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Enhanced degradation of bioremediation residues in petroleum-contaminated soil using a two-liquid-phase bioslurry reactor

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ABSTRACT

A study was performed to determine the potential of two-liquid-phase (TLP) bioslurry reactors using silicon oil as solvent for degradation of residual contaminants in petroleum-contaminated soil. The residues were characterized by gas chromatography–mass spectrometry and electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry. This allowed for the identification of a mixture of residual biomarkers, metabolic byproducts, oxygenated and hetero-polynuclear aromatic hydrocarbons in the contaminated soil. The removal amount of total extractable organics (TEO) was 15 900 mg kg^{-1} soil in the TLP reactor within 12 weeks. However, TEO remained intact in the bioslurry reactor without the addition of silicon oil for the duration of the experiment, due to high toxicity of metabolites to the microorganisms. The availability of TEO was calculated using a mild extraction with Triton X-100, and the amount of TEO extracted was in accord with the amount of biodegraded TEO. Significantly reduced toxicity in soil was observed at week 12 through TLP remediation. Dehydrogenase activity in the bioslurry reactor was strongly suppressed. Fluorescein diacetate was significantly hydrolyzed by the composition of bioremediation residues in the contaminated soil. Microbial adhesion to the solvent was revealed by the determination of microbial activity in the water-immiscible-liquid.

1. Introduction

Petroleum is an important source of energy and feedstock for the synthesis of organic chemicals. It is also one of the most prevalent soil pollutants in China. This contamination causes significant environmental impacts and presents substantial hazards to human health.

Bioremediation is currently one of the most widely used and cost-effective treatment technologies for petroleum-contaminated soils with low to moderate levels of hydrocarbon contamination and suitable climate and soil conditions (Huesemann et al., 2002). Despite its popularity, bioremediation is often unable to decrease contaminant levels below the stringent cleanup standards dictated by environmental regulations (Huesemann et al., 2002).

Most of the interest in the biodegradation of petroleum hydrocarbons has been dedicated to the removal of the parent compounds. However, considering that it contains multitudinous compounds of varying structural complexities, petroleum can not be completely metabolized to CO_2 and H_2O, and always leaves more or less complex residues depending on oil composition and remediation efficiencies (Atlas, 1995). These residues consist of recalcitrant compounds and oxidation products. This is especially important for toxicologically relevant polycyclic aromatic hydrocarbons (PAHs), for some oxygenated PAHs (oxy-PAHs) are more toxic than their parent PAHs (Gibson and Subramanian, 1984). Most importantly, these organic compounds often become increasingly less available to microorganisms, as indicated by the remarkably declining rates of biodegradation in soils with the passing of time due to their low solubility in water and their sequestration by soil and sediments (Alexander, 1995).

The principal approach to increase the bioavailability of hydrophobic organic compounds is based on enhancing solubilization of contaminants, which can be achieved by the addition of surfactants (Allan et al., 2007; Alcántara et al., 2008). However, the use of synthetic surfactants causes many problems in the application of bioremediation. They can inhibit biodegradation due to their toxicity to microorganisms or because of sequestration of target compounds within surfactant micelles (Guha and Jaffé, 1996). Moreover, they are often costly, poorly specific toward contaminants, difficult to biodegrade, and may adsorb onto soil particles (Marcoux et al., 2000). Microbially produced surfactants represent a promising alternative to chemical surfactants, but they also exhibit many of limitations encountered with chemical surfactants and their features and the mechanisms are still insufficiently understood (Marcoux et al., 2000). Therefore, alternative methods to enhance the bioavailability and biodegradation of hydrophobic chemicals will be required.

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Recently, there has been increasing interest in the application of two-liquid-phase (TLP) bioreactors to enhance the degradation of hydrophobic organics with poor bioavailability or toxicity to microorganisms (Marcoux et al., 2000; Zilouei et al., 2008). Usually, TLP systems consist of an aqueous phase and a water-immiscible-liquid (WIL) phase to increase the substrate bioavailability or reduce substrate toxicity. Also, the aqueous phase can be composed of water phase and slurry phase. For example, García-Rivero et al. (2007) used toluene as the solvent in a slurry phase system and hydrocarbon consumption was increased 2-fold in comparison to the control without solvent.

The present study focused on the removal process of bioremediation residues in petroleum-contaminated soil by combining the soil slurry concept with a TLP system.

2. Materials and methods

2.1. Chemicals and soil samples

Dichloromethane was chromatographic grade. All other organic solvents and chemicals were of reagent quality. Silicone oil was 20 $mm^2\ s^{-1}$ and had a density of 0.95 $g\ mL^{-1}$. The contaminated soil was initially collected from the adjacent areas of oil wells in Dagang Oilfield (Tianjing, China, latitude 38°34′N and longitude 116°43′E). The soil had been biotreated using composting for two years in our previous study. The initial concentration of total petroleum hydrocarbon (TPH) in the soil before composting was 60 500 $mg\ kg^{-1}$. The treatment process was as follows: the soil was stacked with sawdust in separate piles of approximately 30–35 cm height and 1.5 m width; NPK fertilizer (20:20:20), composed of urea phosphate, potassium nitrate and ammonium nitrate, was applied to fertilize all the treatments; the ambient temperature during the preparation work was 15–30 °C; a mixed culture of microorganisms isolated from crude oil-contaminated soil was used as inoculum.

In order to obtain a homogeneous soil-contaminant mixture, the soil was mixed with tap water at a ratio of 1:1 (w/w) to form a slurry, and then the slurry was stirred in a mud agitator for 12 h. After homogenizing, the slurry was sieved to remove particles and sawdust larger than 0.2 mm. Tap water was added to make a slurry with a solids concentration of 25%. Then the slurry was stored at 4 °C until used. The sieved soil was classified as moderately alkaline and saline-sodic, which had a clay texture, pH of 7.8 (1:2 ratio of dry soil to distilled water). The dry soil contained ($mg\ kg^{-1}$) 26 500 of total dichloromethane-extractable organics (TEO).

2.2. Biodegradation experiments

All biodegradation experiments in this study were carried out in a series of identical glass-made vessels which had a total volume of 7 L. Each reactor received 5 L of 25% slurry. A mechanical mixer set at 600 rpm provided mixing. Aeration was via a diffuser stone situated at the bottom of the reactor. The rate of air flow was adjusted manually to maintain the dissolved oxygen level above 2 $mg\ L^{-1}$. The temperature of slurry was controlled between 25 and 30 °C with a thermostat jacket. The slurry was supplemented with nutrients (NH_4NO_3 and K_2HPO_4). Slurry samples were analyzed every 3 d for nitrogen and phosphate concentrations in order to maintain 100 $mg\ L^{-1}$ ammonia nitrogen and 50 $mg\ L^{-1}$ phosphate in the slurry. Water lost in the reactor via evaporation was replaced daily.

Two sampling ports were located along the side-wall of the reactor at 5 and 30 cm from the bottom of the reactor. Two treatment conditions were evaluated to obtain information on the rate and extent of bioremediation residues removal. They included: (a) bioslurry treatment, using the prepared slurry with the addition of nutritional supplement; and (b) TLP bioslurry treatment, using the prepared slurry with the addition of nutritional supplement, and 750 mL of silicon oil (15%, v/v).

For sampling, slurry samples in the bioslurry reactor were directly withdrawn. In the TLP reactor, the mixture of slurry and silicon oil was centrifuged at 3000 *g* for 15 min and then the WIL and slurry were taken periodically.

2.3. Analytical methods

Residues in slurry samples were extracted by the conventional Soxhlet extraction as follows: slurry samples were oven dried at 45 °C for 24 h. These samples were then further dried with anhydrous sodium sulfate and extracted with dichloromethane for 8 h using a Soxhlet apparatus. Solvent was evaporated at 30 °C under a nitrogen stream. TEO was determined gravimetrically after solvent evaporation. For analysis of residues in the TLP reactor, centrifuged slurry was dried and extracted using the same method as was the case for slurry in the bioslurry reactor. After evaporation of dichloromethane, the extract was determined gravimetrically. An aliquot (50 mg) of the extract was then extracted three times with 20 mL of methanol each, and the pooled methanolic extract was isolated after centrifugation and evaporated under vacuum at 45 °C. TEO was determined gravimetrically after methanol evaporation. The silicon oil level in the soil was calculated gravimetrically as the difference between the dichloromethane extract and the methanol extract. TEO in the silicone oil was extracted and determined using the above technique.

An aliquot of TEO was dissolved in dichloromethane and then analyzed by gas chromatography–mass spectrometry (GC–MS), using a Thermo-Finnigan SSQ710 GC–MS with HP-5MS elastic silica capillary columns (60 m × 0.25 mm × 0.25 μm). The injection volume was 1 μL. The carrier gas was helium at 37 kPa. Flow velocity was 1 $mL\ min^{-1}$. The analytical conditions were: initial temperature of 50 °C, with isothermal operation for 1 min; heating to 120 °C at a constant rate of 20 °C min^{-1}; and heating to a final temperature of 310 °C at a constant rate of 4 °C min^{-1}, with a 30 min isothermal. Mass spectrometer conditions were: electron impact, electron energy 70 eV; filament current 100 μA; multiplier voltage, 1200 V; full scan. Deuterated bisbenzothiazole and *n*-$C_{24}D_{50}$ were used as internal standards. Mass spectra were analyzed with Auto Mass Spectral Deconvolution and Identification System, and NIST MS Search Program (software version 2.0). In MS database search results, compounds with probability above 60% were determined. Quantification was performed by summing peak areas relative to that of the internal standards for selected compounds.

In order to identify heteroatom-containing compounds in the soil, an aliquot of initial TEO was examined by electrospray ionization (ESI) Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS). For FT-ICR MS analysis, the sample was dissolved in dichloromethane at a concentration of 0.1 $mg\ mL^{-1}$. A 1-mL aliquot of this solution was diluted with 1 mL of methanol, and then concentrated with 10 μL of 35% (v/v) conc. NH_4OH to facilitate deprotonation of acidic compounds by negative-ion electrospray. The analysis was performed on an Apex-ultra FT-ICR MS (Bruker Daltonics, USA) equipped with a 9.4 T actively shielded magnet. Ions were generated by negative-ion electrospray equipped with a 50 μm id fused silica ESI needle. Samples were infused at a flow rate of 250 $\mu L\ h^{-1}$. The operating software was XMASS version 6.0 (Bruker Daltonics, USA). Each spectrum was composed of 64 scans.

2.4. Biochemical analyses

Total hydrolytic activity in slurry was measured by fluorescein diacetate (FDA) hydrolysis using a modification of Green et al.

(2006). Briefly, 0.5 mL of slurry was incubated with 50 mL of 60 mM sodium phosphate buffer (pH 7.6), and 0.5 mL of 7.2 mM FDA lipase substrate (dissolved in acetone) at 37 °C for 3 h in the dark while being shaken horizontally at 150 rpm. After incubation, 2 mL of acetone was added to terminate FDA hydrolysis. The slurry suspension was centrifuged at 8000*g* for 5 min, filtered through filter-slow paper, and the filtrate was measured at a wavelength of 490 nm on a spectrophotometer (Unico 7072B, China). Controls were performed with each slurry sample analyzed to measure the color not derived from the hydrolysis of FDA and the color obtained from non-microbial hydrolysis of FDA. To perform controls, follow the procedure described for the FDA assay, but add 0.5 mL of autoclaved slurry (121 °C, 60 min) instead of viable slurry. Results were expressed as μg fluorescein g^{-1} soil 3 h^{-1}. FDA activity in the WIL phase was determined following the same method as described above, but using 1 mL of autoclaved silicon oil as the control, and results were expressed as μg fluorescein mL^{-1} solvent 3 h^{-1}.

Microbial dehydrogenase activity (DHA) in slurry and WIL samples was determined with 2,3,5-triphenyltetrazolium chloride (TTC) adopting an optimized method described by Casida (1977). Briefly, 1 mL of slurry or 1 mL of silicon oil was mixed with 2 mL TTC solution (4 mg mL^{-1}), 2 mL of 1% glucose solution, and 2 mL Tris–HCl (pH 8.4) in a 50-mL Erlenmeyer flask. The mixture was incubated at 37 °C for 6 h in the dark on a shaker (150 rpm) with H_2SO_4 to terminate the reaction, then extracted with chloroform and measured by absorbance at 485 nm. Controls were prepared with autoclaved samples (121 °C, 60 min) and treated like the samples. Results were expressed as μg triphenyl tetrazolium formazan (TPF) g^{-1} soil 6 h^{-1} or μg TPF mL^{-1} solvent 6 h^{-1}.

2.5. Estimation of bioavailability

Bioavailability of bioremediation residues in the soil was carried out using mild extraction in 100-mL Erlenmeyer flasks. Each flask contained 1 g of dry soil and 15 mL of 4% (w/v) Triton X-100 solution including 1 g L^{-1} $HgCl_2$. Flasks were closed with a Teflon-liner cap and shaken horizontally at 150 rpm, 25 °C and shielded from light. After a shaking time of 1, 2, 3, 4, 6, and 8 d, the extraction was stopped by centrifuging the slurry at 8000*g* for 30 min to separate soil from aqueous extraction solution. Fresh extraction solution was added to soil after each centrifugation. Supernatant was liquid–liquid extracted three times using 10 mL of hexane, for we found TEO could be completely dissolved in hexane. For the Triton X-100 interference with the hexane extraction could be prevented by addition of ethanol during the extraction procedure (Volkering et al., 1998). All three extracts were combined, dehydrated by percolation through anhydrous Na_2SO_4, evaporated by rotary evaporation under N_2. The amount of extracted residues was determined gravimetrically.

2.6. Toxicity assessment

Toxicity assessment was performed using the lettuce emergence test, following the method described by Greene et al. (1998). Triplicate soil dilutions were each planted with 40 lettuce seeds in Petri dishes. Water was added to provide 75% of soil water-holding capacity, and the seeds were incubated at 24 °C for 2 d in the dark followed by 3 d on a 16:8 h light:dark schedule at 6000 Lx. Lettuce emergence was determined after 5 d of exposure. LC_{50} and corresponding 95% confidence intervals were calculated by probit analysis on serial dilutions of reference materials.

2.7. Statistical analysis

All experiments were carried out in triplicate to get reliable data and the results presented here represent average of three independent measurements. All results of slurry were expressed on an oven-dry (105 °C, 24 h), dichloromethane-extracted soil mass basis.

3. Results and discussion

3.1. Characterization of bioremediation residues

The residues in the soil had been heavily weathered and biodegraded in the previous remediation process, suggested by large area of the unresolved complex mixture "hump" under the resolved peak envelope and the absence of linear alkanes and aromatic hydrocarbons (figure not shown). As shown in Table 1, residual contaminants were characterized by a wide variety of compounds. Among these compounds, oxy-PAHs have drawn special attention. Oxy-PAHs may be formed through chemical oxidation, photooxidation, or biological transformation of PAHs in the environment. Many oxy-PAHs have been found at significant levels in bioremediation processes (Saponaro et al., 2002; Lundstedt et al., 2003, 2006). In some cases, it may lead to the generation of new, even more toxic contaminants in the remediated material. Moreover, there existed a variety of heterocyclic analogues of PAHs (hetero-PAHs) in the residues containing nitrogen, sulfur, or oxygen. A number of hetero-PAHs have been reported to display toxicity, mutagenicity, and carcinogenicity even presenting in low concentrations (Meyer et al., 1999). Sometimes the hetero-PAHs are even more toxic than the parent compounds. Unfortunately, because of the highly complexity in chemical structure, the thermal lability, excessive low volatility, or strong interaction between the carbonyl groups and the stationary phase in the GC column, some intermetabolites could not be successfully analyzed by GC–MS (Letzel et al., 2001).

In contrast to GC–MS, FT-ICR MS has an ultra-high resolving power, and provides an ultra accurate mass determination of many compounds in complex mixtures. Molecular formulas of compounds contained in complex mixtures can be estimated by FT-ICR MS without chromatographic fractionation of the sample. Furthermore, ESI is amenable to polar compounds without the need for derivatization, eliminating the need to correct for isotope distributions in the derivatizing agent. Recently, FT-ICR MS has been developed to characterize the acidic and neutral polar nitrogen, sulfur, and oxygen compounds in a suite of biodegraded oils (Kim et al., 2005). The calibrated negative-ion FT-ICR mass spectrum of initial residues samples is presented in Fig. 1. The distribution of peaks spans the mass range from 200 to 650 Da. The chemical formulae for all these compounds are unambiguously determined by FT-ICR accurate mass measurements. The spectrum has a primary mode in its peak distribution at 360 Da and a secondary mode at 450 Da.

3.2. FDA activity

As shown in Fig. 2, measures of FDA hydrolysis showed a marked increase in enzymatic activity right after reactor start-up. After 2 weeks of treatment, FDA activity in the bioslurry reactor gradually decreased to the background level and remained unchanged. At week 4, FDA activity reached its maximum (5560 μg fluorescein g^{-1} soil 3 h^{-1}) in the TLP reactor, and then decreased. FDA activity in silicon oil reached its peak at week 3 and kept stable. Microbial activity significantly increased more and remained higher in TLP soils than in bioslurry ones ($p < 0.05$). The presence of silicon oil seemed to have stimulated and maintained enzymatic activity by absorbing contaminants that can cause significant toxicity to the microflora. Although the degradation of bioremediation

Table 1
Identity of selected hydrocarbons, metabolites, oxy-PAHs, and hetero-PAHs in the GC–MS analysis (results are expressed as the mean of triplicate samples ± standard deviations).

Compound	Concentration (mg kg^{-1} soil)	Compound	Concentration (mg kg^{-1} soil)
4-Hydroxy-benzaldehyde	28 ± 1	1-Ethylidene-1H-indene	13 ± 1
Decahydro-4,4,8,9,10-pentamethylnaphthalene	169 ± 5	α-Isobutyl-2,4,5-trimethyl-benzyl alcohol	80 ± 3
1H-indene,octahydro-2,2,4,4,7,7-hexamethyl-,trans-	321 ± 12	3-(2-Methyl-propenyl)-1H-indene	128 ± 3
2,6,10,14-Tetramethyl-pentadecane (pristane)	327 ± 11	2,3,6-Trimethyl-naphthalene	76 ± 3
2,6,10,14-Tetramethyl-hexadecane (phytane)	193 ± 5	1,2,3,3a,4,5,5a,6,7,8-Decahydropyrene	117 ± 4
9-Octadecenoic acid (Z)-, methyl ester	160 ± 5	Allogibberic acid	91 ± 2
Tert-hexadecanethiol	96 ± 4	2,3-Dihydro-2-methyl-7-phenyl-benzofuran	49 ± 2
Sterane	253 ± 8	Danthron	139 ± 4
17-Pentatriacontene	86 ± 4	17-Noranthiaergostan-5,7,9-trien-3-ol,17-acetyl	141 ± 3
Oleic acid, 3-(octadecyloxy)propyl ester	387 ± 11	1,4,9(11)-Pregnatriene-3,20-dione,21-acetoxy-17-hydroxy-	148 ± 5
Undecylenic acid	627 ± 15	9-Ethyl-9,10-dihydro-10-hydroxy-anthracene	129 ± 3
3-Cyclohexen-1-ol,4-methyl-1-(1-methylethyl)-	403 ± 13	9H-carbazole	28 ± 2
Baccharane	215 ± 7	Methyl(1-O-retinyl-2,3,4-triacetyl-β-D-glucopyran)uronate	103 ± 4
2,6,10,15,19,23-Hexamethyl-tetracosapentaene	704 ± 18	5-Hydroxy-6,7,8-trimethoxy-2,3-dimethyl-chromone	144 ± 4
7,8-Epoxylanostan-11-ol, 3-acetoxy-	142 ± 4	Dibenzothiophene	74 ± 3
Squalane	415 ± 12	Astaxanthin	153 ± 4
Sum	6140 ± 185		

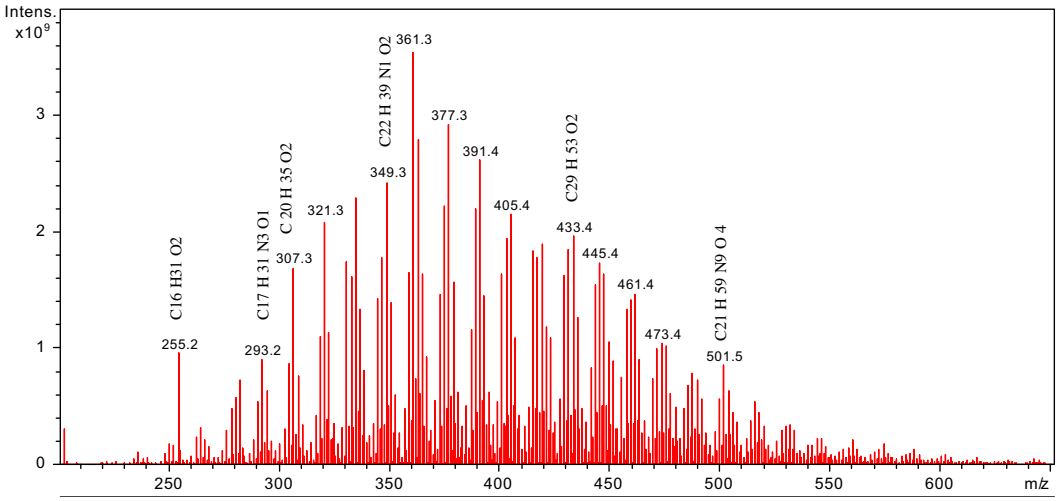

Fig. 1. Broadband negative-ion electrospray ionization FT-ICR mass spectrum of the bioremediation residues.

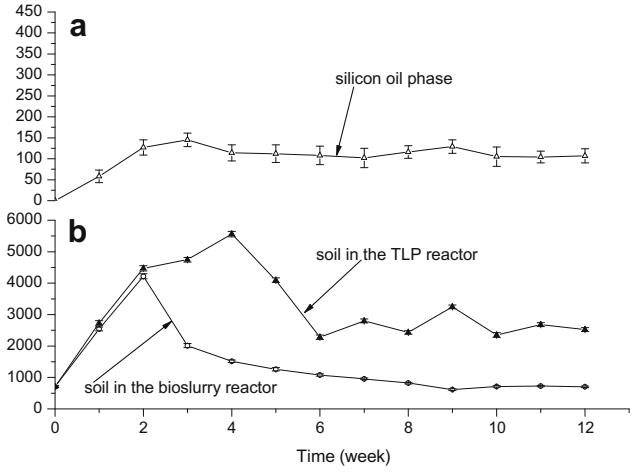

Fig. 2. FDA activity in soil and silicon oil samples during 12 weeks bioremediation. (a) Silicon oil phase, results are expressed as μg fluorescein mL^{-1} solvent 3 h^{-1}. (b) Soil, results are expressed as μg fluorescein g^{-1} soil 3 h^{-1}.

residues was little in the bioslurry reactor, soil humus may nurture and propagate the microbes, and increased FDA activity.

There are three possible modes for microbial substrate uptake from the WIL, and Déziel et al. (1999) has discussed and summarized these. In the first mode, only the substrate dissolved or pseudosolubilized in the aqueous phase is bioavailable, making the degradation rate dependent on the mass-transfer rate of the contaminant from the WIL to the aqueous phase. In the second mode, microbial cells produce biosurfactants, which form small micelles around hydrophobic particles that can be directly assimilated. In the third mode, cells come into direct contact with the WIL, concentrating at the WIL-aqueous phase interface, and obtain the substrate directly from the WIL. One, two, or all three of these modes can occur in a TLP bioreactor, consecutively or simultaneously, and the characteristics of the substrate and the microbial population will determine which mode is predominant. In our experiment, there existed a wide variety of microorganisms in the soil, and these microorganisms might perform differently in the uptake mode of substrate from the WIL. We could conclude that there existed a certain amount of microorganisms attached onto the WIL, although we did not use microscopy to examine these cells and determine which uptake mode was predominant. Otherwise, the relatively high FDA activity cannot be observed in the separated silicon oil. We cannot expect that abiotic substances absorbed into silicon oil may produce such a relatively high activ-

ity, because the autoclaved silicon oil was used as a control in the determination of microbial activity, and this approach excluded possible overestimation of total microbial activities. In fact, it was just the determination of microbial activity in the WIL that let us indirectly know the performance of attached microflora. FDA is hydrolyzed by a number of different enzymes such as proteases, lipases, and esterases and it has been applied to determine amounts of active fungi and bacteria (Gaspar et al., 2001). The FDA activity remained constant might indicate the microbial number of attached microorganisms was constant.

FDA hydrolysis is widely accepted as an accurate and simple method for the determination of total microbial activity in a range of environmental samples, and a variety of determination methods have been proposed (Schnürer and Rosswall, 1982; Stubberfield and Shaw, 1990; Adam and Duncan, 2001; Gaspar et al., 2001; Green et al., 2006). These authors found the hydrolysis of FDA was not significantly influenced by soil matrix and organics. Riis et al. (1998) found the blank of steam-sterilized soil (121 °C, 20 min) was about 2% of the value of the original soil and thought it was thus negligible. However, our study showed that 0.1 g of autoclaved soil had an $A_{490\ nm}$ 0.350 which significantly exceeded the sum of spontaneous hydrolysis (0.035) and background color of the soil (0.016). The sterilized blank absorbance was approximately 10% of that of the unautoclaved soil, but not in the burned soil (muffle furnace at 550 °C for 4 h). This may be attributed to interfering factors, such as the composition of petroleum metabolites, and/or the presence of inhibiting compounds, which may influence FDA hydrolysis in contaminated soils. Further study is needed to elucidate the precise mechanism linking chemical hydrolysis of FDA with organic compounds.

3.3. Dehydrogenase activity

DHA in particular is of interest as it provides a measure of overall microbial activity and consequently indicates whether stimulation or inhibition of the microbial communities present (either in the product or indigenous to the soil environment) are occurring as a result of the selected remediation strategy (Claassens et al., 2006). DHA in the bioslurry reactor dropped substantially after reactor start-up. A statistically significant ($p < 0.05$) inhibition of DHA was observed after week 4, and DHA was not detected from then on (Fig. 3). Conversely, DHA in TLP soil increased to 394 μg TPF g^{-1} soil 6 h^{-1} at week 5 and remained over 150 μg TPF g^{-1} soil 6 h^{-1} during the process. DHA in silicon oil also remained over 50 μg TPF mL^{-1} solvent 6 h^{-1} from week 2. Several trends in enzyme activity were observed in this investigation. Enzyme activities were generally higher in the TLP reactor than in the bioslurry reactor. DHA was heavily suppressed by toxic compounds in the bioslurry reactor, but FDA activity was not inhibited completely. FDA hydrolysis was indicative of the onset of biodegradation in contaminated soil, and it was not considered a representative measure of actual biodegradation, because FDA hydrolysis did not decline after biodegradation had decreased (Lee et al., 2008). DHA was also useful to indicate the onset of the biodegradation process but decreased rapidly after the biodegradation rate had declined (Margesin et al., 1999). Relatively constant DHA in the WIL phase suggested that biodegradation activity of adhering microorganisms was stable.

3.4. Biodegradation of residual contaminants

The changes in TEO level are shown in Fig. 4. The concentration of TEO in the bioslurry reactor increased from 26 500 mg kg^{-1} soil at the beginning, to 32 800 mg kg^{-1} soil at week 2, and ranged from 32 900 to 34 200 mg kg^{-1} soil thereafter (Fig. 4a). This increase was a result of microbial release of non-extractable contaminants which resulted in some bound residues remobilized. Since the solvents used to determine the extractable contaminants only extracted a small amount of the soil organic matters, the sorption or sequestration of residues might be desorbed from the humic and fulvic fraction in aged contaminated soil (Macleod and Semple, 2000). An increase in the extractability of the bound residues in slurry biotreatment was also reported by Molnár et al. (2007). Bioslurry reactors were able to substantially increase the rates of contaminant degradation because the availability of contaminants, electron acceptors, nutrients, and other additives to the microbial populations were significantly enhanced in the slurry. However, in this study, the bioslurry technology was not efficient in case of soil contaminated by high concentration of bioremediation residues. In a parallel experiment, Triton X-100 (from 200 to 2000 mg L^{-1} slurry) and yeast extract (1 g L^{-1} slurry) were added to the bioslurry reactor at week 4, but degradation of contaminants was still not observed. Moreover, the count of hydrocarbon degraders was found above 10^8 colony-forming units g^{-1} soil (data not shown). These findings demonstrate that microbial factors may be responsible for the persistence of residual contaminants despite their ready bioavailability even after intense bioremediation treatment. The inability of microorganisms to continually decompose the residues was explained by the accumulation of toxic intermediate metabolites in the previous bioremediation compost process. Casellas et al. (1997) described that 9-fluorenone and 1-hydroxy-9-fluorenone, which inhibit the degradation of this PAH, were produced by metabolism of a non-productive branch of fluorine.

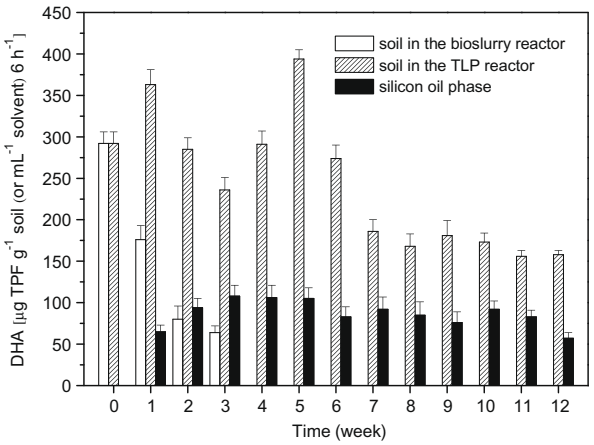

Fig. 3. DHA in soil and silicon oil samples during 12 weeks of bioremediation.

The TLP system, however, exhibited excellent efficacy in degrading contaminants against the bioslurry reactor. TEO in the soil decreased continuously to 7320 mg kg^{-1} soil at week 12 (Fig. 4b). TEO in silicon oil declined from 12 100 mg L^{-1} solvent at week 2, to 7260 mg L^{-1} solvent at week 12 (Fig. 4c). Finally, total removal amount of TEO was 15 900 mg kg^{-1} soil (Fig. 4d). The level of silicon oil in soil was kept at approximately 1800 mg kg^{-1} soil after week 2. In addition, we observed emulsification of silicon oil at week 1, which lasted thereafter for the entire duration of the experiment.

Most organic contaminants can be detoxified and mineralized by microorganisms. However, even if microorganisms with the required degrading capability are present and environmental conditions for growth and degradation are adequate, the applicability of bioremediation is sometimes restricted due to limited bioavailability and toxicity. Firstly, the pollutant may be insufficiently available to microorganisms. For example, some organic contaminants have very low aqueous solubilities, however only the water-dissolved fraction of chemicals is usually assumed to be available to

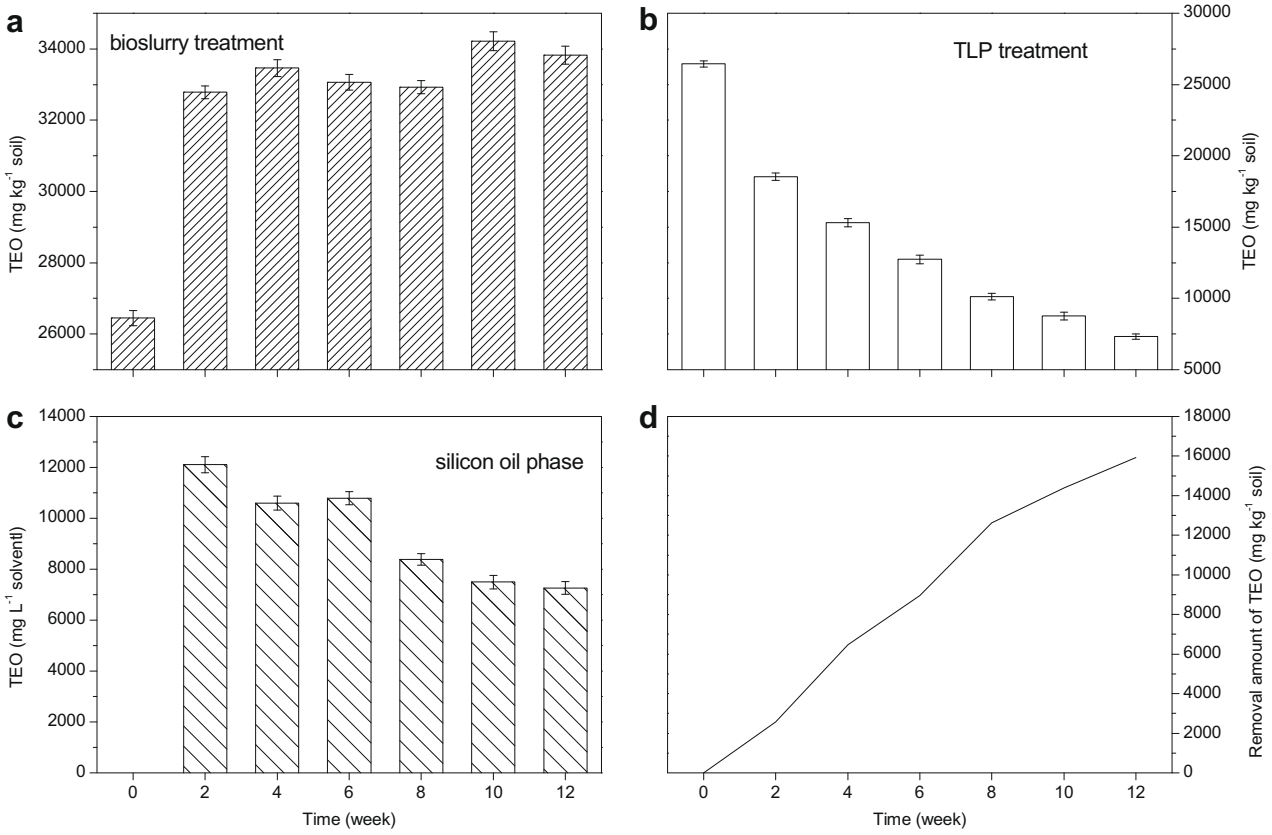

Fig. 4. Concentrations of TEO in soil and silicon oil samples during 12 weeks of bioremediation. The removal amount of TEO in soil equals the value that the initial content of TEO subtracts the sum of TEO content in soil and silicon oil. The total mass of soil is 1.7 kg, and total volume of silicon oil is 0.75 L.

Table 2
Concentrations of selected compounds in soil and WIL phase during the TLP treatment (presented as mg kg^{-1} for soil samples, and mg L^{-1} for silicon oil samples. Removal efficiency = 100% × (1 − (concentration in soil + concentration in silicon oil × 0.75/1.7)/initial concentration in soil). In the TLP reactor, the total mass of soil is 1.7 kg, and total volume of silicon oil is 0.75 L. Results are expressed as the mean of triplicate samples ± standard deviations).

Compound	Week 2		Week 6		Week 12	
	Soil	Silicon oil	Soil	Silicon oil	Soil	Silicon oil
Pristane	132 ± 7	247 ± 17	43 ± 3	161 ± 14	16 ± 2	75 ± 6
Phytane	81 ± 4	138 ± 10	25 ± 2	83 ± 7	13 ± 1	41 ± 3
Baccharane	106 ± 5	183 ± 7	73 ± 3	135 ± 10	42 ± 4	95 ± 7
Squalane	263 ± 11	341 ± 18	148 ± 12	258 ± 18	115 ± 9	183 ± 15
2,3,6-Trimethyl-naphthalene	32 ± 2	79 ± 4	17 ± 1	52 ± 4	8 ± 1	21 ± 2
2,3-Dihydro-2-methyl-7-phenyl-benzofuran	26 ± 2	37 ± 3	13 ± 1	28 ± 2	ND	19 ± 2
9-Ethyl-9,10-dihydro-10-hydroxy-anthracene	74 ± 3	106 ± 6	48 ± 4	75 ± 6	21 ± 2	48 ± 3
9H-carbazole	ND	55 ± 3	ND	31 ± 2	ND	25 ± 3
Dibenzothiophene	45 ± 3	53 ± 3	26 ± 2	48 ± 3	17 ± 2	23 ± 2

ND, not detected. Removal efficiencies for these compounds are 85%, 84%, 40%, 53%, 77%, 68%, 67%, 61%, and 63%, respectively.

microorganisms. Secondly, some pollutants are toxic to microorganisms, and growth inhibition at high substrate concentration prevents the effective biodegradation of these contaminants (Déziel et al., 1999). TLP bioreactors have the potential to resolve these two limitations of biotreatment technologies by controlling the mass-transfer rate and aqueous phase concentrations of hydrophobic/toxic compounds. By careful selection of the WIL and the operating conditions, the WIL phase of a TLP bioreactor can provide inhibitory substrates continuously via diffusion into the aqueous phase. The concentration within the aqueous phase is maintained at a low or non-toxic level, depending on phase ratio, types of microorganisms and contaminants. This is especially interesting when it is widely recognized that opposite solutions were usually required for toxicity and limited bioavailability (Déziel et al., 1999). Villemur et al. (2000) used oil as solvent in TLP bioslurry reactors to remediated high-molecular-weight PAHs-contaminated soil and achieved excellent outcome. Zilouei et al. (2008) successfully developed a TLP bioreactor for mineralization of pentachlorophenol (PCP) at a concentration of 10 g L^{-1} based on aqueous phase and the degradation of 10 g PCP was completed in less than 100 h. In our study, emulsification of WIL in the TLP bioreactor suggested that microorganisms were producing biosurfactants. This emulsion formation increased the interfacial area between the two liquid phases, promoted the partitioning of contaminants in the aqueous phase and also favored adhering microorganisms. Furthermore, hydrophobicity of the cell surface assisted in the attachment of microorganisms at the WIL surface, which promoted the uptake of pollutant molecules directly from the WIL. But if the

emulsion is too stable, this may represent a major problem in sampling of the two phases for analysis purposes (Déziel et al., 1999). In our experiment, fortunately, by centrifugation at 3000 *g* for 15 min, soil and silicon oil were well separated.

Several selected compounds were further degraded in the TLP treatment (Table 2). Both pristane and phytane were significantly degraded, although their initial concentrations were relatively high. Pristane and phytane were widely used as conserved markers against which the biodegradation of oil could be assessed. However, pristane and phytane served as a suitable internal reference marker only for a limited time period (up to a few months), because they could be rapidly and extensively degraded under certain circumstances (Fedorak and Westlake, 1981; Atlas, 1995). In bioremediation experiments, secondary metabolites accumulated over time and may have reached the critical level. At this stage, the remaining contaminants are resistant to biodegradation, due to the toxicity of these metabolic byproducts to microorganisms. How to eliminate this inhibiting effect so as to further remediate the contaminated soil is a key to the future generalization of efficient and cost-effective bioremediation approaches.

3.5. Bioavailability of bioremediation residues in soil

Extraction of residues with Triton X-100 consisted of two phases: after a rapid initial extraction, the amount of residues extracted decreased progressively during the following extractions (data not shown). Eventually, 16,600 mg TEO kg^{-1} soil was extracted. Extensive research has been put into the development of non-exhaustive extraction methods for the measurement of PAH bioavailability. These methods use various extractants comprising solvent mixtures, surfactants, cyclodextrins, as well as solid-phase extraction. Volkering et al. (1998) applied Triton X-100 to predict bioavailability of mineral oil in soil and found slightly higher concentrations of mineral oil (especially for *n*-alkanes C_{12}–C_{18}) after surfactant extraction than after 10 weeks of biodegradation. However, the use of Triton X-100 has been deemed too rigorous for the prediction of bioavailability of PAHs. Cuypers et al. (2002) found that residual PAH concentrations after Triton X-100 extraction were considerably lower than the residual PAH concentrations after biodegradation, particularly for the 5, 6-ring PAHs. In our study, TEO concentration after a mild extraction (Triton X-100) was in good agreement with that obtained after 12 weeks of biodegradation. We thought that the WIL phase enhanced desorption of contaminants sorbed on soil, and the microbial contact with contaminants and mass-transfer were greatly increased by the formation of silicon oil droplets. Most importantly, the inhibition of microbial activity, caused by the toxicity of some metabolites, was significantly alleviated through the absorption of toxic contaminants from aqueous phase into silicon oil.

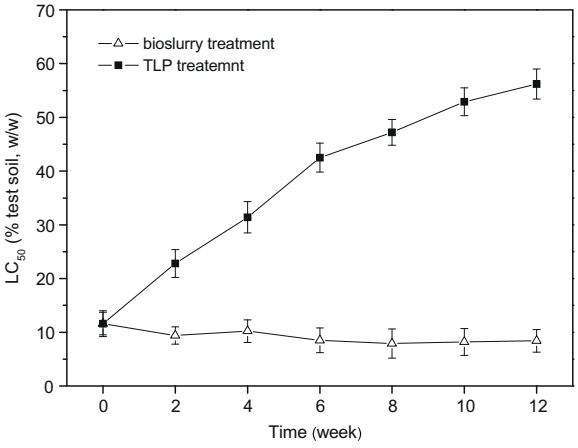

Fig. 5. Lettuce germination LC_{50} values for soils sampled during bioremediation. Data represent LC_{50} values for single microcosms with error bars corresponding to 95% confidence intervals calculated by probit analysis.

3.6. Soil toxicity during bioremediation

The lettuce emergence test was performed over the course of the study to monitor toxicity levels of the treated soil to determine if toxicity declined in the treatment process. As shown in Fig. 5, the toxicity of soil in the bioslurry reactor increased after reactor start-up and remained stable. This was interpreted as an increase in contaminant levels due to enhanced mobility and availability, and thus toxicity, in the soil. Fortunately, the toxicity of soil in the TLP system declined markedly over the 12 week period. This was explained by the fact that a large amount of toxic contaminants were absorbed into the WIL phase, hence reduced toxic compound levels in the aqueous phase, and an overall reduction in contaminant levels due to biodegradation and thus, soil toxicity.

4. Conclusions

This paper has attempted to utilize an extremely efficient approach, TLP biotreatment, for degradation of toxic bioremediation residues. This TLP bioreactor consisted of a 5-L slurry phase and 750 mL of silicon oil. Due to high toxicity to the microflora, the residues could not be further degraded using ordinary bioslurry treatment means. However, in the TLP system, the levels of residual contaminants and toxicity in soil were significantly reduced, which indicated TLP treatment was a promising technology to remediate soils contaminated by toxic/recalcitrant organics. TLP treatment increases the interfacial area between contaminants and microorganisms, which enhances the mass-transfer rate of contaminants, and sequesters toxic compounds into a separate solvent phase, thus maintains their aqueous concentrations below inhibitory levels. Future investigations should be focused on optimization of key operational and design parameters, especially in the later steps of a treatment process such as enriched specific microflora recovery, final phase separation and solvent recycling.

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