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ScienceDirect
Journal of Hazardous Materials 150 (2008) 21–26
Journal of Hazardous Materials
www.elsevier.com/locate/jhazmat

Biodegradation of aged polycyclic aromatic hydrocarbons (PAHs) by microbial consortia in soil and slurry phases

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Received 15 December 2006; received in revised form 13 April 2007; accepted 16 April 2007
Available online 20 April 2007

Abstract

Microbial consortia isolated from aged oil-contaminated soil were used to degrade 16 polycyclic aromatic hydrocarbons (15.72 mg kg^{-1}) in soil and slurry phases. The three microbial consortia (bacteria, fungi and bacteria–fungi complex) could degrade polycyclic aromatic hydrocarbons (PAHs), and the highest PAH removals were found in soil and slurry inoculated with fungi (50.1% and 55.4%, respectively). PAHs biodegradation in slurry was lower than in soil for bacteria and bacteria–fungi complex inoculation treatments. Degradation of three- to five-ring PAHs treated by consortia was observed in soil and slurry, and the highest degradation of individual PAHs (anthracene, fluoranthene, and benz(a)anthracene) appeared in soil (45.9–75.5%, 62–83.7% and 64.5–84.5%, respectively) and slurry (46.0–75.8%, 50.2–86.1% and 54.3–85.7%, respectively). Therefore, inoculation of microbial consortia (bacteria, fungi and bacteria–fungi complex) isolated from in situ contaminated soil to degrade PAHs could be considered as a successful method.
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Keywords: PAHs; Microbial consortia; Soil; Slurry; Degradation

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants in the environment and their fates in nature are of great environmental concern due to their potential toxicity, mutagenicity, and carcinogenicity [1]. Although PAHs may undergo chemical oxidation, photolysis, bioaccumulation, volatilization and adsorption, microbial degradation is the major process affecting PAH persistence in nature [2,3]. Recently, bioremediation, which is expected to be an economic and efficient alternative method to other remediation processes such as chemical or physical ones, has been developed as a soil clean-up technique [4,5]. However, the bioavailability of PAHs to microorganisms in soil is a primary limiting factor, which may be resulted from PAH molecule stability and hydrophobicity, soil properties and soil–PAH contact time (i.e., aging) [6–10].

Many researches were conducted on evaluating the degradative ability of microorganisms. Some species of microorganisms have been well documented, which include bacteria (*Mycobacterium* sp., *Pseudomonas* sp., *Sphingomonas*, and *Rhodococcus* sp.) [11–15] and fungi (*Chrysosporium P.*, *Bjerkandera adusta*, *Irpex lacteus* and *Lentinus tigrinus*) [16–18]. Generally, the functions and the strategy of the biodegradation of PAHs are dissimilar between bacteria and fungi. It seems that intracellular oxidation and hydroxylation of PAHs in bacteria are the initial steps preparing ring fission and carbon assimilation, whereas in fungi it is an initial step in detoxification [19]. The release of biosurfactants and extracellular polymeric substances (EPS) in order to increase the availability of PAHs is the primary remediation strategies for the bacteria and fungi [20,21]. Therefore, inoculation of fungal–bacterial co-cultures can improve the degradation of PAHs. The fungal hyphae may act as vectors to mobilize bacteria upon fungal growth, and the creation of voids and provision of continuous surfaces by fungal hyphae could facilitate the displacement of bacteria in soil [22,23]. Boonchan et al. [24] found that inoculation of fungal–bacterial co-cultures into PAH-contaminated soil could significantly improve the degradation of high molecular-weight PAHs and benzo[*a*]pyrene mineralization (53% of added [14C] benzo[*a*]pyrene was recovered as $^{14}CO_2$ in 100 days), compared

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doi:10.1016/j.jhazmat.2007.04.040

with the indigenous microbes and soil amended with only axenic inocula.

In order to remove PAHs, degraders should be able to get enough biomass and ideally mineralize and grow on PAHs as carbon and energy source in contaminated soil [25,26]. The aim of this study was to test the method of using microbial consortia isolated from oil-contaminated soil to degrade PAHs in soil. Differences of PAHs biodegradation in soil and slurry were also investigated to evaluate the availability of microbial consortia for in situ bioremediation.

2. Material and methods

2.1. Soil

Soil samples were collected from the surface layer (0–10 cm) in Shenfu Irrigation Area, Liaoning Province, China (41°50′46″N, 123°44′43″E), which had been contaminated for more than 30 years due to the irrigation with oil–sewage water. The soil had the following physical characteristics: pH 6.25, organic matter 3.58%, sand 20.92%, silt 62.88%, clay 16.2%, water holding capacity 48%, and contained approximately 15.72 mg PAHs (16 EPA-PAHs) kg^{-1} soil (Table 1). Soil samples were air dried in the dark, passed through 2 mm sieve, and stored at 4 °C until use.

2.2. Chemicals

Phenanthrene and Pyrene used in this study were obtained from Supelco Corporation (America), and other chemicals were purchased from Concord Corporation of TianJin, China.

2.3. Preparation of culture and microbial consortia

The method to isolate the PAH-degrading microorganisms was adjusted from the Boonchan et al. [24]. Cultures preparation: (1) A 100 ml medium (0.1% grape sugar, 0.05% yeast extract, 0.05% peptone, 0.5% NaCl, 1.0% NH_4NO_3, 0.5% K_2HPO_4, 0.5% KH_2PO_4, 0.02% $MgSO_4{\cdot}7H_2O$) was added to a 500 ml Erlenmeyer flask and autoclaved at 121 °C for 30 min, and pH was adjusted to 7.2–7.5 for bacteria and 6.0 for fungi. Phenanthrene and pyrene (each 100 mg L^{-1}), which were dissolved previously in acetone were added into the medium. After acetone evaporated completely, 10 g of contaminated soil was added and the culture medium was shaken on a reciprocal shaker (175 rpm) at 28 °C for one week. (2) The enrichment culture (0.05% yeast extract, 0.01% NaCl, 0.1% NH_4NO_3, 0.1% K_2HPO_4, 0.1% KH_2PO_4, 0.02% $MgSO_4{\cdot}7HO_2$, 0.01% $CaCl_2$, 0.002% $FeCl_3$) was prepared by the addition of 10 ml of the prepared culture in (1) instead of 10 g contaminated soil.

The degrading microorganisms consisting of bacteria and fungi were isolated from sole carbon mediums with phenanthrene and pyrene. The bacteria–fungi complex was mixed with bacteria and fungi (1:1, v/v).

In the contaminated soil used, three– and four– ring PAHs accounted for 65% of total 16 EPA-PAHs. Some studies indicated that while the lower-molecular-weight (<four rings) PAHs

Table 1
16 EPA-PAHs concentration (mg kg^{-1} of dry soil) of aged contaminated soil before and after 30-day inoculation[a]

	Initial	Soil				Slurry			
		CK	B	F	B + K	CK	B	F	B + K
Acenaphtene	0.24 ± 0.08	0.57 ± 0.13	0.35 ± 0.10	0.31 ± 0.10	0.27 ± 0.02	0.36 ± 0.10	0.29 ± 0.05	0.19 ± 0.02	0.69 ± 0.28
Acenaphtylene	0.04 ± 0.01	0.40 ± 0.32	0.09 ± 0.04	0.09 ± 0.01	0.07 ± 0.07	0.38 ± 0.36	0.06 ± 0.03	0.14 ± 0.15	0.08 ± 0.06
Napthalene	0.00	0.00	0.00	0.02 ± 0.02	0.03 ± 0.06	0.02 ± 0.03	0.00	0.04 ± 0.08	0.00
Anthracene	4.04 ± 0.16	1.85 ± 0.32	1.44 ± 0.47	1.34 ± 0.13	1.57 ± 0.29	1.75 ± 0.16	1.73 ± 0.46	1.38 ± 0.35	1.82 ± 0.40
Fluorene	0.12 ± 0.04	0.29 ± 0.20	0.12 ± 0.05	0.14 ± 0.06	0.24 ± 0.19	0.37 ± 0.13	0.13 ± 0.05	0.14 ± 0.14	0.19 ± 0.06
Phenanthrene	0.58 ± 0.12	1.44 ± 0.11	0.62 ± 0.28	0.48 ± 0.09	0.63 ± 0.05	1.22 ± 0.38	0.64 ± 0.02	0.54 ± 0.19	0.91 ± 0.20
Chrysene	1.61 ± 0.07	0.81 ± 0.03	0.85 ± 0.13	0.84 ± 0.07	0.73 ± 0.12	0.91 ± 0.08	1.01 ± 0.15	0.75 ± 0.25	0.99 ± 0.26
Fluoranthene	3.57 ± 0.54	1.28 ± 0.11	1.06 ± 0.23	0.97 ± 0.14	0.75 ± 0.24	1.42 ± 0.11	1.36 ± 0.60	0.83 ± 0.34	1.04 ± 0.14
Pyrene	0.45 ± 0.10	0.28 ± 0.11	0.37 ± 0.11	0.32 ± 0.12	0.58 ± 0.09	0.42 ± 0.11	0.37 ± 0.08	0.19 ± 0.11	0.38 ± 0.28
Benz(a)anthracene	1.53 ± 0.16	0.51 ± 0.01	0.44 ± 0.10	0.43 ± 0.08	0.37 ± 0.16	0.63 ± 0.06	0.53 ± 0.12	0.37 ± 0.13	0.51 ± 0.12
Benzo(a)pyrene	0.95 ± 0.09	0.49 ± 0.10	0.52 ± 0.09	0.51 ± 0.02	0.53 ± 0.27	0.60 ± 0.01	0.63 ± 0.12	0.47 ± 0.17	0.61 ± 0.24
Benzo(b)fluoranthene	0.97 ± 0.13	0.47 ± 0.18	0.67 ± 0.11	0.66 ± 0.05	0.56 ± 0.12	0.56 ± 0.15	0.83 ± 0.15	0.58 ± 0.26	0.67 ± 0.40
Benzo(k)fluoranthene	0.85 ± 0.06	0.32 ± 0.04	0.47 ± 0.13	0.44 ± 0.09	0.45 ± 0.06	0.26 ± 0.16	0.64 ± 0.08	0.41 ± 0.17	0.64 ± 0.07
Dibenz(a,h)anthracene	0.12 ± 0.03	1.93 ± 0.44	0.19 ± 0.12	0.28 ± 0.09	0.28 ± 0.08	1.68 ± 0.57	0.26 ± 0.01	0.10 ± 0.18	0.32 ± 0.10
Benzo(ghi)perylene	0.26 ± 0.07	0.70 ± 0.10	0.53 ± 0.10	0.54 ± 0.02	0.44 ± 0.16	1.11 ± 0.40	0.84 ± 0.42	0.44 ± 0.27	0.75 ± 0.47
Indeno(1,2,3-cd)pyrene	0.37 ± 0.09	0.40 ± 0.08	0.50 ± 0.07	0.50 ± 0.04	0.43 ± 0.15	0.48 ± 0.05	0.56 ± 0.12	0.43 ± 0.17	0.57 ± 0.24

[a] Mean values ± S.D. for three replicates; B, bacteria; F, fungi; B + F, bacteria–fungi mixtures; CK, control.

were degraded, five- and six-ring PAHs might also be degraded by co-metabolism with lower-molecular-weight PAHs as substrates, which could stimulate the growth of some specific cells able to degrade five- to six-ring PAHs [27]. Thus, the medium was enriched with phenanthrene and pyrene.

2.4. PAH degradation

To assess the abilities of the microbial consortia (bacteria, fungi, and bacteria–fungi mixtures) to degrade PAHs, experiments were conducted in soil and slurry phases, respectively.

(1) PAHs degradation in soil: Culture tubes (50 ml) containing 5 g of air-dried soil were inoculated with 40% abiosalt medium and 2% of the microbial consortia at room temperature; (2) PAHs degradation in slurry: The slurry samples (soil–water 1:2, W/W) were put into tubes with sterilized tier gauze covered, and inoculated similar to (1). After inoculation, all tubes were shaken in an incubation shaker in the dark at room temperature for 30 days. Distilled water was supplied by weight every three days. Controls (added culture without inoculation) and all treatments were conducted with three replications.

2.5. PAHs analysis

Extraction of PAHs was performed according to Song et al. [28]. After 30-d incubation, 20 ml of dichloromethane was added to each air-dried soil sample in the culture tube, then, samples were extracted in an ultrasonic extractor for 2 h. Slurry was centrifuged at 4000 rpm for 10 min, and the particles were allowed to deposit for 5 min, and 5 ml of supernatant was removed and loaded into a cleanup column with silica gel and anhydrous sodium sulfate (5 ml supernatant was obtained, and passed through column with a silica gel and anhydrous sodium sulfate). Extracts were condensed by evaporation of the dichloromethane under a stream of nitrogen, and remains were dissolved in 1 ml Hexane. The moisture was determined [by ISO 11465:1993] to allow data presented on a dry matter basis.

The concentrations and profiles of PAHs were analyzed by an Agilent 6890(+) gas chromatography (GC), equipped with a flame ionization detector. The capillary column used was a DB-5 (30 m × 0.32 mm i.d. × 0.25 μm film thickness). The initial column temperature of 80 °C for 1 min, 15 °C/min to 255 °C for 1 min, 1 °C/min to 265 °C, and then 2.5 °C/min to 295 °C for 5 min. Temperatures of injector and detector were both 300 °C. The carrier gas was nitrogen at a constant flow rate of 1.0 ml/min [29].

Identification and quantification of 16 PAH compounds were based on matching their retention time with a mixture of PAH and individual PAH standards. The procedural blank was determined by going through the same extraction. The mean recovery of the PAHs was 80.3%. For the biodegradation experiments, the standard curves were linear in the concentration range of 0.01–15 mg L^{-1}.

The percentage of PAHs degradation ($D\%$) was given by the formula: $D\% = 100\,(\mathrm{MI} - \mathrm{MF})\mathrm{MI}^{-1}$, in which MI was the initial concentration of PAHs; MF was the finial concentration in each treatment after 30-d incubation.

2.6. Data analysis

All the data obtained in the study were subjected to statistical analysis of two ways ANOVA, and post hoc Turkey test with SPSS Version 13.0.

3. Results

3.1. PAH biodegradation in soil

The initial and final concentrations of PAHs were shown in Table 1 and Fig. 1. With the exception of two-ring and six-ring PAHs, the degradation of the other three was positive and the range was 4.3–70.2%. The degradation of PAHs in treatments incubated with microbial consortia was 53.9–56.3%, which was 20–25% higher than the control (32.7%). However, there was no significant difference in PAHs degradation between these three treatments ($P < 0.05$). Significant differences were observed in PAHs degradation among the four-ring PAHs, the three-ring and five-ring PAHs ($P < 0.05$), and the highest degradation of individual PAHs were anthracene, fluoranthene, and benz(a)anthracene (45.9–75.5%, 62–83.7% and 64.5–84.5%, respectively).

3.2. PAH biodegradation in slurry

Table 1 and Fig. 2 presented the initial and final concentrations of PAHs in slurry after 30 days incubation. As obtained from the soil, the degradation of total three-ring, four-ring and five-ring PAHs was positive and the range was 9.8–79.6%. The degradation of PAHs among the incubations with bacteria, fungi, and bacteria–fungi mixtures were 44.4–60.5% with 12–28% increment compared with the control (32.1%). Significant differences in PAHs degradation were observed between fungal inocula and the control ($P < 0.05$). The differences in PAHs degradation among these three rings PAHs in slurry were similar to those in soil. The highest degradation of individual PAHs were

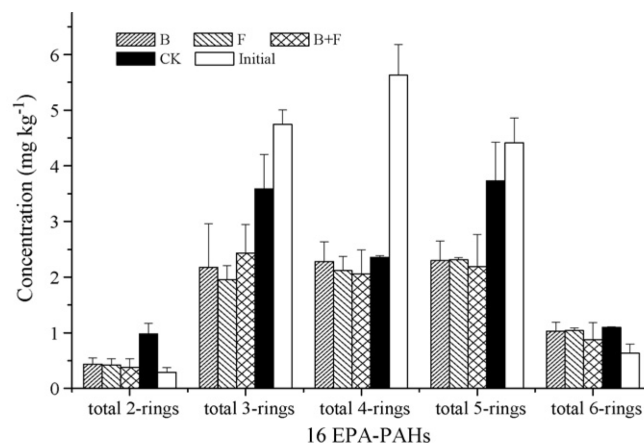

Fig. 1. Concentrations of two- to six-ring PAHs in non-sterile soil before and after 30 days' incubation with bacteria, fungi, or bacteria–fungi mixtures. Mean values ± S.D. for three replicates; B, bacteria; F, fungi; B + F, bacteria–fungi mixtures; CK, control; Initial, initial concentration in soil.

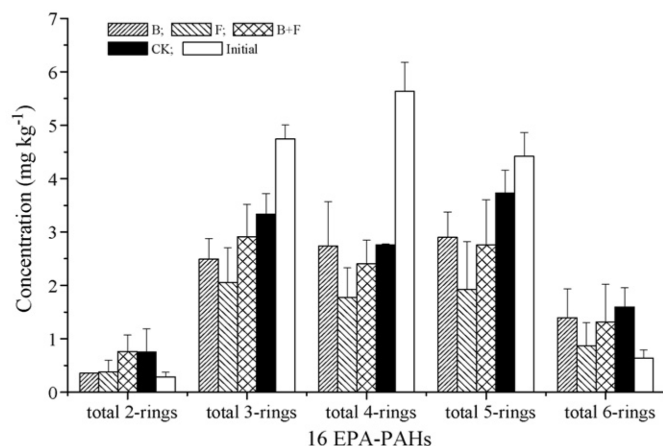

Fig. 2. Concentrations of two- to six-ring PAHs in non-sterile slurry before and after 30 days' incubation with bacteria, fungi, or bacteria–fungi mixtures. Mean values ± S.D. for three replicates; B, bacteria; F, fungi; B + F, bacteria–fungi mixtures; CK, control; Initial, initial concentration in soil.

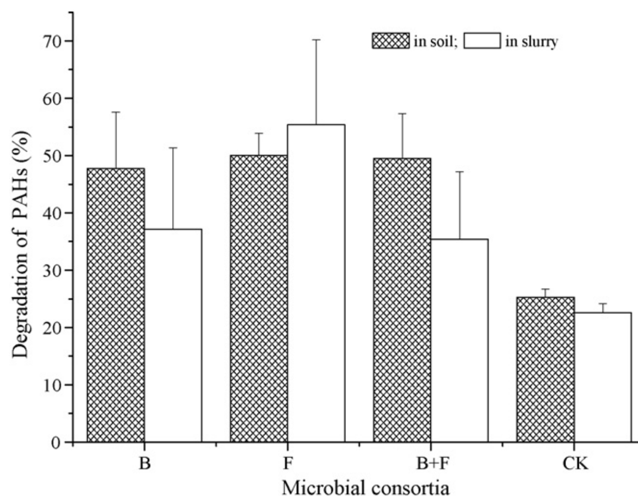

Fig. 3. Degradation of PAHs in soil and slurry by different microbial consortia. Mean values ± S.D. for three replicates; B, bacteria; F, fungi; B + F, bacteria–fungi mixtures; CK, control.

anthracene, fluoranthene, and benz(a)anthracene (46.0–75.8%, 50.2–86.1% and 54.3–85.7%, respectively).

4. Discussion

It was accepted that indigenous microorganisms screened from polluted soils were often more effective to metabolize PAHs than organisms obtained from elsewhere in bioremediation. However, previous studies mainly focused on the use of some identified microbes to degrade single or combined PAH rather than 16 EPA-PAHs in soil or slurry [4,11,14–16,30]. Due to the hydrophobic of PAHs, some biostimulating strategies, including surfactants or nutrients added, were employed to improve the desorption of PAHs and microbial biomass [15,31–33]. Furthermore, the degradation of high-molecular-weight PAHs by combined bacterial and fungal strain could occur in culture [24]. In this assay, non-sterile soil or slurry and microbial consortia isolated from in situ contaminated soil were used so as to improve the practicality of bioremediation.

The microbial consortia isolated from oil-contaminated soil were used directly in non-sterile contaminated soil and slurry, and they enhanced the biodegradation of PAH in this study significantly ($P < 0.05$). The degradation of PAHs was shown in Fig. 3. Compared to those in controls (22.6–25.3%), the degradation of total PAHs in the treatments inoculated with microbial consortia showed a significant increase after 30 days of incubation ($P < 0.05$). Results from the treatments of inoculation with the consortia of fungi presented that about 55.4% of PAHs was removed though there were no statistically significant differences among the treatments. The soil was air-dried before the inoculation of the microbial consortia. This might allow the growth of both indigenous and exogenous microorganisms. The fungi mycelium had a high surface area, which can maximize both mechanical and enzymatic contact with insoluble substrates and invade a larger volume of soil [4,34]. These reasons might explain the high level of PAHs degradation observed with the fungi consortia. The total PAH recoveries (74.7–77.4%) in the controls were lower than those reported by Canet et al. (2004) [35] and Potin et al. (2004) [4], in which 16.7% and 13% (on an average) of the total PAH initially present, respectively, was removed from the untreated soil at the end of a 32-week or 4-week incubation. The depletions of PAHs within microbial inocula in soil and slurry were in agreement with the results of Potin's study, which indicated that the degradation of the 16 EPA-PAHs was 13.6–27.5% in contaminated soils with mycelial inoculum [36].

The biodegradation of low molecular weight (two- and three-ring) PAHs occurred much more rapidly and extensively than that of the high molecular weight (four-, five- and six-ring) hydrocarbons [37,38]. However, high degradation of the latter would have been obtained if some pretreatments had been done. In this study, the degradation of three-ring PAHs was similar to that of five-ring PAHs, and was lower than that of four-ring PAHs. This indicated that, besides chemical pre-oxidation, the particular microbial consortia screening using typical rings contaminants might be one of the other pretreatment methods [39]. The more the initial amount of individual PAHs, the higher the percentage degradation was obtained. This result was consistent with previous findings in which the highest percentage degradation was obtained from benzo(a)pyrene for which the initial concentration was the highest in the soil, thus could be explained by the fact that PAH concentration gradients influenced the mass transfer rate of PAHs to microorganisms and lower the toxicity for aged contaminants [36,40]. These results were especially important for high molecular weight PAHs and could be a real advantage for remediation of aged, contaminated soils.

Some studies reported that slurry reactors were able to substantially increase the rates of contaminants' biodegradation because the availability of contaminants, electron acceptors, nutrients, and other additives to the microbial populations were enhanced in the slurry [41,42]. However, in this study, the degradation of PAHs in slurry was less than that in soil except for the fungal inocula. The degradation of some PAHs in this experiment was much lower than those reported by Zappi et al., in

which the level of naphthalene dropped from 1.1 mg kg^{-1} soil to <0.05 mg kg^{-1} by the 21st day. However, the degradation decreased with an increase in the number of benzene rings [43]. No significant reductions of pyrene and benzo(a)pyrene were observed during the course of that experiment. These discrepancies might be due to limited additives (oxygen, nutrients), soil:water ratios and aged contaminant soil [44,45].

The processes by which organic compounds become increasingly desorption-resistant in soil resulted from sequestration, which originated from the slow diffusion of organic compounds within solid organic matter components, the entrapment within nanopores in soil aggregates, and the formation of strong bonds between organic compounds and the soil [46]. PAHs are preferentially sequestered in a separable, low-density fraction at levels not predictable by the equilibrium partitioning theory. Furthermore, the low-density fraction apparently controls whole-sediment PAHs release [47]. Mineralization of PAHs in aged soils appears to be controlled by mass transfer rather than the biodegradation rate [48]. In this study, with the exception of all the two- and six-ring PAHs, the recoveries of some three- to five-rings PAHs were higher than the initial concentration after 30 days' inoculation. This was agreed with Hwang who presented that higher solution-phase concentrations of pyrene and phenanthrene were maintained for the aged soil than those of the freshly spiked soil before the 18th day [49].

In this study, there was a little difference for two- and six-ring PAHs, the concentrations of which were higher than those of the initial after the inoculation with microbial consortia. It was similar to some studies which used aged contaminated soil in bioremediation experiment [50,51]. Further study need to be done to explain the reason. However, we might account for this according to some studies. For two-ring PAHs, the anomaly might be from the recovery of measuring method used in this study which was only 65–70% for two-ring PAHs. For six-ring PAHs, this anomaly might occur due to the following. First, it was generally assumed that the spiked contaminant had not reached equilibrium in the relatively short contact time used in the laboratory as compared to the contact time of long-time aged contaminants. Less than 15% of the spiked contaminant in the very slow fraction could be observed, whereas for the native contaminant approximately 95% was in this fraction [52]. Second, there were microbial consortia, which could use some PAHs as a sole source of carbon for growth in aged contaminated soil, and the amounts of nutrients might be the limitation on the degradation of PAHs in native environment [4]. When the nutrients were added, microorganism would grow quickly, and improved the degradation of the PAHs. However, the nutrients would be used up very rapidly for cell growth, followed by deficiency of some nutrients especially nitrogen which always had a positive effect on biomineralization. The deficiency of nitrogen was probably remedied by that derived from the humic substances which resulted in some bound residues remobilized [53]. Since the solvents used to determine the extractable contaminants only extracted a small amount of the soil organic matters (SOM). The sorption or sequestration of six-ring PAHs might be desorped from the humic and fulvic fraction in aged contaminated soil [54]. An increase in the extractability of the previously bound residues that were independent of the presence of earthworms was also reported by Gevao et al. [55].

5. Conclusions

The results obtained in the present study showed that the inoculation of microbial consortia (bacteria, fungi and bacteria–fungi mixtures) to degrade PAHs in oil-contaminated soil could be considered as a potential method. The highest PAH removal was observed in the inoculation with fungal consortia, both in the soil and in the slurry. Similar degradation of three- to five-ring PAHs among the microbial consortia might have resulted from the phenanthrene and pyrene culture and the high concentration of anthracene, fluoranthene, and benz(a)anthracene in the polluted medium. Therefore, it was an alternative method to use microbial consortia isolated from contaminated soil to remediate the original contaminated soil.

Acknowledgements

This research was supported by funds provided by National Basic Research Program of China ("973" Program), National Natural Science Foundation of China (20337010) and Postdoctoral Foundation of Liaoning Province.

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