Original Article

Impact of Anthracene Addition on Microbial Community Structure in Soil Microcosms from Contaminated and Uncontaminated Sites*

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Abstract

Objective This paper aims to investigate the impact of anthracene addition on microbial community in agricultural soil irrigated with tap water or reclaimed wastewater.

Methods The changes of microbial community were characterized by terminal restriction fragment length polymorphism in combination with 16S rRNA gene clone library analysis.

Results A significant change in microbial community composition was observed during the biodegradation of anthracene, with dominantly enriched members from the genus *Methylophilus*.

Conclusion This work might be useful for developing techniques for the isolation of novel putative PAH degrader.

Key words: Biodegradation; Microbial community; *Methylophilus*; Reclaimed wastewater; Polycyclic aromatic hydrocarbons (PAHs)

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INTRODUCTION

There has been an increasing interest over the past decades in wastewater reclamation as an additional source of water supply in many countries, particularly in arid or semi-arid regions. Reclaimed wastewater has been used for agricultural irrigation, ground-water recharge, car washing, toilet flushing, urban lawn watering and recreational amenities, and road cleaning, etc[1-2]. Agricultural irrigation is the major user in many regions where wastewater is reused[1-2]. Impacts of land application on public health have recently drawn increasing attention. In some areas, a high level of polycyclic aromatic hydrocarbons (PAHs) has been detected and associated with land application of reclaimed wastewater[3-5].

Microbial degradation has been known to be the primary process for removing and eliminating PAHs from contaminated sites[6-7]. The impact of contaminants on bacterial communities is dependent on the pollution history of contaminated sites[8-10]. Although many studies have mainly focused on the composition of bacterial communities in contaminated sites or on its changes during the bioremediation process, few investigations have been conducted to assess the impact of PAH addition[10-11]. Moreover, previous works concerning the impact of the reclaimed wastewater application on bacterial communities in soils have usually neglected its association with PAHs[12-13]. For the reclaimed wastewater application, the impacts of PAH addition on bacterial communities in agricultural soils are still poorly understood. Additionally, as reclaimed wastewater containing PAHs will be applied to agricultural irrigation in a large scale in many regions (e.g. Chinese northern regions), it is also necessary to explore the impact of PAH addition

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on microbial ecosystems in pristine soils. However, information in this aspect is still lacking.

Anthracene has a high pollution potential and causes health risks. It is commonly studied together with the issues of PAH degradation[14]. In this study, the impact of anthracene addition on microbial communities was investigated for soil irrigated with tap water or reclaimed wastewater. The changes of microbial communities were characterized by terminal restriction fragment length polymorphism (TRFLP) coupled with 16S rRNA gene clone library analysis.

MATERIALS AND METHODS

Microcosm Experiment Set-up

Soils were collected from two different sites. Site 1 has received four-year irrigation with reclaimed wastewater, but Site 2 was irrigated with tap water. The soils had no link to exposure of any other known source of PAH contamination except that the soil from Site 1 was contaminated by reclaimed wastewater. The preliminary research indicated that the soil from Site 2 did not contain detectable PAH species regulated by the United States Environmental Protection Agency, while some PAH species including anthracene (less than 10 μg/kg) were detected in the soil from Site 1 (data not shown). After the soil samples (each 10 kg) were collected, they were dried, homogenized, sieved through a 0.18-mm screen, fully mixed and stored at 4 °C until use.

Anthracene (99%, J&K China Chemical) dissolved in acetone was added to empty microcosm chambers (150 mL serum bottle) with 200 μg anthracene in each chamber. After the acetone evaporated, 2 g (dry weight) soil was added to each microcosm along with 10 mL phosphate buffered mineral media, as previously described[15]. The bottles were then sealed with rubber stoppers and aluminum seals to retain anthracene in the microcosms. The microcosms were incubated at 25 °C and usually sacrificed at 5-d interval to measure the concentrations of anthracene in solid phase. For every set of measurements, two replicate microcosms were used for the anthracene analysis and the other two for soil DNA extraction.

Anthracene Extraction and Analysis

The water-soil mixture in sacrificed microcosms was transferred to a 100-mL centrifuge tube. After centrifugation at 5 000 rpm for 10 min, the supernatant was discarded. The moist soil retained in tube was collected and dried with a freeze drier. 1 g dry soils were extracted three times with 10 mL acetone, by using a 300 W ultrasonic processor. The mixture was vigorously shaken and then centrifuged at 5 000 rpm for 10 min. 0.2 mL of the supernatant was collected into a GC vial and diluted by 0.8 mL of methanol. The mixture was filtered with a 0.45-μm syringe filter. The anthracene analysis was conducted according to the standard method[16].

TRFLP Analysis

DNA was extracted from the duplicate microcosm samples by using the UltraClean DNA extraction kit (Mobio Laboratories) following the manufacturer's instructions. Duplicate samples were not pooled, and the entire analysis for each sample was carried out individually. Bacterial 16S rRNA genes were amplified by using eubacterial primers 27F-FAM (5'-GAGTTTGATCMTGGCTCAG-3', 5' end-labeled with carboxyfluorescine) and 1492R (5'-GGTTACCTTGTTACGACTT-3') with the following PCR program: 94 °C (5 min); 94 °C (30 s); 55 °C (30 s); 72 °C (1.5 min) (30 cycles); and 72 °C (5 min)[17]. PCR products were purified with QIA quick PCR purification kit (Qiagen Inc., German), following the manufacturer's instructions, and digested with *Hae*III. Additional digests (*Hha*I, *Msp*I) for TRFLP analyses were also conducted to correlate the TRFLP fragment lengths to the in silico cut sites of the cloned 16S rRNA gene sequences[18]. The fragment pattern was detected with an ABI 3 730 DNA Analyzer (Applied Biosystems) set at the Genescan mode.

The TRFLP profile was standardized according to the previous work[19]. Richness (*S*) equaled to the total number of distinct terminal fragments in a profile. The Shannon diversity index (*H*) and evenness (*E*) were calculated[19-21]. Bray-Curtis similarity index was calculated by using PRIMER 5.0 software to evaluate the compositional similarity for microcosm samples[22].

Cloning and Sequencing

The DNA templates used for constructing clone libraries were the same DNA preparations from which 16S rRNA genes for TRFLP analysis were amplified. The clone libraries were constructed according to the previous work[19]. Sequences were checked for chimeras with CHECK_CHIMERA software of Ribosomal Database Project II[23]. All clones displaying chimeric profiles were discarded without any further analysis. 67- 85 clones from each sample were obtained. The partial 16S rRNA gene

sequences determined in this study were deposited with GenBank under accession numbers HQ011509-HQ011824. The partial 16S rRNA gene sequences were compared with reported sequences in public databases by using NCBI Blast. Sequences showing highest matches in the Blast search were obtained from GenBank database.Taxonomic identity of the dominant fragment was determined[24].

RESULTS

Biodegradation

Anthracene concentration in microcosms declined significantly during the experiment, while in the autoclaved controls, the percentages of decline were limited only to 7%-27%, which confirmed existence of a biological removal mechanism (Figure 1). For the microcosm constructed with contaminated soil from Site 1, the remaining anthracene on Day 30 was only 35.6% of that on Day 0. On Day 32, a nearly complete degradation was observed. In contrast, relatively less biodegradation (only about 30%) was observed on Day 30 in the microcosm constructed with uncontaminated soil from Site 2. The remaining anthracene was 38% on Day 40 and 15.6% on Day 45. These results were in consistent with previous studies showing that prior exposure to PAHs could increase the rate and extent of PAH degradation[10,25].

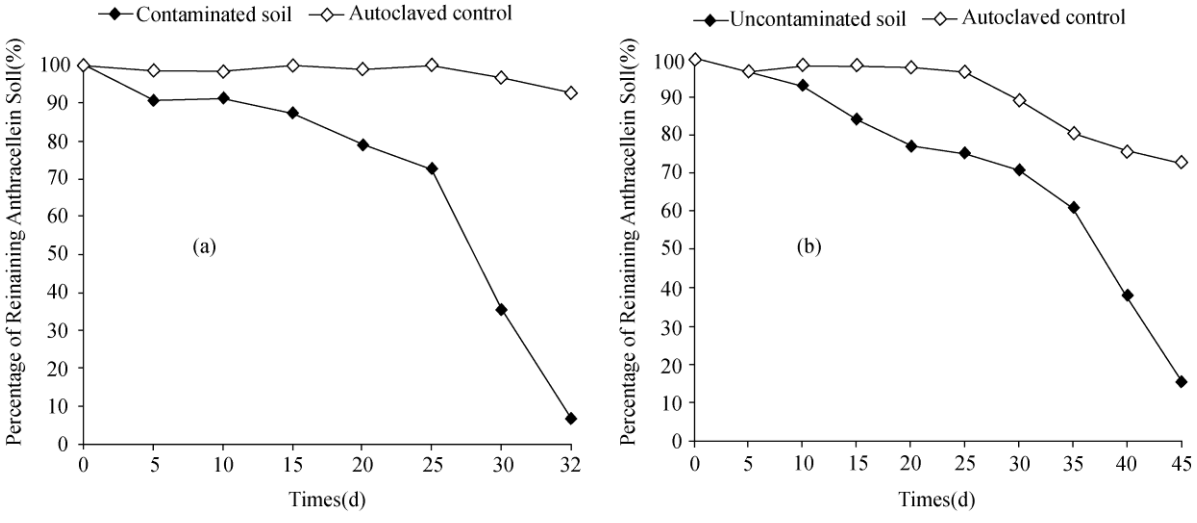

Figure 1. Time series of anthracene concentration in controls and in microcosms constructed with contaminated site soil (a), or uncontaminated site soil (b). The duplicate result illustrated the same trend.

TRFLP Analysis

In this study, DNA samples for the molecular analysis were extracted in duplicate from four microcosms including: (a)microcosm constructed with contaminated site soil on Day 0 ($microcosm_{contaminated\text{-}day0}$), (b) microcosm constructed with contaminated site soil on Day 32 ($microcosm_{contaminated\text{-}day32}$), (c) microcosm constructed with uncontaminated site soil on Day 0 ($microcosm_{uncontaminated\text{-}day0}$), and (d)microcosm constructed with uncontaminated site soil on Day 45 ($microcosm_{uncontaminated\text{-}day45}$). After an experimental period of 32 or 45-day, the relative abundances of fragment 218 bp (HaeIII digest) increased significantly from below 1% to 25.3% and 49.1% for contaminated soil and uncontaminated soil, respectively (Figure 2).

For the two microcosms analyzed on Day 32 or Day 45, Shannon-Weiner index, and evenness decreased (Table 1). The calculated Bray–Curtis similarity index showed a 56.8% similarity for $microcosm_{contaminated\text{-}day0}$ and $microcosm_{contaminated\text{-}day32}$ (Table 2). However, the similarity between $microcosm_{uncontaminated\text{-}day0}$ and $microcosm_{uncontaminated\text{-}day45}$ was even lower (35.8%). Overall, these indices indicated large variations in microbial community structure over the experimental period.

Table 1. Comparison of Diversity and Evenness Indices for the TRFLP Profiles (*Hae*III digest) from the Four Microcosm Samples

Samples	S	H	E
Microcosmcontaminated-day0	17	2.283	0.806
Microcosmcontaminated-day32	9	1.169	0.759
Microcosmuncontaminated-day0	20	2.281	0.761
Microcosmuncontaminated-day45	14	1.853	0.702

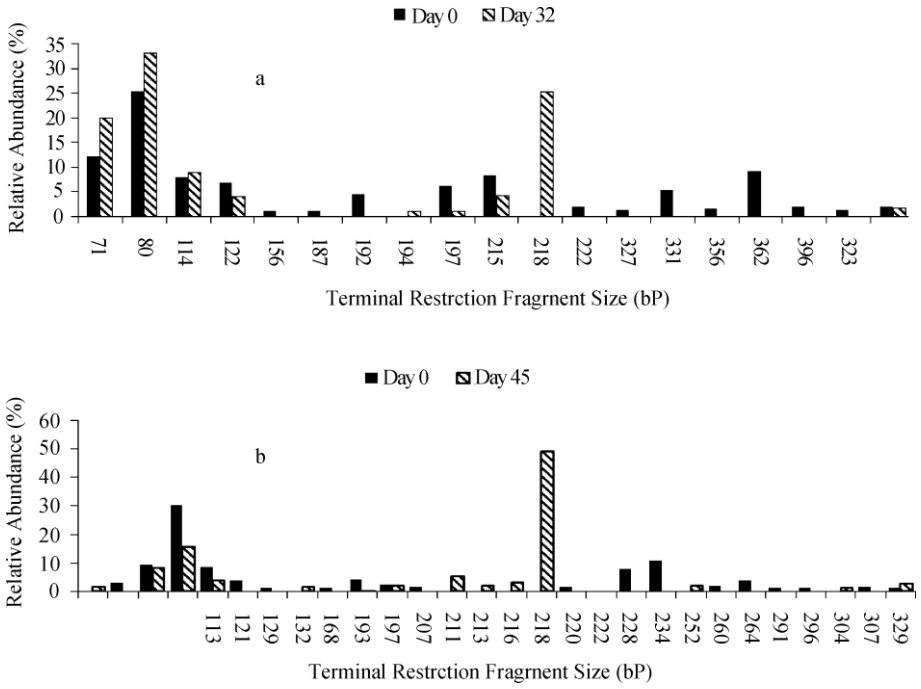

Figure 2. Terminal restriction fragments (*Hae*III digest) and their abundances in the microcosm$_{contaminated\text{-}day0}$ and microcosm$_{contaminated\text{-}day32}$ (a), or microcosm$_{uncontaminated\text{-}day0}$ and microcosm$_{uncontaminated\text{-}day45}$ (b).

Table 2. Bray–Curtis Similarity Index for the TRFLP Profiles (*Hae*III digest) from the Four Microcosm Samples

Samples	Microcosm Contaminated-day0	Microcosm Contaminated-day32	Microcosm Uncontaminated-day0	Microcosm Uncontaminated-day45
Microcosm contaminated-day0	100			
Microcosm contaminated-day32	56.8	100		
Microcosm uncontaminated-day0	34.2	33.4	100	
Microcosm uncontaminated-day45	28.8	50.7	35.8	100

Phylogeny

In this study, many known phyla (Bacteroidetes, Proteobacteria, Actinobacteria, Acidobacteria, Planctomycetes, Nitrospira, Gemmatimonadetes, Firmicutes, Chloroflexi), and some unclassified bacteria were detected, although only α-, β-, γ-proteobacteria and unclassified Proteobacteria were found in all microcosm samples (Figure 3). For microcosm$_{contaminated\text{-}day0}$, the 67 clones recovered from soil DNA sample were distributed across phyla as follows: δ-proteobacteria 20.9%, β-proteobacteria 13.4%, Acidobacteria 11.9%, Unclassified bacteria 10.4%, Bacteroidetes 7.5%, α-proteobacteria 9.0%, Planctomycetes 4.5%, Actinobacteria 4.5%, Unclassified Proteobateria 3.0%, Nitrospira 3.0%, Gemmatimonadetes 1.5%. However, the major phylum type of the 85 clones recovered from the microcosm$_{contaminated\text{-}day32}$ DNA sample was β-proteobacteria (75.3%). Other phyla and sub Proteobacteria pyla (α-, γ-, δ-proteobacteria and Unclassified Proteobacteria) had the relative abundance below 6%.

For microcosm$_{uncontaminated\text{-}day0}$, the major phylum types of the 81 clones were Actinobacteria (33.3%) and Firmicutes (27.2%), indicating a quite different phylum composition from that of microcosm$_{contaminated\text{-}day0}$. However, in the 83 clones recovered from the microcosm$_{uncontaminated\text{-}day45}$ DNA sample, β-proteobacteria (96.4%) predominated. Only α- and γ-proteobacteria and Unclassified Proteobacteria were detected with minor abundance (1.2%).

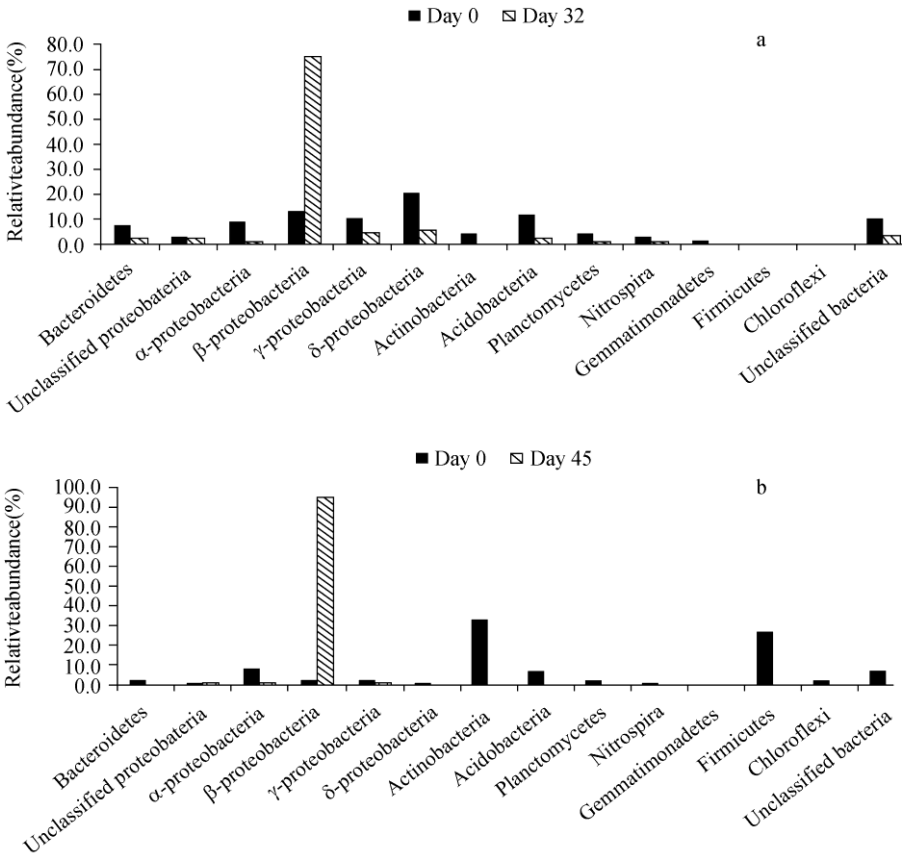

Figure 3. Comparison of the quantitative contribution of the clones affiliated with different phyla and sub-phyla to the total number of clones from (a) microcosmcontaminated-day0 and microcosmcontaminated-day32 DNA samples, and (b) microcosmuncontaminated-day0 and microcosmuncontaminated-day45 DNA samples. Unclassified bacteria refer to clones not classified in this study.

Identity of Dominant Enriched TRFLP Fragments

Dominant TRFLP fragments obtained from microcosm$_{contaminated\text{-}day32}$ and microcosm$_{contaminated\text{-}day45}$ with two additional restriction enzymes were used to provide a positive identification of the *Hae*III TRFLP enriched fragment (218 bp). The dominant fragments obtained from all TRFLP restriction enzymes were compared with those obtained from in silico digests to determine the 16S rRNA sequence of the enriched fragment[18]. A comparison of TRFLP cut site and in silico cut site was presented in Table 3. The slight differences (2-3 bases) between the measured fragment lengths and those predicted with sequence data have been previously noted[17,26-27].

The taxonomic identity of 218 bp (*Hae*III digest) in either microcosm$_{contaminated\text{-}day32}$ or microcosm$_{uncontaminated\text{-}day45}$ was classified into the genus *Methylophilus*, affiliated with *β-Proteobacteria.* Among the 83 and 85 clones recovered from DAN samples, 54 and 72 clones, for microcosm$_{contaminated\text{-}day32}$ and microcosm$_{contaminated\text{-}day45}$, respectively, belonged to the genus *Methylophilus* (data not shown). However, no clone classified as *Methylophilus* species was detected in either microcosm$_{uncontaminated\text{-}day0}$ or microcosm$_{contaminated\text{-}day0}$. This was consistent with the observed relative abundances of 218 bp, i.e, below 1% (Figure 2). Therefore, the genus *Methylophilus* was significantly

Table 3. Comparison of Dominant Enriched TRFLP Fragments with Clone Restriction Enzyme Cut Sites Predicted from Sequence Analyses to Confirm the Identity

Samples	Restriction Enzyme	TRFLP	Sequence Data
Contaminated Soil	*Hae*III	218 (25%)[a]	219
	*Msp*I	487 (27%)[a]	490
	*Hha*I	367 (39%)[a]	367
Uncontaminated Soil	*Hae*III	218 (49%)[a]	219
	*Msp*I	487 (77%)[a]	490
	*Hha*I	366 (52%)[a]	367

Note. [a]The relative abundance of the dominant enriched TRFLP fragments with different digests.

enriched with the biodegradation of anthracene.

DISCUSSION

The structure of bacterial communities changes during the bioremediation of PAH and/or petroleum hydrocarbon-contaminated soils[9,28-29]. However, limited information is available concerning the impact of PAH or other petroleum derived molecule addition on bacterial communities in contaminated sites. Muckian et al. reported the significant change of the microbial communities in fluoranthene- or phenanthrene-amended soils over a 28-day period of study, with biodegradation in advance[10]. The composition variations of the microbial mat community in heavily polluted sites and pristine sites for different time periods following petroleum hydrocarbons addition have also been observed in other studies[30-31]. However, to the authors' knowledge, this was the first reported work to explore the impact of PAH addition on microbial ecosystems in uncontaminated soils. In this study, the diversity of the microbial communities greatly decreased with the biodegradation of anthracene.

The phylogenetic description of the change of dominant bacterial groups is also important for studying the impact of PAH addition. In this study, 16S rRNA clone libraries were constructed for microcosm samples to provide the phylogenetic information on microbial communities. There was a clear difference in phylum groups between $microcosm_{contaminated\text{-}day0}$ and $microcosm_{uncontaminated\text{-}day0}$, which was consistent with the results from TRFLP analysis. Moreover, the predominance of β-proteobacteria occurred with the degradation of anthracene. In Beijing, Guo found different phylum structures in grassland soils that were collected from the same garden but had been irrigated differently, specifically, with reclaimed wastewater or with tap water[12]. Two earlier works using denaturing gradient gel electrophoresis (DGGE) indicated that, compared to the sample geographical origin, the contamination history of samples plays a more important role in determining the functional or species diversity within soil bacterial communities[32-33]. Shifts in α-, β-, γ-proteobacteria have been observed in aromatic hydrocarbon bioremediation[30,34].

Many different classes of bacteria have been isolated from PAHs-contaminated soil[35]. In this study, the genus *Methylophilus* was dominantly enriched with the significant biodegradation of anthracene. *Methylophilus* has been linked to the metabolization of biphenyl[36], and phenol and humic matter[37]. These compounds have a phenolic hydroxyl which is also present in intermediate compounds proposed in anthracene degradation pathways[38].Therefore, *Methylophilus* may play roles in the degradation of intermediates, and thus get enriched dominantly after significant degradation of anthracene. In conclusion, TRFLP coupled with 16S rRNA clone library could effectively monitor the shift of microbial communities associated with anthracene addition. The anthracene addition had significant impacts on the microbial community structure. *Methylophilus* might play an important role in the biodegradation process of anthracene. However, further studies in this regard are necessary to clarify the mechanism involved.

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