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Biodegradation of the low concentration of polycyclic aromatic hydrocarbons in soil by microbial consortium during incubation

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ABSTRACT

The biodegradation of polycyclic aromatic hydrocarbons (PAHs) (8.15 mg PAHs kg^{-1} soil) in aged contaminated soil by isolated microbial consortium (five fungi and three bacteria) during the incubation of 64 d is reported. The applied treatments were: (1) biodegradation by adding microbial consortium in sterile soils (BM); (2) biodegradation by adding microbial consortium in non-sterile soils (BMN); and (3) biodegradation by *in situ* "natural" microbes in non-sterile soils (BNN). The fungi in BM and BMN soils grew rapidly 0–4 d during the incubation and then reached a relative equilibrium. In contrast the fungi in BNN soil remained at a constant level for the entire time. Comparison with the fungi, the bacteria in BNN soils grew rapidly during the incubation 0–2 d and then reached a relative equilibrium, and those in BM and BMN soils grew slowly during the incubation of 64 d. After 64 d of incubation, the PAH biodegradations were 35%, 40.7% and 41.3% in BNN, BMN and BM, respectively. The significant release of sequestrated PAHs in aged contaminated soil was observed in this experiment, especially in the BM soil. Therefore, although bioaugmentation of introduced microbial consortium increased significantly the biodegradation of PAHs in aged contaminated soil with low PAH concentration, the creation of optimum of the environmental situation might be the best way to use bioremediation successfully in the field.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are an important class of environmental contaminants because some of them are toxic, mutagenic and resist biodegradation [1]. Possible fates of PAHs released into the environment include volatilization, photooxidation, chemical oxidation, bioaccumulation, and adsorption on soil particles [2]. Soil clean-up may be accomplished using different remediation technologies. Among these, bioremediation is an effective and low-cost alternative that has garnered widespread use [3].

Two processes have been found to increase the activity of microorganisms during bioremediation: biostimulation and bioaugmentation. Biostimulation involves the addition of nutrients and/or a terminal electron acceptor to increase the scant activity of indigenous microbial populations. Bioaugmentation involves the addition of external microbial strains (indigenous or exogenous) which have the ability to degrade the target toxic molecules [4]. The added specific PAHs degrader, which has a competitive capacity to become dominant species with indigenous microbial strains or grow simultaneously with indigenous microbial strains, may greatly increase the rate of PAHs biodegradation [5,6]. Numerous studies have focused on this latter process of biodegradation and have reported some useful species of microorganisms including bacteria (*Mycobacterium* sp., *Pseudomonas* sp., and *Sphingomonas* sp.) [7–9] and fungi (*Chrysosporium* P., *Bjerkandera adusta*, *Irpex lacteus*, *Agrocybe* sp. CU-43, and *Lentinus tigrinus*) [10–12]. Uyttebroek et al. [13] reported that the combined action of an introduced enriched consortium and indigenous microorganisms could degrade fluoranthene and phenanthrene to a greater extent than either of them alone. Hamdi et al. [14] found that adding aged PAH-contaminated soil containing activated indigenous degraders produced higher PAH dissipation rates than those observed in unamended PAH-spiked soils (3000 mg PAHs kg^{-1} dry soil), especially for anthrance and pyrene (>96%).

However, the application of bioaugmentation with microbial consortium is comparatively less studied in the long-term contaminated soils with low PAH concentration (i.e., total 16 EPA-PAH concentration below 10 mg PAHs kg^{-1} [15,16]). Consequently the

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Table 1
Concentrations of PAHs in contaminated soil units: mg kg^{-1}.

Name	Concentration	
	Non-sterile soil	Sterile soil
Acenaphthene	0.62 ± 0.09	1.09 ± 0.11
Acenaphthylene	0.07 ± 0.05	0.13 ± 0.06
Anthracene	1.20 ± 0.11	1.20 ± 0.05
Naphthalene	–	–
Fluorene	1.00 ± 0.12	0.92 ± 0.28
Phenanthrene	0.64 ± 0.09	0.50 ± 0.05
Chrysene	0.68 ± 0.02	0.75 ± 0.04
Fluoranthene	0.22 ± 0.10	0.24 ± 0.11
Pyrene	0.47 ± 0.14	0.48 ± 0.12
Benz(a)anthracene	0.42 ± 0.02	0.44 ± 0.15
Benzo(a)pyrene	0.56 ± 0.03	0.54 ± 0.06
Benzo(b)fluoranthene	0.69 ± 0.05	0.60 ± 0.09
Benzo(k)fluoranthene	0.47 ± 0.09	0.48 ± 0.10
Dibenz(a,h)anthracene	0.16 ± 0.07	–
Benzo(g,h,i)perylene	0.47 ± 0.03	0.45 ± 0.02
Indeno(1,2,3-cd)pyrene	0.48 ± 0.03	0.51 ± 0.08
Total	8.15 ± 0.07	8.33 ± 0.09

(–) Not detected.

aims of this study are to (1) evaluate the ability of previously isolated strains of bacteria and fungi to degrade PAHs in aged contaminated soil with low PAH concentration; (2) identify the changes of the numbers of bacteria and fungi in aged contaminated soil during biodegradation.

2. Materials and methods

2.1. Soil

Soil samples were collected from the surface layer (0–20 cm) in Shenfu Irrigation Area (41°50′30″N, 123°44′7″E), Liaoning Province, China, which had been contaminated for more than 50 years due to the irrigation with oil–sewage water. The soil had the following physico-chemical characteristics: pH 6.25, organic matter 5.18%, sand 61.42%, silt 28.53%, clay 10.04%, water holding capacity 48%, and contained approximately 8.15 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. Some of these soils were autoclaved three times at 121 °C for 1.5 h at the beginning of the experiment.

2.2. Chemicals

16 EPA-PAHs used in this study were obtained from Supelco Corporation (America), and other chemicals were purchased from Concord Corporation of TianJin, China.

2.3. Experimental design

2.3.1. Preparation of culture and microbial consortium

A potent PAHs degrading microbial consortium added in contaminated soils has been isolated previously from PAH-contaminated soil. This consortium is composed of five fungi (*Phanerochaete chrysosporium*, *Cuuninghamella* sp., *Alternaria alternata* (Fr.) Keissler, *Penicillium chrysogenum*, and *Aspergillus niger*) and three bacteria (*Bacillus* sp., *Zoogloea* sp., and *Flavobacterium* sp.) [17,18]. Cultures were maintained on nutrient broth agar (NBA) slants for bacteria and potato dextrose agar (PDA) slants for fungi at 0–4 °C and subcultured at 28 °C for 24 h for further use. The subculture medium (0.05% yeast extract, 0.01% NaCl, 0.1% NH_4NO_3, 0.1% K_2HPO_4, 0.1% KH_2PO_4, 0.02% $MgSO_4{\cdot}7H_2O$, 0.01% $CaCl_2$, and 0.002% $FeCl_3$) with subcultured microorganism consortium mixing equal proportions of pure microorganism cultures was shaken on a reciprocal shaker (175 rpm) at 28 °C for one week [19].

2.3.2. PAHs degradation

To assess the efficacy of microbial consortium in PAH removal, the following treatments were applied to the aged PAH-contaminated soil: (1) biodegradation by added microbial consortium in the sterile soils (BM); (2) biodegradation by added microbial consortium in the non-sterile soils (BMN); and (3) biodegradation by nature microbe in the non-sterile soils (BNN). Culture tubes (50 ml) containing 15 g of air-dried soil were inoculated with 40% (v/w) sterile distilled water (the best moisture for the growth of the microbial consortium) and 5% of the microbial consortium (5% sterile culture medium in the third treatment) in sterile chamber. All tubes closed with sterile cotton plugs were cultured at 28 °C for 64 d. Sterile distilled water was supplied by weight every day. All treatments were conducted with three replications.

2.4. Soil sampling and analytical methods

2.4.1. Soil sampling

Soil samples from each treatment were collected after 0, 2, 4, 8, 16, 32 and 64 d. 10 g wet soil from collected sample was used to analyze the microbial numbers immediately, and the air-dried residue was used to analyze the PAH concentration as described below.

2.4.2. PAHs analysis

Extraction of PAHs was performed according to Song et al. [15]: 20 ml of dichloromethane was added to each air-dried soil sample in the culture tube, then, samples were extracted by sonication 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 clean-up 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 temperature program comprised 80 °C for 1 min, 15 °C min^{-1} to 255 °C for 1 min, 1 °C min^{-1} to 265 °C, and then 2.5 °C min^{-1} to 295 °C for 5 min. Temperatures of both injector and detector were 300 °C. The carrier gas was nitrogen at a constant flow rate of 1.0 ml min^{-1} [20].

Identification and quantification of 16 EPA-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}.

2.4.3. Microbial numbers

For soil samples of 0–32 d, the isolation and enumeration of soil microorganisms were performed using the plate-count techniques with nutrient broth agar (NBA) for bacteria and potato dextrose agar (PDA) for fungi. Aqueous suspensions of the microbial population of 10 g soil sample were serially diluted. Plates were incubated at 28 °C for 2 or 3 d prior to counting colony forming units (CFU) [21].

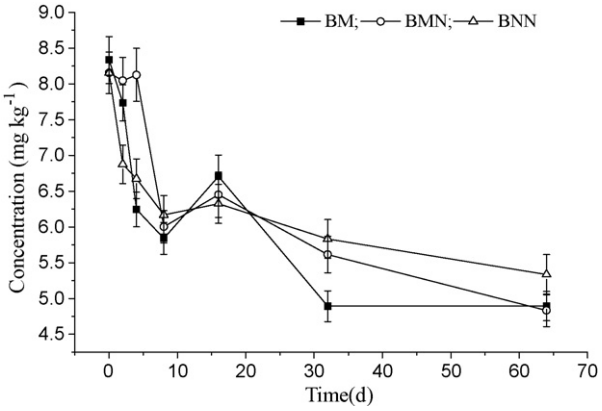

Fig. 1. Concentrations of PAHs during biodegradation in aged PAH-contaminated soils. BM, sterile soils inoculated with microbial consortium; BMN, non-sterile soils inoculated with microbial consortium; BNN, non-sterile soils.

2.5. Data analysis

All the data obtained in the study were subjected to statistical analysis of one way ANOVA, and post hoc Tukey's test with SPSS Version 13.0.

The percentage of PAHs biodegradation ($D\%$) was given by the formula: $D\% = 100(MI - MF)MI^{-1}$, in which MI was the initial concentration of PAHs; MF was the concentration at calculated time.

3. Results

3.1. PAH biodegradation in soil

The concentrations of PAHs in aged PAH-contaminated soils during biodegradation were presented in Fig. 1. The initial PAH concentration (8.33 mg kg^{-1}) in sterile soil was higher than that in non-sterile soil (8.15 mg kg^{-1}) in this study. In contrast, the study of Mueller and Shann [22] found that there were no significant differences in total PAH concentration before and after sterilization in soil. After inoculation, PAHs were rapidly degraded from soils of three treatments in the first 8 d, in which period 25–30% of PAHs were biodegraded for all treatments, then followed by a slower decreasing. No significant difference existed between BM and BMN soils, in both of soils only 41.3% and 40.7% of PAHs were biodegraded, respectively ($P < 0.05$), at 64 d after inoculation. The PAH concentration in BNN soil was significantly higher than that in other two treatments at 64 d after inoculation ($P < 0.05$). Notably, the degradation of PAHs in BMN soil was not significant as compared to BM and BNN soils from 0 to 4 d. There was a significant increase for PAH concentrations in BM soil compared to BMN and BNN from 8 to 16 d ($P < 0.05$).

3.2. Changes of the microbial community

Fig. 2 shows the numbers of fungi and bacteria in aged PAH-contaminated soils over the process of 32 d biodegradation. The 32 d of incubation selected was from references in which the biodegradation experiment of PAHs was usually in one month [19,23]. Compared to those in BNN soil, the numbers of fungi and bacteria had a similar trend during the biodegradation except those in BM soil on the 2 d. The indigenous fungi detected in BNN soil remained approximately at a constant level (7.13×10^2 CFU g^{-1} of soils) throughout the experiment. The numbers of fungi in BM soil were higher than those in BMN soil after day 4 and reached 5.6×10^4 CFU g^{-1} of soils after 32 d incubation. The indigenous bacteria in BNN soil increased rapidly after inoculation and remained at a constant level (4.9×10^6 CFU g^{-1} of soils) from 2 to 8 d, and then decreased before relative equilibrium was arrived. The numbers of bacteria in BM and BMN soils began to decrease when they reached peak values (1.7×10^7 and 1.9×10^7 CFU g^{-1} of soils) on 4 and 2 d, and increased again after 16 and 8 d, respectively. By the 32 d incubation experiment the numbers of bacteria in BM, BMN and BNN soils were 1.7×10^7, 1.3×10^7 and 3.4×10^6 CFU g^{-1} of soils, respectively.

The microflora in indigenous soil needed about 20–50 d to repopulate after autoclaving soil, because soil microorganisms could not be completely destroyed [24]. The fungi in BM and BMN soils still exhibited significant growth just in the first 4 d incubation, which was similar to the study of Andersson et al. [25]. The autoclaving process facilitated the growth of the inoculated fungi in two ways. Firstly, the autoclaving process could kill most of the indigenous microorganisms, which benefits the inoculated fungi to compete and survive in the soil. Secondly, the autoclaving process could modify the physical/chemical environment in the soil, leading to an increase in the concentration of soluble nutrients and organic matter [26]. However, the indigenous fungi in BNN soil remained approximately at a constant level, which might be explained by the inability of the fungi indigenous to compete with the indigenous bacteria, or by the inexistence or inactivity of the necessary extracellular enzymes for fungi growth [27].

4. Discussion

Although some species of microorganisms able to enhance PAH degradation in soils have been well documented [9,10,28],

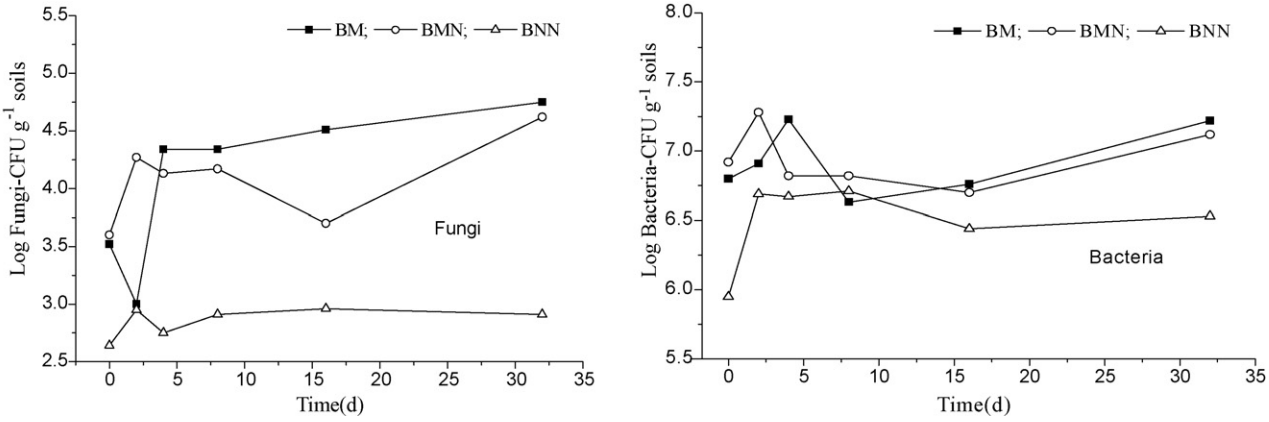

Fig. 2. Microorganism numbers during biodegradation in aged PAH-contaminated soils. BM, sterile soils inoculated with microbial consortium; BMN, non-sterile soils inoculated with microbial consortium; BNN, non-sterile soils.

a few successful examples in field-scale bioaugmentation was reported, because of the catabolic properties and survival ability of introduced microorganisms in the target environment [29]. In this study, the microbial consortium isolated from aged PAH-contaminated soil was used to evaluate the bioaugmentation efficiency of introduced microorganisms in the long-term contaminated soil with low PAH concentration in order to reasonably arrange the inoculation frequency and optimize the cultivation system *in situ*.

It is accepted that indigenous microorganisms in aged contaminated soils are able to effectively metabolize PAHs. However, *in situ* environmental conditions such as nutrient, water content, temperature and aeration might limit PAHs degradation. The soil in this study had been continuously irrigated by oil sewage during a period of almost 50 years, and then ceased for additional 10 years prior to this study being conducted.

Therefore, the residue of low level PAH concentration (8.16 mg PAHs (16 EPA-PAHs) kg^{-1} soil) detected was not due to the lack of the PAH degrader, but due to the environmental conditions. This conclusion is substantiated by finding that 35% of PAHs were degraded in non-sterile soil after 64 d incubation in optimal conditions (given nutrition, fitful moisture and temperature) (Fig. 1). Cunliffe and Kertesz [30] also reported that 21% of initial PAH concentration (5981 $\mu g\, g^{-1}$) was decreased in the absence of strain B1 after 30 d, which was lower than the 30% degradation of PAHs in this study (Fig. 1), and higher than that (13%) reported by Potin et al. [31] during the same period. Compared to 25% degradation in our former study [19], the PAH degradation in this study in the non-sterile soil increased almost 3%, which could be explained by the different initial concentration of PAHs and/or the optimal temperature and moisture applied in this study. Therefore, these results show how very important it is to improve the environmental conditions of microorganisms in aged contaminated soil with low PAH concentration in order to implement an *in situ* bioaugmentation program successfully.

Although the indigenous microorganisms in aged contaminated soil could degrade PAHs significantly, this study still showed that the introduced microbial consortium as an agent in the bioaugmentation had the capability to significantly enhance PAH biodegradation (41.3%) in soils, especially after 30 d incubation ($P < 0.05$). The PAH biodegradation in the treatments (BM, BMN) inoculated microbial consortium was significantly higher than that in BNN. The PAH degradation in BM soil was the highest among the three treatments (in the period 16–32 d) due to the highest increase of PAH concentration during the incubation from 8 to 16 d (Fig. 1). The increase of PAH concentrations during the biodegradation in aged contaminated soil had also been reported in some other studies [19,32]. Usually, the bioavailability of aged contaminants in soil would decrease with increasing aging time [33,34], and mineralization of PAHs in aged soils appeared to be controlled by the increase of non-bioavailable fraction [6,35]. However, the contaminants in non-bioavailable fraction might be released during the biodegradation and could affect ecological health potentially [36]. The increase in the extractability of the previously bound residues had been reported by Gevao et al. [37]. Li et al. [19] also reported that the increased concentrations of 6-ring PAHs in aged PAH-contaminated soil occurred compared to 3–5 rings PAHs during the 32 d incubation. Fig. 3 presents the concentration of PAHs in aged contaminated soil during 8–16 d incubation in this study. The concentrations of 2–4 rings PAHs increased by 24%, 22% and 20% in BM, BMN and BNN soil, respectively. The concentrations of 5 and 6 rings PAHs in all the treatments decreased significantly except the 5-ring PAHs in BM soils. The changes of 3–6 rings PAH concentrations were dissimilar to those of some studies [28,38]. The reasons might be the different growth conditions of microorganisms and the different initial concentration of PAHs in aged contaminated soils. Therefore,

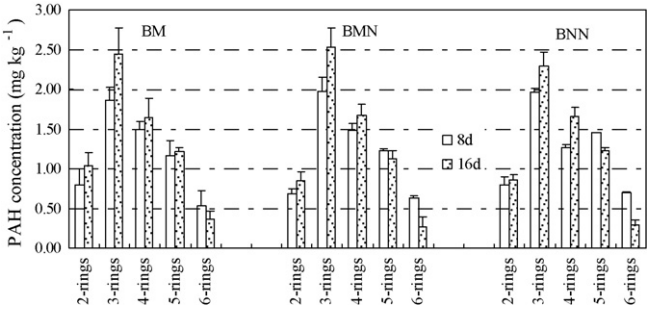

Fig. 3. Concentrations of PAHs in aged contaminated soils during 8–16 d incubation. BM, sterile soils inoculated with microbial consortium; BMN, non-sterile soils inoculated with microbial consortium; BNN, non-sterile soils.

the release of sequestrated contaminants might be very important during the bioremediation of aged contaminated soil.

For different rings PAHs, the molecular weights substantially affected their biodegradation. The biodegradation of 5–6 rings PAHs was significantly higher than that of 2–4 rings PAHs especially in non-sterile soil (Fig. 4). These results were different from other studies, in which the higher rings of PAHs were, the lower degradation was [39]. The reason might be explained in two ways. Firstly, even though many studies had shown that the sequestrated contaminants in soil were unavailable to the microorganisms due to the adsorption of organic matters and sequestration of soil structures [40]. It was also known that sterilization could alter soil structure and properties [26], which resulted in some PAHs released and some entrapped (sequestrated) due to the appearance of new micropores [41,42]. This might be supported by the increase of PAH concentration after the sterilization in this study (Fig. 1). Secondly, the composition of PAHs contacting with microorganisms was different in different treatments. The growth characteristics of fungi in sterile soil and of bacterial in non-sterile soil indicated the difference of carbon and energy sources of microorganisms among these treatments in this study. The concentration and ring structure of PAHs present *in situ* had appreciable effects on microbial community structure in soils [36]. Generally, the easily bioavailable PAHs would be used firstly by microorganism in soil. Released 2-ring PAHs during soil incubation in BM could stimulate the mycelial growth of fungi, which might therefore penetrate contaminated soil to reach PAHs and subsequently enhance the contact between insoluble compounds and fungi [43]. Compared to the sterile soil in this study, the contaminants contacted easily by microorganisms in non-sterile soil might be composed of the high rings PAHs due to the long exposure to the microorganisms, and might stimulate the growth of bacteria which needs to be examined in further studies. The presence of particular microbial community structures might be highly significant in determining whether *in situ* bioremediation strategies would be successful at a practical level. Thereby, it was

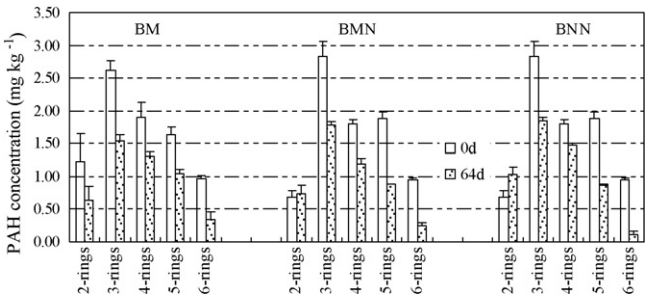

Fig. 4. PAH concentrations in aged contaminated soils at the initial and end of 64 d incubation. BM, sterile soils inoculated with microbial consortium; BMN, non-sterile soils inoculated with microbial consortium; BNN, non-sterile soils.

very important to define the effective microbial community on PAH degradation in low-level contaminated soil.

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