Journal of Industrial Microbiology & Biotechnology (2000) 24, 132–139

www.nature.com/jim

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

TM Phillips[1], D Liu[2], AG Seech[3], H Lee[1] and JT Trevors[1]

[1]*Department of Environmental Biology, University of Guelph, Guelph, ON, Canada N1G 2W1;* [2]*Canada Centre for Inland Waters, Burlington, ON, Canada L7R 4A6;* [3]*GRACE Bioremediation Technologies, Mississauga, ON, Canada L5C 4P9*

**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 $^{14}$C-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.

**Keywords:** bioremediation; creosote; microbiology; soil contamination; soil toxicity

## 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, $O_2$ 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 $^{14}$C-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

Correspondence: JT Trevors or H Lee, Department of Environmental Biology, University of Guelph, Guelph, ON, Canada N1G 2W1. E-mail: jtrevors@uoguelph.ca or hlee@uoguelph.ca
Received 14 June 1999; accepted 12 November 1999

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 and incubated at 10°C or 22°C in the dark. Flasks 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°C, 1–2 days) and thawing (22°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 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 foetida* 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 *Latuca 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 ($P \leq 0.05$), by Dunnett's analysis using Sigmaplot software (SPSS, Inc, Chicago, IL, USA).

The Toxi-Chromotest was performed using the Toxi-Chromopad™ Analytical Toxicity Test Kit, version 3.1 (Environmental Biodetection Products Inc (EBPI), Brampton, ON, Canada). Positive controls consisted of serial dilutions of kanamycin (100 $\mu$g 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

Reporting Software (version 7.8, Microbics Corp, Carlsbad, CA, USA).

A previously outlined [24] adaptation of the red blood cell (RBC) haemolysis assay developed by Negrete *et al* [26] was used to measure shifts in RBC lysis curves in the presence of soil extracts. Cell lysate absorbance at 540 nm was measured with a BIO-RAD Model 3550-UV Microplate reader. $EC_{50}$ values (the concentration of Simmel's solution at which 50% RBC lysis occurred) and 95% confidence intervals were determined using Probit analysis. The shift in $EC_{50}$ for each soil extract was calculated by subtracting the control $EC_{50}$ from that of the sample.

## Results and discussion

### *Soil analysis*

Soil pH, texture, organic carbon and mineral contents are shown in Table 1. NB soil, a gravelly sandy loam, contained the highest total phosphorus (P) at 7 ppm. TI soil, a loamy sand, contained the highest total nitrogen (N) at 4.34 ppm. The AC and NB soils had organic carbon concentrations of 6.92 and 5.31%, respectively, two to three times higher than in TI soil (2.45% each). Soil pH values (6.5–8.1) were within a reasonable range for microbial activity in all three soils.

### *$^{14}$C-Pyrene mineralization*

We performed preliminary mineralization experiments to find treatment conditions that enhanced hydrocarbon degradation and which could be applied to the soil toxicity study. The $CO_2$ recovery curves for the best treatment of each soil are shown in Figure 1. The rate of $^{14}$C-pyrene mineralization differed for each of the three soils; however, mineralization always proceeded more rapidly at 22°C than at 10°C, and at 85% WHC, as opposed to 50%. It was expected that degradation would increase with increasing temperature between 10°C and 30°C in soil contaminated with petroleum hydrocarbons [6]. Both temperature and moisture content have been inversely related to sorption of contaminants in soil [28]. Decreased sorption may increase the bioavailability of contaminants and, therefore, the degradation rates, as temperature or moisture content increase. It was not surprising therefore, that the $^{14}$C-pyrene was mineralized more quickly at 22°C and 85% of WHC than at 10°C and 50% of WHC.

The nutrient amendment conditions that enhanced $^{14}$C-pyrene mineralization were different for each soil. In the TI soil, mineralization was observed in only one treatment, 22°C, 85% of WHC with P amendment (Figure 1). Mineralization was not observed in the unamended control microcosm (data not shown); however, mineralization in microcosms amended with P indicated the presence of viable PAH-degrading microorganisms.

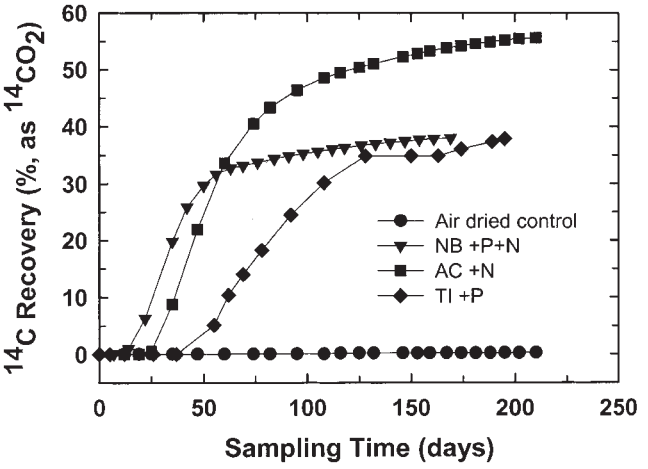


**Figure 1** Mineralization of $^{14}$C-pyrene in each of the historically contaminated soils, at the following amendment and incubation conditions: NB soil +P+N freeze/thawed, AC soil +N and TI soil +P, all incubated at 22°C, 85% of WHC. Each data point represents the average mineralization in two microcosms. Error bars representing SD are too small to detect. Air-dried control soil data are shown for the AC soil.

In the NB soil, mineralization was observed at all temperatures and moisture contents; however, mineralization at 10°C was slow (10% mineralization was not achieved until after 100 days). At 22°C, amendment with N and P together enhanced mineralization and the soil that had been previously frozen performed slightly better than the unfrozen soil (Figure 1). Amendment with only N inhibited mineralization and addition of P, alone, had no effect.

In the AC soil, at 22°C and 85% of WHC, amendment with N alone or N and P together enhanced mineralization resulting in 53% and 46% recoveries, respectively, compared to 13% and 18% recoveries for unamended or P treatments, respectively.

The responses of the TI, NB and AC soils to nutrient amendment were not predictable based on soil mineral nutrient levels. Furthermore, although amendment with P generally enhances hydrocarbon degradation in soil and N amendment typically has no effect or adverse effects [10,23], we observed exceptions to this pattern, confirming that the biodegradation of soil contaminants may also be affected by other, undefined parameters [13].

Between 0–25% of the initial radiolabel was recovered as $^{14}CO_2$ after 150 days of incubation in the unamended control microcosms of each soil. Higher percent recoveries, over 30% by day 60, were obtained from amended NB and AC soils. These results are comparable to literature values [13]. Half-lives of four-ring PAHs in laboratory soil microcosms have been estimated at >200 days [37]. With optimum nutrient amendment and incubation temperatures, we observed shorter half-lives for the $^{14}$C-pyrene spike in our microcosms, yet we did not observe complete mineralization of the tracer to $^{14}CO_2$. Mineralization may have

**Table 1** Mineral nutrient levels, soil texture, and pH in the NB, AC, and TI soils

| Soil | Soil texture (% sand/silt/clay) | pH | N (ppm)[a] | P (ppm) | K (ppm) | Mg (ppm) | organic C (%) |
|---|---|---|---|---|---|---|---|
| NB | gravelly sandy loam (75/16/9) | 6.5 | 4.10 | 7 | 89.9 | 64 | 5.31 |
| AC | sandy loam (61/24/15) | 7.5 | 2.11 | 6 | 129.8 | 335 | 6.92 |
| TI | loamy sand (76/21/3) | 8.1 | 4.34 | 4 | 25.8 | 47 | 2.45 |

[a]Total N measured as $NH_4^+$ and $NO_3^-$.

slowed due to sorption and reduced bioavailability of remaining $^{14}$C-pyrene. Herbes [15] reported that during mineralization of high molecular weight PAHs in previously contaminated sediments, bound $^{14}$C generally exceeded the amount evolved as $^{14}CO_2$. Alternatively, products of pyrene degradation containing the labelled carbon atom might have been incorporated into the cellular biomass instead of being mineralized to $^{14}CO_2$.

### *Soil toxicity during bioremediation*

Soil TPH and PAH concentrations were measured before, during and after bioremediation in the 4-L microcosm jars (Figure 2). TPH concentrations in the TI soil decreased at each consecutive sampling, from 17 400 to 11 800 $\mu g\ g^{-1}$ after 125 days, but did not appear to change overall in the AC and NB soils. PAH concentrations did not change overall in the AC or TI soils, but were reduced from 42 to 25 $\mu g\ g^{-1}$ in the NB soil, after 60 days of bioremediation.

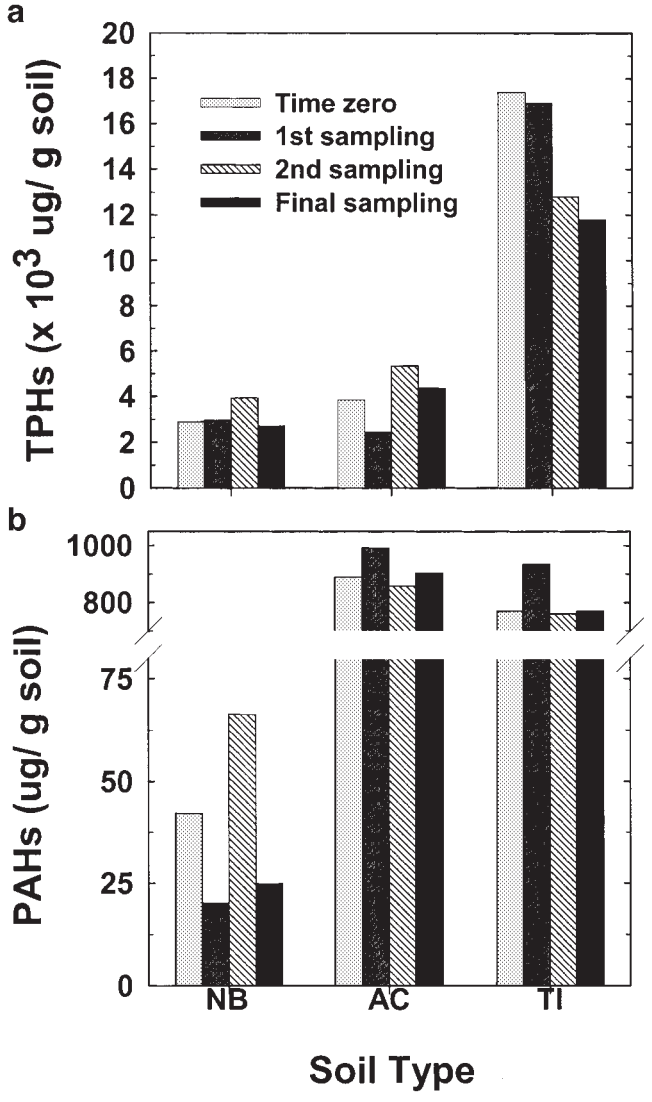


**Figure 2** (a) Total petroleum hydrocarbon (TPH) and (b) polycyclic aromatic hydrocarbon (PAH) concentrations in the NB, AC and TI soils during bioremediation in microcosms. Each data point represents the analysis of a single composite sample.

Increased contaminant levels were measured in some soil samples, particularly NB and AC soils at the second sampling time. These apparent increases in PAH concentrations are likely attributable to hot spots (small area of high concentration of pollutants) in the soil, and the fact that only single samples from each microcosm were analysed.

We evaluated the ability of six toxicity tests to detect changes in soil toxicity due to bioremediation. Toxicity test data could not always be correlated directly to changes in PAH or TPH concentrations, and a number of complications arose from using solid soil as the test matrix. Each test is discussed below and evaluated for its usefulness in the context of this study. Results are shown only for tests where changes in toxicity were observed.

Using the SOS-chromotest, genotoxicity and cytotoxicity were not detected in the soils relative to clean reference soils of similar texture (data not shown). Our previous work suggests that the solid-phase SOS-chromotest is unreliable for detecting changes in hydrocarbon concentrations [20,24]. Results from the Toxi-chromotest indicated no overall change in toxicity in any of the soils as a result of treatment (data not shown). Variations in $EC_{100}$ and NOEC values for the TI soil were observed, but the trends indicated by each type of observation contradicted one another, and the differences were only one dilution apart. Thus, we suspect that the variation was due to the subjective nature of the test (visual determination of colour intensity).

The earthworm survival assay may have been sensitive to changes in TPH concentrations in the TI soil as the toxicity decreased. This was demonstrated by an increase in the $LC_{50}$ of approximately 6% (Figure 3). An initial increase in toxicity was detected by this and other tests with the TI soil (see below) and may have been due to formation of intermediary metabolites of hydrocarbon degradation more toxic than parent compounds. Earthworm survival was 100% in the 50% diluted NB soil (50% artificial soil) at

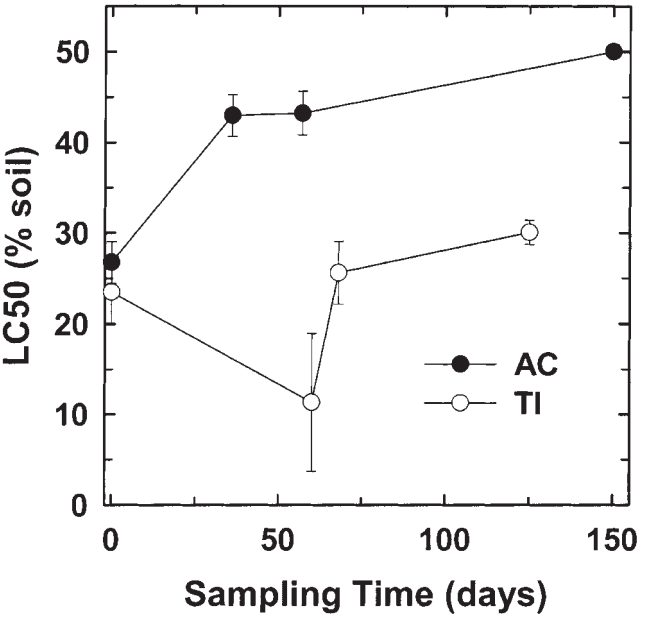


**Figure 3** Earthworm survival $LC_{50}$ values for the AC and TI soils during bioremediation in microcosms. Error bars represent 95% confidence intervals obtained using the Trimmed Spearman–Karber method ($\alpha = 10$).

time zero. Soil moisture content was too high to apply the test at higher concentrations, since the protocol for the test specifies soil moisture content be set at 75% of WHC, and the bioremediation microcosms were hydrated to 85% of WHC. PAH concentrations in the NB soil were less than 100 ppm and may have been too low to have an acute toxic effect on the earthworms. Sediment creosote levels below 550 ppm have low toxicity to the species *Eisenia foetida* [1]. Earthworm survival data for the AC soil suggested that toxicity decreased after 36 days of treatment. This trend continued up to 150 days but cannot be explained based on PAH or TPH concentrations.

Seed germination ($LC_{50}$) and emergence ($EC_{50}$) data are presented in Figure 4a and b respectively. $LC_{50}$ values for the TI soil indicated increased soil toxicity as a result of bioremediation. These data corroborate the earthworm data for early sampling times, and suggest the presence of toxic metabolites. In the NB soil, $LC_{50}$ and $EC_{50}$ values increased from 13% and 10%, to 20% and 19%, respectively. These data suggest that lettuce seed and seedling toxicity were reduced by decreasing PAH concentrations. Although contaminant concentrations did not change in the AC soil, changes in toxicity to lettuce were observed. Variation between replicates and from one sampling time to the next may have resulted from the texture of this clay soil. The soil did not mix well with the seed germination test sand, likely resulting in hot spots within each petri dish, and inconsistent results.

Average seedling root lengths for soil concentrations in which >25% emergence was measured are presented in Figure 5. Data are not shown for the TI soil due to insufficient emergence at the concentrations tested. Differences in mean root lengths were evaluated for statistical significance ($P \leq 0.05$), by Dunnett's analysis, using Sigmaplot software (SPSS Inc). Mean root lengths in the control sand were significantly different from one day to the next, therefore we did not compare root lengths between days at any soil concentration.

In the NB soil, root lengths were significantly longer in 5% soil than in the control sand alone (0% test soil). The lengths then decreased as soil concentration increased. Root growth may have been greater as a result of soil nutrients and/or other factors, introduced when the test soil was mixed with the control sand. However, as the soil concentration increased, toxic effects from contaminants could be observed. This trend appeared not to have changed at later sampling times, indicating no change in toxicity to roots as a result of PAH degradation.

Significant ($P < 0.001$) differences in root length were observed between different concentrations of AC soil at each sampling event. In this soil, root lengths were higher (20–27 mm) in the control sand than in the lowest soil concentrations tested (2.5%; 2–6 mm). At day zero, root lengths continued to decrease as soil concentration increased. At later sampling times, root lengths in 5, 10 and higher percent soil were all longer than in 2.5% soil. The positive effect of soil nutrients on root growth might have outweighed the inhibitory effects of contaminants at lower test soil concentrations. This effect grew stronger over time, suggesting that soil toxicity may have decreased over time.

Solid-phase Microtox $EC_{50}$ values for the three soils are presented in Figure 6. The data for the NB soil indicated no change from day 0 to day 60 but a slight increase in toxicity on days 25 and 35. This trend may be due to the formation of toxic metabolites of PAH degradation or hotspots in the soil where contaminant concentrations were higher. The latter is probable considering the small amount of soil (1 g) and serial dilution methodology that are used in preparing the bioassay tubes. $EC_{50}$ values for the TI soil at days zero and 60 were 0.23 and 0.15% soil, respectively (Figure 6). The apparent increase in toxicity corroborates earthworm and seed germination data. Toxicity did not change from days 60 to 125, although there were further reductions in TPHs in the TI soil. In the AC soil, $EC_{50}$ values ranged from 0.2% to 0.4% soil, with no obvious

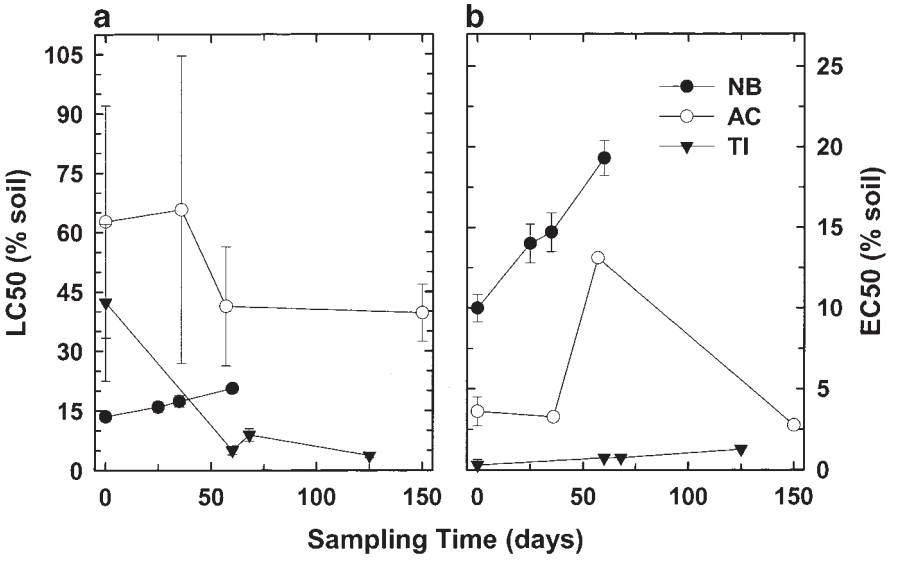


**Figure 4** Seed germination $LC_{50}$ (a) and seedling emergence $EC_{50}$ (b) values for the NB, AC and TI soils during bioremediation in microcosms. Each data point represents the value obtained using probit analysis of total seedlings germinated or emerged on triplicate petri dishes. Error bars represent 95% confidence intervals.

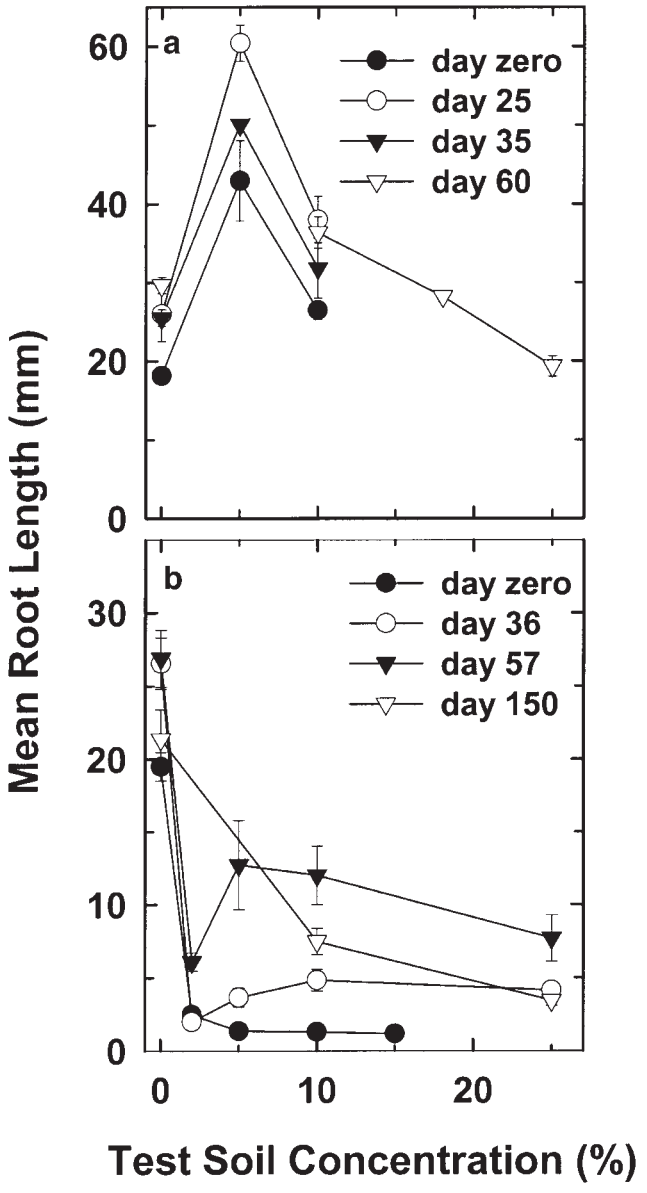


**Figure 5** Mean root lengths (±SD) for the NB (a) and AC (b) soils during bioremediation in microcosms. Each data point represents the average root length measured on triplicate petri plates where greater than 25% of seedlings emerged.

trend. Again, data fluctuations for this soil might be caused by the texture of the soil, which was difficult to mix effectively.

Earthworm survival, Microtox and seed germination tests are sensitive to changes in soil toxicity during bioremediation of PAH and petroleum hydrocarbon-contaminated soils [1,7,20,24]. We found that the NB soil, which was not toxic in the earthworm test, could also be considered initially non-toxic according to the solid-phase Microtox test according to criteria suggested by Kwan and Dutka [21] who reported that an $EC_{50}$ value $\geq 2$ is indicative of a non-toxic or 'clean' soil. The three assays were in agreement in suggesting that toxicity had initially increased in the TI soil. We attribute the inconsistencies in the AC soil data to soil texture.

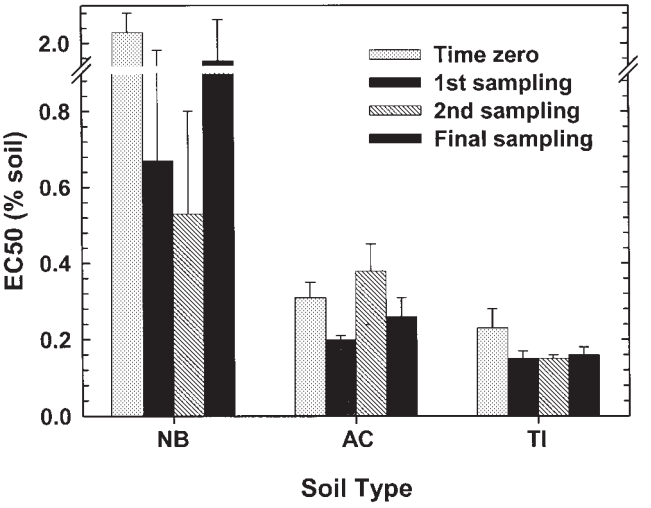


**Figure 6** Solid-phase Microtox $EC_{50}$ values for the NB, AC and TI soils during bioremediation in microcosms. Each data point represents the $EC_{50}$ of a single analysis obtained using Microtox Data Capture and Reporting software. Error bars represent 95% confidence intervals.

The amount of error in the solid-phase Microtox results (measured as 95% confidence) increased when $EC_{50}$ values rose above 0.4% soil. The precision of the solid-phase Microtox test is lower at high concentrations of test soil [5,20,24] and the efficiency of serial dilutions may be affected by the number, size and variability in soil particles [3]. Cook and Wells [5] found that $EC_{50}$ values decreased in a test soil with particle sizes between 20–40 $\mu$M, high organic carbon, high N and high bacterial numbers. Bacterial cell counts showed a correlation between Microtox test bacteria in the aqueous suspension and soil silt-clay concentration. It is believed that bacteria adsorb to the silt particles and are filtered out of suspension, resulting in lower luminescence than would correctly represent the level of soil toxicity [3,30]. Apparent changes in toxicity may also be caused by other unknown reactions between the test bacteria and natural soil components [5,17].

The aqueous Microtox test, when applied to soil extracts, corroborates analytically determined reductions in soil PAH concentrations [31,33]. However, this test appears to be sensitive to other factors in the soil [1]. Without comparison to an uncontaminated soil of identical texture, it is difficult to determine the extent to which toxicity in the contaminated soil was reduced, using solid-phase Microtox test results. Users must decide if the benefits of testing solid soil outweigh the complications presented by interference of solid particles [31].

The results of the RBC lysis assay are shown in Figure 7. A negative shift in $EC_{50}$ indicates that blood cells exposed to test soils were protected from lysis at lower osmotic strengths than control cells. $EC_{50}$ values shifted in the negative direction in the presence of extracts from all three soils, but less for AC soil extract than for TI and NB. The negative shift was interpreted as increased toxicity [20,24]. We suggest that the increased negative shift may have been caused by increased bioavailability of contaminants either due to the surfactant effects of added P amendment, or higher water solubility of intermediary metabolites of biodegradation.

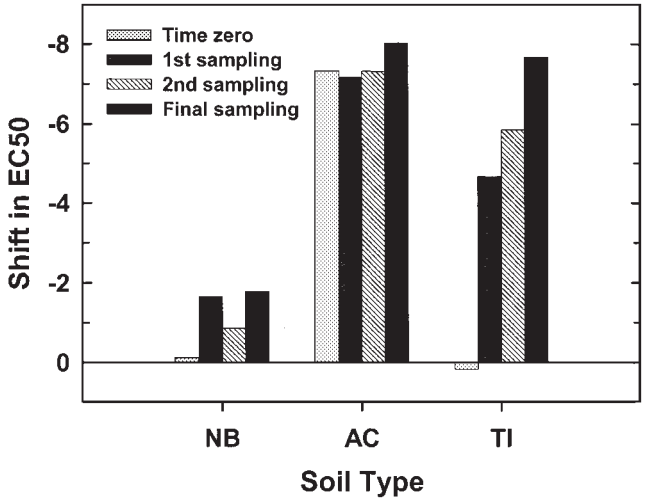


**Figure 7** Red blood cell lysis curve $EC_{50}$ shifts for the NB, AC and TI soils during bioremediation in microcosms. Each data point represents the $EC_{50}$ shift calculated by subtracting the average of triplicate control $EC_{50}$ values from the average of triplicate sample $EC_{50}$ values. $EC_{50}$ values were obtained by Probit analysis.

The toxicity test data obtained in this study did not indicate that bioremediation had successfully reduced soil toxicity, although the treatment conditions were chosen based on successful mineralization of a $^{14}$C-pyrene tracer in previous experiments. Erickson *et al* [9] studied PAH degradation in manufactured gas plant soils and observed that several compounds, including pyrene, naphthalene and phenanthrene, were not degraded under a number of conditions of temperature, moisture and nutrients even when inoculated with bacterial cultures known to degrade PAHs. Freshly added naphthalene and phenanthrene, however, were quickly degraded. This and other reports suggest that the bioavailability, degradation and toxicity of soil contaminants are all influenced by sorption, which is influenced by time and the physico-chemical properties of individual soils [9,22,23,35,36]. A measure of total soil PAHs during the mineralization study, to determine whether degradation of the aged contaminants had occurred concurrently with mineralization of the added radiolabel, would have given a better indication of the effects of different treatments. Furthermore, if the scaled-up toxicity portion of our experiment had been prolonged to a point where contaminant levels were even further reduced, we might have seen a more consistent trend of reduced toxicity for each soil.

We have studied this battery of toxicity tests in different soils contaminated with PAHs, petroleum hydrocarbons and chlorophenols, undergoing bioremediation treatment in a number of different environments [20,24,27]. No obvious trends have emerged from these studies to suggest that one test is more reliable or sensitive to changes in a particular contaminant, without interference by other factors such as soil nutrient levels or composition. Although there are advantages to testing solid soil, each soil is unique in the response it induces, and each toxicity test unique in its ability to detect different contaminant levels in different soils. Therefore it appears that, although soil toxicity tests can be used to monitor bioremediation, chemical and toxicity data do not always corroborate one another, nor do the results of each toxicity test in a battery always agree. This study stresses the importance of using a battery of toxicity tests, in parallel with chemical analysis, when assessing the progress of a bioremediation program.

## Acknowledgements

This research was supported by Environment Canada, GRACE Bioremediation Technologies and the University Research Incentive Fund (URIF) administered by the Ontario Ministry of Universities and Colleges. Research by JT and HL are also supported by NSERC operating grants.

## References

1 Athey LA, JM Thomas, WE Miller and JQ Word. 1989. Evaluation of bioassays for designing sediment cleanup strategies at a wood treatment site. Environ Toxicol Chem 8: 223–230.
2 Atlas RM and R Bartha (eds). 1981. Microbial Ecology: Fundamentals and Applications. Addison Wesley Publishing, London.
3 Benton MJ, ML Malott, SS Knight, CM Cooper and WH Benson. 1995. Influence of sediment composition on apparent toxicity in a solid-phase test using bioluminescent bacteria. Environ Toxicol Chem 14: 411–414.
4 Cerniglia CE. 1992. Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation 3: 351–368.
5 Cook NH and PG Wells. 1996. Toxicity of Halifax Harbour sediments: an evaluation of the Microtox solid-phase test. Water Qual Res J Canada 31: 673–708.
6 Coover MP and RC Sims. 1987. The effect of temperature on polycyclic aromatic hydrocarbon persistence in an unacclimated agricultural soil. Haz Waste Haz Mat 4: 69–82.
7 Dorn PB, TE Vipond, JP Salanitro and HL Wisniewski. 1998. Assessment of the acute toxicity of crude oils in soils using earthworms, Microtox, and plants. Chemosphere 37: 845–860.
8 Dutka BJ, K Teichgraber and R Lifshitz. 1995. A modified SOS-Chromotest procedure to test for genotoxicity and cytotoxicity in sediments directly without extraction. NWRI Contribution No. 95–53.
9 Erickson DC, RC Loehr and EF Neuhauser. 1993. PAH loss during bioremediation of manufactured gas plant site soils. Wat Res 27: 911–919.
10 Graham DW, VH Smith and KP Law. 1995. Application of variable nutrient supplies to optimize hydrocarbon biodegradation. In: Bioremediation of Recalcitrant Organics (RE Hinchee, DB Anderson, and RE Hoeppel, eds), Battelle Press, Columbus, Ohio.
11 Greene JC, CL Bartels, WJ Warren-Hicks, BR Parkhurst, GL Linder, SA Peterson and WE Miller. 1988. Protocols for short term toxicity screening of hazardous waste sites. US Environmental Protection Agency Report No. 600/3–88/029.
12 Grosser RJ, D Warshawsky and JR Vestal. 1991. Indigenous and enhanced mineralization of pyrene, benzo[a]pyrene, and carbazole in soils. Appl Environ Microbiol 57: 3462–3469.
13 Grosser RJ, D Warshawsky and JR Vestal. 1995. Mineralization of polycyclic and *N*-heterocyclic aromatic compounds in hydrocarbon-contaminated soils. Environ Toxicol Chem 14: 375–382.
14 Heitzer A and GS Sayler. 1993. Monitoring the efficacy of bioremediation. Trends Biotechnol 11: 334–343.
15 Herbes SE. 1981. Rates of microbial transformation of polycyclic aromatic hydrocarbons in water and sediments in the vicinity of a coal-coking wastewater discharge. Appl Environ Microbiol 41: 20–28.
16 Hubert JJ. 1992. Bioassay, 3rd edn. Kendall/Hunt Publishing Co, Dubuque, IA.
17 Hund K and W Traunspurger. 1994. Ecotox-evaluation strategy for soil bioremediation exemplified for a PAH-contaminated site. Chemosphere 29: 371–390.
18 Keith LH and WA Telliard. 1979. Priority pollutants I—a perspective view. Environ Sci Technol 13: 416–423.
19 Kiyohara H, N Takizawa and K Nagao. 1992. Natural distribution of bacteria metabolizing many kinds of polycyclic aromatic hydrocarbons. J Ferment Bioeng 74: 49–51.
20 Knoke KL, TM Marwood, MB Cassidy, D Liu, AG Seech, H Lee and

JT Trevors. 1999. A comparison of five bioassays to monitor toxicity during bioremediation of pentachlorophenol-contaminated soil. Water Air Soil Pollut 110: 157–159.
21 Kwan KK and BJ Dutka. 1992. Evaluation of Toxi-chromotest direct sediment toxicity testing procedure and Microtox solid-phase testing procedure. Bull Environ Contam Toxicol 49: 656–662.
22 Loehr RC and MT Webster. 1996. Behavior of fresh *vs* aged chemicals in soil. J Soil Contam 5: 361–383.
23 Manilal VB and M Alexander. 1991. Factors affecting the microbial degradation of phenanthrene in soil. Appl Microbiol Biotechnol 35: 401–405.
24 Marwood TM, K Knoke, K Yau, A Suchorski-Tremblay, CA Flemming, V Hodge, D Liu, AG Seech, H Lee and JT Trevors. 1998. Comparison of toxicity detected by five bioassays during bioremediation of diesel-spiked soils. Environ Toxicol Water Quality 13: 117–126.
25 Mueller JG, PJ Chapman and PH Pritchard. 1989. Creosote-contaminated sites. Eng Sci Technol 23: 1197–1201.
26 Negrete JC, EA Hosein, H Lee and U Goehlert. 1985. The haemolysis resistance test (HRT) in chronic excessive drinkers: a pilot study. Brit J Addic 80: 77–82.
27 Phillips TM, D Liu, AG Seech, H Lee and JT Trevors. 1999. Bioremediation in field box plots of a soil contaminated with wood-preservatives: a comparison of treatment conditions using toxicity testing as a monitoring technique. Water Air Soil Pollut (in press).
28 Providenti MA, H Lee and JT Trevors. 1993. Selected factors limiting the microbial degradation of recalcitrant compounds. J Ind Microbiol 12: 379–395.
29 Quillardet P and M Hofnung. 1993. The SOS chromotest: a review. Mutat Res 297: 235–279.
30 Ringwood AH, ME DeLorenzo, PE Ross and AF Holland. 1997. Interpretation of Microtox solid-phase toxicity tests: the effects of sediment composition. Environ Toxicol Chem 16: 1135–1140.
31 Tay KL, KG Doe, SJ Wade, DA Vaughan, RE Berrigan and MJ Moore. 1992. Sediment bioassessment in Halifax harbour. Environ Toxicol Chem 11: 1567–1581.
32 Wang X and R Bartha. 1990. Effects of bioremediation on residues, activity and toxicity in soil contaminated by fuel spills. Soil Biol Biochem 22: 501–505.
33 Wang X, X Yu and R Bartha. 1990. Effect of bioremediation on polycyclic aromatic hydrocarbon residues in soil. Eng Sci Technol 24: 1086–1089.
34 Weir SC, SP Dupuis, MA Providenti, H Lee and JT Trevors. 1995. Nutrient-enhanced survival of and phenanthrene mineralization by alginate-encapsulated and free *Pseudomonas* sp UG14Lr cells in creosote-contaminated soil slurries. Appl Microbiol Biotechnol 43: 946–951.
35 Weissenfels WD, H-J Klewer and J Langhoff. 1992. Adsorption of polycyclic aromatic hydrocarbons (PAHs) by soil particles: influence on biodegradability and biotoxicity. Appl Microbiol Biotechnol 36: 689–696.
36 White JC and M Alexander. 1996. Reduced biodegradability of desorption-resistant fractions of polycyclic aromatic hydrocarbons in soil and aquifer solids. Environ Toxicol Chem 15: 1973–1978.
37 Wilson SC and KC Jones. 1993. Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs): a review. Environ Pollut 81: 229–249.