Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
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Changes in bacterial community of anthracene bioremediation in municipal solid waste composting soil*

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Received Dec. 20, 2010; Revision accepted Mar. 8, 2011; Crosschecked Aug. 18, 2011

Abstract: Polycyclic aromatic hydrocarbons (PAHs) are common contaminants in a municipal solid waste (MSW) composting site. Knowledge of changes in microbial structure is useful to identify particular PAH degraders. However, the microbial community in the MSW composting soil and its change associated with prolonged exposure to PAHs and subsequent biodegradation remain largely unknown. In this study, anthracene was selected as a model compound. The bacterial community structure was investigated using terminal restriction fragment length polymorphism (TRFLP) and 16S rRNA gene clone library analysis. The two bimolecular tools revealed a large shift of bacterial community structure after anthracene amendment and subsequent biodegradation. Genera *Methylophilus*, *Mesorhizobium*, and *Terrimonas* had potential links to anthracene biodegradation, suggesting a consortium playing an active role.

Key words: Anthracene, Microbial community, Biodegradation, Bioremediation
doi:10.1631/jzus.B1000440 **Document code:** A **CLC number:** X172

1 Introduction

With rapid urbanization, municipal solid waste (MSW) constitutes an immediate and serious environmental problem in many developing countries. In many Chinese urban and suburban regions, a great amount of collected MSW is dumped on land in a more or less uncontrolled manner. Leachate from MSW composting site may contain a large number of xenobiotic organic compounds (Baun *et al*., 2004; Xu *et al*., 2008). Therefore, inadequate MSW disposal can lead to the contamination of soil, and surface and ground waters in the vicinity of the site. Polycyclic aromatic hydrocarbons (PAHs) are a group of chemical compounds consisting of carbon and hydrogen, whose ubiquitous existence in the various environments has aroused great concerns. Leachate from an unmanaged MSW composting site can cause heavy PAH contamination in soil in the vicinity of the site (Han *et al*., 2009).

Microbial degradation plays the primary role in PAH reduction in contaminated sites (Yuan *et al*., 2001; Yousefi Kebria *et al*., 2009). The knowledge about the microbial community structure in MSW composting soil is very important for development of bioremediation technology. Numerous studies have investigated the bacterial community structure in contaminated aquifers near MSW composting sites (Röling *et al*., 2000; 2001; Tian *et al*., 2005; Yu *et al*., 2010). However, these previous works have not investigated the links of PAHs to the bacterial communities. Also, little is known about the microbial community in MSW soil. The concentration of PAHs in soils near MSW compositing site usually increases with time (Han *et al*., 2009). Unfortunately, very few investigations have been undertaken to assess the impact of prolonged PAH contamination or PAH amendment on soil microbial community (Muckian *et al*., 2009). As a consequence, the microbial community in the MSW composting soil and its change associated

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* Project (No. 50979002) supported by the National Natural Science Foundation of China

with prolonged exposure to PAHs and subsequent biodegradation remain largely unknown.

Anthracene has been listed as one of the priority environmental pollutants by the United States Environmental Protection Agency. Anthracene is also a model compound for PAH degradation studies (Müncnerová and Augustin, 1994; Krivobok *et al*., 1998). An average level of 1208.5 μg/kg total PAHs (including 88.2 μg/kg anthracene) has even been observed in an MSW composting soil (Han *et al*., 2009). Therefore, the aim of current study was to investigate the bacterial community in the MSW composting soil, and to explore the change associated with anthracene amendment and subsequent biodegradation. The genera with potential PAH degradation capabilities were also investigated.

2 Materials and methods

2.1 Microcosm experiment set-up

Soil was collected from the vicinity of an unmanaged MSW composting site in Changzhou, a southeast city in China. Following sample collection, soil was air-dried, homogenized, and sieved through a 0.18-mm screen, and stored at 4 °C until use. Anthracene (99%, J & K China Chemical) dissolved in acetone was added to each empty microcosm chamber (150 ml serum bottle) with a total mass of 200 μg anthracene per chamber. After the acetone evaporated, 2 g dry soil was added to each microcosm along with 10 ml phosphate buffered mineral media, as previously described (Mu and Scow, 1994). The bottles were sealed with rubber stoppers and aluminum seals. The sterile controls were obtained by autoclaving (121 °C, 1 h) repeatedly (three times). Microcosms were maintained at 1 °C in a refrigerator and stirred periodically (manually stirred for 2 min, every 30 min) for 48-h equilibration. Microcosms were incubated at 25 °C (Day 0). Microcosms were sampled on Days 0, 10, and 15. In each sampling time, three replicate microcosms were used for anthracene analysis and the other three for soil DNA extraction.

2.2 Anthracene extraction and analysis

In order to determine anthracene in solid phase, the water/soil mixture in microcosms was transferred to a 100-ml centrifuge tube. After 10-min centrifugation at 5000 r/min, the supernatant was collected. A total of 1 g dry soils were extracted three times with 10 ml acetone, using a 300-W ultrasonic processor. The mixture was vigorously shaken and then centrifuged at 5000 r/min for 10 min. Then 0.2 ml of the supernatant was collected into a gas chromatography (GC) vial and diluted by 0.8 ml methanol (Zhang *et al.*, 2011). The mixture was filtered with a 0.45-μm British Nylon 66 syringe filter provided by Tianjin Jingteng Corp., China. The anthracene analysis was conducted according to the standard method (Huang Y. *et al*., 2010).

2.3 Terminal restriction fragment length polymorphism (TRFLP) analysis

Soil DNAs from Samples A (from microcosm on Day 0) and B (from microcosm on Day 15) were used for further molecular analysis. DNA was extracted using the UltraClean DNA extraction kit (Mo Bio Laboratories). Bacterial 16S rRNA genes were amplified using the bacterial primers 27F-FAM (5′-GAG TTT GAT CMT GGC TCA G-3′, 5′ end-labeled with carboxyfluorescein) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′). The polymerase chain reaction (PCR) program was as previously described (Cupples and Sims, 2007; Zhang *et al*., 2011). A total of 300 ng fluorescently labeled PCR products were purified with QIA quick PCR purification kit (Qiagen Inc., Germany) and digested with *Hae*III with a 6-h incubation period. *Hha*I and *Msp*I were also used to confirm the identity of the greatly enriched fragments. The same purified PCR product was used for digestion with *Hae*III, *Hha*I, and *Msp*I. The clone sequence was first identified, the in silico cut site (*Hae*III digest) of which matched the length of the greatly enriched fragment (*Hae*III digest). If the clone restriction enzyme (*Hha*I or *Msp*I) cut site predicted from sequence also matched the observed length of one abundant fragment (*Hha*I or *Msp*I digest) in Sample B, the taxonomic identity of the greatly enriched fragment (*Hae*III digest) could be confirmed (Cupples and Sims, 2007; Zhang *et al*., 2011). The fragment pattern was detected using an ABI 3730 DNA analyzer set at the GeneScan mode together with an internal lane standard (GeneScan-500 LIZ Size Standard, Applied Biosystems).

Replicate profiles from separate DNA extractions and PCR reactions for each sample were compared to

identify the subset of reproducible fragment sizes. The average area of each reproducible peak was calculated. Terminal fragments smaller than 50 bp or under 200 fluorescent units were excluded from the further analysis. Fragments differing by less than 1 bp length were clustered. The standardized binning criteria used to identify the subset of reproducible peaks were as previously described (Dunbar *et al*., 2001; Mills *et al*., 2003). Ribotype richness (*S*) equals to the total number of distinct fragments in a profile. The Shannon diversity index (*H*) and evenness (*E*) were calculated according to the standard methods (Mills *et al*., 2003; Falk *et al*., 2009). Bray-Curtis similarity index was calculated using PRIMER 5.0 software to evaluate the similarity for two microcosm samples (Clarke and Warwick, 2001). Similarity percentage (SIMPER) analysis was further used to identify the fragments that were mainly responsible for the dissimilarity between samples (Clarke and Warwick, 2001; Rees *et al*., 2004).

2.4 DNA cloning and sequencing

The PCR conditions were the same as the above-mentioned, except that the forward primer was unlabeled. The PCR products were cloned into pMD19-T vector (TaKaRa Co., Japan) following the manufacturer's instruction. The white colonies were verified by PCR with primers M13 F (5′-TGT AAA ACG ACG GCC AGT-3′) and M13 R (5′-AAC AGC TAT GAC CAT G-3′). Clones containing an insert of the correct size were bidirectionally sequenced. The 69–72 clones recovered from each sample were successfully sequenced in this study. The partial 16S rRNA gene sequences were deposited with GenBank under accession Nos. HQ015161-HQ015301.

Sequences for chimerism were checked using the Chimera Check program available at the Michigan State University (MSU) Center for Microbial Ecology. All clones displaying chimeric profiles were discarded from the further analysis. Sequences that were over 97% similar were grouped into an operational taxonomic unit (OTU) by manual comparison. The representative GenBank sequences to the clones of interest were extracted from the National Center for Biotechnology Information (NCBI) database and included in further phylogenetic analyses using MEGA Version 4.0 (Tamura *et al*., 2007). The neighbor-joining trees were constructed using *p*-distance with 1000 replicates to produce bootstrap values. Alignment of the sequences was carried out using ClustalW (http://www.ebi.ac.uk/clustalw/). The Ribosomal Database Project (MSU Center for Microbial Ecology) analysis tool "classifier" (http://rdp.cme.msu.edu/classifier/classifier.jsp) was utilized to assign taxonomic identity (Wang *et al*., 2007).

3 Results

3.1 Biodegradation

In this study, the total amount of anthracene in supernatant was below 3% of that in the solid phase over the whole experiment (data not shown). Anthracene concentration in soil declined greatly after a 15-d experiment period. In contrast, only a limited decline in soil was observed in the autoclaved control, possibly due to aging effect. This confirmed a biological removal mechanism in microcosms (Table 1).

Table 1 Anthracene (ANT) remaining in soil samples over the experimental period

Time (d)	Remaining ANT (%)	
	Sterile control with ANT	Microcosm with ANT
0	97.5	97.2
10	80.9	44.8
15	77.0	11.4

3.2 TRFLP analysis

The total average numbers of terminal restriction fragments (*Hae*III digest) in Samples A and B were 35 and 29, respectively (Fig. 1). Shannon diversity index and evenness did not differ much between Samples A and B (Table 2). However, the low Bray-Curtis similarity (average 38%) reflected a big difference of microbial community structure between Samples A and B.

Table 2 Comparison of diversity and evenness indices for the TRFLP profiles (*Hae*III digest) from the different samples

Samples	*S*	*H*	*E*
A	35	3.191	0.897
B	29	3.145	0.934

S: ribotype richness; *S*=total number of bands in profile. *H*: Shannon diversity index; $H=-\sum(p_i)(\log_2 p_i)$, where p_i is the individual peak area; $H_{max}=\log_2 S$. *E*: evenness; $E=H/H_{max}$

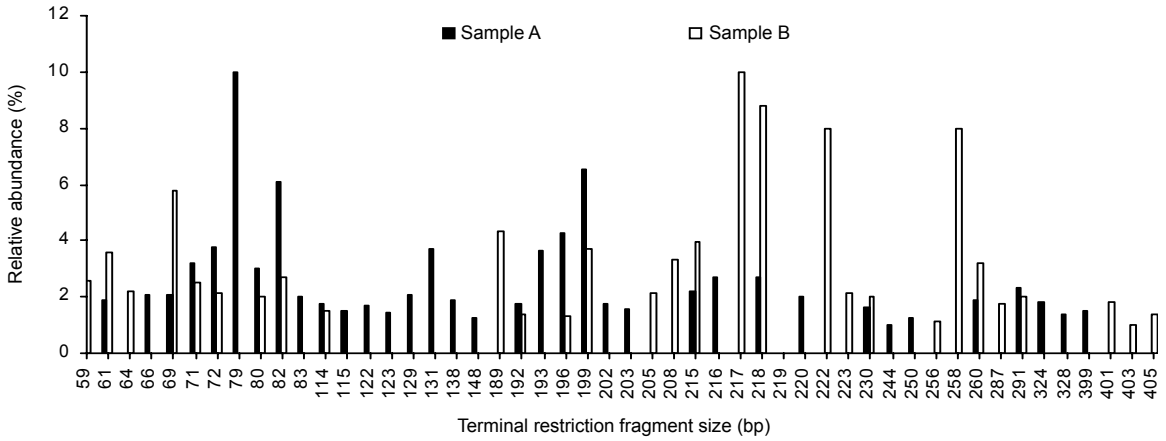

Fig. 1 Terminal restriction fragments (*Hae*III digest) and their abundances in the Samples A and B

The SIMPER analysis revealed that the differences of TRFLP profiles (*Hae*III digest) between Samples A and B were driven primarily by variation in 217, 79, 222, and 258 bp (Table 3). The 79 bp was abundant in Sample A. These abundant fragments (217, 222, and 258 bp) in Sample B were not detected in Sample A (Fig. 1), which indicated these fragments were largely enriched with anthracene biodegradation.

Table 3 Results of SIMPER analysis of TRFLP profiles of two samples

Fragment (bp)	Mean relative abundance (%)[a]		Fragment contribution (%)[b]
	Sample A	Sample B	
217	0	10	6
79	10	0	6
222	0	8	5
258	0	8	5

[a] Mean relative abundance of each fragment (*Hae*III digest) as a percentage of total fragment abundance; [b] Fragment contribution as a dissimilarity percentage between the two groups. Lists are truncated to include only those fragments that contribute no less than 5% to the differences between samples

3.3 Phylogeny

In this study, many known phyla (Bacteroidetes, Proteobacteria, Verrucomicrobia, Acidobacteria, TM7, Cyanobacteria, Gemmatimonadetes) were detected, although only Bacteroidetes and Proteobacteria were shared between both samples (Fig. 2). For Sample A, the 72 recovered clones were distributed across phyla as follows: β-proteobacteria 43.1%, γ-proteobacteria 15.3%, α-proteobacteria 12.5%, unclassified bacteria 8.3%, δ-proteobacteria 6.9%, Gemmatimonadetes 6.9%, Bacteroidetes 4.2%, Acidobacteria 2.8%. However, the major phylum types (10% or more) of 69 clones recovered from the Sample B were γ-proteobacteria (44.9%), β-proteobacteria (23.2%), and Bacteroidetes (14.5%). Compared with Sample A, Bacteroidetes and γ-proteobacteria achieved a large increase in relative abundance (no less than 10%). Unclassified Proteobacteria, Verrucomicrobia, TM7, and Cyanobacteria were also detected in Sample B. These results show a shift of bacterial community structure in MSW composting soil.

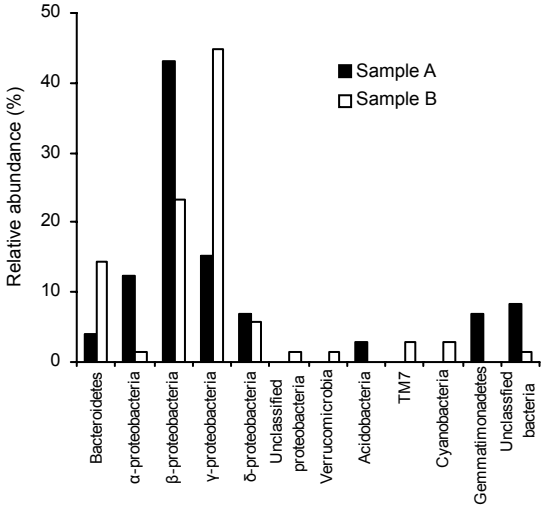

Fig. 2 Percentages of the clones affiliated with different phyla and sub-phyla to the total number of clones from Sample A or B

Clones not classified to any known phylum are included as unclassified bacteria

Moreover, the 72 clones in the bacterial library constructed with Sample A could be further divided into 46 OTUs (14 OTUs had two or more clones). However, the 69 clones in library constructed with Sample B were grouped into 39 OTUs (11 OTUs had two or more clones). Therefore, in agreement with the TRFLP analysis, the bacterial community structure reflected by the clone library analysis also indicated a big change with PAH biodegradation.

3.4 Identity of enriched TRFLP fragments

The taxonomic identity of 217, 222, or 258 bp (*Hae*III digest) was also investigated. Comparisons of TRFLP fragments and in silico cut sites are presented in Table 4. The taxonomic identity of each enriched fragment is presented in Table 5. The comparisons of TRFLP fragments and in silico cut sites to determine the enriched fragments have also been noted elsewhere (Cupples and Sims, 2007; Luo *et al*., 2009). 217, 222, and 258 bp (*Hae*III digest) obtained from Sample B were identified as *Methylophilus*, *Mesorhizobium*, and *Terrimonas*, respectively. The numbers of clones classified within genera *Methylophilus*, *Mesorhizobium*, and *Terrimonas* in Sample B were 6, 2, and 7, respectively. However, only one *Methylophilus* clone and one *Terrimonas* clone were detected in Sample A. Therefore, similar to TRFLP results (Fig. 1; Table 3), the enrichment of the three genera was also demonstrated.

Phylogenetic tree of two representative sequences in each genus (*Methylophilus*, *Mesorhizobium*, or *Terrimonas*) was constructed using MEGA Version 4.0 (Fig. 3). Clones L5 and L6 were identified

Table 4 Comparison of TRFLP fragments to clone restriction enzyme cut sites predicted from sequence analyses to confirm the identity

Enriched fragment	Restriction enzyme	TRFLP	Sequence data
1	*Hae*III	217	219
	*Msp*I	487	490
	*Hha*I	367	367
2	*Hae*III	222	225
	*Msp*I	128	128
	*Hha*I	58	61
3	*Hae*III	258	260
	*Msp*I	90	93
	*Hha*I	69	67

Table 5 Phylogenetic affiliation of each enriched fragment (*Hae*III digest) as determined with the Ribosomal Database Project analysis tool "classifier"

Fragment (bp)	Phylum	Class	Order	Family	Genus
217	Proteobacteria	β-proteobacteria	Methylophilales	Methylophilaceae	*Methylophilus*
222	Proteobacteria	α-proteobacteria	Rhizobiales	Phyllobacteriaceae	*Mesorhizobium*
258	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Chitinophagaceae	*Terrimonas*

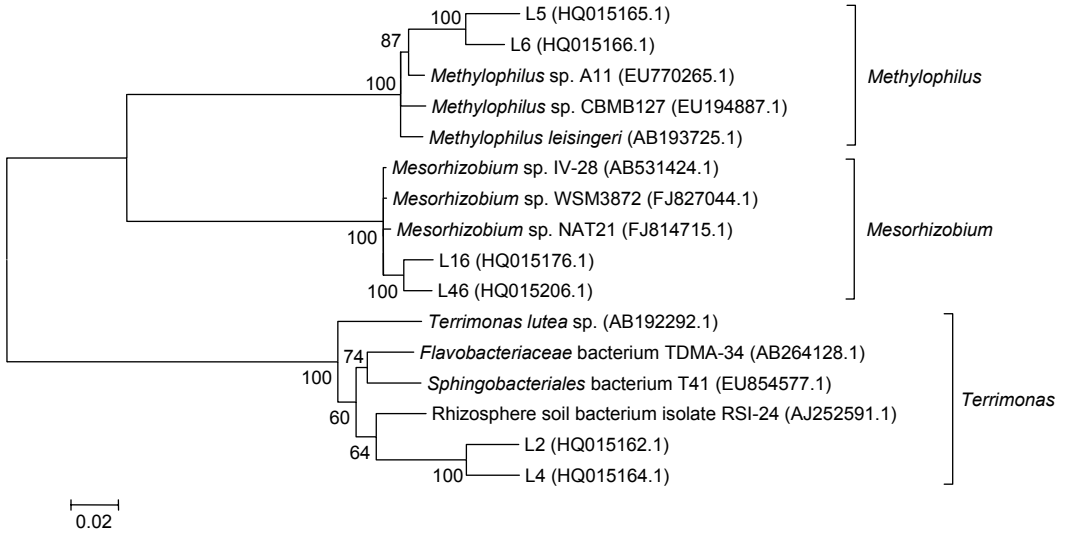

Fig. 3 Phylogenetic tree of representative bacterial 16S rRNA gene sequences (beginning with 'L') within the genera of *Methylophilus*, *Mesorhizobium*, and *Terrimonas* from Sample B and reference sequences from GenBank
Data in parentheses are GenBank accession numbers. Numbers at the nodes indicate the levels of bootstrap support based on neighbor-joining analysis of 1000 resampled datasets

as the members of genus *Methylophilus*. Their three closest cultivated matches were obtained from human organs (AB193725.1, 99% identity), microcystis colonies from Taihu Lake (EU770265.1, 99% identity), and field grown rice cultivars (EU194887.1, 99% identity). Clones L16 and L46 were classified within genus *Mesorhizobium*. Their three closest cultivated matches were obtained from grassland soil (AB531424.1, 99% identity), legume genus *Biserrula* growing in Eritrea (FJ827044.1, 99% identity), and root nodules (FJ814715.1, 99% identity). Clones L2 and L4 were identified as *Terrimonas* species. The two clones illustrated 95% similarity to a *Terrimonas lutea* sp. species (AB192292.1) isolated from soil. They were also related to several cultured sequences, such as fresh water *Flavobacteriaceae* species (AB264128.1, 96%), soil *Sphingobacteriales* species (EU854577.1, 96%), and soil rhizosphere species (AJ252591.1, 95%).

4 Discussion

Considerable attention has been paid to the diversity of indigenous microorganisms capable of degrading pollutants in various environments (Abed *et al*., 2002). Some other profiling techniques, such as community-level physiological profiling, restriction fragment length polymorphism, denaturing gradient gel electrophoresis, and phospholipid fatty acid profiling, have been applied to investigate the composition of bacterial community in contaminated aquifers near MSW composting sites (Röling *et al*., 2000; 2001; Tian *et al*., 2005; Yu *et al*., 2010). Among available DNA profiling techniques, TRFLP has been used to monitor structural changes of various soil microbial communities (Dunbar *et al*., 2000; Singh *et al*., 2006; Grant *et al*., 2007). However, to the best of our knowledge, this is the first report to investigate the change of the bacterial community in the MSW composting soil in response to anthracene amendment and subsequent biodegradation.

The bacterial community structure usually changes during the bioremediation of PAH-contaminated soils (MacNaughton *et al*., 1999; Vinas *et al*., 2005; Gandolfi *et al*., 2010). Muckian *et al*. (2009) have revealed the significant change of the microbial community associated with fluoranthene or phenanthrene amendment and subsequent biodegradation. Piskonen *et al*. (2005) have also shown selection in the microbial community as a result of naphthalene biodegradation. In this study, both TRFLP and 16S rRNA gene clone library analysis revealed a big shift of microbial community structure with anthracene biodegradation.

The phylogenetic description of the bacterial community structure is also important. Tian *et al*. (2005) has reported the dominance of *Firmicutes* and *Fusobacteria* in the leachate-contaminated aquifers. Although the literature on the microbial community in MSW composting soil is still lacking, the dominance of Proteobacteria has been observed in the soil/water system for PAH biodegradation (Chang *et al*., 2007), and in aged PAH-contaminated soil (Cébron *et al*., 2009). Our preliminary research indicated that the soil used to construct microcosms contained high concentration of anthracene (about 80 μg/kg). Therefore, the abundance of Proteobacteria in Sample A might be partly attributed to the high level of PAHs in MSW composting soil.

Many different bacterial genera previously isolated from PAH-contaminated sites belong to *Sphingomonas* (Pinyakong *et al*., 2000), *Paracoccus* (Teng *et al*., 2010), *Mycobacterium* (Luo, 2008), *Comamonas* (Goyal and Zylstra, 1996), *Pseudomonas* (Jacques *et al*., 2005; Santos *et al*., 2008), *Burkholderia* (Juhasz *et al*., 1997), *Janibacter* (Zhang *et al*., 2009), and *Sphingobium* (Cunliffe and Kertesz, 2006). Enrichment of PAH-degraders is a common phenomenon during the course of PAH biodegradation (Guo *et al*., 1997; Piskonen *et al*., 2005). Therefore, in this study, the large enrichment of three TRFLP fragments might also be linked to the enrichment of some PAH-degraders. The greatly enriched fragments (217, 222, and 258 bp) (*Hae*III digest) obtained from Sample B belong to *Methylophilus*, *Mesorhizobium*, and *Terrimonas*, respectively. However, surprisingly, to the best of our knowledge, no isolates from these genera have previously been founded in PAH-contaminated soils or linked to PAH biodegradation.

Some species of *Methylophilus* have been linked to degradation or utilization of various compounds including dichloromethane (Nikolausz *et al*., 2006), methylated amines (Large and Haywood, 1981), methanol (Jenkins *et al*., 1987), glucose, and galactose

(Doronina and Trotsenko, 1994). Two recent reports have also linked *Methylophilus* to utilization of biphenyl (Uhlik *et al*., 2009), phenol, and humic matter (Hutalle-Schmelzer *et al*., 2010). Interestingly, these compounds have a common phenolic hydroxyl proposed as intermediate compounds in anthracene degradation pathways (Pinyakong *et al*., 2003). Therefore, *Methylophilus* may play roles in the degradation of intermediates of anthracene degradation, thus leading to its enrichment.

Mesorhizobium species are usually isolated from root nodules (Lin *et al*., 2009; Chen *et al*., 2010). Recently, some species of *Mesorhizobium* have been linked to sulfur oxidization (Ghosh and Roy, 2006), nitrile biotransformation (Feng *et al*., 2008), oxamyl degradation (Osborn *et al*., 2010), high arsenic resistance (Huang A.H. *et al*., 2010), chromium reducing (Wani *et al*., 2009), and acetonitrile biodegradation (Feng and Lee, 2009). However, little is known about the environmental significances of *Terrimonas*. One recent report has revealed the dominance of *Terrimonas* in methyl tert-butyl ether degrading culture (Liu *et al*., 2009). Therefore, the roles of *Methylophilus*, *Mesorhizobium*, and *Terrimonas* in PAH biodegradation still remained largely unclear.

5 Conclusions

Both TRFLP and 16S rRNA gene clone library analysis revealed the big shift of bacterial community structure in MSW composting soil after anthracene amendment and subsequent biodegradation. Three genera, *Methylophilus*, *Mesorhizobium*, and *Terrimonas*, had potential links to anthracene biodegradation, suggesting a consortium playing an active role. Further study will be necessary to explore the biodegradation mechanism of anthracene in MSW composting soil. This is very important because the presence of particular microorganisms or a consortium may determine the bioremediation strategies for PAHs in MSW composting soil in practice.

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