Monitoring bioremediation in creosote-contaminated soils using chemical analysis and toxicity tests

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Three soils with a history of creosote contamination (designated NB, TI and AC) were treated in bench-scale microcosms using conditions (nutrient amendment, moisture content and temperature) which had promoted mineralization of 14C-pyrene in a preliminary study. Bioremediation was monitored using the solid-phase Microtox test, seed germination and earthworm survival assays, SOS-chromotest, Toxi-chromotest and a red blood cell (RBC) haemolysis assay. Contaminant concentrations in the AC soil did not change after 150 days. Polycyclic aromatic hydrocarbon (PAH) concentrations decreased in the NB soil, and toxicity decreased overall according to the earthworm, seed germination and Microtox tests. Although total petroleum hydrocarbons (TPHs) in the TI soil were reduced following treatment, results of the earthworm, seed germination, RBC and Microtox tests suggested an initial increase in toxicity indicating that toxic intermediary metabolites may have formed during biodegradation. Toxicity testing results did not always correlate with contaminant concentrations, nor were the trends indicated by each test consistent for any one soil. Each test demonstrated a different capacity to detect reductions in soil contamination. Journal of Industrial Microbiology & Biotechnology (2000) 24, 132–139.

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Introduction

Creosote is a complex mixture of chemical compounds, primarily polycyclic aromatic hydrocarbons (PAHs), many of which are toxic and included on the list of US EPA priority pollutants [18]. Biodegradation can be an effective and inexpensive approach to remediating soils which contain PAHs and other hydrocarbon compounds, provided a population of microorganisms is present which can degrade them and the soil conditions are conducive to biodegradation of the contaminants. Microorganisms capable of degrading some PAHs found in creosote have been isolated [4,12,19,25]. Degradation of chemical contaminants in soil is affected by environmental factors (moisture, pH, temperature, O2 levels and contaminant bioavailability) or metabolic constraints such as bacterial nutrient requirements and acclimation of the population [25,28]. Some of these factors may be manipulated during a bioremediation program to enhance biodegradation rates.

Bioremediation is often monitored by following target contaminant concentrations, reductions of which are not always indicative of decreased soil toxicity [20,24,27]. Incomplete degradation and formation of toxic intermediary metabolites may result in increased soil toxicity during bioremediation [14,32,33]. A combination of chemical analysis, for target contaminant levels, and toxicity testing is recommended for monitoring the progress of bioremediation. Two tests for measuring soil toxicity are the seed germination and earthworm survival assays [11]. Other tests for water toxicity have been adapted for soil, such as the solid-phase Microtox test [5,30], SOS-chromotest [8,29] and Toxi-chromotest [21]. Different toxicity tests are expected to respond differently to individual toxicants. Furthermore, soil physical, chemical and biological parameters also affect toxicity to a particular test organism. A battery of toxicity tests may be useful to provide an overall assessment of the progress of bioremediation in contaminated soils [17,24].

We investigated the effects of nutrient amendments, temperature and soil moisture content on mineralization of 14C-pyrene in soils with a history of creosote contamination. Using the optimized amendment and incubation conditions for each soil, we then studied bioremediation in larger-scale microcosms and evaluated the ability of six soil toxicity tests to indicate the success of bioremediation, based on their correlation with data from chemical analysis for target compounds. Soil toxicity was measured using the following tests: the solid-phase Microtox test, seed germination and earthworm survival assays, the SOS-chromotest, Toxi-chromotest and a red blood cell (RBC) haemolysis assay.

Materials and methods

Soil preparation and analysis

Three soils with a history of contamination, from different wood-treatment facilities in Canada, were provided by GRACE Bioremediation Technologies (Mississauga, ON, Canada). These were designated by the initials AC, NB and TI, so as not to identify the sources. Each soil was passed through a 4.75-mm sized screen (number 4 mesh, USA Standard Testing Sieve), mixed thoroughly, and stored in sealed containers at 4°C in the dark. The moisture content...
and water-holding capacity (WHC) were determined for each soil by the method of Atlas and Bartha [2]. Soil texture, pH, and nutrient levels (P, N, K, Mg) were determined by the Analytical Services Laboratory of the Department of Land Resource Science, University of Guelph (Guelph, ON, Canada). The soils were analysed for total petroleum hydrocarbon (TPH) and polycyclic aromatic hydrocarbon (PAH) content by the Water Technology International (WTI) Corporation, Organic Chemistry Section (Burlington, ON, Canada) using standard methods.

$^{14}$C-Pyrene mineralization

The effects of nutrient amendments, temperature and moisture content on the ability of indigenous soil microflora to degrade a freshly-added PAH spike were measured by monitoring mineralization of $^{14}$C-pyrene in soil microcosms. Each microcosm consisted of a 250-ml glass Erlenmeyer flask containing the equivalent of 25 g dry soil. Controls contained air-dried soil and treated soils were hydrated to 50 or 85% of WHC using distilled water. Nutrient amendments (N and/or P) were added at concentrations of 1000 ppm each, as previously described [24]. Microcosms for each treatment were prepared in duplicate. Each microcosm was spiked with $1.3 \times 10^5$ dpm (4,5,9,10-$^{14}$C)-pyrene in 0.25 ml absolute ethanol (specific activity 32.3 mCi mmol$^{-1}$; Sigma Chemical Co, St Louis, MO, USA) applied dropwise via a syringe. The flasks were sealed with rubber stoppers for 10 min, and then were aerated by removing the rubber stopper for 10 min, twice weekly for those at 22°C and once weekly for those at 10°C (flasks at 22°C were expected to have a higher metabolic rate and, therefore, to require more O$_2$). Each microcosm was weighed bi-weekly and distilled water was added if necessary, to replace lost moisture. Some treatments were also prepared using soil which had been subjected to freezing ($-20^\circ$C, 1–2 days) and thawing ($22^\circ$C) before the addition of nutrient amendments and the tracer. The amount of evolved $^{14}$CO$_2$ in each microcosm was measured as described by Weir et al [34], using caustic (2 N NaOH) traps and a model LS6500 liquid scintillation counter (Beckman Instruments Inc, Fullerton, CA, USA) programmed for a 3-min counting time.

Soil toxicity and chemical analysis

Microcosms were prepared in 4-L glass jars containing the equivalent of 1500 g dry weight of soil. Four microcosm jars were prepared per soil. Nutrient amendments (nitrogen and/or phosphorus) were added to the soil at a concentration of 1000 ppm each, as previously described [24]. The nutrient amendments and incubation conditions for each soil were those which enhanced mineralization of $^{14}$C-pyrene the most, during the preliminary study: 22°C and 85% of WHC, with P, N and P+N amendments for the TI, AC and NB soils, respectively (see Results). Since the highest mineralization was observed in NB soil subjected to freezing and thawing, NB microcosms were frozen at –20°C for 24 h and thawed at 22°C for 24 h, prior to the start of the study. The microcosms were sealed, incubated in the dark and aerated weekly for 20 min by removal of the lids. One microcosm of each soil was removed from incubation per sampling event and the soil was stored at 4°C in sealed plastic bags prior to toxicity testing. Sampling was done on day zero, at the time corresponding to 10% mineralization in the previous study, the time corresponding to maximum mineralization and one sampling time midway between 10% and maximum mineralization. These sampling times were different for each soil: NB, days 25, 35 and 60; AC, days 36, 57 and 150; TI, days 60, 68 and 125. Individual composite samples, consisting of soil taken from three different locations within each microcosm jar were submitted to the Water Technology International (WTI) Corporation, Organic Chemistry Section (Burlington, ON, Canada) for TPH and PAH analysis using standard methods.

The 14-day earthworm survival assay was performed using *Eisenia fetida* obtained from the Salmon River Worm Farm (Shannonville, ON, Canada) and the method described by Greene et al [11]. Briefly, soils with a pH greater than 8 were adjusted to a value between 5 and 8 (as dictated in the protocol), by dropwise addition of 1 N HCl. The test soil was mixed with artificial soil to create a range of test soil concentrations where 50% mortality was expected to occur. The assay was performed in triplicate, in standard US quart-size mason jars. Ten worms, 0.3–0.5 g each, were added to each jar. Mortality was recorded at 7 and 14 days. Earthworm mortality LC$_{50}$ values and 95% confidence intervals were determined using the Trimmed Spearman–Karber method [16].

The seed germination assay, Toxi-chromotest, SOS-chromotest, solid-phase Microtox and RBC lysis assays were performed as described previously [24] and are outlined briefly below. The lettuce seed germination assay was performed in triplicate using *Lactuca sativa* var Paris Is. 318 MI (Stokes Seeds, St Catherines, ON, Canada). After 5 days incubation, replicates were examined for the total number of emerged lettuce seedlings (at or above soil surface), germinated seeds (beneath soil surface) and the root lengths of emerged seedlings. Probit analysis was used to determine LC$_{50}$ and EC$_{50}$ values for germination and emergence, respectively. Differences in mean root lengths, on each plate where >25% seedling emergence was measured, were evaluated for statistical significance ($P < 0.05$), by Dunnett’s analysis using Sigmmaplot software (SPSS, Inc, Chicago, IL, USA).

The Toxi-Chromotest was performed using the Toxi-Chromotest Analytical Toxicity Test Kit, version 3.1 (Environmental Biodetection Products Inc (EBPI), Brampton, ON, Canada). Positive controls consisted of serial dilutions of kanamycin (100 μg ml$^{-1}$) in the reaction mixture and the negative control was reaction mixture incubated in the absence of contaminants. An EC$_{100}$ value was obtained by determining the highest soil concentration that completely inhibited colour development. To detect mutagenicity, we employed a modified version of the SOS Chromotest as previously described [24]. The highest soil concentration tested was 50 mg soil per 0.5 ml bacterial suspension, prepared using commercially available buffer and lyophilized cells (EBPI). Genotoxicity and cytotoxicity values were calculated as in Dutka et al [8]. A modified solid-phase Microtox test [21] used *Photobacterium phosphoreum* to monitor acute toxicity of soil samples. EC$_{50}$ data were determined using Microtox Data Capture and