Bacterial communities predominant in the degradation of $^{13}\text{C}_4$ -4,5,9,10-pyrene during composting

Jingjing Peng^{a,1}, Yuan Zhang^{a,b,1}, Jianqiang Su^a, Qiongfen Qiu^c, Zhongjun Jia^{d,*}, Yong-Guan Zhu^{a,e,*}

^a Key Laboratory of Urban Environment and Health Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, China

^b Suzhou University of Science and Technology, Suzhou 215009, China

^c School of Marine Science, Ningbo University, Ningbo 315211, China

^d State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, Nanjing 210008, China

^e Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

HIGHLIGHTS

- The $^{13}\text{C}_4$ -pyrene dissipation rates were significantly different at different composting stage.
- Significant bacterial community shift was observed during the composting process.
- *Streptomyces* was the dominant $^{13}\text{C}_4$ -pyrene degraders at 55 °C in the composting.

ARTICLE INFO

Article history:

Received 14 March 2013

Received in revised form 9 June 2013

Accepted 12 June 2013

Available online 19 June 2013

Keywords:

Community structure

Composting

Pyrene degradation

Stable isotope probing (SIP)

Temperature

ABSTRACT

An in-vessel composting bioremediation of $^{13}\text{C}_4$ -4,5,9,10-pyrene and unlabeled pyrene spiked soil amended with fresh wastes was investigated by DNA-based stable isotope probing (SIP) of active bacteria involved. Highest dissipation of $^{13}\text{C}_4$ -pyrene was detected at 55 °C after 42 days composting. The active bacterial communities in the composting changed over time, showing a distinct difference among different stages. α -, β -, γ -*Proteobacteria*, and *Actinobacteria* were detected mainly involving in pyrene degradation at 38 °C over 14 days composting. *Streptomyces* appeared to dominate the pyrene degradation at 55 °C. β - and γ -*Proteobacteria* and *Actinobacteria* were the dominant pyrene degraders at 70 °C after 42 days composting and at 38 °C after 60 days composting. The results of this study suggest the pyrene degradation was performed by phylogenetically distinct bacterial guilds from the phyla *Actinobacteria* and *Proteobacteria* during in-vessel composition processes.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Composting has shown to be effective in biodegrading polycyclic aromatic hydrocarbons (PAHs). It could achieve a higher dissipation of PAHs after the addition of fresh green wastes than the addition of mature compost in contaminated soil (Antizar-Ladislao and Galil, 2006; Zhang et al., 2011). Previous investigations indicated that temperature and soil/waste amendment ratio are important operating parameters for composting-bioremediation treatment of contaminated soils (Antizar-Ladislao et al., 2005a,b). However, microbial communities which plays a key role in the PAHs elimination strongly affected the compost stability (Antizar-Ladislao, 2010). Hence many studies suggested that

microbiological parameters can serve as indicators of compost maturity (Tiquia, 2005). So far, only few studies have monitored the microbial community changes during conventional windrow-composting (Bolta et al., 2003; Steger et al., 2005) and in-vessel composting system (Antizar-Ladislao et al., 2008; Zhang et al., 2011) using phospholipids fatty acid (PLFA) analysis.

Most microorganisms that have been isolated and characterized as using pyrene as the sole carbon and/or energy source are fast-growing *Mycobacterium*, among the *Actinobacteria* (DeBruyn et al., 2007; Heitkamp et al., 1988), but other studies indicated that the primary pyrene degraders in bioreactors were uncultivated, β - and γ -*Proteobacteria* by SIP (Jones et al., 2011; Jones et al., 2008). Therefore, microorganisms responsible for the degradation of pyrene might be different in the other systems. It is believed that composting can improve significantly the pyrene removal (Potter et al., 1999; Sayara et al., 2009), but the active microbial communities at each composting phase have not yet been characterized in details.

* Corresponding authors. Address: Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China. Tel./fax: +86 592 6190997 (Y.-G. Zhu). Tel.: +86 25 86881311; fax: +86 25 86881000 (Z. Jia).

E-mail addresses: jia@issas.ac.cn (Z. Jia), ygzhu@rcees.ac.cn (Y.-G. Zhu).

¹ These authors have contributed equally to this work.

Incorporation of stable-isotope-labeled substrates into cellular biomarkers (DNA, RNA, PLFA) of microorganisms actively degrading the labeled substrate reveals linked phylogenetic and functional information about the organisms that assimilated these compounds (Lueders et al., 2004; Neufeld et al., 2007). In order to address the question which microorganisms are responsible for the degradation of pyrene in the composting soil, by the $^{13}\text{C}_4$ -pyrene was used. The aim of this study was to: (i) determine the potential for losses of the $^{13}\text{C}_4$ -pyrene from PAHs contaminated soil during composting; (ii) identify the bacterial populations that were actively involved in vessel composting for the removal of $^{13}\text{C}_4$ -pyrene in soil using DNA-SIP and T-RFLP methods.

2. Methods

2.1. Soils characterization

Soil samples were collected from a depth of 20 cm from a site planted with ryegrass in Sanming (26°15.398N; 117°36.505E). The soil contained 5.21% clay, 27.88% silt, and 66.91% sand. Prior to introduction to the bioreactors, the soil was sieved through a 2 mm wire-mesh before being returned to storage in the dark at 4 °C. The initial total concentration of 16 USEPA PAHs (ΣPAHs) was 6.69 mg kg⁻¹ soil (on dry wt. basis), and the concentration of pyrene was 0.85 mg kg⁻¹ soil (on dry wt. basis). The contaminated soil was moistened with sterile distilled water to the level of 40% of water holding capacity and incubated at room temperature (25 °C) for 2 weeks to reactivate the soil microorganisms before the composting (Maliszewskakordybach, 1993).

2.2. Experimental design

The soils were spiked with 1000 mg L⁻¹ solution of $^{13}\text{C}_4$ -pyrene (99 at.% ^{13}C) and unlabeled pyrene, respectively, in acetone to a final concentration at 100 mg kg⁻¹ dry. Briefly, a sub-sample of 10% was spiked with the $^{13}\text{C}_4$ -pyrene and unlabeled pyrene solution, respectively, and was mixed by hand shaking in a 1000 ml glass beaker. After evaporation of the acetone (overnight at 25 °C), the spiked sub-sample (10%) was mixed with the rest (90%) of the soil, and shaken thoroughly. All the reagents in this study were the highest purity (>97%). All solvents were HPLC grade. The atomic percentage of $^{13}\text{C}_4$ -pyrene accounts higher than 95% in contaminated soil finally due to the dilution by background PAHs.

The wastes included leaves, sawdusts and biowastes consisting of green vegetables (72%, 13.5% and 14.5% on dry weight basis) (see more details in Table S1). All wastes were treated as reported previously (Zhang et al., 2011). The vegetables and tree leaves were roughly ground to millimeter size (<2 mm) before the mixture. The TC content in the initial mixture was 43.78 ± 0.09%. The initial C/N ratio range between 20% and 45% to provide stable composting conditions. The C/N ratio of the initial soil/waste mixture was 28.99%.

The wastes were added to the contaminated soil with a ratio of 0.8:1 (dry wt. soil/dry wt. wastes) as Antizar-Ladislao et al. (2005a,b) used. The moisture content of the mixtures was adjusted to 50% of water holding capacity. The initial total concentration of 16 USEPA PAHs (ΣPAHs) was 92.44 ± 10.68 mg kg⁻¹ dry wt. of mixture. All the mixtures were placed in beakers with a volume of 1L in water-baths to control the temperature. The bioreactors were setup as previous study (Zhang et al., 2011). All the beakers were sealed with removing cover by Aluminum paper. Constant air-flow to the composting reactors was provided with the optimal aeration during the active phase varied from 3 to 25 L h⁻¹ kg⁻¹ dry wt.

The variation temperature setup was days 0–14 at 38 °C, days 15–35 at 55 °C, days 36–42 at 70 °C, and days 43–60 at 38 °C according to the reference (Antizar-Ladislao et al., 2005b), which

showed an optimum temperature profile for soil composting process. The moisture content in treatments was maintained by adding distilled and sterile water into the bioreactors. $^{13}\text{C}_4$ -pyrene and unlabeled pyrene (as the control) treatments were carried out in triplicates in three separate beakers, respectively.

2.3. Composting soil sampling and PAHs analysis

Sampling was carried out in triplicates for each treatment after day 14, 35, 42, and 60. Each beaker was thoroughly mixed and sub-samples were collected in triplicates. All the composting mixture was frozen at -80 °C for analysis of PAHs and subsequent molecular analysis. Then composting mixture pH was determined in a 1:1 mixture:water mixture. The samples were dried in an oven at 105 °C to obtain the dry weight. PAHs were extracted from 2 g soils which were dried by lyophilization and measured in triplicate by accelerated solvent extractor (Dionex ASE-300). Analysis of PAHs followed the procedure described earlier (Zhang et al., 2011). Briefly, 10 mg kg⁻¹ of fluoranthene-d10 and phenanthrene-d10 were used as substitute and internal standards, respectively. Concentrated (1 ml) soil extracts were analyzed using an Agilent gas chromatograph (7890A) equipped with a mass-selective detector (model 5975) and 7683B series autosampler (Agilent Technologies): Helium was used as a carrier gas (constant velocity of 30 cm s⁻¹) at 250 °C. The GC oven conditions followed: 50 °C 1 min, a 10 °C min⁻¹ ramp to 200 °C and then a ramp of 8 °C min⁻¹ to final temperature of 280 °C (hold for 10 min). Mass spectrometer detection was used with the selected ion monitoring system. Each treatment was carried out in triplicate.

2.4. DNA extraction and SIP gradient fractionation

Total DNA was extracted from 0.5 g of the mixture (dry weight) in both treatments at different time points with a FastDNA Spin kit for soil (MPBIO101) as described by the manufacturer. DNA quantity and purity were determined by Nanodrop (NanoDrop Technologies, Wilmington, DE, USA). DNA from the different replicates was combined for SIP gradient fractionation. Both of ^{12}C -DNA from the control treatment (unlabeled pyrene treatment) and ^{13}C -DNA from $^{13}\text{C}_4$ -pyrene treatment were density resolved by equilibrium density gradient centrifugation in CsCl (Amersham Biosciences) following a similar protocol described previously (Xia et al., 2011). Briefly, 2.0 µg of each soil DNA extract was mixed well with CsCl solution to achieve an initial CsCl buoyant density of 1.725 g ml⁻¹. Centrifugation conditions were 177,000 g for 44 h at 20 °C. The DNA fractionation was achieved by displacing the gradient medium with water at the top of the tube using an NE-1000 single syringe pump (New Era Pump Systems Inc., Farmingdale, NY, USA) with a precisely controlled flow rate of 0.38 ml min⁻¹. After centrifugation, 15 or 16 DNA gradient fractions were generated with equal volumes of about 380 µL, and a 65 µL aliquot of each fraction was as determined refractometrically, and DNA was precipitated and resuspended in 30 µL TE.

2.5. T-RFLP analyses

PCR amplification of bacterial 16S rRNA gene was performed using primers 27f and 907r (Lu et al., 2006). The 5' end of the forward primer was labeled with 6-carboxyfluorescein (FAM). The thermal profile for bacterial amplification was: 94 °C for 5 min, 35 cycles at 94 °C for 1 min, 56 °C for 45 s, 72 °C for 1 min, followed by a final elongation of 72 °C for 7 min. The 50 µL PCR reaction mixture contained the following components: 2 µL of DNA template, 25 µL of Premix Ex Taq (TaKaRa), 1.5 µL of each 50 µM primer (Invitrogen), 2 µL of 10 mg L⁻¹ BSA. The PCR product of bacterial DNA was digested at 37 °C for 4 h by *HaeIII* (Takara).

Table 1
Evolution of the characteristics for $^{13}\text{C}_4$ -pyrene.

Time	Dry mass (g/ beaker)	pH value	Pyrene concentration (mg kg ⁻¹ dry wt. soil)
0	70 ± 0	7.24 ± 0.07	92.44 ± 10.68
7	36.54 ± 3.48	7.42 ± 0.39	87.32 ± 5.87
14	30.09 ± 3.62	8.04 ± 0.09	84.88 ± 1.97
35	19.32 ± 3.76	8.62 ± 0.07	49.44 ± 11.42
42	15.38 ± 3.43	7.88 ± 0.25	20.55 ± 3.66
60	15.32 ± 2.45	8.58 ± 0.02	17.91 ± 2.63
Degradation rate	78.11%		80.62%

The DNA fragments were size separated using a 3130xl Genetic Analyzer (Applied Biosystems). DNA from both of the control treatment and $^{13}\text{C}_4$ -pyrene treatment were analyzed.

2.6. Cloning, sequencing and phylogenetic analysis

Six bacterial clone libraries were constructed from a 'heavy' DNA (BD = 1.735 g ml⁻¹) (WT14H) and a 'light' DNA (BD = 1.722 g ml⁻¹) obtained from the $^{13}\text{C}_4$ -labeled pyrene treatment (WT14L) at day 14, a 'heavy' DNA (BD = 1.741 g ml⁻¹) obtained from the $^{13}\text{C}_4$ -labeled pyrene treatment at day 35 (WT35H), a 'heavy' DNA (BD = 1.739 g ml⁻¹) obtained from the $^{13}\text{C}_4$ -labeled pyrene treatment at day 42 (WT42H), a 'heavy' DNA (BD = 1.738 g ml⁻¹) obtained from the $^{13}\text{C}_4$ -labeled pyrene treatment at day 60 (WT60H) and a 'heavy' DNA (BD = 1.738 g ml⁻¹) obtained from the control treatment (WK60H). Here, WK represents treatment with addition of unlabeled pyrene. WT represents treatment with addition of $^{13}\text{C}_4$ -labeled pyrene. For cloning and sequencing, PCR amplification used the same primer sets as above. The amplicons were ligated into PMD19-T Vector and transformed into DH5 α competent cells and clones randomly selected were sequenced with an ABI3730xl sequencer (Applied Biosystems). 16S rRNA gene based sequences were classified from phylum down to the genus level using the RDP Classifier (version 2.2) and a 90% confidence cutoff. The sequence data were analyzed using the MEGA4 software. The sequences were compared with bacterial 16S rRNA gene sequences from the Genbank (NCBI) database using BLAST. The phylogenetic tree was constructed according to chimera's two-parameter method and neighbor-joining method. Data sets were bootstrapped with 1000 random replicates. The total sequences obtained from this study have been deposited in the EMBL nucleotide sequence database under the accession number from HE965808 to HE966401.

3. Results and discussion

3.1. Dry mass

The dry mass losses were mainly occurred during the first 14 days but varied among composting replicates at different stages of composting. A dry mass loss of 78.1% was observed in the treatments after 2 months of composting (Table 1). The losses can range from 20% to 60% of initial organic matter (Atkinson et al., 1996; Canet and Pomares, 1995), while the present result was higher than some other observations during the composting of organic wastes (Doublet et al., 2011; Lashermes et al., 2012). The significant loss of dry mass in the treatment can be explained by the significant decomposition of organic matters in which the green wastes represents more than 70% of mixtures. The diminution of organic matters is quite variable and depends on the composting conditions and its duration.

3.2. pH

The soil pH was monitored during composting (Table 1). Our results showed that initial wastes were acidity slightly and stronger than the finished composts. An increase in the pH from 7.24 to 8.62 was observed at day 35 (Table 1). The pH increased substantially from the beginning to day 35, implying that organic acids are degraded leading to an alkalization of the compost and as consequence of the continued decomposition process, and possibly that the release of ammonia increased (Steger et al., 2007a). The latter pH decrease was probably due to the immobilization of ammonium in the microbial biomass.

3.3. Removal of $^{13}\text{C}_4$ -pyrene

Composting process resulted in a significant decrease in total $^{13}\text{C}_4$ -pyrene removal, and the majority of $^{13}\text{C}_4$ -pyrene was removed by the end of the composting treatment resulting in an overall removal of 8.2% at 38 °C (day 14), 46.5% at 55 °C (day 35), 77.8% at 70 °C (day 42) and 80.6% at 38 °C (day 60) (Table 1). $^{13}\text{C}_4$ -pyrene removal was significantly higher at 70 °C or 55 °C than at 38 °C, and this increased with the age of the compost (15–60 days). The degradation of $^{13}\text{C}_4$ -pyrene may be due, at least in part to the increase of temperature resulting in pyrene bioavailability (Maliszewskakordybach, 1993; Pignatello and Xing, 1996) and the stimulation of specific microorganisms responsible for pyrene degradation over a sufficiently extended incubation period (Tang et al., 2008).

Fig. 1. Relative abundance of bacterial taxa in the different clone library: 'heavy' DNA fractions at day 14 (WT14H), day 35 (WT35H), day 42 (WT42H), day 60 (WT60H) and 'light' DNA fractions at day 14 (WT14L) in $^{13}\text{C}_4$ -pyrene spiked composting soil, one is from 'heavy' DNA fractions (WK60H) at day 60 in pyrene spiked composting soil.

Fig. 2. T-RFLP fingerprints of the bacterial 16S rRNA genes retrieved from density-resolved gradient fractions of nonlabeled control DNA at day 14 (a), and ^{13}C -labeled DNA at day 14 (b). The CSCI buoyant densities (g ml^{-1}) of the DNA fractions are shown in brackets. The bp lengths and changes of T-RFs with DNA buoyant density are indicated with arrows.

Fig. 3. T-RFLP fingerprints of the bacterial 16S rRNA genes retrieved from density-resolved gradient fractions of nonlabeled control DNA at day 35 (a), and ^{13}C -labeled DNA at day 35 (b). The CSCI buoyant densities (g ml^{-1}) of the DNA fractions are shown in brackets. The bp lengths and changes of T-RFs with DNA buoyant density are indicated with arrows.

Fig. 4. T-RFLP fingerprints of the bacterial 16S rRNA genes retrieved from density-resolved gradient fractions of nonlabeled control DNA at day 42 (a), and ^{13}C -labeled DNA at day 42 (b). The CSCI buoyant densities (g ml^{-1}) of the DNA fractions are shown in brackets. The bp lengths and changes of T-RFs with DNA buoyant density are indicated with arrows.

3.4. Phylogenetic identification of ^{13}C -labeled bacteria involved in pyrene degradation

These sequences were classified according to bacterial taxonomy using the RDP Classifier. Most of DNA reads associated with five bacterial phyla, *Actinobacteria*, α -*Proteobacteria*, γ -*Proteobacteria*, β -*Proteobacteria*, and *Firmicutes* in the heavy fractions retrieved from $^{13}\text{C}_4$ -pyrene treatments (Fig. 1). The percentage of *Actinobacteria* is notably highest at 38 °C for 14 days, while only 20% correspondent to 70 °C. This indicated that the growth of *Actinobacteria* was favored at lower temperatures (Steger et al., 2007a). In addition, there was a shift from predominance of *Streptomyces* species at 38 °C (day 14) to *Thermobifida* and *Streptomyces* at 55 °C (day 35). Then *Ralstonia*, *Acinetobacter*, and *Thermobifida* were major species at 70 °C (day 42). At the final stage, *Thermobifida* and *Streptomyces* became dominant again at day 60 (Table S2).

Bacterial community of the unlabeled control (added with non-labeled pyrene) did not change across DNA buoyant density gradients at day 14 (Fig. 2a). For the labeled sample at day 14, T-RFLP patterns similar to those of the control were obtained. However, there was already evidence of a tendency of the 63-, 91-, 190-, 198-, 248-, and 331-bp T-RFs in heavy DNA fractions to increase (Fig. 2b), based on relative abundance calculation. T-RFLP patterns changed markedly among DNA fractions of different buoyant density gradients and higher community richness was also observed. T-RFs of 63- and 222-bp were dominant, with the relative abundance being higher than 38%. A combination of in silico sequence analyses and T-RFLP fingerprinting of the representative clones showed that not all T-RFs could be assigned to a single lineage (Table S3, Fig. S1a). The abundance of 63-bp which represent mostly of *Pseudoxanthomonas*, *Enterobacteriales* which belongs to γ -*Proteobacteria*, few of *Paracoccus*, *Phenyllobacterium*, *Brevundi-*

monas which belongs to α -*Proteobacteria*, increased significantly in the heavy fraction in the labeled sample (Table S3, Fig. S1a). The 91-bp showed the 16S rRNA gene similarity of 100% to *Nocardiopsis*, *Corynebacterium*, and *Nocardia*. There was already evidence of *Corynebacterium*, and *Nocardia* can degrade PAHs (Pineda-Flores et al., 2004; Zeinali et al., 2007). Organisms characterized by T-RFs of 190-bp showed the 16S rRNA gene similarity of 100% to *Rhizobium* and *Ochrobactrum* (Table S3, Fig. S1a). Only two clones represent 198-bp which related to *Hydrogenophaga* and *Delftia*. *Delftia* group was found to degrade fluorene previously (Castro-Gutierrez et al., 2012). Both of 222- and 248-bp represents *Streptomyces* (Fig. S1a), while the relative abundance of 248-bp increased in heavy fractions. Therefore, *Streptomyces* accounts a lot in bacterial communities and maybe one of the dominant pyrene degraders in this stage. Previous studies also found that pyrene can be degraded by *Streptomyces* (Chaudhary et al., 2011) 331-bp which related to *Bacillus* increased in heavy fractions maybe also involved in the $^{13}\text{C}_4$ -pyrene degradation (Table S3). The relative abundance of *Actinobacteria* which was characterized by 91- and 248-bp (increasing with the buoyant) was highest in this stage according to the calculation of the T-RFs. γ -*Proteobacteria* was also the second predominant group. Both of *Actinobacteria* and γ -*Proteobacteria* highly related to $^{13}\text{C}_4$ -pyrene degradation. *Actinobacteria* are believed to play a major role in organic matter degradation and humification processes in composts (Steger et al., 2007a). Previous study also showed that the bacterial diversity in the large-scale composting of source-separated household waste was exclusively dominated by γ -*Proteobacteria* (Sundberg et al., 2011). Hence, the composting system can favor the growth of *Actinobacteria* and γ -*Proteobacteria* to involve the pyrene degradation.

After 21 days composting at 55 °C, 68-bp became the dominant group in the control and labeled sample, respectively (Fig. 3a and

Fig. 5. T-RFLP fingerprints of the bacterial 16S rRNA genes retrieved from density-resolved gradient fractions of nonlabeled control DNA at day 60 (a), and ^{13}C -labeled DNA at day 60 (b). The CSCI buoyant densities (g ml^{-1}) of the DNA fractions are shown in brackets. The bp lengths and changes of T-RFs with DNA buoyant density are indicated with arrows.

b). The 68-bp belongs to *Actinobacteria*, which has a similarity of 100% to *Thermobifida*, *Thermomonospora*, *Actinomadura* (Table S3). The growth optimum of these organisms is often around 50–60 °C (Digital Atlas of Actinomycetes; <http://www0.nih.go.jp/saj/DigitalAtlas/index.htm>). These organisms have previously been found in compost system (Steger et al., 2007b). The relative abundance of 68-bp did not change in control and labeled sample. Hence these organisms may be not related to pyrene degradation. However, there was a tendency of 222- and 248-bp T-RFs which represents *Streptomyces* to increase in heavy fractions of the ^{13}C -labeled DNA treatment, based on relative abundance calculation in the labeled sample (Fig. 3b). In addition, pyrene removal increased significantly in this stage. Hence this indicated that *Streptomyces* actively involved in the degradation of $^{13}\text{C}_4$ -pyrene at this stage.

After 7 days of composting at 70 °C, for the unlabeled control sample, T-RFLP fingerprints did not change across DNA buoyant density gradients. The 71-, 190- and 230-bp were the dominant peaks in the control samples (Fig. 4a). For the labeled sample, the 206-, 217-, and 252-bp T-RFs became exclusively dominant while the 71-, 190, and 230-bp decreased significantly in heavy fractions (Fig. 4b). This indicated that $^{13}\text{C}_4$ -pyrene degraders were closely related to *Ralstonia* (206-bp), *Burkholderia* (217-bp), and *Acinetobacter* and *Saccharomonospora* (252-bp) (Table S3, Fig. S1b). Here 230-bp was also mainly related to *Ralstonia* (14 clones), only one clone related to *Acinetobacter* which mainly represents 252-bp. All these organisms were characteristic of β -*Proteobacteria*, γ -*Proteobacteria* and *Actinobacteria* (Table S3, Fig. S1b). This result is partly in accordance with the clone library. β - and γ -*Proteobacteria* was abundant pyrene degraders in the composting system, based on relative abundance calculation. However, previous study found that the primary pyrene degraders in the bioreactor were unculti-

vated, low-abundance β - and γ -*Proteobacteria* by qPCR and DNA-SIP method (Jones et al., 2011; Singleton et al., 2006). Presently, compost is used as a soil conditioner. The different system may result in the change of microbial diversity.

At day 60, there was no significant change in the unlabeled control across the buoyant density gradients (Fig. 5a). For the ^{13}C -labeled DNA treatment, 198-bp, 217-bp, and 252-bp increased significantly with the DNA buoyant density gradients and became dominant in the heavy DNA fractions. These T-RFs were mainly related to β -*Proteobacteria*, γ -*Proteobacteria* and *Actinobacteria* (Fig. 5b, Fig. S1c). In addition, a decrease in $^{13}\text{C}_4$ -labeled pyrene degradation after a thermophilic phase was observed. This could be explained by the limitation of recovery of surviving populations and/or by the depletion of the nutrients for microbial activity (Cooper et al., 2002). However, *Firmicutes* was not found to relate to the major T-RFs. This also showed that *Firmicutes* was the dominant group but not the pyrene degraders at this stage.

4. Conclusions

This study indicated that in-vessel composting can reduce $^{13}\text{C}_4$ -pyrene by 80.6% after 60 day continuous composting. To our best knowledge this is the first report that DNA-SIP method was used to analyze the bacterial community responsible for $^{13}\text{C}_4$ -labeled pyrene degradation in a composting system. The shifts in bacterial communities were closely related to the $^{13}\text{C}_4$ -labeled pyrene contamination. Members of α -*Proteobacteria*, β -*Proteobacteria*, γ -*Proteobacteria*, and *Acitinobacteria* were active in the $^{13}\text{C}_4$ -labeled pyrene degradation during composting. The study provides insights about dominant bacteria involved in $^{13}\text{C}_4$ -labeled pyrene degradation, and will be useful for defining bio-indicators and optimizing bioremediation of pyrene-contaminated soil in composting.

Acknowledgements

We thank Prof. Yahai Lu's lab at China Agricultural University for the assistance in T-RFLP analysis, and Chen Yan at Institute of Soil Science for the assistance in isotope analysis. This research was financially supported by the Ministry of Science and Technology, China (2011DFB91710).

References

- Antizar-Ladislao, B., 2010. Bioremediation: working with bacteria. *Elements* 6, 389–394.
- Antizar-Ladislao, B., Galil, N.I., 2006. Biodegradation of 2,4,6-trichlorophenol and associated hydraulic conductivity reduction in sand-bed columns. *Chemosphere* 64, 339–349.
- Antizar-Ladislao, B., Lopez-Real, J., Beck, A.J., 2005a. In-vessel composting-bioremediation of aged coal tar soil: effect of temperature and soil/green waste amendment ratio. *Environ. Int.* 31, 173–178.
- Antizar-Ladislao, B., Lopez-Real, J., Beck, A.J., 2005b. Laboratory studies of the remediation of polycyclic aromatic hydrocarbon contaminated soil by in-vessel composting. *Waste Manage.* 25, 281–289.
- Antizar-Ladislao, B., Spanova, K., Beck, A.J., Russell, N.J., 2008. Microbial community structure changes during bioremediation of PAHs in an aged coal-tar contaminated soil by in-vessel composting. *Int. Biodeter. Biodegr.* 61, 357–364.
- Atkinson, C.F., Jones, D.D., Gauthier, J.J., 1996. Biodegradabilities and microbial activities during composting of municipal solid waste in bench-scale reactors. *Compos. Sci. Util.* 4, 14–23.
- Bolta, S.V., Mihelc, R., Lobnik, F., Lestan, D., 2003. Microbial community structure during composting with and without mass inocula. *Compos. Sci. Util.* 11, 6–15.
- Canet, R., Pomares, F., 1995. Changes in physical, chemical and physicochemical parameters during the composting of municipal solid-wastes in 2 plants in Valencia. *Bioresour. Technol.* 51, 259–264.
- Castro-Gutierrez, V.M., Rodriguez-Rodriguez, C.E., Vargas-Azofeifa, I., 2012. Hydrocarbon degrading microflora in a tropical fuel-contaminated aquifer: assessing the feasibility of PAH bioremediation. *Int. J. Environ. Res.* 6, 345–352.
- Chaudhary, P., Sharma, R., Singh, S.B., Nain, L., 2011. Bioremediation of PAH by *Streptomyces* sp. *Bull. Environ. Contam. Toxicol.* 86, 268–271.
- Cooper, J.N., Anderson, J.G., Campbell, C.D., 2002. How resilient are microbial communities to temperature changes during composting? *Microb. Compos.* 3–16.
- DeBruyn, J.M., Chawning, C.S., Sayler, G.S., 2007. Comparative quantitative prevalence of mycobacteria and functionally abundant *nidA*, *nahAc*, and *nagAc* dioxygenase genes in coal tar contaminated sediments. *Environ. Sci. Technol.* 41, 5426–5432.
- Doublet, J., Francou, C., Poirereaud, M., Houot, S., 2011. Influence of bulking agents on organic matter evolution during sewage sludge composting; consequences on compost organic matter stability and N availability. *Bioresour. Technol.* 102, 1298–1307.
- Heitkamp, M.A., Freeman, J.P., Miller, D.W., Cerniglia, C.E., 1988. Pyrene degradation by a *Mycobacterium* sp. – identification of ring oxidation and ring fission-products. *Appl. Environ. Microb.* 54, 2556–2565.
- Jones, M.D., Crandell, D.W., Singleton, D.R., Aitken, M.D., 2011. Stable-isotope probing of the polycyclic aromatic hydrocarbon-degrading bacterial guild in a contaminated soil. *Environ. Microbiol.* 13, 2623–2632.
- Jones, M.D., Singleton, D.R., Carstensen, D.P., Powell, S.N., Swanson, J.S., Pfander, F.K., Aitken, M.D., 2008. Effect of incubation conditions on the enrichment of pyrene-degrading bacteria identified by stable-isotope probing in an aged, PAH-contaminated soil. *Microb. Ecol.* 56, 341–349.
- Lashermes, G., Barriuso, E., Le Villio-Poitrenaud, M., Houot, S., 2012. Composting in small laboratory pilots: performance and reproducibility. *Waste Manage.* 32, 271–277.
- Lu, Y.H., Rosencrantz, D., Liesack, W., Conrad, R., 2006. Structure and activity of bacterial community inhabiting rice roots and the rhizosphere. *Environ. Microbiol.* 8, 1351–1360.
- Lueders, T., Manefield, M., Friedrich, M.W., 2004. Enhanced sensitivity of DNA- and rRNA-based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. *Environ. Microbiol.* 6, 73–78.
- Maliszewskakordybach, B., 1993. The effect of temperature on the rate of disappearance of polycyclic aromatic hydrocarbons from soils. *Environ. Pollut.* 79, 15–20.
- Neufeld, J.D., Wagner, M., Murrell, J.C., 2007. Who eats what, where and when? Isotope-labelling experiments are coming of age. *ISME J.* 1, 103–110.
- Pignatello, J.J., Xing, B.S., 1996. Mechanisms of slow sorption of organic chemicals to natural particles. *Environ. Sci. Technol.* 30, 1–11.
- Pineda-Flores, G., Boll-Arguello, G., Lira-Galeana, C., Mesta-Howard, A.M., 2004. A microbial consortium isolated from a crude oil sample that uses asphaltenes as a carbon and energy source. *Biodegradation* 15, 145–151.
- Potter, C.L., Glaser, J.A., Chang, L.W., Meier, J.R., Dosani, M.A., Herrmann, R.F., 1999. Degradation of polynuclear aromatic hydrocarbons under bench-scale compost conditions. *Environ. Sci. Technol.* 33, 1717–1725.
- Sayara, T., Sarra, M., Sanchez, A., 2009. Preliminary screening of co-substrates for bioremediation of pyrene-contaminated soil through composting. *J. Hazard. Mater.* 172, 1695–1698.
- Singleton, D.R., Sangaiah, R., Gold, A., Ball, L.M., Aitken, M.D., 2006. Identification and quantification of uncultivated proteobacteria associated with pyrene degradation in a bioreactor treating PAH-contaminated soil. *Environ. Microbiol.* 8, 1736–1745.
- Steger, K., Ekland, Y., Olsson, J., Sundh, I., 2005. Microbial community growth and utilization of carbon constituents during thermophilic composting at different oxygen levels. *Microb. Ecol.* 50, 163–171.
- Steger, K., Jarvis, A., Vasara, T., Romantschuk, M., Sundh, I., 2007a. Effects of differing temperature management on development of actinobacteria populations during composting. *Res. Microbiol.* 158, 617–624.
- Steger, K., Sjogren, A.M., Jarvis, A., Jansson, J.K., Sundh, I., 2007b. Development of compost maturity and actinobacteria populations during full-scale composting of organic household waste. *J. Appl. Microbiol.* 103, 487–498.
- Sundberg, C., Franke-Whittle, I.H., Kauppi, S., Yu, D., Romantschuk, M., Insam, H., Jonsson, H., 2011. Characterisation of source-separated household waste intended for composting. *Bioresour. Technol.* 102, 2859–2867.
- Tang, J.X., Petersen, E., Weber, W.J., 2008. Development of engineered natural organic sorbents for environmental applications. 4. Effects on biodegradation and distribution of pyrene in soils. *Environ. Sci. Technol.* 42, 1283–1289.
- Tiquia, S.M., 2005. Microbiological parameters as indicators of compost maturity. *J. Appl. Microbiol.* 99, 816–828.
- Xia, W.W., Zhang, C.X., Zeng, X.W., Feng, Y.Z., Weng, J.H., Lin, X.G., Zhu, J.G., Xiong, Z.Q., Xu, J., Cai, Z.C., Jia, Z.J., 2011. Autotrophic growth of nitrifying community in an agricultural soil. *ISME J.* 5, 1226–1236.
- Zeinali, M., Vossoughi, M., Ardestani, S.K., Babanezhad, E., Masoumian, M., 2007. Hydrocarbon degradation by thermophilic *Nocardia otitidiscaviarum* strain TSH1: physiological aspects. *J. Basic Microb.* 47, 534–539.
- Zhang, Y., Zhu, Y.G., Houot, S., Qiao, M., Nunan, N., Garnier, P., 2011. Remediation of polycyclic aromatic hydrocarbon (PAH) contaminated soil through composting with fresh organic wastes. *Environ. Sci. Pollut. Res.* 18, 1574–1584.