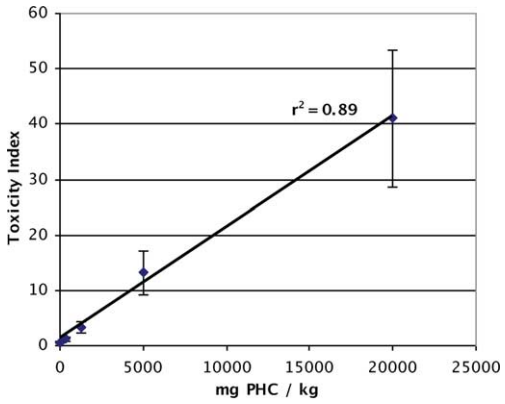

Fig. 2. Toxicity of amylase-treated γ-CD extracts of soil spiked with PHC.

Variance

The response of *V. fischeri* to toxicants varies a little from one batch of supplied freeze-dried bacteria to another; $EC_{50}(5)$ values for phenol reference standards are acceptable if they fall between 13 and 26 mg L^{-1} (WCMUC 1994), as was always the case in our tests.

In our method, sample heterogeneity can affect the amount of PHC recovered by aqueous CD from individual soil samples, and there could also be variance in amylase efficacy. We made and tested CD extracts of new sub-samples from the stored jars of control and PHC-spiked soils, taken on five occasions spread over 3 mo from the time of spiking. There was no trend in toxicity with time; the standard error in the toxicity index was typically 20% of the mean of the five results (error bars in Fig. 2).

CONCLUSIONS

A 2-h toxicity assessment technique for screening PHC-contaminated soil is proposed that avoids quantifying PHC, employing instead extraction with aqueous γ-CD, centrifugation, α-amylase treatment of the centrifugate, and finally a Microtox bioassay. The resulting index of toxicity was correlated with the concentration of PHC in the soil used in our tests. Relative proportions of F2, F3 and F4 hydrocarbons in contaminated field soils (and the presence of other CD-soluble compounds) could affect their toxicity to biota. Planned future work will use soils from various oil-contaminated sites, at which sequestering reactions may have had time to reduce the bio-availability of solvent-extractable PHC. Microtox bioassay results for amylase-treated γ-CD extracts will be compared with results of 56-d earthworm reproduction bioassays, in order to assess the correlation of the chronic toxicity of these soils to earthworms with the acute toxicity of the treated soil CD extracts to *V. fischeri*.

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