Journal of Hazardous Materials 183 (2010) 395–401

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Bioremediation of crude oil-contaminated soil: Comparison of different biostimulation and bioaugmentation treatments

Yaohui Xu[a,*], Mang Lu[b]

[a] Department of Geochemistry, Yangtze University, Nanhuan Road, Jingzhou 434023, China
[b] School of Materials Science and Engineering, Jingdezhen Ceramic Institute, Jingdezhen 333001, Jiangxi Province, China

ARTICLE INFO

Article history:
Received 30 April 2010
Received in revised form 8 July 2010
Accepted 9 July 2010
Available online 15 July 2010

Keywords:
Peanut hull
Dehydrogenase activity
Bulking
Toxicity
Fourier transform ion cyclotron resonance mass spectrometry

ABSTRACT

Biostimulation with inorganic fertilizer and bioaugmentation with hydrocarbon utilizing indigenous bacteria were employed as remedial options for 12 weeks in a crude oil-contaminated soil. To promote oil removal, biocarrier for immobilization of indigenous hydrocarbon-degrading bacteria was developed using peanut hull powder. Biodegradation was enhanced with free-living bacterial culture and biocarrier with a total petroleum hydrocarbon removal ranging from 26% to 61% after a 12-week treatment. Oil removal was also enhanced when peanut hull powder was only used as a bulking agent, which accelerated the mass transfer rate of water, oxygen, nutrients and hydrocarbons, and provided nutrition for the microflora. Dehydrogenase activity in soil was remarkably enhanced by the application of carrier material. Metabolites of polycyclic aromatic hydrocarbons were identified by Fourier transform ion cyclotron resonance mass spectrometry.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

During the production and transportation of crude oil, unsuitable operation and leakage may result in contamination of soil with petroleum hydrocarbons. Petroleum contamination causes significant environmental impacts and presents substantial hazards to human health.

There is a variety of remedial options available for cleaning up contaminated sites including incineration, solidification/stabilization, soil vapor extraction, soil washing, etc. These methods can be relatively expensive because the extracted contaminants or incinerated soil must often be further treated or disposed of.

During the past decade, bioremediation of petroleum-contaminated soil has been a hot issue in environmental research, and many bioremediation strategies have been developed and improved to clean up soils polluted with petroleum and its derivatives [1–3]. Bioremediation is an efficient, economic and versatile alternative to physiochemical treatments [4]. Soil bioremediation may be broadly divided into in situ and ex situ strategies. In situ bioremediation refers to the biological treatment of contaminated soil without excavating prior to treatment [5]. Ex situ treatments (including landfarming, composting, bioslurry and biopiling) using tilling, turning or continuously mixed slurries to apply oxygen and nutrients, and is performed in a prepared bed or reactor.

Composting of organic contaminants has been successfully demonstrated at both laboratory and field-scales especially for petroleum hydrocarbons [1,6–9]. In generally, composting involves the addition of bulking agents and organic amendments, such as bark chips [6], wheat bran [7], sawdust [8], and the mixture of municipal and green wastes [9]. These enhance aeration and microbial activity, and hence the biodegradation rate of contaminants. Nutrient addition to the pile and microbial augmentation may as well accelerate the rate of biodegradation in the piles [7].

Indigenous microorganisms in the soil can degrade a wide range of hydrocarbons, but their population and activity are affected when toxic contaminants are present at higher concentrations. The introduction of microorganisms after enrichment of indigenous microorganisms or exogenous microorganisms can degrade the contaminants efficiently and have a higher tolerance to toxicity [10,11]. In many cases, bioaugmentation is a feasible strategy for contaminant removal and site remediation [12,13]. One of the key points for bioaugmentation is maintaining high biomass of bacterial populations. Obuekwe and Al-Muttawa [14] used sawdust, styrofoam and wheat bran as carriers for incubation of two hydrocarbon-degrading bacteria and found that immobilized cells had a good utilization of hydrocarbons in liquid medium.

The aim of the present work was to compare the efficiency of various biostimulation and bioaugmentation strategies in reducing soil petroleum contamination under laboratory conditions. Peanut

* Corresponding author. Tel.: +86 716 8060406.
E-mail address: yaohuixu@hotmail.com (Y. Xu).

0304-3894/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jhazmat.2010.07.038

hull powder was used as a bulking agent and carrier material to improve oxygen diffusion and to immobilize a greater quantity of bacteria cells. Soil microtoxicity and dehydrogenase activity (DHA) were used as sensitive indicators to assess the impact of pollution remediation on soil quality. The degradation degree of specific contaminants was determined by gas chromatography–mass spectrometry (GC–MS). The principal intermediates of polycyclic aromatic hydrocarbons (PAHs) were identified by electrospray ionization (ESI) Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS).

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

All solvents used were of analytical or glass-distilled grade and were obtained from commercial supplier (Beijing Chemical Company, China).

2.1.2. Soil

The contaminated soil was collected from a crude oil spill site in Liaohe Oilfield, Liaoning Province, China. During the collection of the contaminated soil, surface letter was removed and soil samples were collected to a depth of 30 cm. The soil was air-dried and sieved through a 2-mm mesh sieve, homogenized by hand with shovels, and then stored at 4 °C in the dark until required. Soil pH was determined with a 1:2 soil to water ratio. Total nitrogen and phosphorus were analyzed by the methods described by Bryant et al. [15]. The total petroleum hydrocarbon (TPH) content of the soil was determined as described below. In the present study, the texture of the raw soil was classified as a clay loam, and its physicochemical characteristics were as follows: sand, 25.9%; silt, 47.6%; clay, 26.5%; pH, 7.2; total nitrogen, 15.62 mg kg^{-1}; total phosphorus, 3.15 mg kg^{-1}; TPH, 29,500 mg kg^{-1}.

2.1.3. Biocarrier

Peanut hull powder was used as a carrier material to immobilize bacteria owing to its properties of being highly granular, absorbent, biodegradable and inexpensive. Peanut hull was harvested at maturity, air-dried and mechanically ground to obtain a particle size of approximately 0.5–1.5 mm. Before use, peanut hull powder was sterilized by autoclaving at 121 °C for 20 min. The carrier was pretreated by soaking in dilute sodium hydroxide solution (1%) for 2 days, rinsing 3 times with distilled water, drying at 110 °C for 10 h, cooling to room temperature and finally soaking in sterilized water to saturation.

2.2. Bacterial consortium and immobilization

An indigenous hydrocarbon-degrading bacterial consortium was developed in minimal salt medium, with crude oil as the sole source of carbon and energy, from a sample of the contaminated soil, following procedures described in detail elsewhere [3]. Cells of the mixed consortium grown in 10 L of Bushnell-Haas medium (BH) [16] containing 2% glucose up to an optical density (OD) of 1 were harvested, washed 3 times with phosphate buffer (pH 6.5) containing 1 M NaCl, resuspended in BH medium to get approximately 10^{10} cells per mL, and mixed with 2000 g of peanut hull powder. The carrier culture was incubated for 4 days at room temperature. After incubation, the culture was withdrawn and dried in a sterile hood in a stream of dry air at room temperature for 4 days.

The stability of cells immobilized on peanut hull powder was checked according to Labana et al. [17] with some modifications. Briefly, the dry powder containing immobilized cells was kept at 4 °C and room temperature for 2, 4, 8, 12, and 16 weeks, respectively. After that, the powder was divided into two groups. One group (50 mg) was suspended in 5 mL of sterile 0.9% NaCl solution and the mixture was agitated vigorously on a vortex mixer for 10 min to suspend the adhered cells. Subsequently, suspension was allowed to settle down. The resulting supernatant fluid was diluted serially and plated on nutrient agar plates. Colony-forming units (CFU) were counted after 3 days of incubation at 28 °C. Moreover, another group was used to determine total hydrolytic activity in the powder by fluorescein diacetate (FDA) hydrolysis using the method described previously [18]. Results were expressed as μg fluorescein g^{-1} carrier.

Table 1
Experimental setup for treatment of crude oil contaminated soil (dry weight basis in brackets).

Set no.	Treatment units	Description
A	Soil	Biostimulated
B	Soil + bacteria solution	Bioaugmented
C	Soil + peanut hull powder (15% w/w, no immobilized cells)	Biostimulated
D	Soil + peanut hull powder (15% w/w, with immobilized cells)	Bioaugmented

2.3. Experimental design and treatments

Experimental units consisted of 10 L (40 cm length, 25 cm width, and 10 cm height) plastic trays. A total of 12 trays were used in the study. A summary of treatment and control trays tested are presented in Table 1. Each treatment was carried out in triplicate. For each tray, 5 kg of soil and the corresponding materials (peanut hull powder or bacteria solution) were added and mixed thoroughly. For treatment B, the bacteria solution was obtained as indicated above. Soil was maintained at 18–20% moisture content by the daily addition of distilled water.

Fertilizer application was designed to optimize nutrient levels for microbial degradation of hydrocarbons. A C:N ratio of 10:1 was considered ideal for microbial communities [19]. Inorganic nutrients such as $(NH_4)_2SO_4$ and K_2HPO_4 was added to the treatment units A, B, C, and D to give a final C:N:P ratio of 100:10:1. Each unit was thoroughly mixed every alternate day to allow good aeration. The trays were incubated in a greenhouse at 25–30 °C.

2.4. Sampling and instrumental analyses

The sampling dates were 0 and 12 weeks after beginning of treatment. The samples were taken by mixing six subsamples from six sites of the tray, spanning the whole profile (from top to bottom), and homogenized. The samples were stored at −20 °C and thawed 3 days before analyses. This storage procedure was recommended for soil enzyme measurements and guaranteed a high recovery of soil enzyme activities [20].

Residual oil in soil was extracted using the conventional Soxhlet extraction. In brief, 15 g of dry soil were placed in a cellulose thimble mixed with 2 g anhydrous magnesium sulfate to remove the moisture. The thimble and its contents were extracted with dichloromethane for 16 h in a Soxhlet apparatus. The extract was condensed to 1 mL in a rotary evaporator and fractionated by silica-gel column chromatography to separate the different fractions of crude oil i.e., saturate, aromatic and polar fractions, following the methods given by Bastow et al. [21]. The different elute was evaporated to dryness under nitrogen, and TPH was calculated gravimetrically. All TPH concentrations were expressed on an ash weight basis (mg kg^{-1}). This removes potential bias due to dilution by organic bulking agents. Ash content was determined using a loss-on-ignition procedure. Triplicate 5 g samples were dried for

24 h at 105 °C and then transferred to a muffle furnace held at 550 °C for 6 h to burn the organic matter. Ash content was calculated from the ratio of pre- and post-ignition sample weights. Ash content results were averaged, and the averaged values were used to calculate TPH concentrations in the soil as mg kg^{-1}.

The hydrocarbon fraction was analyzed by 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.

In order to identify metabolites of PAHs, an aliquot of extracted oil without fractionation was examined by ESI FT-ICR MS. For ESI 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 μL h^{-1}. The operating software was XMASS version 6.0 (Bruker Daltonics, USA). Each spectrum was composed of 64 scans [18].

2.5. Soil toxicity testing

The toxicity of soil elutriate was determined using the Microtox® bioassay mentioned by Robidoux et al. [22]. Sampled soil (5 g dry weight) was mixed with sterile water (20 mL) in a 100-mL flask and shaken for 24 h (150 rpm) at 25 °C in the dark. The slurry was centrifugated at 2000 rpm for 20 min at room temperature, and filtered through filter paper then 0.22 μm membrane filter to obtain soil elutriate. Aliquots (20 μL) of the bacterial reagent (*Photobacterium phosphoreum*) were added to a series of dilutions of elutriate. The salinity of the samples was adjusted to 2% with reagent-grade NaCl. The luminescence of bacteria was measured after 15 min of exposure at 15 °C, using a Microtox M500 Analyzer. The analyzer, reagents and freeze-dried bacteria were obtained from Azur Environmental (Carlsbad, CA). Toxicity values are the average of five replicates of each filtrate sample, expressed as EC_{50}, which was defined as the effective nominal concentration of elutriate (volume percent) that reduces the intensity of light emission by 50%.

2.6. Dehydrogenase activity

Dehydrogenase activity in soil samples was measured with 2,3,5-triphenyltetrazolium chloride according to the method of Lu et al. [18]. Results were expressed as μg triphenyl tetrazolium formazan (TPF) g^{-1} soil.

2.7. Statistical analysis

The variance and significant differences of TPH concentration, microtoxicity and dehydrogenase activity among various treatments were analyzed by Student's *t*-test. The statistical significance in this analysis was defined at $p < 0.05$.

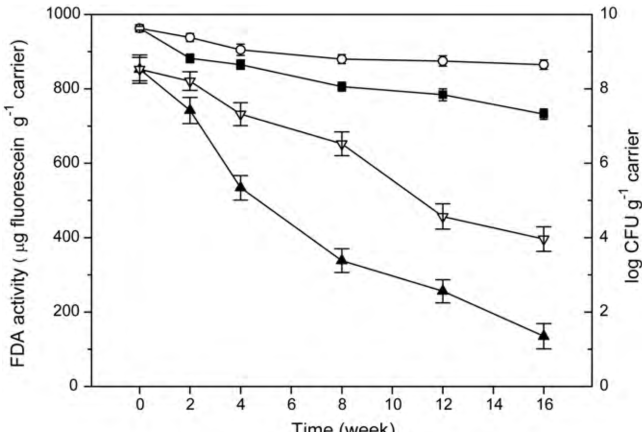


Fig. 1. Stability of cells of the test bacterial consortium immobilized on peanut hull powder indicated by the changes in the viable number (○: log CFU, 4 °C; ■: log CFU, room temperature), and FDA activity (▽: 4 °C; ▲: room temperature) of immobilized cells as a function of time during storage.

3. Results and discussion

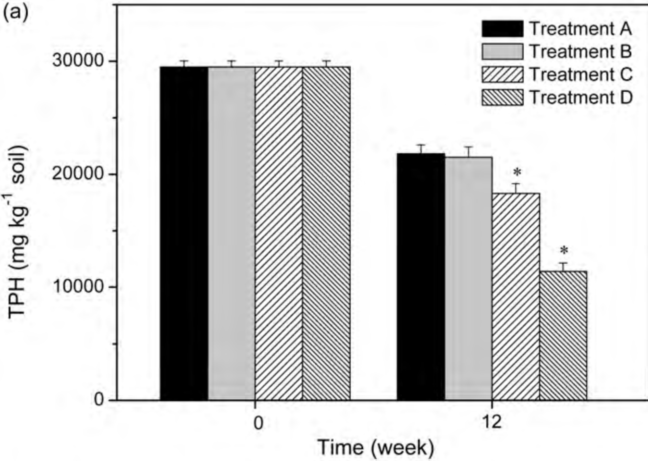
3.1. Survival of immobilized cells under storage

The stability of the immobilized bacteria under storage was assessed by viability at different times when stored at room temperature and 4 °C. Fig. 1 shows the changes in the number of viable cells. It was observed that storage of immobilized cells at room temperature resulted in a significant decrease of about 2 log units after 16 weeks. However, at 4 °C, the cells immobilized on peanut hull powder were stable, as the log CFU decreased only approximately by 1 unit after 16 weeks.

FDA hydrolysis was selected in an attempt to measure bacterial activity parameters in carrier stored at room temperature and 4 °C versus time. As shown in Fig. 1, the FDA hydrolytic activity of immobilized cells decreased with time during storage. Moreover, it can be observed that FDA activity decreased more significantly than did microbial counts during storage. FDA hydrolysis is widely accepted as an accurate and simple method for measuring total microbial activity in a range of environmental samples such as soil and sediment [23]. Colorless fluorescein diacetate is hydrolyzed by both free and membrane bound enzymes, releasing a colored end product fluorescein which can be measured by spectrophotometry. However, microbial counts did not always correlate significantly with biological activities [24]. In the case of stored bacteria, long-term dormancy or starvation can lead to low enzymatic activity, even though these cells are viable.

3.2. Petroleum hydrocarbon degradation

In this study we compared the efficacy of biostimulation processes of oil-contaminated soil with that induced by bioagumentation processes. During time-course incubations significant changes of TPH were observed (Fig. 2a). Bioremediation experiments enhanced significantly the biodegradation yields of hydrocarbons. After 12 weeks of biodegradation, TPH concentrations in soil were 21,800, 21,500, 18,300, and 11,400 mg kg^{-1} for treatments A, B, C, and D, respectively. The total removal efficiencies of TPH were 26%, 27%, 38%, and 61% for treatments A, B, C, and D, respectively, during 12 weeks of bioremediation. This result suggested that the addition of bacteria immobilized on peanut hull powder to the soil was the most effective treatment for the hydrocarbon removal, whereas the other treatments displayed a lower efficiency.



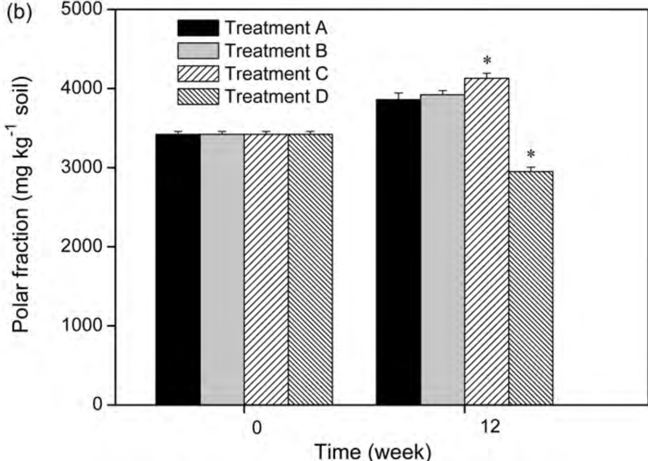


Fig. 2. Changes in concentrations of petroleum fraction during bioremediation. (a) TPH; (b) the polar fraction. Treatment A: soil; treatment B: soil + bacteria solution; treatment C: nutrient + peanut hull powder; treatment D: nutrient + peanut hull powder + immobilized cells. Values in each column labelled with an asterisk (*) indicated significant ($p < 0.05$) differences between treatment A and other treatments at week 12.

Bioaugmentation (treatment B) with an enrichment culture at a cell density of 10^8 cells per g of soil did not enhance the total amount of TPH removed at the end of the treatment period ($p < 0.05$), as compared with biostimulation (treatment A). However, the addition of peanut hull powder (treatments C and D) significantly enhanced TPH removal. One possible explanation for the lack of a stimulatory effect of the inoculum in this study is that nutrition or aeration, other than microbial counts, was the limitation factor of continuous degradation of petroleum hydrocarbons. Using bulking agents can lower the contaminated soil bulk density, increase porosity and oxygen diffusion and help to form water stable aggregate. These enhance the mass transfer rate of water, oxygen, nutrients, hydrocarbons, and microbial activity, and hence biodegradation. Moreover, although we regularly added inorganic solution to soils, nutrition deficiency might still arise during quick biodegradation of petroleum hydrocarbons. The major components of peanut hull are cellulose, hemicellulose, and lignin with small amounts of protein, starch, and lipids. These organic substances can continuously nurture microorganisms in soil during decomposition of peanut hull. It has been reported that the tolerance ability to difficult conditions of immobilized cells was improved due mainly to enhancement modifications of the cell membrane [25,26]. In some cases, microbial metabolism of petroleum hydrocarbons may produce toxic metabolites such as naphthenic acids, which can hamper subsequent biodegradation due to their toxicity that represses microbial metabolism [3]. In this study, immobilization of bacteria might enhance microbial tolerance ability to some inhibitory metabolites during biodegradation.

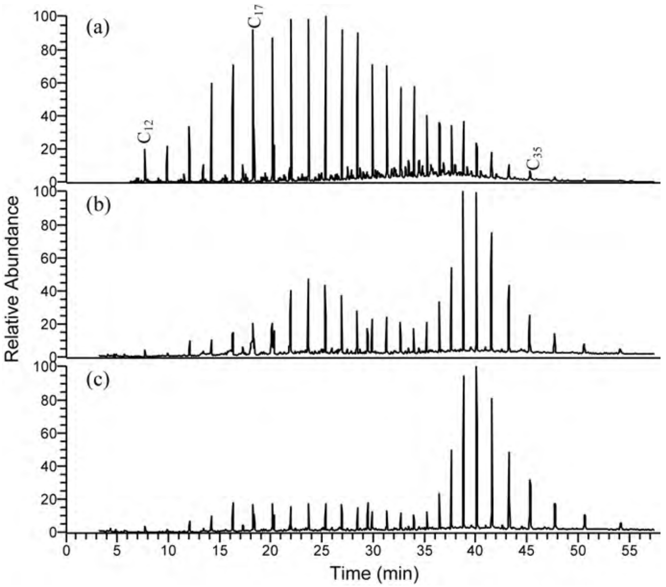


Fig. 3. Gas chromatography fingerprinting of saturate fraction (a) time zero, (b) after 12 weeks of biodegradation from treatment C (nutrient + peanut hull powder), and (c) after 12 weeks of biodegradation from treatment D (nutrient + peanut hull powder + immobilized cells).

As shown in Fig. 2b, there was a real increase in the polar fraction as a result of bioremediation during treatment without immobilized cells, suggested that polar fraction appeared to be resistant to biodegradation. The increase in the polar fraction may be the result of the accumulation of undegraded metabolic byproducts. Polar fractions in crude oil have been shown to be partially or completely resistant to microbial assimilation [27]. A temporary or permanent increase in the polar fraction is often observed in batch liquid cultures as a result of the accumulation of unassimilated metabolites [28]. However, it has been reported that in optimal cultures, a 30% maximum biodegradation of polar compounds can occur [29]. By contrast, in this experiment, the concentration of the polar fraction in soil was decreased from 3420 to 2950 mg kg^{-1} soil during immobilization-based bioremediation (treatment D). Our results suggest that the present application of bioaugmentation combined with immobilization technology can enhance the biodegradation of not only saturates but also of the polar fraction in crude oil.

3.3. GC–MS analysis

Gas chromatography of the saturate fraction from microcosm study has been depicted in Fig. 3, which indicated that oil was degraded better with the help of immobilized consortium. The hydrocarbons involved in the saturate fraction were identified in the range of C_{12} to C_{35} at the initial time of incubation (Fig. 3a). After 12 weeks of biodegradation, the aliphatic fraction analyzed showed that chromatographic profiles of this fraction had a different degradation pattern. Treatments C and D showed a preferable removal of C_{12} to C_{29} compounds, as compared with the relative accumulation of saturates above C_{29} at the end of the incubation period (Fig. 3b and c). Moreover, in treatment D (with added microbial carrier) a slightly different chromatographic profile was observed (Fig. 3c); in this case there was a greater extent of consumption of C_{19} to C_{23} compounds than in treatment C. The hydrocarbon removal regularly occurs on a specific group of hydrocarbons, specificity occurs due to the capacity of the involved microorganisms and

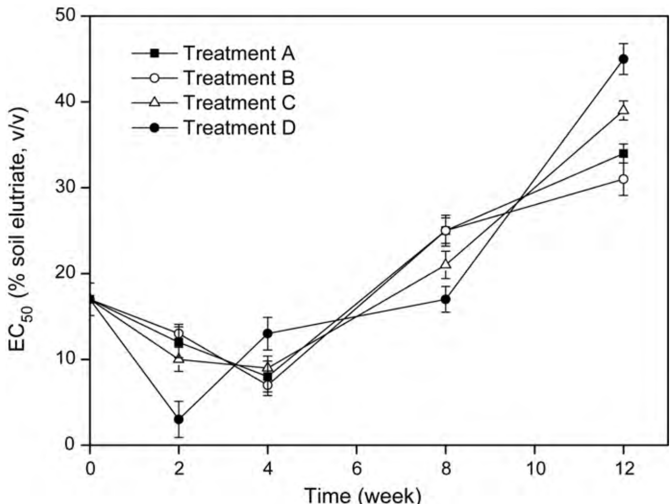


Fig. 4. Microtox EC_{50} values for soils sampled during bioremediation. Data represent EC_{50} values for single microcosms with error bars corresponding to 95% confidence intervals calculated using Microtox Data Capture and Reporting software (Ver. 7.8). Treatment A: soil; treatment B: soil + bacteria solution; treatment C: nutrient + peanut hull powder; treatment D: nutrient + peanut hull powder + immobilized cells.

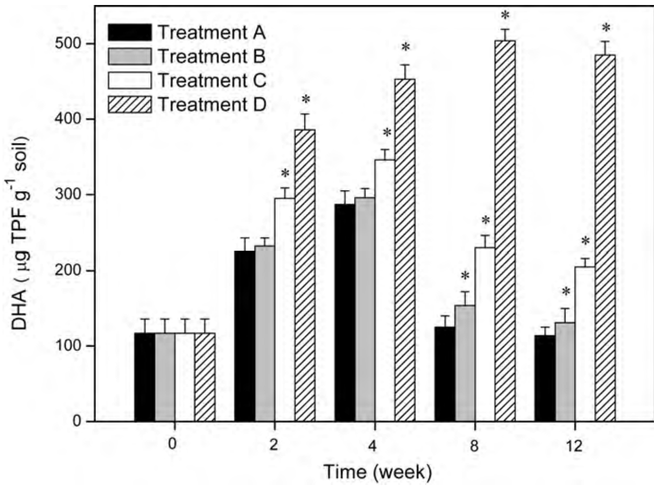


Fig. 5. DHA in soil samples during 12 weeks of bioremediation. Treatment A: soil; treatment B: soil + bacteria solution; treatment C: nutrient + peanut hull powder; treatment D: nutrient + peanut hull powder + immobilized cells. Values in each column labelled with an asterisk (*) indicated significant ($p < 0.05$) differences between treatment A and other treatments in the same sampling time.

according to its degrading enzyme system, including the chemical nature of the hydrocarbons [30]. For alkanes of equal carbon number, the more highly branched alkanes have a somewhat higher recalcitrance to microbial attack. For alkanes of different carbon number, alkanes with more carbon numbers have more isomers. In the present study, the greater removal of C_{19} to C_{23} aliphatic compounds in treatment D is attributed to the characteristics of microorganisms involved and not to the treatment. This is concluded because both treatments (C and D) had the same process. One possible reason of previous situation could be related to the fact that some special consortium was enriched and immobilized onto carrier during previous experiments, as indicated by the broad-scale metabolic specificity of microorganisms during hydrocarbon degradation [31].

3.4. Microtox analysis

Monitoring contaminant levels by analytical methods can indicate removal of contaminants, but such methods do not directly show the effect of residual contaminants and metabolites in the treated soil on ecological systems. Ecotoxicity assessment gives refined information on the changes in soil quality. The end point measured by Microtox is a decrease in light intensity generated by the luminescent bacteria. Microtox analysis was performed over the course of the study to monitor toxicity levels of the bioremediated soil and the results are shown in Fig. 4. Toxicity first increased during the initial phase of biodegradation, but started to decrease after week 4 for treatments A, B, and C (after week 2 in the case of treatment D). Toxicity was higher but lasted for a shorter time by bioaugmentation with immobilized bacteria (treatment D). Higher EC_{50} values at week 12 suggested an overall decrease in soil toxicity for all treatments. Compared with other treatments, significantly lower toxicity in soil ($p < 0.05$) was observed in bioaugmented composting. The enhanced toxicity was interpreted as an increase in contaminant levels and thus, toxicity, in the aqueous extracts. We propose that hydrocarbon degradation reactions generated toxic intermediary metabolites, such as aldehydes. These compounds would typically be more hydrophilic than hydrocarbons and, therefore, more efficiently extracted in aqueous solution [32]. Increased concentrations of toxic compounds in the aqueous extracts would be reflected in higher apparent toxicity of the sample. Subsequent degradation of the intermediary metabolites may be responsible for the reduced toxicity observed at later sampling times.

The Microtox assay provides a rapid, economical toxicity assay that has been used to measure the response of the luminescent bacteria to chemical agents in bulk water, soil, and sediments. The Microtox test appears to be less sensitive and more variable than the earthworm bioassay. The Microtox test was found sensitive to toxic components of crude oil and was used successfully to monitor oil residues toxicity during bioremediation, but appeared less sensitive or too variable in some other circumstances [33].

3.5. 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. Changes in soil DHA during bioremediation are shown in Fig. 5. In all treatments, DHA increased substantially after beginning of the incubation. The highest soil DHA of treatments A, B, and C were observed after 4 weeks of incubation when the highest activity of the inoculated soil occurred at week 8 for treatment D (bioaugmentation with carrier material). From then on, DHA decreased with increasing incubation time. DHA in the soil of treatment D was remarkably higher than that of other soils. The observed increases of DHA after start of treatment can be explained by increased substance conversions and mineralization. One possible reason for the decrease of biological activities at the later stage of bioremediation is the accumulation of inhibiting intermediates and of recalcitrant high-branched aromatics and condensates.

The efficiency of microbial releases for bioremediation processes can be optimized by using carrier materials, which may provide a protective niche to microbial inoculants in soil via provision of a protective surface or pore space creating protective microhabitats [34]. Labana et al. [17] also demonstrated that immobilization of *p*-nitrophenol-degrading bacteria on corncob powder maintained a high degree of viability of cells. Microbial carriers improve the adjacent conditions for inoculated bacteria by protecting them from predation and contaminant toxicity, and therefore enhance microbial activities in soils [35]. In the present study,

Table 2
Elemental composition and structure assignments of oxygenated PAHs identified in oil sampled from treatment D after 12-week bioremediation. These compounds were not found in the oil before bioremediation.

Peak number	Peak location *m*/*z*	Elemental composition	Error (+/−ppm)	Proposed structure	Parent PAH
1	267.08267	$C_{20}H_{12}O_1$	0.6		Benzo[a]pyrene
2	209.05282	$C_{14}H_{10}O_2$	−0.5		Anthracene
3	241.05162	$C_{14}H_{10}O_4$	0.8		Anthracene

peanut hull powder, which was used as carrier material for immobilizing bacteria, is an inexpensive and easily available agricultural byproduct. It has several useful properties such as low-weight high volume, high water holding capacity, and is a good soil conditioner.

3.6. Metabolite identification by ESI FT-ICR MS

The calibrated negative-ion ESI FT-ICR mass spectra of oil samples are presented in Fig. 6. The mass spectra clearly showed that the molecular weight distribution of polar compounds was greatly altered by biodegradation. The weight-average molecular weight was relatively constant at 526 Da for sample a and decreased with biodegradation to 435 Da for sample b. Numerous species of polar compounds containing N, S, and O were identified in these spectra. In this study, we focused on identifying PAH metabolites through use of ultrahigh mass resolution and ultrahigh mass accuracy of ESI FT-ICR MS.

As shown in Table 2, we listed three peaks and their corresponding possible representative structures. Benzo[a]pyrene is the most thoroughly studied PAH for it is one of the most carcinogenic compounds known. The initial step in the metabolism of benzo[a]pyrene involves the multifunctional P-450 enzyme system forming different epoxides through the addition of one oxygen atom across a double bond. The epoxides are short-lived compounds and may rearrange spontaneously to phenols or undergo hydrolysis to dihydrodiols. These products may then be conjugated with glutathione, glucuronic acid or sulfuric acid, to form products that can be excreted by the procaryotic microorganisms [36,37].

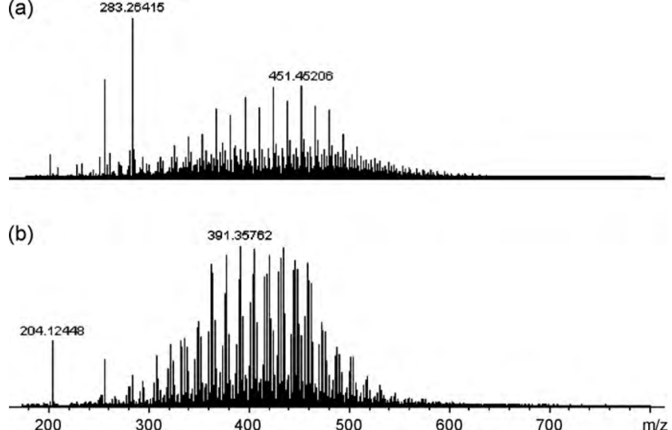


Fig. 6. Broadband negative-ion ESI FT-ICR mass spectra of oil sampled at: (a) before treatment; (b) after 12-week biodegradation in treatment D (nutrient + peanut hull powder + immobilized cells).

The bacterial degradation of anthracene generally begins with a dioxygenase attack on one of the aromatic rings to form a *cis*-dihydrodiol, which is subsequently dehydrated to catechol. Catechol is a key intermediate from which ring cleavage can occur. The aromatic ring is cleaved between the hydroxyl groups. Successive ring degradation may then occur, so that the structure is ultimately degraded to molecules that can enter the central metabolic pathways of the bacteria [36,38,39].

The polar components including metabolites in biodegraded oils are not easily analyzed by GC methods because this information is easily lost because they are not GC-amenable. Moreover, GC cannot provide enough resolving power to separate all components, and a large 'hump' can be seen from GC. 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 precisely estimated by FT-ICR MS without chromatographic fractionation or derivatization of the sample.

4. Conclusions

This study has demonstrated that bioaugmentation with indigenous bacteria immobilized on peanut hull powder enhanced degradation of crude oil under laboratory conditions. Peanut hull powder was chosen as the carrier for immobilizing microorganisms because of its porous structure, large surface area and strong adsorption capability. The use of biocarrier was shown to be a more effective way of removing crude oil compared to the use of free-living bacteria. However, long-term monitoring experiments still need to be performed to further examine application of the method, especially in field scale.

Acknowledgements

This work is supported by Hubei Provincal Department of Education (Project No. B200712002) and China Offshore oil company Ltd. (CNOOC2C2003-012).

References

[1] K. Van Gestel, J. Mergaert, J. Swings, J. Coosemans, J. Ryckeboer, Bioremediation of diesel oil-contaminated soil by composting with biowaste, Environ. Pollut. 125 (2003) 361–368.
[2] R. Xu, A.N.L. Lau, Y.G. Lim, J.P. Obbard, Bioremediation of oil-contaminated sediments on an inter-tidal shoreline using a slow-release fertilizer and chitosan, Mar. Pollut. Bull. 51 (2005) 1062–1070.

[3] M. Lu, Z. Zhang, W. Qiao, X. Wei, Y. Guan, Q. Ma, Y. Guan, Remediation of petroleum-contaminated soil after composting by sequential treatment with Fenton-like oxidation and biodegradation, Bioresour. Technol. 101 (2010) 2106–2113.
[4] R.M. Atlas, Microbial hydrocarbon degradation-bioremediation of oil spills, J. Chem. Technol. Biotechnol. 52 (1991) 149–156.
[5] R.L. Rhykerd, B. Crews, K.J. McInnes, R.W. Weaver, Impact of bulking agents, forced aeration and tillage on remediation of oil-contaminated soil, Bioresour. Technol. 67 (1999) 279–285.
[6] K.S. Jørgensen, J. Puustinen, A.M. Suortti, Bioremediation of petroleum hydrocarbon-contaminated soil by composting in biopiles, Environ. Pollut. 107 (2000) 245–254.
[7] N. Vasudevan, P. Rajaram, Bioremediation of oil sludge-contaminated soil, Environ. Int. 26 (2001) 409–411.
[8] A. Godoy-Faúndez, B. Antizar-Ladislao, L. Reyes-Bozo, A. Camaňo, C. Sáez-Navarrete, Bioremediation of contaminated mixtures of desert mining soil and sawdust with fuel oil by aerated in-vessel composting in the Atacama Region (Chile), J. Hazard. Mater. 151 (2008) 649–657.
[9] I. Gandolfi, M. Sicolo, A. Franzetti, E. Fontanarosa, A. Santagostino, G. Bestetti, Influence of compost amendment on microbial community and ecotoxicity of hydrocarbon-contaminated soils, Bioresour. Technol. 101 (2010) 568–575.
[10] D.E. Ellis, E.J. Lutz, J.M. Odom, R.J. Buchanan Jr., C.L. Bartlett, Bioaugmentation for accelerated in situ anaerobic bioremediation, Environ. Sci. Technol. 34 (2000) 2254–2260.
[11] S. Barathi, N. Vasudevan, Bioremediation of crude oil contaminated soil by bioaugmentation of *Pseudomonas fluorescens* NS1, J. Environ. Sci. Heal. A 38 (2003) 1857–1866.
[12] L. Ruberto, S.C. Vazquez, W.P. Mac Cormack, Effectiveness of the natural bacterial flora, biostimulation and bioaugmentation on the bioremediation of a hydrocarbon contaminated Antarctic soil, Int. Biodeter. Biodegr. 52 (2003) 115–125.
[13] R.J.S. Jacques, B.C. Okeke, F.M. Bento, A.S. Teixeira, M.C.R. Peralba, F.A.O. Camargo, Microbial consortium bioaugmentation of a polycyclic aromatic hydrocarbons contaminated soil, Bioresour. Technol. 99 (2008) 2637–2643.
[14] C.O. Obuekwe, E.M. Al-Muttawa, Self-immobilized bacterial cultures with potential for application as ready-to-use seeds for petroleum bioremediation, Biotechnol. Lett. 23 (2001) 1025–1032.
[15] J.P. Bryant, G.D. Wieland, T. Clausen, P. Kuropat, Interactions of snowshoe hare and feltleaf willow in Alaska, Ecology 66 (1985) 1564–1573.
[16] R.C. Wyndham, J.W. Costerton, Heterotrophic potentials and hydrocarbon potentials of sediment microorganisms within the Athabasca oil sands deposit, Appl. Environ. Microbiol. 41 (1981) 783–790.
[17] S. Labana, G. Pandey, D. Paul, N.K. Sharma, A. Basu, R.K. Jain, Pot and field studies on bioremediation of *p*-Nitrophenol contaminated soil using *Arthrobacter protophormiae* RKJ100, Environ. Sci. Technol. 39 (2005) 3330–3337.
[18] M. Lu, Z. Zhang, S. Sun, Q. Wang, W. Zhong, Enhanced degradation of bioremediation residues in petroleum contaminated-soil using a two-liquid phase bioslurry reactor, Chemosphere 77 (2009) 161–168.
[19] M.H. Huesemann, Guidelines for land-treating petroleum hydrocarbon-contaminated soils, J. Soil Contam. 3 (1994) 299–318.
[20] M. Stemmer, M.H. Gerzabek, E. Kandeler, Invertase and xylanase activity of bulk soil and particle-size fractions during maize straw decomposition, Soil Biol. Biochem. 31 (1999) 9–18.
[21] T.P. Bastow, B.G.K. van Aarssen, D. Lang, Rapid small-scale separation of saturate, aromatic and polar components in petroleum, Org. Geochem. 38 (2007) 1235–1250.
[22] P.Y. Robidoux, P. Gong, M. Sarrazin, G. Bardai, L. Paquet, J. Hawari, C. Dubois, G.I. Sunahara, Toxicity assessment of contaminated soils from an antitank firing range, Ecotox. Environ. Safe. 58 (2004) 300–313.
[23] G. Adam, H. Duncan, Development of a sensitive and rapid method for the measurement of total microbial activity using fluorescein diacetate (FDA) in a range of soils, Soil Biol. Biochem. 33 (2001) 943–951.
[24] D. Miethe, V. Riis, W. Babel, The relationship between the microbial activity of the autochthonous microorganisms of pristine and contaminated soils and their potential for the degradation of mineral oil hydrocarbons, Acta Biotechnol. 14 (1994) 131–140.
[25] M.K. Kim, I. Singleton, C.R. Yin, Z.X. Quan, M. Lee, S.T. Lee, Influence of phenol on the biodegradation of pyridine by freely suspended and immobilized *Pseudomonas putida* MK1, Lett. Appl. Microbiol. 42 (2006) 495–500.
[26] Y. Wang, Y. Tian, B. Han, H. Zhao, J. Bi, B. Cai, Biodegradation of phenol by free and immobilized *Acinetobacter* sp. strain PD12, J. Environ. Sci. 19 (2007) 222–225.
[27] S.J.T. Pollard, M. Whittaker, G.C. Risden, The fate of heavy oil wastes in soil microcosms. I, a performance assessment of biotransformation indices, Sci. Total Environ. 226 (1999) 1–22.
[28] F. Chaillan, A. Le Flèche, E. Bury, Y. Phantavong, P. Grimont, A. Saliot, J. Oudot, Identification and biodegradation potential of tropical aerobic hydrocarbon-degrading microorganisms, Res. Microbiol. 155 (2004) 587–595.
[29] F. Chaillan, C.H. Chaîneau, V. Point, A. Saliot, J. Oudot, Factors inhibiting bioremediation of soil contaminated with weathered oils and drill cuttings, Environ. Pollut. 144 (2006) 255–265.
[30] E. Cervantes-González, L.I. Rojas-Avelizapa, R. Cruz-Camarillo, N.G. Rojas-Avelizapa, Effect of keratinous waste addition on improvement of crude oil hydrocarbon removal by a hydrocarbon-degrading and keratinolytic mixed culture, Int. Biodeter. Biodegr. 63 (2009) 1018–1022.
[31] R.M. Atlas, Microbial degradation of petroleum hydrocarbons: an environmental perspective, Microbiol. Mol. Biol. Rev. 45 (1981) 180–209.
[32] P.J. Vuorinena, M. Keinänena, H. Vuontisjärvia, J. Baršienėb, K. Broegc, L. Förlind, J. Gerckene, J. Kopeckaf, A. Köhlerc, J. Parkkonend, J. Pempkowiakf, D. Schiedek, Use of biliary PAH metabolites as a biomarker of pollution in fish from the Baltic Sea, Mar. Pollut. Bull. 53 (2006) 479–487.
[33] E. Pelletier, D. Delille, B. Delille, Crude oil bioremediation in sub-Antarctic intertidal sediments: chemistry and toxicity of oiled residues, Mar. Environ. Res. 57 (2004) 311–327.
[34] J.A. van Veen, L.S. van Overbeek, J.D. van Elsas, Fate and activity of microorganisms introduced into soil, Microbiol. Mol. Biol. Rev. 61 (1997) 121–135.
[35] J.D. Elsas, C.E. Heijnen, Methods of introduction of bacteria into soil: a review, Biol. Fertil. Soils 10 (1990) 127–133.
[36] C.E. Cerniglia, Biodegradation of polycyclic aromatic hydrocarbons, Biodegradation 3 (1992) 351–368.
[37] S. Lundstedt, Analysis of PAHs and their transformation products in contaminated soil and remedial processes, Ph.D. thesis, Umea University, Solfjädern Offset AB, Sweden, 2003.
[38] J.D. Moody, J.P. Freeman, D.R. Doerge, C.E. Cerniglia, Degradation of phenanthrene and anthracene by cell suspensions of *Mycobacterium* sp. strain PYR-1, Appl. Environ. Microb. 67 (2001) 1476–1483.
[39] R. Van Herwijnen, D. Springael, P. Slot, H.A.J. Govers, J.R. Parsons, Degradation of anthracene by *Mycobacterium* sp. strain LB501T proceeds via a novel pathway, through *o*-phthalic acid, Appl. Environ. Microb. 69 (2003) 186–190.