

Bacterial and Archaeal Community Structures in Phenanthrene Amended Aquifer Sediment Microcosms Under Oxic and Anoxic Conditions

Zhang, S.Y., Wang, Q.F. and Xie S.G.*

College of Environmental Sciences and Engineering, Peking University, Beijing 100871, China

Received 12 Nov. 2011;

Revised 4 May 2012;

Accepted 14 May 2012

ABSTRACT: In this study, oxic and anoxic microcosms were constructed with aquifer sediment collected from the vicinity of a municipal solid waste composting site. Phenanthrene was used as a model PAH compound. The changes of bacterial and archaeal communities in microcosms were characterized with terminal restriction fragment length polymorphism. 16S rRNA gene clone library analysis was also used to investigate phylogenetic composition of the microbial communities when phenanthrene significantly depleted. Results showed that the phenanthrene could be significantly degraded under both oxic and anoxic conditions. Phenanthrene addition had significant impact on bacterial and archaeal community structures, depending on redox conditions. After a significant depletion of phenanthrene, the bacteria in the oxic microcosm were mainly composed of β -proteobacteria, α -proteobacteria and δ -proteobacteria, in contrast, γ -proteobacteria was the major bacterial type in the anoxic microcosm. Moreover, *Euryarchaeota* was detected as the major archaeal community in the anoxic microcosm, however, sample from the oxic microcosm was not successfully amplified with archaea-specific primers. Bacterial family *Enterobacteriaceae* and archaeal genus *Methanosarcina* might play important roles in phenanthrene degradation under anoxic conditions. This work does add new insights on how to bioremediate PAHs in aquifer sediment.

Key words: Aquifer, Biodegradation; Bioremediation; Microbial community; Polycyclic aromatic hydrocarbons (PAHs)

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a group of toxic compounds composed of carbon and hydrogen, arranged in the form of two or more aromatic rings (Zhang and Wang, 2011; Oluseyi *et al.*, 2011; Castro-Gutierrez *et al.*, 2012). High concentration of PAHs is usually present in landfill leachate (Marttinen *et al.*, 2003). Inadequate disposal of landfill waste can lead to the contamination of soil and aquifer (Maqbool *et al.*, 2011; Hyun Il *et al.*, 2011; Nouri *et al.*, 2011; Abdoli *et al.*, 2012; Shafieiyoun *et al.*, 2012; Mahmoudkhani *et al.*, 2012). The remediation of contaminated groundwater typically involves microbial degradation. Aerobic PAH-degrading bacteria have been well documented (Meckenstock *et al.*, 2004). However, leachate-contaminated aquifers may become anoxic with a redox gradient along the groundwater flow path. In the anoxic zones, anaerobic bacteria play a greater role in contaminant reduction than the aerobic ones. Several works have demonstrated anaerobic PAH degradation under nitrate-, ferric iron-, or sulfate-reducing conditions (Meckenstock *et al.*, 2004). Unfortunately, only very few anaerobic nitrate- and

sulfate-reducing bacterial degraders have been isolated (Haritash and Kaushik, 2009). The information on PAH degraders under methanogenic condition is still lacking. The traditional culture-dependent isolation approaches typically tend to identify the microorganisms which are able to compete well under laboratory conditions (Xie *et al.*, 2010). Therefore, the knowledge about the microbial community structure in contaminated groundwater and its change associated with PAH biodegradation is very important. Molecular techniques have been widely applied to identify members of communities without isolating an individual species. Recently, bacterial community structures in leachate-contaminated aquifers have been widely investigated (Tian *et al.*, 2005; Yu *et al.*, 2010). However, all these previous works have not investigated the links of the bacterial communities to PAH biodegradation. Therefore, the bacteria involved in the biodegradation of PAHs in subsurface environments are largely unknown. The information for archaeal community structure in leachate-contaminated aquifer is still not available. As a powerful DNA profiling technique, terminal restriction

*Corresponding author E-mail: xiesg@pku.edu.cn

fragment length polymorphism (TRFLP) has been widely employed to monitor the shift of bacterial community structure during the biodegradation of hydrocarbon contaminants (Grant *et al.*, 2007; Zhang *et al.*, 2011a, b; Wang *et al.*, 2011). With analysis of clone library, phylogenetic information on microbial community structure can also be obtained. Therefore, the aim of this study was to investigate the microbial community structures in landfill leachate-contaminated aquifer sediment and their changes in microcosms with PAH biodegradation under oxic or methanogenic condition. The bacterial and archaeal communities were characterized by TRFLP in combination with 16S rRNA gene clone library analysis. Phenanthrene was selected as a model PAH compound. This work will add new insights on how to bioremediate PAHs in aquifer sediment.

MATERIAL & METHODS

Shallow aquifer sediment sample, 5 m below the ground surface, was collected from a borehole, in the vicinity of a municipal solid waste composting site. Following sample collection, aquifer sediment was air-dried, homogenized and sieved through a 0.18-mm screen. Each microcosm consisted of mineral salt medium (10 mL), as previously described (Yang and McCarty, 1998), and sediment (2 g) in serum bottles (150 mL). Phenanthrene (J&K China Chemical) stock was first prepared in methanol. A volume of methanol containing the desired mass (200 µg) of phenanthrene was added to the serum bottle and the solvent was allowed to evaporate. Medium and sediment were then added to the phenanthrene crystals in the bottles. No supplementary electron acceptor was added to the cultures in anoxic microcosms. The bottles were sealed with rubber stoppers and aluminum seals. The strictly anoxic microcosms were constructed, as previously described (Li *et al.*, 2010). The sterile controls were obtained by autoclaving at 121°C repeatedly (1 h, three times). Microcosms were incubated on a horizontal shaker (~100 rpm) at 25°C. The oxic microcosms were sacrificed at 10-day interval to measure the concentrations of phenanthrene in solid phase, while the anoxic microcosms were sacrificed nearly at a four-week interval. All samples were prepared in triplicates. Dry sediment (1 g) was extracted for phenanthrene analysis and determined according to the standard method (Zhang *et al.*, 2011a). The remaining sediment was used for DNA extraction (see below). In order to confirm the methanogenic condition in anoxic microcosms, gas headspace samples from anoxic microcosms were taken every 30-day and analyzed for methane with a gas chromatograph. The operational temperature of the column was 40°C, and the carrier gas (N₂) was at a flow rate of 30 mL/min (He *et al.*,

2008). Sediment DNA was extracted using the UltraClean DNA extraction kit (Mobio Laboratories, Carlsbad, USA). TRFLP analyses were performed for triplicate DNA samples from raw sediments, oxic microcosm on day 30 (OM_{d30}), and anoxic microcosm on day 116 (AM_{d116}). Triplicate samples were not pooled, and the entire analysis for each was carried out separately. Bacterial 16S rRNA genes were amplified using bacterial primers 27F-FAM (5'-GAGTTTGATCMTGGCTCAG-3', 5' end-labeled with carboxyfluoresceine) and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Huang *et al.*, 2011; Zhang *et al.*, 2011a). Archaeal 16S rRNA genes were amplified with specific archaeal primers A109F (5'-ACKGCTCAGTAACACGT-3') and A934R-FAM (5'-GTGCTCCCCCGCCAATTCCT-3', 3' end-labeled with carboxyfluoresceine) (Großkopf *et al.*, 1998). PCR conditions were as follows: 94°C (5 min); 94°C (30 s); 55°C (30 s); 72°C (1.5 min) (30 cycles); 72°C (5 min). 300 ng PCR products were purified with QIA quick PCR purification kit (Qiagen Inc., German) and digested with *Hae*III (Saibaisheng Gene Tech, China) at 37°C with a 6-hour incubation period. The fragment pattern was detected using an ABI 3730 DNA Analyzer (Applied Biosystems, USA). Bray-Curtis similarity index was calculated using PRIMER 5.0 software to evaluate the compositional similarity for microcosm samples (Clarke and Warwick, 2001). Similarity percentage analysis (SIMPER) was further used to identify the fragments that were mainly responsible for the dissimilarity between samples (Clarke and Warwick, 2001; Rees *et al.*, 2004). To obtain a phylogenetic knowledge of microbial communities, bacterial 16S rRNA gene clone libraries were constructed with samples OM_{d30} and AM_{d116} respectively, while archaeal clone library was only constructed for sample AM_{d116}. The PCR conditions were the same as the above-mentioned, except the primers were unlabeled. The PCR purified products were cloned into pMD19-T vector (Takara Corp, Japan) following the manufacturer's instruction. *E. coli* clones were grown on Luria-Bertani (LB) medium solidified with 15 g/L agar with 50 µg/L ampicillin for 16 h at 37°C. The white colonies were verified by PCR with primers M13 F (5'-TGAAAACGACGGCCAGT-3') and M13 R (5'-AACAGCTATGACCATG-3'). Clones with an insert of the correct size were sequenced at SinoGenoMax Co., Ltd. (Beijing). Sequences were checked for chimeras with CHECK_CHIMERA software of Ribosomal Database Project II (Maidak *et al.*, 2001). Sequences that were over 97% similar were grouped into an operational taxonomic unit (OTU) by manual comparison. The most similar and representative GenBank sequences to the representative clones in each OTU were extracted from

the National Center for Biotechnology Information database and included in further phylogenetic analyses using MEGA version 4.0 (Tamura *et al.*, 2007). Alignment of the sequences was carried out using ClustalW (<http://www.ebi.ac.uk/clustalw/>). The Ribosomal Database Project II analysis tool “classifier” was utilized to obtain taxonomic identity (Wang *et al.*, 2007). The partial 16S rRNA sequences obtained in this study were submitted to GenBank under accession numbers HQ015302 to HQ015349 (bacterial library with the oxic microcosm sample), HQ438761 to HQ438830 (bacterial library with the anoxic microcosm sample), and HQ438700 to HQ438760 (archaeal library with the anoxic microcosm sample).

RESULTS & DISCUSSION

Methane production in anoxic microcosms was observed during the whole biodegradation experiment, while the amount was negligible in the sterile controls, indicating the occurrence of methanogenesis in microcosms (data not shown). Our preliminary research indicated that the sediment used to construct microcosms contained 50 µg/kg phenanthrene (data not shown). In this study, the lack of a lag time was observed either in the oxic microcosm or in the anoxic microcosm (Table 1 and 2), which is indicative of the presence of microbial population acclimated to phenanthrene biodegradation. Prior exposure to PAHs can reduce the length of the acclimation period associated with PAH degradation (Zhang *et al.*, 2011a). On day 116, a significant decline of phenanthrene (79% reduction) in the anoxic microcosm was observed, but decline was limited in the autoclaved controls (5% decrease), confirming a biological reduction mechanism (Table 2). In contrast, in the oxic microcosm, the degradation rate was much higher. The reduction rates were 67% and 88% on day 20 and day 30 respectively (Table 1). Rapid mineralization of naphthalene and phenanthrene was also observed in aerobic microcosms inoculated with coal-tar contaminated aquifer sediment (Rogers *et al.*, 2007). The information about biodegradation of PAHs under anoxic condition

is still very few. One previous report showed disappearance of phenanthrene in methanogenic culture initiated with marine sediment after a 150-day incubation (Chang *et al.*, 2005a).

Many previous works have shown that PAH addition has significant impact on bacterial community structure in various environments. Changes in hydrocarbon content in soils usually resulted in the shift of bacterial community structure (Vinãs *et al.*, 2005; Wiinsche *et al.*, 1995). Gu *et al.* (2009) found that there was a significant correlation among the change of PAH concentration and the number of PAH-degrading bacteria in marine sediment. Tian *et al.* (2008) revealed that there was a remarkable shift in the bacterial community structure due to the PAH addition in enrichment batch culture inoculated with mangrove surface sediments. In this study, the abundant terminal fragments (relative abundance equals to or above 5%) in raw sediment sample were 73 bp, 80 bp, 81 bp, 199 bp, and 220 bp. After a 30-day experiment, the abundant bacterial fragments in the oxic microcosm were much different (Fig. 1). Bray–Curtis similarity index showed sample Omd30 and raw sediment only had 37.6% similarity. The SIMPER analysis revealed that the differences of bacterial TRFLP profiles between sample Omd30 and raw sediment were driven primarily by variation in 219 bp and 81bp (Table 3). 81 bp was abundant in raw sediment but disappeared in sample Omd30. 219 bp was greatly enriched with phenanthrene biodegradation although it was not detected in raw sediment. The low Bray–Curtis similarity (33.5%) between sample AM_{d116} and raw sediment also reflected a big change in the anoxic microcosm. The SIMPER analysis revealed that variation in 220 bp and 79 bp was mainly responsible for the differences of bacterial TRFLP profiles between sample AMd116 and raw sediment (Table 4). The authors' previous work also indicated that phenanthrene and anthracene amendment also had significant impact on bacterial community structure in landfill leachate-contaminated soil under oxic condition (Zhang *et al.*, 2011a, b).

Table 1. Percentages of the remaining phenanthrene in solid phase with time in controls and in microcosms under oxic condition

Time (d)	10	20	30
Sterile control	98±0.6	97±1.4	96±0.3
Oxic microcosm	74±2.0	33±2.3	12±1.5

Table 2. Percentages of the remaining phenanthrene in solid phase with time in controls and in microcosms under anoxic condition

Time (d)	27	55	86	116
Sterile control	98±0.8	96±1.0	95±0.8	95±1.1
Anoxic microcosm	86±1.4	62±1.7	53±1.1	21±0.9

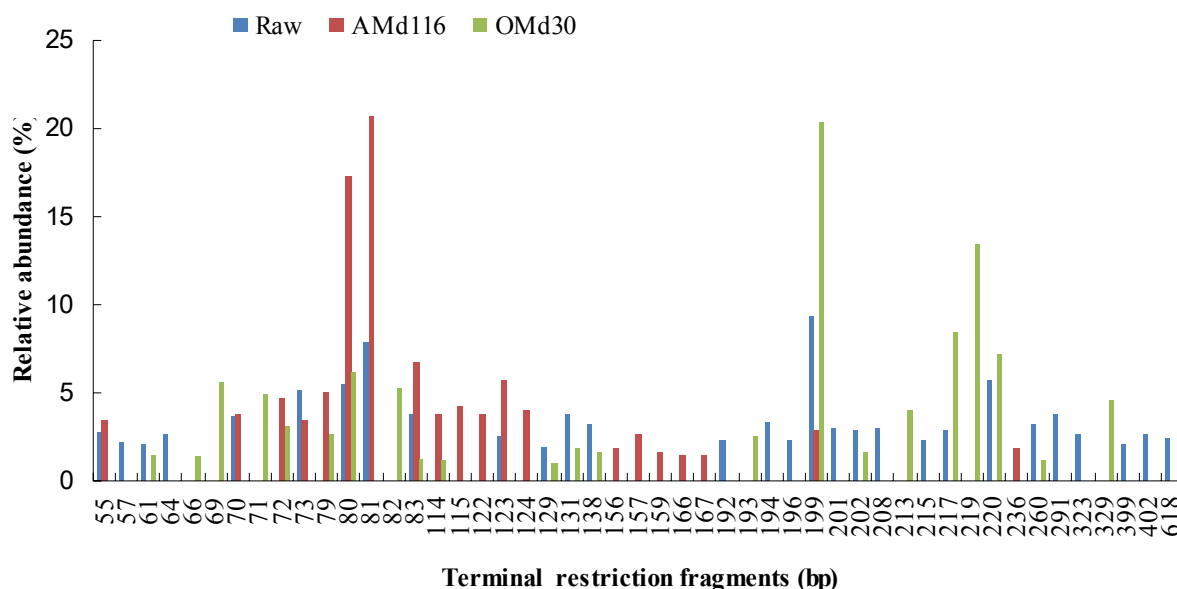


Fig. 1. Representative TRFLP profiles of bacterial communities in raw sediment, samples OMd30 and AMd116

Table 3. Results of SIMPER analysis of bacterial TRFLP profiles of raw sediment and OM_{d30} samples

Fragments (bp)	Mean relative abundance ^a	Mean relative abundance ^a	% ^b
	Raw	OM _{d30}	
219	0	13	8
81	8	0	6

^aMean relative abundance of each fragment as a percentage of total fragment abundance.

^bFragment contribution as a percentage dissimilarity between the two groups. Lists are truncated to include only those fragments that contribute no less than 5% to the differences between samples.

Table 4. Results of SIMPER analysis of bacterial TRFLP profiles of raw sediment and AM_{d116} samples

Fragments (bp)	Mean relative abundance ^a	Mean relative abundance ^a	% ^b
	Raw	AM _{d116}	
220	6	0	6
79	0	5	5

^aMean relative abundance of each fragment as a percentage of total fragment abundance.

^bFragment contribution as a percentage dissimilarity between the two groups. Lists are truncated to include only those fragments that contribute no less than 5% to the differences between samples.

The bacterial community structure in sample OM_{d30} was much different from that in sample AM_{d116} (23.4% similarity). It seems that the structure of bacterial community degrading contaminants was dependent on redox conditions. Molecular analysis of water samples, obtained from different parts of a model wetland supplied with *cis*- and *trans*-1,2-dichloroethenes - contaminated groundwater, revealed that changes of the bacterial community structure coincided with a succession of the hydrochemical conditions in the

wetland, from oxic towards anoxic conditions (Imfeld *et al.*, 2010). Sediment microcosms exposed to leachate-contaminated groundwater also revealed a shift in bacterial community structure, in consistence with the transition from oxic condition to anoxic environment (Mouser *et al.*, 2010). Archaeal community in a petroleum-contaminated soil was much different from that in an adjacent uncontaminated site (Kasai *et al.*, 2005). Gilles *et al.* (2010) found that petroleum contamination could induce significant shifts in the

composition of archaeal communities inhabiting anoxic coastal marine infralittoral sediments. It seems that changes in hydrocarbon content could also lead to the shift of archaeal community structure. However, the information about the impact of PAH addition on archaeal community structure is still scarce. Chang *et al.* (2005a) found that addition of PAHs could also largely shift the structure of archaeal community in marine sediment under methanogenic condition. In this study, the sample OMD₃₀ was not successfully amplified with archaea-specific primers, possibly because the prolonged exposure to oxygen would reduce the archaeal populations to a level below detection by PCR amplification. The most abundant fragments in raw sediment and sample AM_{d116} were 368 bp (46%) and 125 bp (81%) respectively (Fig. 2). After a 116-day experiment of anoxic phenanthrene biodegradation, the low Bray–Curtis similarity (28.9%) between sample AM_{d116} and raw sediment showed a big shift in the archaeal community structure. The SIMPER analysis revealed that the differences of archaeal TRFLP profiles between sample AM_{d116} and raw sediment were driven primarily by variation in 125 bp, 368 bp, 306 bp, 96 bp

and 124 bp (Table 5). The phylogenetic description of major groups is of great importance for better understanding of microbial community. Numerous previous works have usually shown the dominance of phylum *Proteobacteria* in various oxic environments degrading PAHs. *Proteobacteria* was the dominant group of a PAH-degrading microbial consortium in liquid culture (Vinas *et al.*, 2005). Rapid mineralization of naphthalene and phenanthrene in aerobic laboratory microcosms could lead to the significant enrichment of β - and γ - *proteobacteria* (Rogers *et al.*, 2007). However, the dominant bacterial phylum in PAH-degrading environment is not always *Proteobacteria*. For example, Vinãs *et al.* (2005) found that *Bacteroidetes* was also one of the dominant groups in heavily creosote-contaminated soil degrading PAHs. In this study, the major bacterial types (relative abundance more than 10%) in the sample OMD₃₀ were β -*proteobacteria* (54.2%), α -*proteobacteria* (14.6%) and δ -*proteobacteria* (10.4%) (Fig. 3).

The 48 clones in bacterial libraries constructed with sample OMD₃₀ could further divided into 33 OTUs,

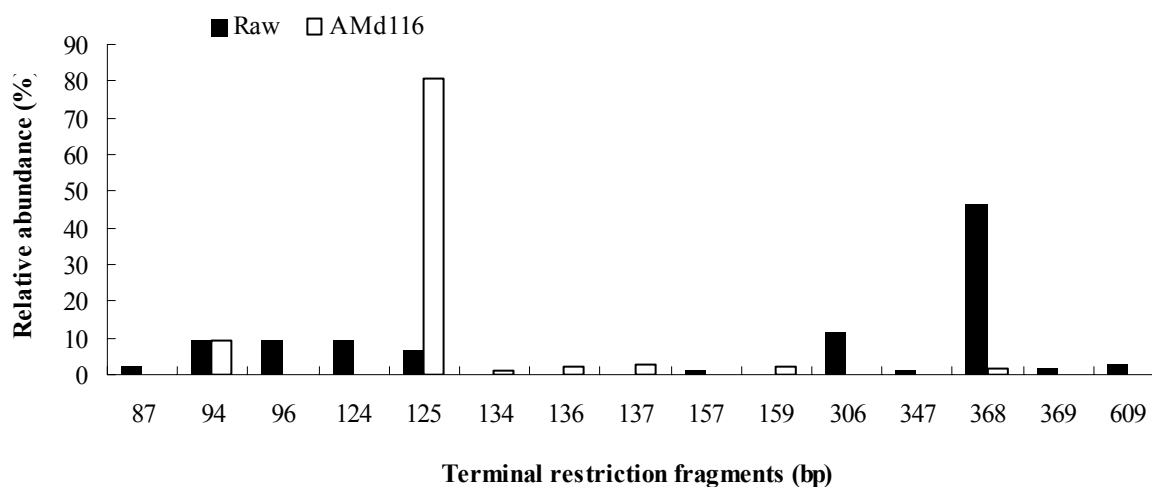


Fig. 2. Representative TRFLP profiles of archaeal communities in raw sediment and AM_{d116}

Table 5. Results of SIMPER analysis of archaeal TRFLP profiles of raw sediment and AM_{d116} samples

Fragments (bp)	Mean relative abundance ^a	Mean relative abundance ^a	% ^b
	Raw	AM _{d116}	
125	6	81	19
368	46	2	16
306	12	0	10
96	9	0	9
124	9	0	9

^aMean relative abundance of each fragment as a percentage of total fragment abundance.

^bFragment contribution as a percentage dissimilarity between the two groups. Lists are truncated to include only those fragments that contribute no less than 5% to the differences between samples.

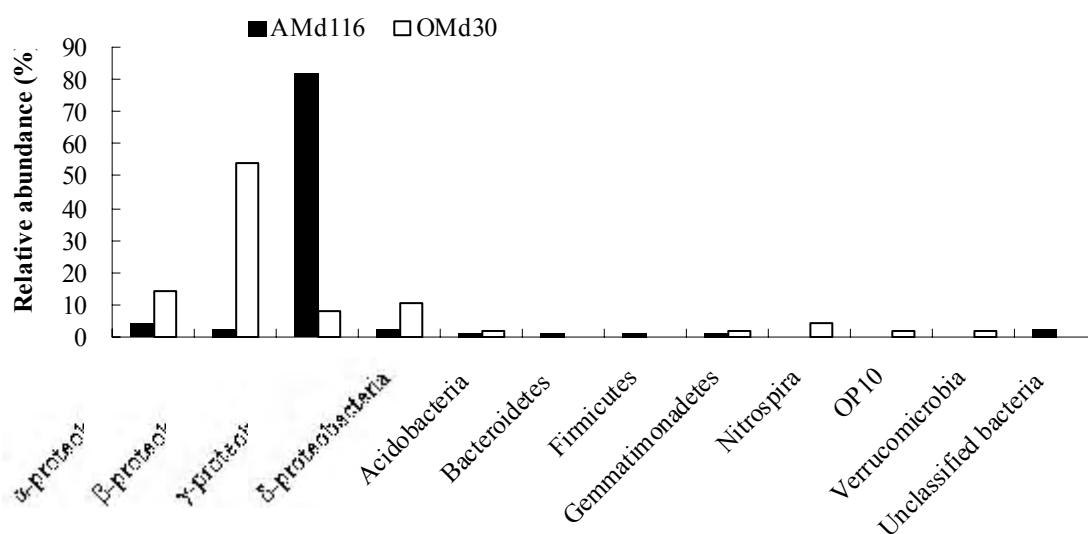


Fig. 3. Bacterial phylum composition in samples OM_{d30} and AM_{d116}

indicating the great diversity of clone sequences. Only five OTUs had two or more clones. Phylogenetic tree of one or two representative bacterial 16S rRNA gene sequences in each one of the five OTUs were constructed using MEGA version 4.0 (Fig. 4). In this study, the bacterial OTU recovered from sample OM_{d30} was referred to as OMOTU. Clones PHE16 and PHE24 (OMOTU1, with a total of three clones in the OTU) were unclassified *Rhodospirillaceae* species within order *Rhodospirillales*, class *α-proteobacteria*. They were closely related with 99% identity to uncultured clone sequences EF651658.1, obtained from vertisol, and GQ860111.1, obtained from river sediment, respectively. Clones PHE9 and PHE11 (OMOTU2, with two clones), were also unclassified *Rhodospirillaceae* species. The closest match of clone PHE9 was sequence EU133438.1 (97% identity), originating from tallgrass prairie soil, while clone PHE11 also show 97% identity to sequence FJ936882.1, obtained from environmental samples from volcano mud. The role of family *Rhodospirillaceae* in reduction of contaminants remains largely unknown. However, interestingly, a recent work showed that family *Rhodospirillaceae* was one of major groups in marine sediments enriched with crude oil (Cui *et al.*, 2008).

Clones PHE19 and PHE26 (OMOTU3, five clones), fall into genus *Methylothenera* within family *Methylophilaceae*, order *Methylophilales*, class *β-proteobacteria*. They were closely related with 99% identity to sequence FJ802334.1, obtained from river sediment at iron-reducing condition. Clones PHE4 and PHE21 (OMOTU4, nine clones) were unclassified *Methylophilaceae* species. The 16S rRNA gene sequence of clones PHE4 and PHE21 illustrated little similarity (92% or 94% identity respectively) to known sequence within Genbank. Genus *Methylothenera* has

been linked to methanol uptake in freshwater lake sediment (Kalyuzhnaya *et al.*, 2009). One species of genus *Methylothenera* was a methylamine-utilizing bacterium, isolated from Lake Washington sediment (Kalyuzhnaya *et al.*, 2006). However, the role of family *Methylophilaceae* in reduction of contaminants also remains largely unknown. Clone PHE15 (OMOTU5, two clones) was classified into genus *Nitrospira* within the phylum *Nitrospira*. Genus *Nitrospira* is well-known nitrifying bacteria. The closest match of clone PHE15 was uncultured sequence EU160342.1 (99% identity), obtained from rhizosphere soil samples. Moreover, the most similar cultured sequence (Y14644.1, 96% identity) originated from a nitrite-oxidizing bioreactor.

Sample AM_{d116} was mainly composed of *γ-proteobacteria* (81.4%). The bacterial composition of sample AM_{d116} was much different from that of sample OMd30 (Fig. 3). In agreement with TRFLP profiles analysis, the structure of bacterial community was dependent on redox conditions. Oxidic/anoxic conditions also affected the phylogenetic composition of the bacterial communities transforming polychlorinated biphenyl in a river sediment (D'Angelo and Nunez, 2010). Roling *et al.* (2001) revealed a clear difference between the bacterial community structures inside and outside contaminant plume in the landfill leachate-polluted aquifers along the redox gradient. They found that *β-* and *γ-proteobacteria* dominated upstream of the landfill and beneath the landfill respectively. Further downstream the abundance of *γ-proteobacteria* decreased, while the contribution of *δ-proteobacteria* strongly increased.

The 70 clones in bacterial libraries constructed with sample AM_{d116} were grouped into 18 OTUs.

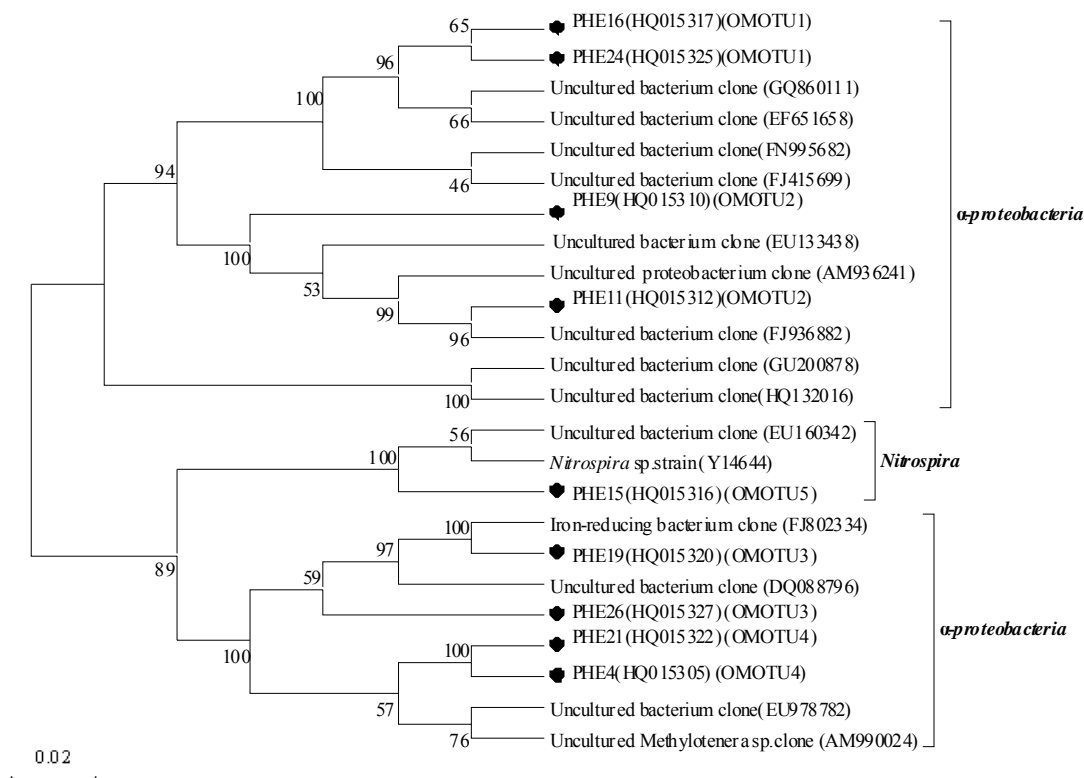


Fig. 4. Phylogenetic tree of one or two representative bacterial 16S rRNA gene sequences in each bacterial OTU (beginning with 'PHE') from sample OM_{d30} and reference sequences from GenBank. Data in parentheses are GenBank accession numbers. Numbers at the nodes indicate the levels of bootstrap support based on neighbor-joining analysis of 1000 resampled datasets. Bootstrap values < 50 are not shown

However, only three OTUs had two or more clones. Phylogenetic tree of two representative bacterial 16S rRNA gene sequences in each one of the three OTUs were constructed (Fig. 5). All the sequences among the three OTUs belonged to family *Enterobacteriaceae*, order *Enterobacteriales*, class *γ-proteobacteria*. In this study, the bacterial OTU recovered from sample AM_{d116} was referred to as AMBOTU. Clones ACE3 and ACE18 (AMBOTU1, seven clones) could be further classified within genus *Enterobacter*. The closest match of clone ACE3 was sequence AF371852.1 (97% identity), obtained from swine intestine. Clone ACE3 also had 97% identity to several cultured sequences, such as AM184254.1 from river water, and AM421978.1 from surface soil. Clone ACE18 was highly related with 100% identity to several cultured sequences such as AM184254.1 from river water, GU549440.1 from interior of the plant leaf, and HM165189.1 from soil. Recently, several species of genus *Enterobacter* have been linked to biodegradation of PAHs (Arulazhagan *et al.*, 2010; Lors *et al.*, 2010). Therefore, in this study, genus *Enterobacter* might have some links to the phenanthrene degradation under anoxic condition. Clones ACE1 and ACE9 (AMBOTU2, 18 clones) were

further classified within genus *Citrobacter*. The two clones were related with 99% identity to several cultured sequences from various environments, such as DQ294285.1 and DQ29428.1 from garden soil, EU373418.1 from leaf of young radish, EF491831.1 from sterilely-dissected intestines of goldfish, FJ768455.1 from soil, and HQ123576.1 from freshwater farmed flurgeon. Members of genus *Citrobacter* have been linked to biodegradation of simple aromatic compounds (Ammar *et al.*, 2005), and diesel oil (Singh and Lin, 2008). Therefore, the abundance of the *Citrobacter* species might also be associated with phenanthrene biodegradation under anoxic condition. Clones ACE2 and ACE4 (AMBOTU3, 29 clones) were unclassified *Enterobacteriaceae* species. They were also related with 99% identity to several cultured sequences from various environments, such as GQ451698.1 from high biodiversity regions of India, 360072.1 from tropical nepenthes digestive fluid, FJ189785.1 from rice paddy field soil, and FJ472852.1 from activated sludge. Very interestingly, the isolate (FJ472852.1) was a PAH-degrading bacterium. Since all the members in AMBOTU3, as well as AMBOTU1 and AMBOTU2, fall in family *Enterobacteriaceae*, with a

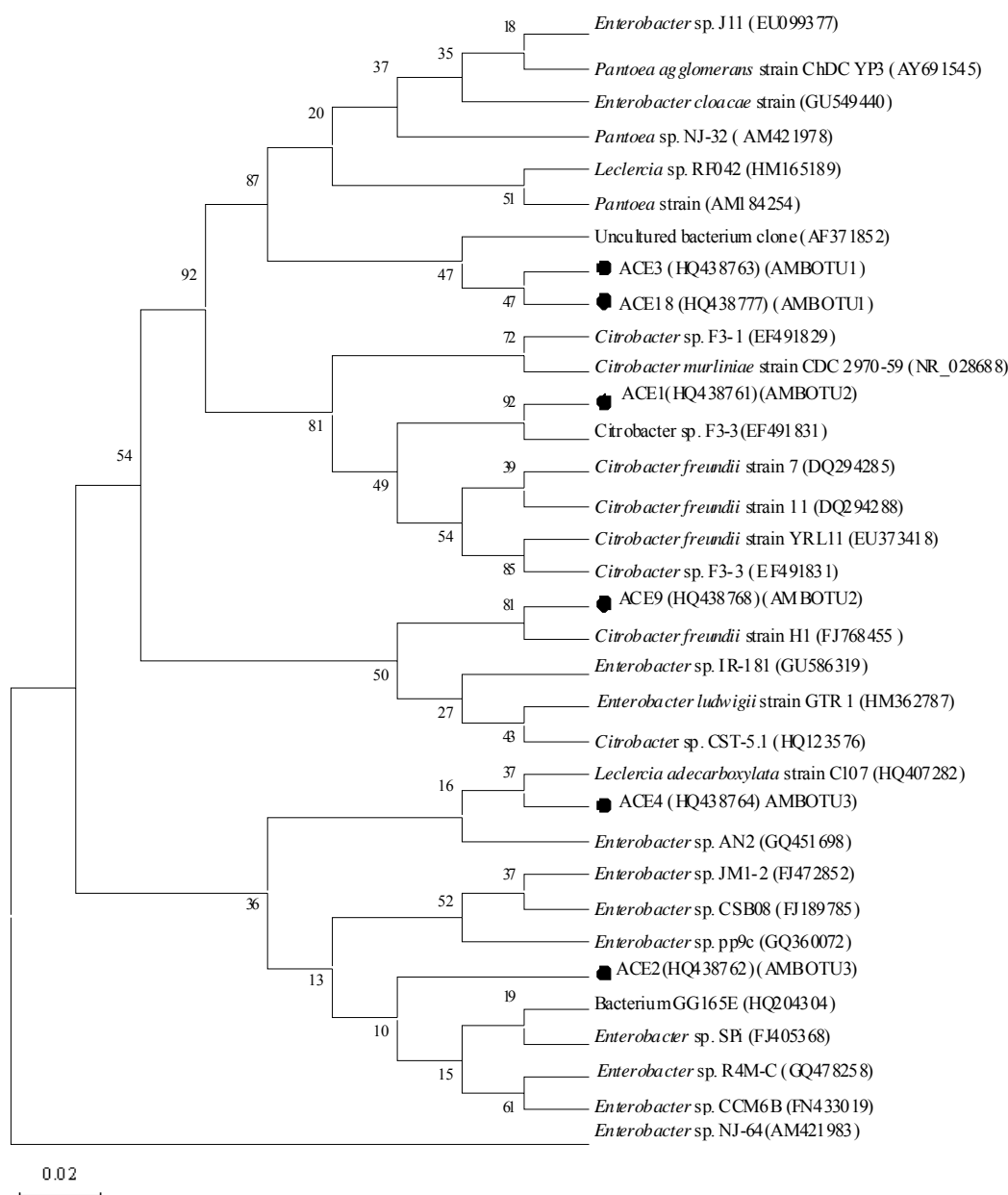


Fig. 5. Phylogenetic tree of two representative bacterial 16S rRNA gene sequences in each bacterial OTU (beginning with 'ACE') from sample AM_{d116} and reference sequences from GenBank. Data in parentheses are GenBank accession numbers. Numbers at the nodes indicate the levels of bootstrap support based on neighbor-joining analysis of 1,000 resampled datasets

contribution of 77% to bacterial clone library, this family might play an important role in phenanthrene biodegradation under anoxic condition. Previous works have shown that *Euryarchaeota* is usually the dominant archaeal phylum in various hydrocarbon-degrading methanogenic environments. Chang *et al.* (2005a, b) found that *Euryarchaeota* predominated in either PAH- or benzene-degrading methanogenic communities. Gilles *et al.* (2010) revealed that *Euryarchaeota* was the dominant archaeal phylum in petroleum amended anoxic coastal marine infralittoral

sediments, while unamended control sediments were dominated by *Crenarchaeota*. Archaeal populations consisted mainly of *Euryarchaeota* in the oil-contaminated soil (Liu *et al.*, 2009). In this study, the archaeal phylum *Euryarchaeota* also predominated (with a 96.7% relative abundance) in the archaeal community in sample AM_{d116}.

The 61 clones in archaeal libraries constructed with sample AM_{d116} were divided into five OTUs, including four OTUs had two or more clones. Phylogenetic tree of two representative archaeal 16S rRNA gene

sequences in each one of the four OTUs were constructed (Fig. 6). In this study, the archaeal OTU originating from sample AM_{d116} was referred to as AMAOTU. Clones ACA4 and ACA9 (AMAOTU1, with 31 clones) were classified within genus *Methanobacterium*, family *Methanobacteriaceae*, order *Methanobacteriales*, class *Methanobacteria*, phylum *Euryarchaeota*. The two clones were closely related with 98% identity to two cultured sequence, AF276958.1 and GU129130.1. The genus *Methanobacterium* was also present in domestic solid waste landfill soils (Chen *et al.*, 2003a). Some *Methanobacterium* species have also been isolated from aquifers (Godsy, 1980; Kotelnikova *et al.*, 1998). However, in this study, the relationship between the dominance of genus *Methanobacterium* in archaeal community and phenanthrene biodegradation remained unclear. Clones ACA16 and ACA29 (AMAOTU2, with two clones) were unclassified *Thermoprotei* within phylum *Crenarchaeota*. The closest match of clone ACA16 was sequence EF020521.1 (96% identity), obtained from trembling aspen rhizosphere. Clone ACA29 was most similar to sequence FJ957959.1 (98% identity), obtained from soil in Ruoergai plateau wetland. However, the two clones are not closely related to any cultured sequence in the GenBank. This illustrated a novel species. A lower abundance clones belonging to *Thermoprote* was also

detected in production water from a high-temperature, water-flooded petroleum reservoir of an offshore oilfield (Li *et al.*, 2007). In this study, the existence of hyperthermophilic class *Thermoprotei* was unclear. Clones ACA1 and ACA2 (AMAOTU3, with 25 clones) were classified as genus *Methanosarcina*, family *Methanosarcinaceae*, order *Methanosarcinales*, class *Methanomicrobia*, phylum *Euryarchaeota*. They are related with 98% identity to cultured sequences AJ002476.1, obtained from a grazing cow, and AB288262.1, obtained from an artesian spring and deep sedimentary aquifers. Methanogenesis was likely involved in anaerobic PAHs biodegradation (Chang *et al.*, 2006; Kim *et al.*, 2008). Methanogens could be indirectly related to the degradation of hydrocarbons by participating in syntrophic consortia with hydrocarbon-degrading acetogenic microorganisms (Gilles *et al.*, 2010). *Methanosarcina* species were capable of carbon tetrachloride and chloroform degradation in groundwater systems (Baeseman and Novak, 2001). Members of genus *Methanosarcina* have also been linked to PAH biodegradation (Kim *et al.*, 2008). Chen *et al.* (2003a, b) reported the dominance of *Methanosarcina* in methanogenic community in the solid waste landfill soils. Therefore, in this study, the abundance of *Methanosarcina* in archaeal community suggest a possible contribution of methanogenic community to phenanthrene degradation.

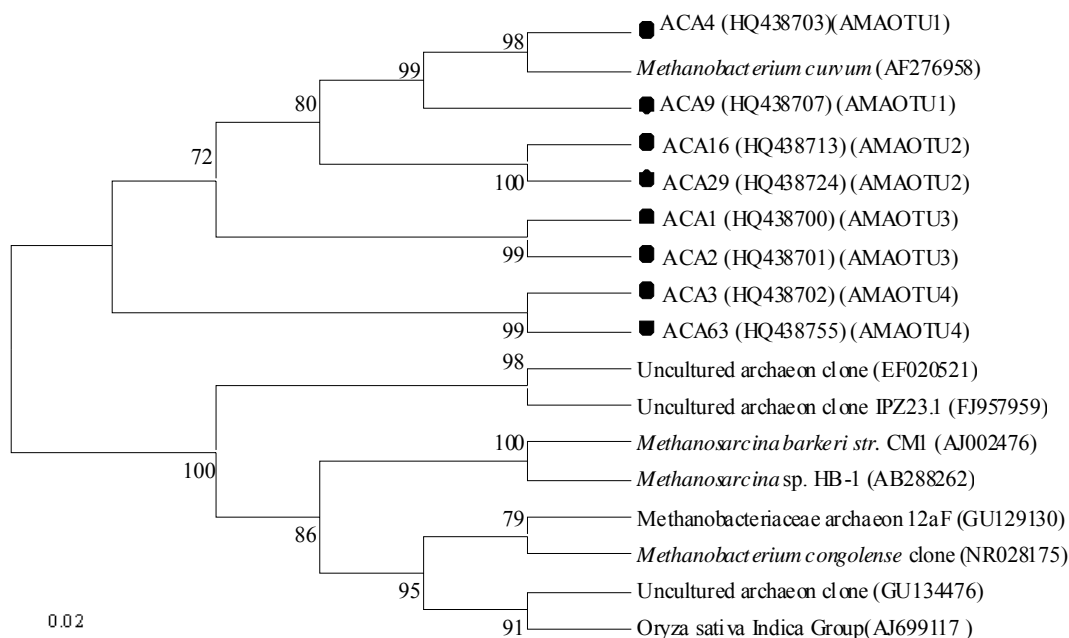


Fig. 6. Phylogenetic tree of two representative bacterial 16S rRNA gene sequences in each archaeal OTU (beginning with 'ACA') from sample AM_{d116} and reference sequences from GenBank. Data in parentheses are GenBank accession numbers. Numbers at the nodes indicate the levels of bootstrap support based on neighbor-joining analysis of 1,000 resampled datasets

Clones ACA3 and ACA63 (AMAOTU4, with two clones) fall within genus *Thermogymnomonas*, family *Thermoplasmatales_incertae_sedis*, *Thermoplasmatales*, class *Thermoplasmata*, phylum *Euryarchaeota*. The closest match of clones ACA3 and ACA63 was sequence GU134476.1 (with 99% identity), and sequence AJ699117.1 (with 100% identity) (Lu *et al.*, 2005), respectively. Each of the two sequences (GU134476.1 and AJ699117.1) originated from rice field soil. However, the two clones are not closely related to any cultured sequence in the GenBank. There is still no information available for roles of *Thermogymnomonas* in reduction of environmental contaminants.

CONCLUSION

The phenanthrene could be biologically degraded under either oxic or anoxic condition. Phenanthrene addition has significant impact on bacterial and archaeal community structures, depending on redox conditions. Both bacterial and archaeal species might be involved in phenanthrene degradation under anoxic condition. However, the mechanism of phenanthrene degradation remains unclear. Further study will be necessary to elucidate the PAH biodegradation in landfill leachate-contaminated aquifer.

ACKNOWLEDGEMENTS

This work was financially supported by National Natural Science Foundation of China (No. 50979002) and State Key Laboratory of Eco-hydraulic Engineering in Northwest Arid Region.

REFERENCES

Abdoli, M. A., Karbassi, A. R., Samiee-Zafarghandi, R., Rashidi, Zh., Gitipour, S. and Pazoki, M. (2012). Electricity Generation from Leachate Treatment Plant. *Int. J. Environ. Res.*, **6** (2), 493-498.

Ammar, E., Nasri, M., and Medhioub, K. (2005). Isolation of *Enterobacteria* able to degrade simple aromatic compounds from the wastewater from olive oil extraction. *World J. Microb. Biot.*, **21**, 253-259.

Arulazhagan, P., Vasudevan, N. and Yeom, I. T. (2010). Biodegradation of polycyclic aromatic hydrocarbon by a halotolerant bacterial consortium isolated from marine environment. *Int. J. Environ. Sci. Tech.*, **7**, 639-652.

Baeseman, J. L. and Novak, P. J. (2001). Effects of various environmental conditions on the transformation of chlorinated solvents by *Methanosarcina thermophila* cell exudates. *Biotechnol. Bioeng.*, **75**, 634-641.

Castro-Gutiérrez, V. M., Rodríguez-Rodríguez, C. E. and Vargas-Azofeifa, I. (2012). Hydrocarbon Degrading Microflora in a Tropical fuel-Contaminated Aquifer: Assessing the Feasibility of PAH Bioremediation. *Int. J. Environ. Res.*, **6** (1), 345-352.

Chang, W., Um, Y. S. and Holoman, T. R. P. (2005a). Molecular characterization of polycyclic aromatic hydrocarbon (PAH)-degrading methanogenic communities. *Biotechnol. Progr.*, **21**, 682-688.

Chang, W., Um, Y. S. and Holoman, T. R. P. (2005b) Molecular characterization of anaerobic microbial communities from benzene-degrading sediments under methanogenic conditions. *Biotechnol. Progr.*, **21**, 1789-1794.

Chang, W., Um, Y. S. and Holoman, T. R. P. (2006) Polycyclic aromatic hydrocarbon (PAH) degradation coupled to methanogenesis. *Biotechnol. Lett.*, **28**, 425-430.

Chen, A. C., Ueda, K., Sekiguchi, Y., Ohashi, A. and Harada, H. (2003a). Molecular detection and direct enumeration of methanogenic Archaea and methanotrophic Bacteria in domestic solid waste landfill soils. *Biotechnol. Lett.*, **25**, 1563-1569.

Chen, A. C., Imachi, H., Sekiguchi, Y., Ohashi, O. and Harada, H. (2003b) Archaeal community compositions at different depth (up to 30 m) of a municipal solid waste landfill in Taiwan as revealed by 16S rDNA cloning analyses. *Biotechnol. Lett.*, **25**, 719-724.

Clarke, K. R. and Warwick, R. M. (2001). Change in marine communities: an approach to statistical analysis and interpretation. 2nd ed PRIMER-E, Plymouth Marine Laboratory, Plymouth, UK.

Cui, Z. S., Lai, Q. L., Dong, C. M. and Shao, Z. Z. (2008). Biodiversity of polycyclic aromatic hydrocarbon-degrading bacteria from deep sea sediments of the Middle Atlantic Ridge. *Environ. Microbiol.*, **10**, 2138-2149.

D'Angelo, E. and Nunez, A. (2010). Effect of environmental conditions on polychlorinated biphenyl transformations and bacterial communities in a river sediment. *J. Soils Sediments*, **10**, 1186-1199.

Gu, T. H., Wang, Z. S., Tian, Y., Huang, B. Q. and Zheng, T. L. (2009). PAH-biodegradation potential of indigenous microorganisms: evidence from the respiratory activity of surface sediments in the Quanzhou Bay in China. *Acta Oceanologica Sinica*, **28**, 120-128.

Gilles, M., Monique, A., Jean-Claude, B. and Philippe, C. (2010). Response of an archaeal community from anoxic coastal marine sediments to experimental petroleum contamination. *Aquat. Microb. Ecol.*, **59**, 25-31.

Godsy, E. M. (1980). Isolation of *methanobacterium-bryantii* from a deep aquifer by using a novel broth-antibiotic disk method. *Appl. Environ. Microbiol.*, **39**, 1074-1075.

Grant, R. J., Muckian, L. M., Clipson, N. J. W. and Doyle, E. M. (2007) Microbial community changes during the bioremediation of creosote-contaminated soil. *Lett. Appl. Microbiol.*, **44**, 293-300.

Großkopf, R., Janssen, P. H. and Liesack, W. (1998). Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. *Appl. Environ. Microbiol.*, **64**, 960-969.

- Haritash, A. K. and Kaushik, C. P. (2009). Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): a review. *J. Hazard. Mater.*, **169**, 1-15.
- He, R., Ruan, A.D., Jiang, C. J. and Shen, D. S. (2008). Responses of oxidation rate and microbial communities to methane in simulated landfill cover soil microcosms. *Bioresour. Technol.*, **99**, 7192-7199.
- Huang, Y., Zou, L., Zhang, S. Y. and Xie, S. G. (2011). Comparison of bacterioplankton communities in three heavily polluted streams in China. *Biomed. Environ. Sci.*, **24**, 140-145.
- Hyun, I. P., Borinara, P. and Hong, K. D. (2011). Geotechnical Considerations for End-Use of Old Municipal Solid Waste Landfills. *Int. J. Environ. Res.*, **5** (3), 573-584.
- Imfeld, G., Aragones, C. E., Fetzer, I., Meszaros, E., Zeiger, S., Nijenhuis, I., Nikolausz, M., Delerce, S. and Richnow, H. H. (2010). Characterization of microbial communities in the aqueous phase of a constructed model wetland treating 1,2-dichloroethene-contaminated groundwater. *FEMS Microbiol. Ecol.*, **72**, 74-88.
- Kalyuzhnaya, M. G., Bowerman, S., Lara, J. C., Lidstrom, M. E. and Chistoserdova, L. (2006). *Methylothermobacter mobilis* gen. nov., sp. nov., an obligately methylamine-utilizing bacterium within the family *Methylophilaceae*. *Int. J. Syst. Evol. Microb.*, **56**, 2819-2823.
- Kalyuzhnaya, M. G., Martens-Habben, W., Wang, T. S., Hackett, M., Stolyar, S. M., Stahl, D. A., Lidstrom, M. E. and Chistoserdova, L. (2009). *Methylophilaceae* link methanol oxidation to denitrification in freshwater lake sediment as suggested by stable isotope probing and pure culture analysis. *Environ. Microbiol. Reports*, **1**, 385-392.
- Kasai, Y., Takahata, Y., Hoaki, T. and Watanabe, K. (2005). Physiological and molecular characterization of a microbial community established in unsaturated, petroleum-contaminated soil. *Environ. Microbiol.*, **7**, 806-818.
- Kim, M., Bae, S. S., Seol, M., Lee, J. H. and Oh, Y. S. (2008). Monitoring nutrient impact on bacterial community composition during bioremediation of anoxic PAH-contaminated sediment. *J. Microbiol.*, **46**, 615-623.
- Kotelnikova, S., Macario, A. J. L. and Pedersen, K. (1998) *Methanobacterium subterraneum* sp. nov., a new alkaliphilic, eurythermic and halotolerant methanogen isolated from deep granitic groundwater. *Int. J. Syst. Bacteriol.*, **48**, 357-367.
- Li, H., Yang, S. Z., Mu, B. Z., Rong, Z. F. and Zhang, J. (2007). Molecular phylogenetic diversity of the microbial community associated with a high-temperature petroleum reservoir at an offshore oilfield. *FEMS Microb. Ecol.*, **60**, 74-84.
- Li, C. H., Wong, Y. S. and Tama, N. F. Y. (2010). Anaerobic biodegradation of polycyclic aromatic hydrocarbons with amendment of iron (III) in mangrove sediment slurry. *Bioresour. Technol.*, **101**, 8083-8092.
- Liu, R. Y., Zhang, Y., Ding, R., Li, D., Gao, Y. X. and Yang, M. (2009). Comparison of archaeal and bacterial community structures in heavily oil-contaminated and pristine soils. *J. Biosci. Bioeng.*, **108**, 400-407.
- Lors, C., Ryngaert, A., Perie, F., Diels, L. and Damidot, D. (2010). Evolution of bacterial community during bioremediation of PAHs in a coal tar contaminated soil. *Chemosphere*, **1**, 1263-1271.
- Lu, Y., Lueders, T., Friedrich, M. W. and Conrad, R. (2005). Detecting active methanogenic populations on rice roots using stable isotope probing. *Environ. Microbiol.*, **7**, 326-336.
- Mahmoudkhani, R., Hassani, A. H., Torabian, A. and Borghei, S. M. (2012). Study on High-strength Anaerobic Landfill Leachate Treatability By Membrane Bioreactor Coupled with Reverse Osmosis. *Int. J. Environ. Res.*, **6** (1), 129-138.
- Maidak, B. L., Cole, J. R., Lilburn, T. G., Parker, C. T., Saxman, P. R., Farris, R. J., Garrity, G. M., Olsen, G. J., Schmidt, T. M. and Tiedje, J. M. (2001). The RDP-II, Ribosomal Database Project. *Nucleic Acids Res.*, **29**, 173-174.
- Maqbool, F., Bhatti, Z. A., Malik, A. H., Pervez, A. and Mahmood, Q. (2011). Effect of Landfill Leachate on the Stream water Quality. *Int. J. Environ. Res.*, **5** (2), 491-500.
- Martinen, S. K., Kettunen, R. H. and Rintala, J. A. (2003). Occurrence and removal of organic pollutants in sewages and landfill leachates. *Sci. Total Environ.*, **301**, 1-12.
- Meckenstock, R. U., Safinowski, M. and Griebler, C. (2004). Anaerobic degradation of polycyclic aromatic hydrocarbons. *FEMS Microbiol. Ecol.*, **49**, 27-36.
- Mouser, P. J., Rizzo, D. M., Druschel, G. K., Morales, S. E., Hayden, N., O'Grady, P. and Stevens, L. (2010). Enhanced detection of groundwater contamination from a leaking waste disposal site by microbial community profiles. *Water Resour. Res.*, **46**, DOI: 10.1029/2010WR009459.
- Nouri, N., Poorhashemi, S. A., Monavari, S. M., Dabiri, F. and Hassani, A. H. (2011). Legal Criteria and Executive Standards of Solid Waste Disposal Subjected to Solid Waste Management Act. *Int. J. Environ. Res.*, **5** (4), 971-980.
- Oluseyi, T., Olayinka, K., Alo, B. and Smith, R. M. (2011). Improved Analytical Extraction and Clean-up Techniques for the Determination of PAHs in Contaminated Soil Samples. *Int. J. Environ. Res.*, **5** (3), 681-690.
- Rees, G. N., Baldwin, D. S., Watson, G. O., Perryman, S. and Nielsen, D. L. (2004). Ordination and significance testing of microbial community composition derived from terminal restriction fragment length polymorphisms: application of multivariate statistics. *Anton. Leeuw. Int. J. G.*, **86**, 339-347.
- Rogers, S. W., Ong, S. K. and Moorman, T. B. (2007). Mineralization of PAHs in coal-tar impacted aquifer sediments and associated microbial community structure investigated with FISH. *Chemosphere*, **69**, 1563-1573.
- Roling, W. F. M., van Breukelen, B. M., Braster, M., Lin, B. and van Verveveld, H. W. (2001). Relationships between

microbial community structure and hydrochemistry in a landfill leachate-polluted aquifer. *Appl. Environ. Microbiol.*, **67**, 4619-4629.

Shafieiyou, S., Ebadi, T. and Nikazar M. (2012). Treatment of Landfill Leachate by Fenton Process with Nano sized Zero Valent Iron particles. *Int. J. Environ. Res.*, **6** (1), 119-128.

Singh, C. and Lin, J. (2007). Isolation and characterization of diesel oil degrading indigenous microorganisms in Kwazulu-Natal, South Africa. *Afr. J. Biotechnol.*, **7**, 1927-1932.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007). MEGA4, molecular evolutionary genetics analysis, MEGA software version 4.0. *Mol. Biol. Evol.*, **24**, 1596-1599.

Tian, Y., Liu, H. J., Zheng, T. L., Kwon, K. K., Kim, S. J. and Yan, C. L. (2008). PAHs contamination and bacterial communities in mangrove surface sediments of the Jiulong River Estuary, China. *Mar. Pollut. Bull.*, **57**, 707-715.

Tian, Y. J., Yang, H., Wu, X. J. and Li, D. T. (2005). Molecular analysis of microbial community in a groundwater sample polluted by landfill leachate and seawater. *J. Zhejiang Univ.-Sci. B*, **23**, 165-170.

Vinas, M., Sabate, J., Guasp, C., Lalucat, J. and Solanas, A. M. (2005). Culture-dependent and -independent approaches establish the complexity of a PAH-degrading microbial consortium. *Can. J. Microbiol.*, **51**, 897-909.

Wang, Q., Garrity, G. M., Tiedje, J. M. and Cole, J. R. (2007). Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.*, **73**, 5261-5267.

Wang, Q. F., Zhang, S. Y., Zou, L. and Xie, S. G. (2011). Impact of anthracene addition on microbial community structure in soil microcosms from contaminated and uncontaminated sites. *Biomed. Environ. Sci.*, **24**, 542-548.

Wiinsche, L., Brüggemann, L. and Babel, W. (1995). Determination of substrate utilization patterns of soil microbial communities: an approach to assess population changes after hydrocarbon pollution. *FEMS Microbiol. Ecol.*, **17**, 295-306.

Xie, S. G., Sun, W. M., Luo, C. L. and Cupples, A. M. (2010). Stable isotope probing identifies novel *m*-xylene degraders in soil microcosms from contaminated and uncontaminated sites. *Water Air Soil Pollut.*, **212**, 113-122.

Yang, Y. and McCarty, P. L. (1998). Competition for hydrogen within a chlorinated solvent dehalogenating anaerobic mixed culture. *Environ. Sci. Technol.*, **32**, 3591-3597.

Zhang, S. Y., Wang, Q. F. and Xie, S. G. (2011a). Microbial community changes in contaminated soils in response to phenanthrene amendment. *Int. J. Environ. Sci. Tech.*, **8**, 321-330.

Zhang, Y., Li, F. and Wang, J. (2011). Determination of Retardation Effect of SOM on Aqueous Leaching of Polycyclic Aromatic Hydrocarbons Using Confocal Laser Scanning Microscope. *Int. J. Environ. Res.*, **5** (4), 999-1008.

Zhang, S. Y., Wang, Q. F., Wan, R. and Xie, S. G. (2011b). Changes in bacterial community of anthracene bioremediation in municipal solid waste composting soil. *J. Zhejiang Univ. Sci. B*, **12**, 760-768.