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CONTENTS

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Microbial community changes in aquifer sediment microcosm for anaerobic anthracene biodegradation under methanogenic condition

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Abstract

The widespread distribution of polycyclic aromatic hydrocarbons (PAHs) in groundwater has become an important environmental issue. Knowledge of microbial community changes could aid in identification of particular microorganisms that are capable of degrading PAHs in contaminated aquifers. Therefore, 16S rRNA gene clone library analysis was used to identify the archaeal and bacterial communities in an aquifer sediment microcosm used for anaerobic anthracene degradation under methanogenic conditions. A remarkable shift of the archaeal community structure occurred after anaerobic anthracene degradation, but the types of the abundant bacterial phyla did not change. However, a decrease of both archaeal and bacterial diversity was observed. Bacterial genera *Bacillus*, *Rhodococcus and Herbaspirillum* might have links with anaerobic anthracene degradation, suggesting a role of microbial consortia. This work might add some new information for understanding the mechanism of PAH degradation under methanogenic conditions.

Key words: polycyclic aromatic hydrocarbons (PAHs); groundwater; sediment; biodegradation; microbial community; Archaea
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Introduction

The widespread distribution of polycyclic aromatic hydrocarbons (PAHs) in groundwater, especially drinking water sources, has become an important public concern, due to their persistence in the environment and potentially deleterious impacts on human health. PAHs are typically thought to be recalcitrant to biodegradation in subsurface environments void of oxygen. Interestingly, several previous works have shown successful anaerobic PAH biodegradation under nitrate- or sulfate-reducing conditions in microcosms inoculated with aquifer materials (Bregnard et al., 1996; Bedessem et al., 1997; Meckenstock et al., 2004), which suggested that anaerobic biodegradation might be enhanced with the amendment of alternative electron acceptors, such as nitrate and sulfate. However, the maximum allowable levels of nitrate and sulfate in drinking water are usually regulated. Improper amendment might lead to undesirable amounts of nitrate and sulfate in groundwater, and subsequent removal of these contaminants would be difficult. Therefore, anaerobic PAH biodegradation under methanogenic conditions is of great interest, especially in the regions where the levels of nitrate and sulfate in groundwater are low. Unfortunately, little is known about PAH biodegradation under methanogenic conditions. However, Chang et al. (2005) showed the disappearance of phenanthrene in a methanogenic culture initiated with marine sediment after a 150-day incubation. A more recent work indicated anthracene could be significantly reduced in methanogenic microcosms inoculated with aquifer sediment in 60 days (Zhang et al., 2012).

Bioaugmentation by introducing microorganisms with high PAH-degradation capability seems to be an ideal way to improve the biodegradation of PAH in contaminated environments (Grosser et al., 1991). Traditionally, identification of PAH degraders has involved isolations. Several anaerobic nitrate- or sulfate-reducing PAH degraders have been documented (Haritash and Kaushik, 2009); however, to the authors' knowledge, information on the isolate of methanogenic PAH degraders is still lacking, which implies the huge uncertainty of efforts to obtain anaerobic isolates. Moreover, the isolates obtained in the laboratory in a pure culture may not be able to perform the same function in contaminated sites. Different culture conditions or habitats may select for specific PAH degraders (Xie et al., 2011). In this case, culture-independent molecular analyses (e.g., clone library) can be very useful, especially for identification of potential anaerobic degraders in the field. For example, a study using clone library analysis showed that Gammaproteobacteria predominated in naphthalene-degrading methanogenic cultures initiated with marine sediments, but Firmicutes was dominant in a phenanthrene-degrading culture (Chang et al., 2005), suggesting that these bacterial groups might play roles in PAH biodegradation.

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In the current study, the aim was to investigate the changes of microbial communities in methanogenic PAH-degrading microcosms constructed with contaminated aquifer sediment. As a three-ring PAH species, anthracene was selected as a model PAH compound, because it could be transported with groundwater over significant distances (Meckenstock et al., 2004).

1 Materials and methods

1.1 Microcosm construction and chemical analyses

A shallow aquifer sediment sample, 5 m below the ground surface, was collected from a borehole near a municipal waste composting site. Our preliminary research indicated the sediment contained 20–30 μg/kg anthracene and 0.51 g/kg total organic carbon. Following sediment sample collection, aquifer sediment was homogenized and sieved through a 0.18-mm screen. Anaerobic sediment microcosms consisted of mineral salt medium (10 mL), as previously described (Yang and McCarty, 1998), 200 μg anthracene (99%, J&K China Chemical, China), and sediment (3 g) in serum bottles (150 mL). These sediment microcosms were constructed in triplicate and maintained under methanogenic conditions according to the literature (Zhang et al., 2012). Sterile controls were obtained by autoclaving repeatedly (three times). Microcosms were incubated on a horizontal shaker (100 r/min) (HZ-9511K, Taicang Kejiao Apparatus Plant, China) at 20°C.

Microcosms were sacrificed every 30 days and sediment samples were dried. Two grams of dried sediment samples were removed for DNA extraction and the remaining sediment was extracted for anthracene analysis using HPLC (Shimadzu LC-10Avp, Agilent Technologies, Japan), as previously described (Zhang et al., 2011, 2012). The gas headspace was analyzed for methane with a gas chromatograph (GC-2008B, Guangzheng Analytical Apparatus Co., Ltd., China) (Zhang et al., 2012).

1.2 Clone library analysis

DNA from raw sediment and sediment samples on day 120 was extracted using the UltraClean DNA extraction kit (Mobio Laboratories, USA). DNA of triplicate samples was pooled together for further molecular analysis. The pooled DNA samples from raw sediment and microcosm sediment samples on day 120 were referred to as Sample R and Sample M, respectively. Bacterial 16S rRNA genes were amplified using primers 27F (5′-GAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Xie et al., 2010; Zhang et al., 2012). Archaeal 16S rRNA genes were amplified with archaeal primers A109F (5′-ACKGCTCAGTAACACGT-3′) and A934R (5′-GTGCTCCCCCGCCAATTCCT-3′) (Grosskopf et al., 1998). PCR conditions were as follows: 94°C (5 min); 94°C (30 sec); 55°C (30 sec); 72°C (1.5 min) (30 cycles); 72°C (10 min). The PCR products were purified with QIA quick PCR purification kit (Qiagen Inc., German) and then cloned into pGEM-T-easy Vector (Takara Corp, Dalian, China). Clones were sequenced at SinoGenoMax Co., Ltd. (Beijing).

Chimeras were excluded from further analyses using Mallard software (Ashelford et al., 2006). Sequences sharing identity greater than 97% identity were grouped into one operational taxonomic unit (OTU) using the DOTUR program. The DOTUR program was also used to calculate the diversity indices including abundance-based coverage estimator (ACE), Chao1, and Shannon (Schloss and Handelsman, 2005). One representative clone was chosen from each selected OTU, and then submitted to the BLAST program to obtain the closest relatives. Phylogenetic trees were constructed using MEGA software version 4.0 (Tamura et al., 2007). Taxonomic identities of the clones in each OTU were assigned using the Ribosomal Database Project (RDP) II analysis tool "classifier" (Wang et al., 2007). The sequences obtained in this study were submitted to GenBank under accession numbers JF793709 to JF793765 (archaeal library with Sample M), JF793766 to JF793823 (bacterial library with Sample M), JF793824 to JF793866 (archaeal library with Sample R), and JF793867 to JF793913 (bacterial library with Sample R).

2 Results and discussion

2.1 Biodegradation

During the whole biodegradation experiment, methane production in the microcosms increased from 0 (day 0) to approximately 150 μmol/L (day 120), in contrast to the negligible amounts in the sterile controls. This confirmed the occurrence of methanogenesis in the microcosms. On day 60, a significant decline of anthracene (average 43.6% reduction) in the microcosm sediment was observed, but decline was limited in the autoclaved controls (average 3.5% decrease) (Table 1). On day 120, an average of 80.7% anthracene reduction occurred in the microcosm sediment, but an average of 3.9% in the autoclaved controls. These results confirmed a biological removal of anthracene in the microcosms. This degradation pattern was similar to a previous microcosm study, although the aquifer sediment used was sampled from a different sampling site (Zhang et al., 2012). Disappearance of phenanthrene was found in a methanogenic culture initiated with marine sediment after a 150-day incubation (Chang et al., 2005).

2.2 Identification of microbial community structures

Under aerobic conditions, PAH amendment could induce significant changes of bacterial community structures (Muckian et al., 2009; Zhang et al., 2011). However, in this study, the types of the abundant bacterial phyla did not change after a 120-day experimental period

Table 1 Ranges for anthracene remaining percentages in the solid phase over the experimental period

Time	30 days	60 days	90 days	120 days
Sterile controls	97.6% ± 0.8%	96.5% ± 1.2%	96.3% ± 1.4%	96.1% ± 1.2%
Microcosms	89.5% ± 1.0%	56.4% ± 2.8%	35.2% ± 4.3%	19.3% ± 4.7%

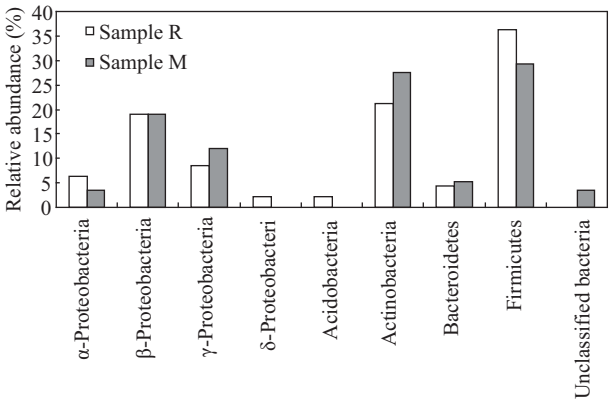

Fig. 1 Comparison of bacterial phylum composition in raw sediment and microcosm sediment on day 120. Clones not classified to any known phylum are included as unclassified Bacteria. Sample R and Sample M are the pooled DNA samples from raw sediment and microcosm sediment samples on day 120, respectively.

(Fig. 1). Both Sample R and Sample M consisted mainly of Firmicutes, Actinobacteria, α-Proteobacteria, and γ-Proteobacteria. Little is known about the impacts of PAH amendment on the archaeal community structure. A previous work showed that PAH amendment could largely shift the structure of the archaeal community in marine sediment under methanogenic conditions (Chang et al., 2005). A remarkable change of the archaeal community structure after anthracene biodegradation was also observed (Fig. 2). Euryarchaeota was the largest group in Sample M, but Crenarchaeota predominated in Sample R.

OTUs and diversity indices were both determined at the 3% sequence difference level using the DOTUR program (Table 2). The 120-day incubation induced the decrease of diversity either of the archaeal community or of the bacterial community. To the authors' knowledge, this was the first report to reveal a change of microbial diversity linked to anaerobic PAH biodegradation.

The archaeal library for Sample R (library RA) had two major OTUs (each with at least 5 archaeal sequences), one identified as genus *Methanobacterium* within phylum Euryarchaeota (with 5 sequences), another as unclassified Thermoprotei within phylum Crenarchaeota (with 9 sequences). The archaeal library for Sample M (library MA) was mainly represented by one OTU classified as genus *Methanosarcina* within phylum Euryarchaeota (with 28 sequences), as well as another OTU of unclassified Archaea (with 7 sequences). Therefore, the compositions of major OTUs in the two archaeal libraries were quite different.

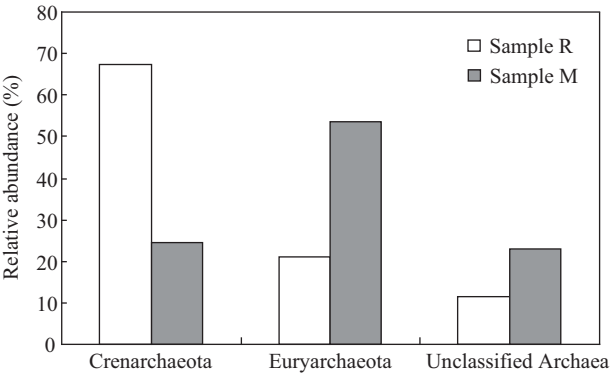

Fig. 2 Comparison of archaeal phylum composition in raw sediment and microcosm sediment on day 120. Clones not classified to any known phylum are included as unclassified Archaea. Sample R and Sample M are the pooled DNA samples from raw sediment and microcosm sediment samples on day 120, respectively.

Table 2 OTUs and diversity indices (calculated at 0.03 difference level) of archaeal and bacterial clone libraries

Library	Number of clones	Number of OTUs	ACE	Chaol	Shannon
RA[a]	43	19	44	28	2.65
MA[b]	57	15	35	24	1.91
RB[c]	47	21	392	89	2.47
MB[d]	58	19	201	45	2.28

[a] Archaeal library with Sample R; [b] archaeal library with Sample M; [c] bacterial library with Sample R; [d] bacterial library with Sample M.

The bacterial library for Sample R (library RB) mainly consisted of three OTUs (each with at least three bacterial sequences), including one OTU (with 6 sequences) affiliated with genus *Bacillus* within phylum Firmicutes, and one OTU (with 5 sequences) belonging to genus *Rhodococcus* within phylum Actinobacteria. The bacterial library for Sample M (library MB) was mainly represented by one OTU (with 14 sequences) classified as genus *Rhodococcus*, one OTU (with 15 sequences) as genus *Bacillus*, one OTU (with 9 sequences) as genus *Herbaspirillum* within class Betaproteobacteria, and one OTU (with 3 sequences) as genus *Azotobacter* within class γ-Proteobacteria. Interestingly, the *Herbaspirillum* and *Azotobacter* species were not detected in library RB. Therefore, the compositions of major OTUs in the two bacterial libraries were also very different.

2.3 Potential significance of the major bacterial OTUs

Figure 3 illustrates the phylogenetic relationship of the representative sequence of each major bacterial OTU (with at least three clones). Sequences MB19 and RB28 belonged to genus *Bacillus* and they had 99% identity. They were both related with 96% identity to several cultured sequences (EU221371.1, AJ315066.1, FN666534.1, and GU397390.1). Many members of the genus *Bacillus* are known to degrade PAHs and most of them have been isolated from contaminated soils (Khanna et al., 2011). Genus *Bacillus* has recently been linked to anaerobic biodegradation of several types of pollutants, such as benzene (Dou et al., 2010), *m*-xylene (Xie et al., 2010), and perchloroethylene (Kalimuthu et al., 2011). Some *Bacillus* species have also been isolated from groundwater (Gao et al., 2010). One *Bacillus* species could degrade PAHs in subsurface sediment (Madsen et al., 1992). Therefore, the abundance of *Bacillus* species in Sample R and Sample M might have links to anthracene biodegradation.

Sequences RB14 and MB41, sharing 99% identity, were *Rhodococcus* species, and were related with 99% identity to several *Rhodococcus* isolates (AF191343.1, FJ539088.1, and HM008918.1). PAH-degrading bacteria belonging to the genus *Rhodococcus* have been isolated from soil (Khanna et al., 2011), freshwater sediment

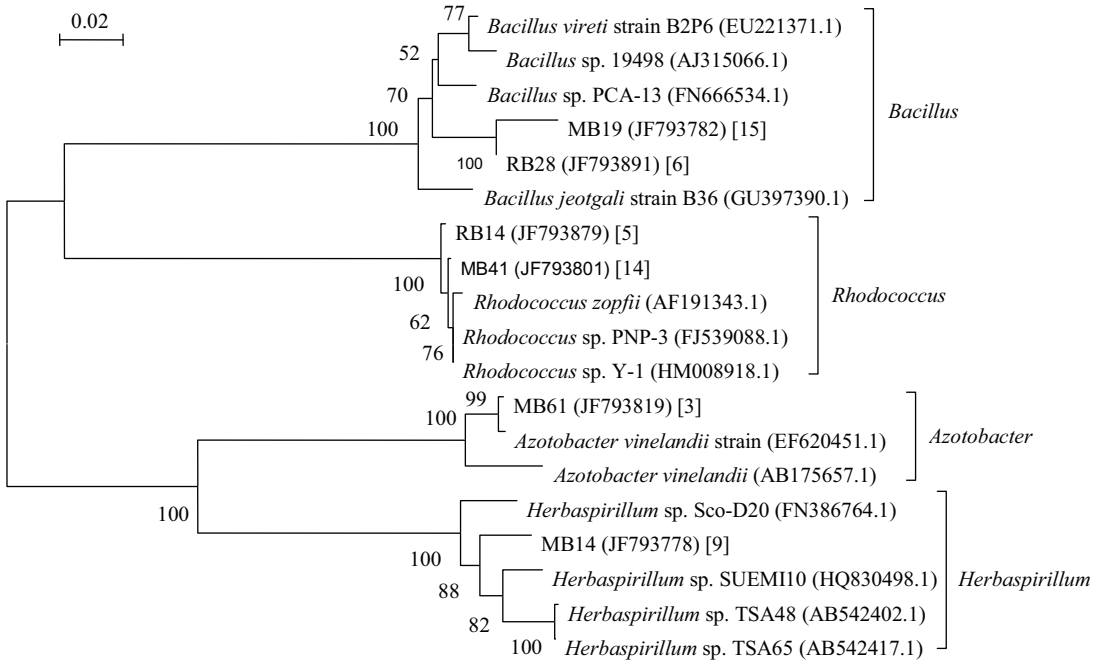

Fig. 3 Phylogenetic tree of representative 16S rRNA gene sequences of each major bacterial OTU, and reference sequences from GenBank. The obtained sequences beginning with 'RB and 'MB' refer to sequences recovered from library RB and library MB, respectively. The numbers in square parentheses represent the numbers of the sequences in the same OTU. Numbers at the nodes indicate the levels of bootstrap support based on neighbor-joining analysis of 1000 resampled datasets. The bar represents 2% sequence divergence.

(Dean-Ross et al., 2002), and marine sediment (Maskaoui et al., 2004). Li et al. (2009) found that one *Rhodococcus* species was an anaerobic PAH-degrading bacterium. Some cultured species from PAH-contaminated aquifers could degrade a variety of environmental chemicals, such as benzene (Fahy et al., 2008), *s*-triazine (Grenni et al., 2008), and 2,6-dichlorobenzamide (Pukkila et al., 2009). Therefore, the abundance of *Rhodococcus* species in Sample R and Sample M might also have links to anthracene biodegradation.

Sequence MB14 belonged to genus *Herbaspirillum* and was with 96% identity related to several isolates (FN386764.1, HQ830498.1, AB542402.1, and AB542417.1). Interestingly, *Herbaspirillum* species was abundant in Sample M, but not detected in Sample R. This showed a significant enrichment of *Herbaspirillum* species after the anthracene biodegradation. Some members of genus *Herbaspirillum* could degrade polymeric galloyl-ester (Franco et al., 2005), 4-chlorophenol (Im et al., 2004), and trifluralin (Bellinaso et al., 2003). The literature on the role of *Herbaspirillum* species in PAH degradation is still scarce. However, a recent work showed one *Herbaspirillum* species could grow well on fluoranthene as sole carbon and energy source (Xu et al., 2011). Therefore, the significant enrichment of *Herbaspirillum* species suggested a potential role in anthracene biodegradation.

Sequence MB61 was an *Azotobacter* species and showed 99% identity to two isolates (EF620451.1 and AB175657.1). Several species of genus *Azotobacter* could degrade glyphosate (Moneke et al., 2010), phenolic compounds (Juárez et al., 2008), and azobenzene (Wackerow-Kouzova, 2005). One *Azotobacter* species could enhance bioremediation of crude oil-contaminated soil as the result of a symbiotic association with a *Pseudomonas* species (Onwurah and Nwuke, 2004). However, to the authors' knowledge, the direct involvement of *Azotobacter* species in PAH degradation has not been documented. Therefore, the role of *Azotobacter* species in Sample M remains largely unclear.

3 Conclusions

Clone library analysis in combination with microcosm study was used to identify the changes of the archaeal and bacterial communities after anaerobic anthracene degradation. The types of the abundant bacterial phyla did not change after anaerobic anthracene degradation, but a remarkable change of the archaeal community structure occurred. However, OTU-based diversity indices indicated the decreased microbial diversity either of the archaeal community or of the bacterial community. Some bacterial genera might have potential roles in anthracene biodegradation. Further works will be necessary to clarify the roles of these microorganisms.

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