

Enrichment of functional microbes and genes during pyrene degradation in two different soils

Songcan Chen¹ · Jingjing Peng² · Guilan Duan¹

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Abstract

Purpose Stimulating microbial degradation is a promising strategy for the remediation of soils contaminated with polycyclic aromatic hydrocarbons (PAHs). To better understand the functional microbial populations and processes involved in pyrene biodegradation in situ, the dynamics of pyrene degradation and functional microbial abundance were monitored during pyrene incubation in soils. We hope our findings will provide new insights into in situ pyrene biodegradation in soils and help to identify functional microbes from soils.

Materials and methods Pyrene (60 mg kg⁻¹) was incubated with two different soils, one is lower PAH-containing agricultural soil (LS), and the other is higher PAH-containing industrial soil (HS). During incubation, triplicate samples were collected on days 0, 3, 7, 14, and 35. Pyrene in soil samples was analyzed using an Agilent gas chromatograph (7890A) equipped with a mass-selective detector (model 5897). DNA in soils was extracted with a FastDNA Spin kit for soil (Bio101, USA). The abundance of functional microbes and genes was monitored by a Taqman or SYBR Green based real-time PCR quantification using an iCycler iQ5 thermocycler (Bio-Rad, USA). The diversity of PAH-RHD_α GP genes was evaluated by constructing clone libraries and sequencing.

Results and discussion In both soils, more than 80 % of the added pyrene was degraded within 35 days. After 35-day incubation, there was a significant enrichment of Gram-positive bacteria harboring PAH-ring hydroxylation dioxygenase (PAH-RHD_α GP) genes, and the abundance of *Mycobacterium* increased significantly. In PAH-RHD_α GP clone libraries from two soils, *Mycobacterium* was detected, while most sequences were closely related to uncultured Gram-positive bacteria. In addition, two pyrene catabolic pathways might be involved in pyrene degradation, as pyrene dioxygenase genes, *nidA* and *nidA3*, were dramatically enriched during incubation. Moreover, the abundance and diversity of potential degraders in two soils showed significantly difference in responding to pyrene stress. This result indicates that soil condition can significantly affect functional microbial populations and biological process for pyrene biodegradation.

Conclusions These results revealed that *Mycobacterium* as well as uncultured Gram-positive PAH-RHD_α genotypes may be the important group of pyrene degraders in soils, and two pyrene catabolic pathways, targeted by *nidA* and *nidA3*, might potentially contribute to in situ biodegradation of pyrene. This study characterized the response pattern of potential pyrene degraders to pyrene stress in two different soils, which would increase our understanding of the indigenous processes of pyrene biodegradation in soil environment.

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✉ Guilan Duan
duangl@rcees.ac.cn

¹ State Key Lab of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

² Key Lab of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, China

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1 Introduction

High molecular weight polycyclic aromatic hydrocarbons (HMW-PAHs), comprised of at least three fused aromatic rings, are of special concern due to their toxicity, mutagenicity,

carcinogenicity, and association with inflammatory vascular responses (Kim et al. 2005). Pyrene, arising from incomplete combustion of hydrocarbons, is a widespread HMW-PAH in the soil environment. Microbial degradation is the main process to remove PAHs from soil environment. Thus, a comprehensive understanding of the microbial degradation of pyrene will facilitate the development of new strategies to improve the remediation of PAH-polluted soils.

Many bacteria using pyrene as the sole carbon source have been isolated from various environments, most of them belong to genera *Mycobacterium* (Heitkamp et al. 1988) and *Rhodococcus* (Walter et al. 1991), which are Gram-positive. Recently, several Gram-negative strains have also been identified as pyrene degrader, such as *Sphingomonas* sp. (Zhong et al. 2011) and *Pseudomonas aeruginosa* RS1 (Ghosh et al. 2014). However, knowledge of pyrene-degrading bacterial populations is still limited and is mainly based on the studies of cultured bacteria. Therefore, the characterization of indigenous microbial populations would be necessary for a better understanding of in situ biodegradation processes of pyrene in complex environments, such as soil.

The initial step of PAH biodegradation commonly occurs via the incorporation of molecular oxygen into the aromatic nucleus by a multi-component terminal aromatic ring hydroxylating dioxygenase (RHD) enzyme system (Kauppi et al. 1998). RHD is composed of a large α subunit (PAH-RHD $_{\alpha}$) and small β subunits (Mason and Cammack 1992). Functional markers encoding PAH-RHD $_{\alpha}$ can be used to specifically target functional microbial populations involved in PAH biodegradation (Dionisi et al. 2004). For example, PAH-RHD $_{\alpha}$ from Gram-positive bacteria (PAH-RHD $_{\alpha}$ GP) were shown to be involved and specialized in the degradation of HMW-PAHs (Baldwin et al. 2003; Zhou et al. 2006; Cebon et al. 2009). Two biochemical pathways of pyrene degradation have been established in pyrene-degrading mycobacterial strains (Kim et al. 2007). *nidA*, which was highly conserved in *Mycobacterium* (Heitkamp et al. 1988), encodes the α -subunits of RHD that adds hydroxyl groups at C-4 and C-5 positions of pyrene in the degradation pathway (Kim et al. 2012). More recently, an alternative pyrene RHD $_{\alpha}$ gene, *nidA3*, has been identified in *Mycobacterium vanbaalenii* PYR-1 (Kim et al. 2006), which was shown to be involved in the initial dihydroxylation step in an alternate detoxification pathway (Kim et al. 2007). Although the biochemical pathways of pyrene biodegradation have been identified in cultivated species, little is known about the in situ biodegradation pathways of pyrene in the soil environment. Moreover, how the abundance and diversity of potential pyrene degraders was influenced by soil environmental factors are not yet fully explored.

Therefore, the aim of the current study was to investigate the functional microbial populations and process involved in pyrene biodegradation in complex soil environment,

including lower PAH-containing agricultural soil (LS) and higher PAH-containing industrial soil (HS). Total bacterial 16S rDNA gene, PAH-RHD $_{\alpha}$ GP gene, and *Mycobacteria* 16S rDNA gene copies were regularly measured by real-time PCR to identify the potential pyrene degraders in soils. The abundance of functional genes, *nidA* and *nidA3*, was also monitored to characterize the likely mechanisms of pyrene biodegradation pathways in two soils.

2 Materials and methods

2.1 Soil property and preparing

Surface soils (0–20 cm) were collected from the suburb of Beijing, China. One was from an agricultural field with less PAH contamination (40° 17.54 N; 116° 06.81 E) (LS). This field is perennially planted with vegetables and corn. When we sampled soil, corn was grown at the field. The other soil was from an industrial green land. This land was long-term contaminated with PAHs, which is near a steel-manufacturing factory (39° 54.57 N; 116° 08.54 E) (HS). When we sampled soil, the land was covered with grass. Both soils are traditional cinnamon soil, which are classified as alfisol according to the US Department of Agriculture soil classification system. After being transported to laboratory, soil samples were air-dried, sieved (2 mm), and stored in the dark at 4 °C. The soil properties were determined following standard procedures and are shown in Table 1. The LS is silty clay loam, and the HS is clay loam. The pH and content of silt, clay, and organic matter of two soils were comparable, while the content of sand of agricultural soil was lower than that of industrial soil. Significantly, the concentration of total PAHs of industrial soil (HS) was 6.3-fold higher than that of agricultural soil (LS), and the pyrene concentration was almost 10-fold higher than that of agricultural soil.

Table 1 Properties of soils from lower PAH-containing soil (LS) and higher PAH-containing soil (HS)

Properties	Lower PAH-containing soil (LS)	Higher PAH-containing soil (HS)
Soil texture	Silty clay loam	Clay loam
Sand (%)	13.32	22.38
Silt (%)	50.23	39.47
Clay (%)	36.46	38.15
pH	8.10	8.03
Organic matter (g kg ⁻¹)	20.81	24.27
Total PAHs (mg kg ⁻¹)	1.097	6.933
Pyrene (mg kg ⁻¹)	0.042	0.409

2.2 Pyrene incubation experiment

In order to re-activate the soil microorganisms, dry soil samples (800 g) in duplicates were placed in separate glass beakers, moistened with sterile distilled water to the level at 50 % of water holding capacity, and incubated at room temperature (25 °C) for 2 weeks. The soils were then spiked with 1000 mg l⁻¹ solution of pyrene in acetone to a final concentration at 60 mg kg⁻¹, following the methods recommended by Brinch et al. (2002) with some adaptations. Briefly, a subsample of 10 % soil was spiked with the pyrene solution and mixed throughout. After evaporation of the acetone (overnight at 25 °C), the spiked subsample (10 %) was mixed with the rest (90 %) of the soil and shaken thoroughly. The moisture content of the samples was adjusted again to 50 % of water holding capacity. For unspiked control, soils were amended with the same amount of acetone as the pyrene-spiked soils. For the abiotic controls, the soils were autoclaved before spiking with pyrene solution (Park and Crowley 2006).

After the treated soils were homogenized, 10-g aliquots were placed into serum bottle and incubated in the dark at 25 °C. During incubation, triplicate samples were collected on days 0, 3, 7, 14, and 35, frozen immediately, and stored at -80 °C until analyses.

2.3 Pyrene quantification

Pyrene was extracted from 2 g soils which were dried by lyophilization and measured by accelerated solvent extractor (Dionex ASE-300). Analysis of pyrene followed the procedure described earlier (Zhang et al. 2011). Briefly, 100 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 5897): Helium was used as a carrier gas (constant velocity of 30 cm s⁻¹) at 250 °C. The GC oven conditions are as follows: 50 °C for 1 min, a 10 °C min⁻¹ ramp to 250 °C, and then a ramp of 5 °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. Analytical procedural blanks and fluorene-d10 (surrogate for pyrene) were used for quality control. Pyrene concentrations of analytical procedural blanks were very low (<0.01 mg kg⁻¹), which were about 4 orders of magnitude lower than that of the soil samples. The average recovery efficiency of fluorene-d10 (surrogate for pyrene) was 89.1±7.2 %. The final sample concentrations were blank corrected, but not surrogate recovery corrected. In addition, the recovery of the pyrene from the freshly spiked soils (on day 0) was 104.6±4.4 %, indicating that the spiking procedures were validated.

2.4 Primer development

To design *nidA3* targeted primer, the DNA sequences of *nidA3* gene from several PAH-degrading *Mycobacterium* strains—PYR-1 (AF249301), 6PYR (AJ494745), JLS (AY330098), KMS (AY330100), and MCS (AY33010)—were obtained from NCBI GenBank (<http://www.ncbi.nlm.nih.gov/>) and aligned by MEGA5 software (Tamura et al. 2011). Conserved regions of *nidA3* were selected from the aligned data, and three sets of real-time PCR primers targeting different regions of the *nidA3* gene were designed by Primer Premier 5.0 software (Premier Biosoft International, USA). Among these, one primer set was chosen for real-time PCR analysis. To ensure specificity of the designed primer, ten cloned products from one of our DNA samples were sequenced, all showing more than 98 % amino acid similarity with PAH-degrading *Mycobacterium* sp. NidA3 (data not shown).

2.5 DNA extraction and real-time PCR analysis

DNA was extracted from 0.5 g of soil (dry weight) with a FastDNA Spin kit for soil (Bio101, USA) according to the manufacturer's instructions. The concentration and quality of the extracted DNA were determined by spectrophotometer analysis (NanoDrop ND-1000, NanoDrop Technologies, Wilmington, DE) and agarose gel electrophoresis.

Total bacterial (16S rDNA), Gram-positive PAH-RHD_α gene (PAH-RHD_α GP), Gram-negative PAH-RHD_α gene (PAH-RHD_α GN), *Mycobacteria* 16S rDNA, and *nidA* and *nidA3* gene copies were evaluated by a Taqman or SYBR Green based real-time PCR quantification using an iCycler iQ5 thermocycler (Bio-Rad, USA), and their primers and amplification protocols were summarized in Table 2. Tenfold serial dilutions of a known copy number of the plasmid DNA were generated to produce the standard curve. Each sample DNA was diluted for minimizing threshold cycle suppression by inhibitors for that sample type (typically 1:10). The real-time PCR efficiencies (87.9~112.5 %) were examined to test for inhibition. *R*² values were more than 0.99 for all calibration curves. All real-time PCR analyses were performed using iCycler software.

2.6 PAH-RHD_α GP gene clone library construction and sequencing

Two clone libraries were constructed from LS and HS at day 35. The PCR amplification using the primers GP-F/GP-R is described in Table 2. PCR products were purified and ligated into the pMD19-T easy vector (Promega, USA) according to the manufacturer's instructions. Plasmids were transformed into *Escherichia coli* JM109 cells (Takara), and more than 50 clones were randomly selected and sequenced for each

Table 2 Quantitative real-time PCR primers in this study

Target group	Primers	Sequences (5'→3')	Length	Annealing temperature	Reference
Bacteria (16S rDNA)	BACT1369F BACT1492R Probe (TM1389F)	CGGTGAATACGTTTCYCGG GGWTACCTTGTTACGACTT FAM-CTTGTACACACCGCCCGTC-TAMRA	103 bp	56 °C	Suzuki et al. 2000
Gram positive PAH-RHD α	PAH-RHD α GP-F GP-R	CGGCGCCGACAAATTYGTNGG GGGGAACACGGTGCCRTGDATRAA	292 bp	54 °C	Cebon et al. 2008
Gram negative PAH-RHD α	GN-F GN-R	GAGATGCATACCACGTKGGTTGGA AGCTGTTGTTCCGGGAAGAYWGTGCMGTT	306 bp	56 °C	Cebon et al. 2008
<i>Mycobacteria</i> (16S rDNA)	Myc-F Myc-R	CATGCAAGTCGAACGGAAA TGTGAGTTTTACGAACA	534 bp	59 °C	Leys et al. 2005
<i>nidA</i>	NidA-F NidA-R	TTCCCAGTACGAGGGATAC TCACGTTGATGAACGACAAA	121 bp	56 °C	DeBruyn et al. 2007
<i>nidA3</i>	NidA3-F NidA3-R	CCTGATGCGACGACAATG GCAACCTAGCCGACTCTT	421 bp	55 °C	This study

clone library. All the sequences and their relatives obtained from the NCBI were aligned by using the MEGA5 software. The sequences with more than 97 % identity were grouped into one operational taxonomic unit (OTU) using the Mothur program (version 1.31.2, USA) by the furthest neighbor approach. Phylogenetic tree was created using the MEGA5 software by neighbor joining (NJ) method. The relative confidence of the tree topologies was evaluated by performing 1000 bootstrap replicates.

3 Results

3.1 Pyrene dissipation

The kinetics of pyrene dissipation in the two soils was monitored during incubation on days 0, 3, 7, 14, and 35. In the unautoclaved soils, pyrene dissipation exhibited similar trends over time in both soils, with an initial lag phase followed by a rapid disappearance (Fig. 1). From day 7, the dissipation of pyrene was noticeable, while no significant difference was observed between two soils. At day 35, more than 80 % of the spiked pyrene was dissipated in both soils. The pyrene dissipation rate was $1.30 \mu\text{g day}^{-1} \text{g}^{-1}$ dry soil in LS; however, the pyrene dissipation rate was significantly higher in HS, about $1.60 \mu\text{g day}^{-1} \text{g}^{-1}$ dry soil. In contrast, for the sterilized soils, there was little pyrene disappearance observed during the whole incubation. Moreover, a slight increase of pyrene levels was observed at the initial incubation phase (at day 3 and day 7), although the increase was not significant (ANOVA, $P > 0.05$). This result suggests that abiotic dissipation of pyrene is not a main route for pyrene removal in soils.

3.2 Quantification of the potential pyrene degraders and functional genes

Total bacterial 16S rDNA gene copies were determined by real-time PCR. For two soils, the amount of bacterial 16S rDNA was relatively constant over time, either spiked with or without pyrene, with the gene copies ranging from 8.9×10^9 to 1.9×10^{10} copies g^{-1} dry weight soil (Fig. 2).

Across the entire incubation times, the quantities of PAH-RHD α GP gene, *Mycobacteria* 16S rDNA gene, *nidA* (from *Mycobacteria*), and *nidA3* (from *Mycobacteria*) increased significantly in both soils spiked with pyrene. For both soils, PAH-RHD α GP gene copies significantly ($P < 0.01$) increased with incubation time (Fig. 2). The copies of *Mycobacteria* 16S rDNA, measured by an assay specific to fast-growing *Mycobacteria*, increased by 5 and 20 times in LS and HS,

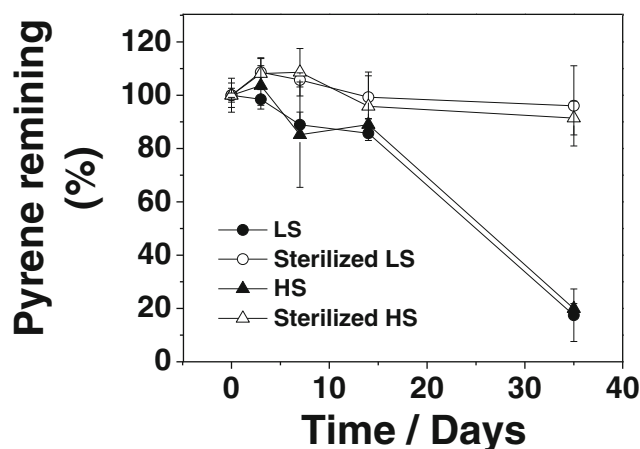
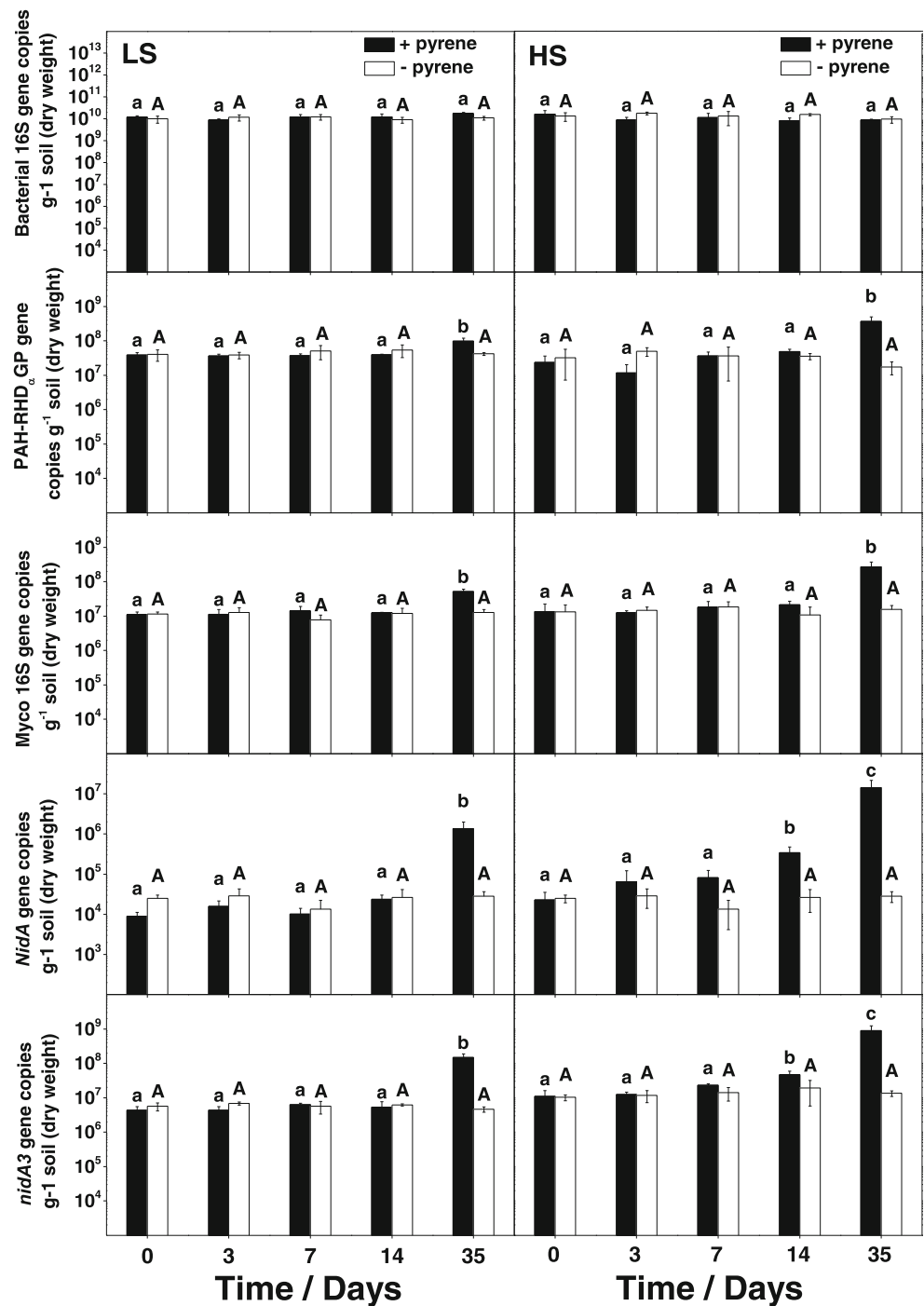


Fig. 1 Pyrene dissipation in the soils during incubation with pyrene. Soils were spiked with 60 mg kg^{-1} pyrene; LS less PAH-contaminated soil, HS higher PAH-containing soil; data are means \pm SE; $n = 3$

Fig. 2 Quantification of bacterial 16S rDNA, PAH-RHD α GP, *Mycobacterium* 16S rDNA, *nidA*, and *nidA3* gene copies during incubation; + pyrene, spiked with 60 mg kg⁻¹ pyrene; - pyrene, unspiked with pyrene; data are means \pm SE; *n*=3



respectively, after 35 days of pyrene incubation (Fig. 2). Copy numbers of genes, *nidA* and *nidA3*, encoding alpha subunits of two different ring hydroxylating dioxygenases, showed approximately 2 orders of magnitude higher at the end of experiment (35 days) in both soils compared with those at the beginning (Fig. 2). In contrast to pyrene-spiked soils, none of these detected genes showed significant changes in both soils without pyrene (Fig. 2). These results suggested that pyrene

incubation increased the abundance of *Mycobacteria* and the expression of *nidA* and *nidA3*.

Between two pyrene-spiked soils sampled at day 35, there was a significant difference in abundances of PAH-RHD α GP gene, *Mycobacteria* 16S rDNA, *nidA*, and *nidA3*: all exhibited significantly higher copies at HS than at LS (Fig. 2). In addition, at the initial time of pyrene incubation (day 0), there was no significant difference in quantities of *Mycobacteria*

16S rDNA or PAH-RHD $_{\alpha}$ GP genes between two soils, while HS had significantly higher *nidA* and *nidA3* copy numbers than LS (Fig. 2). These observations were consistent with the fact that soil HS had experienced the historical contamination and typically exhibited higher concentration of PAHs than LS (Table 1).

3.3 Correlations among catabolic genotypes and pyrene degradation

To determine the relationship between catabolic gene copies which may be related to the degradation potential of the microbial communities, a correlation matrix of Spearman rank correlation coefficients was developed (Table 3). As shown in Table 3, the extent of pyrene degradation was positively correlated to all the target gene copies except bacterial 16S rDNA. In addition, *Mycobacteria* 16S rDNA, *nidA*, and *nidA3* gene copies had significant positive correlations with each other. This is expected as *Mycobacterium* spp. are the primary carriers of *nidA* or *nidA3* genotypes in the contaminated environment (DeBruyn et al. 2012).

3.4 Diversity of PAH-RHD $_{\alpha}$ GP genes

To investigate the diversity of PAH-RHD $_{\alpha}$ GP genes, two clone libraries were constructed from pyrene-spiked LS and HS at day 35, respectively. A total of 10 OTUs were identified in the clone library of the LS (46 sequences analyzed), and 4 OTUs were identified in the clone library of the HS (43 sequences analyzed). The richness indices (Table 4), including Chao 1 estimator, Shannon index, and reciprocal of Simpson index, revealed that a higher diversity of PAH-RHD $_{\alpha}$ GP gene was found in the pyrene-spiked LS than in the HS, and this was consistent with the result of the OTU richness curves

(Fig. 3), which showed representative sampling of each clone library.

The phylogenetic analysis indicated that all of the obtained sequences of PAH-RHD $_{\alpha}$ GP gene from LS and HS could be grouped into three distinct gene clusters (Fig. 4). Cluster I was the largest group and included a total of 80 PAH-RHD $_{\alpha}$ GP gene sequences. Among cluster I, five and four sequences from soil LS and HS, respectively, were closely related to *nidA3* of *Mycobacterium*. However, most sequence from cluster I could not be closely clustered with any functional gene characterized in cultivated microorganisms. They were affiliated with uncultured PAH-RHD $_{\alpha}$ genes recovered from HMW-PAH-contaminated environment and were related to *nidA3* from *Mycobacterium* with approximately 90 % similarity. Cluster II included three sequences from LS and five sequences from HS. These sequences were closely related to an uncultured *Actinobacteria* PAH-RHD $_{\alpha}$ gene and could be clustered with *pdoA2* derived from two cultivated *Mycobacterium* species. Cluster III contained only 1 sequence from LS, which was not closely related (with less than 87 % similarity) to any reported sequences available in public database. This suggested the existence of novel PAH-RHD $_{\alpha}$ GP gene or microorganism in LS.

4 Discussion

4.1 Dissipation of pyrene

Results from this study showed that the majority of added pyrene was dissipated in two soils after 35 days of incubation (Fig. 1). Compared with previous findings (Chen and Ding 2012; Peng et al. 2010; Xu et al. 2006), pyrene dissipation in both soils was relatively more rapid in this study. It was likely due to the rapid enrichment of activated indigenous degraders in response to the presence of pyrene, which would result in higher pyrene degradation (>96 %) in 1-month incubation (Hamdi et al. 2007). However, the rapid pyrene dissipation was not observed in the sterilized soils; this result suggests that pyrene dissipation in soil environment mainly relies on microbial degradation biotic process, rather than abiotic process. It was concerned that, under high content of organic matter and clay condition, abiotic PAH loss might also contribute to pyrene dissipation (e.g., sequestered by soil matrix). However, in our study, no significant (ANOVA, $p > 0.05$) pyrene disappearance was observed in both sterilized soils over the incubation time (Fig. 1). We assumed that it was possible due to the time of aging being relatively short. This observation was consistent with the previously published data obtained from other PAH aging studies using sterilized soils (Luo et al. 2012; Macleod and Semple 2000; Cheung and Kinkle 2001; Mueller 2006).

Table 3 Spearman rank correlation coefficient (r_s) matrix ($n=10$)

Parameter ^a	PyrDeg	16S	Myco	<i>nidA</i>	<i>nidA3</i>	GP
PyrDeg	1.00					
16S	0.28	1.00				
Myco	0.72*	0.20	1.00			
<i>nidA</i>	0.67*	0.04	0.84**	1.00		
<i>nidA3</i>	0.63*	0.14	0.92**	0.98**	1.00	
GP	0.72*	0.02	0.58	0.54	0.50	1.00

Significant correlation is marked with asterisks (* at <0.05 level, ** at <0.01 level)

^a PyrDeg is the percentage of spiked pyrene degradation during incubation; 16S, Myco, *nidA*, *nidA3*, and GP are abundance of bacterial 16S rDNA, *Mycobacteria* 16S rDNA *nidA*, *nidA3*, and PAH-RHD $_{\alpha}$ GP genes, respectively

Table 4 Diversity and richness indices of PAH-RHD_α GP gene sequences from two pyrene spiked soils

Soil	Clone number	OTUs ^a	Chao1 ^b	Shannon ^c	1/Simpson ^d	Coverage (%) ^e
HS	46	10	17.5	1.53	3.39	84.80
LS	43	4	4.0	0.72	1.58	100

OTU operational taxonomic unit, HS higher PAH-containing industrial soil, LS lower PAH-containing agricultural soil

^a OTUs are defined at 3 % nucleotide acid divergence

^b Nonparametric statistical predictions of total richness of OTUs based on the distribution of singletons and doubletons

^c Shannon diversity index. A higher number represents more diversity

^d Reciprocal of Simpson’s diversity index. A higher number represents more diversity

4.2 Enrichment of functional microbes during pyrene degradation

To unravel the response of microbial populations in soils to pyrene pollution, real-time PCR based assay for potential functional microbes and genes was conducted under different soil environments. Specially, we quantified the bacterial 16S rDNA, PAH-RHD_α GP, *Mycobacteria* 16S rDNA, *nidA*, and *nidA3* gene abundances in two pyrene-spiked soils. The amount of bacterial 16S rDNA genes, a proxy for bacterial biomass (Suzuki et al. 2000), was relatively stable over time in both soils regardless of the addition of pyrene or not (Fig. 2). This result indicates that there was no significant change in the overall population density of the total bacteria in soils exposed to pyrene. This observation is similar to previous studies showing that 16S rDNA gene copy numbers were not affected by the addition of PAHs including pyrene, phenanthrene, and naphthalene (Park and Crowley 2006; Peng et al. 2010; Niepceon et al. 2014). However, the relatively constant density for the bacterial biomass did not rule out variations in the species composition of the bacterial community.

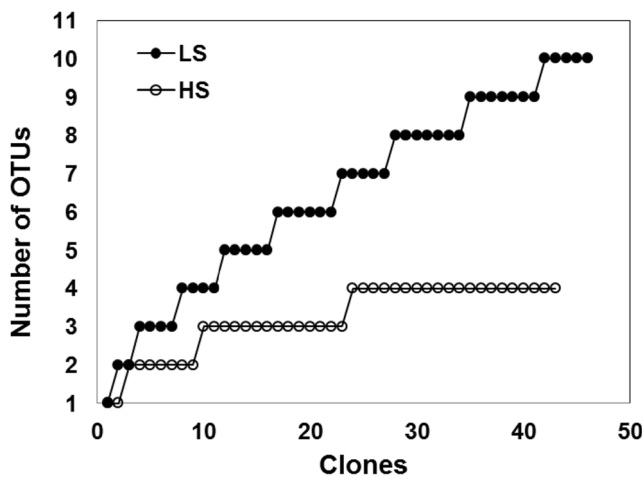


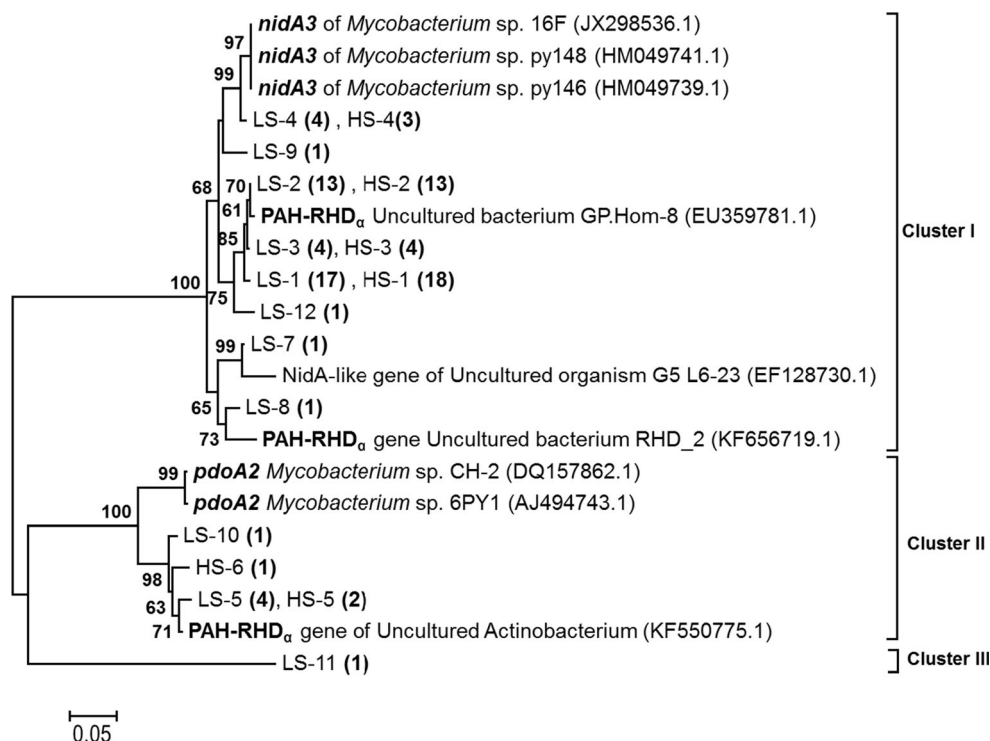
Fig. 3 OTU richness curves of PAH-RHD_α GP gene libraries. The curves were obtained by rarefaction calculations of OTU abundance data and they represent libraries from two pyrene-spiked soils. Curves were calculated for 97 % sequence identity

In order to further study the functional microbial population that might be involved in pyrene biodegradation in two soils, we monitored the bacterial PAH degradation community using primers targeting PAH-RHD_α GP genes from a wide range of bacteria taxa (Gram-positive) (Fig. 2). The increasing abundance of PAH-RHD_α GP gene over the incubation times with pyrene indicated that the Gram-positive bacteria harboring PAH-RHD_α genes might be an important group of pyrene degraders in the test soils. Previous studies have implied that Gram-positive PAH degrader was the predominant PAH degrading microbial group in an industrial soil, where high concentrations of pyrene were detected (Meng and Zhu 2011). Gram-positive degraders might experience advantages in pyrene biodegradation by increasing pyrene bioavailability through forming biofilm directly on hydrophobic pollutants (Johnsen and Karlson 2004).

Sequencing of PAH-RHD_α GP clone library was done to examine the phylogenetic distribution of the potential pyrene degraders in two tested soils (Fig. 4). Several sequences detected in LS and HS were closely related to *nidA3* of *Mycobacterium* with more than 98 % nucleotide sequence identity, indicating the involvement of fast-growing *Mycobacterium* in pyrene biodegradation. However, the majority of retrieved sequences (40 of 46 clones in LS, 39 of 43 clones in HS) were closely related to PAH-RHD_α genes derived from uncultured bacteria (Fig. 4). These genes might be a potential pyrene dioxygenase, as they showed more than 90 % nucleotide acid similarity with either *nidA3* or *pdoA2* from *Mycobacterium* (Fig. 4), which were considered as the key catabolic genes to degrade pyrene (Krivobok et al. 2003; Kim et al. 2006). These results suggested that *Mycobacterium* as well as those uncultured bacteria might play an important role in pyrene degradation. The identity of these uncultured bacteria should be further investigated by cultivation-independent approaches such as stable-isotope probing (SIP) (Peng et al. 2013).

To investigate the response of *Mycobacteria*, detected in PAH-RHD_α GP clone library, to the addition of pyrene, *Mycobacteria* 16S rDNA gene was quantified in two pyrene-spiked soils during incubation times (Fig. 2). Results

Fig. 4 Neighbor-joining tree based on the multiple alignments of PAH-RHD α GP gene sequences. Bootstrap values represent 1000 replicates and only values above 50 % are shown. The scale bar represents the expected number of changes per nucleotide position (5 % nucleotide substitution). Numbers in parentheses (in bold) after the clone names correspond to the analyzed clone numbers



showed that the fast-growing *Mycobacteria* was presented and enriched during the pyrene biodegradation in both soils. This result indicates that *Mycobacteria* were induced to grow and were likely playing an important role in pyrene degradation. Previous studies also showed that fast-growing *Mycobacteria* populations were an important group of HMW-PAH (e.g., pyrene) degrader in PAH-contaminated soils (DeBruyn et al. 2007; DeBruyn et al. 2009). Coincident with the detection of fast-growing *Mycobacteria*, the copy numbers of pyrene dioxygenase genes including *Mycobacteria nidA* and *nidA3* increased significantly during incubation time (Fig. 2), which further suggested that fast-growing *Mycobacteria* were enriched in the test soils and had function genotypes for pyrene conversion. However, we could not exclude the possibility of other *nidA* or *nidA3* carriers, such as *Terrabacter* spp., *Phycococcus* spp., and *Leifsonia* spp. (DeBruyn et al. 2012), which might also be involved in pyrene biodegradation.

4.3 Enrichment of functional gene during pyrene degradation

The positive correlation between pyrene catabolic gene copies (*nidA* and *nidA3*) and pyrene degradation extent indicated that two pyrene catabolic pathways, targeted by *nidA* (degradation pathway) and *nidA3* (detoxification pathway), respectively, might both potentially contribute to the pyrene biodegradation in the tested soils (Table 3). Although the in situ metabolic activity of *nidA* and *nidA3* genes (detected as mRNA

transcripts) remains to be established, it is likely that presence of catabolic genes could serve as markers of their actual function. For example, in the case of PAH-degrading communities, strong positive correlations have previously been found between *nahAc* (α subunit of naphthalene dioxygenase) gene copies and transcripts, indicating that the presence of catabolic genes in a community is related to their activity (Fleming et al. 1993; Yagi and Madsen 2009). Thus, our observations implied that both *nidA* and *nidA3* genes might play metabolic roles in pyrene biodegradation in contaminated soils, and they might provide useful targets for monitoring HMW-PAH degradation. As this is, to our knowledge, the first quantitative detection of *nidA3* in PAH-degrading communities, this also presents the first demonstration of a link between *nidA3* abundance to function (pyrene biodegradation) in indigenous microbial communities.

4.4 Different response patterns of functional microbial community between two soils

Notably, functional microbial community potentially involved in pyrene biodegradation in two soils showed different abundance and diversity after exposure to pyrene, indicating a soil condition dependent response of the functional soil bacteria to pyrene contamination. The abundances of PAH-RHD α GP gene, *Mycobacterium* 16S rDNA, *nidA*, and *nidA3* were approximately 1 order of magnitude higher in HS after spiking with pyrene than in the LS detected at day 35 (Fig. 2). These results might be due to (1) the initial abundance of these

functional genes in HS being already higher than in LS before artificial pyrene spiking, and (2) the more rapidly growing of microbial populations carrying functional genes in HS (Ding et al. 2010). The diversity of the PAH-RHD α GP genes was lower in HS than in LS after 35 days of pyrene incubation based on the value of the Shannon index and reciprocal of Simpson (Table 4). These differences in functional microbial community structure between two soils might attribute to lower initial microbial diversity in HS, which was heavily contaminated by PAHs. Previous studies showed that indigenous microbial communities in heavily PAH-contaminated soils were less diverse than those in less contaminated areas due to the toxicity of PAHs (Smalla et al. 2007).

5 Conclusions

In conclusion, this study revealed that *Mycobacterium* as well as uncultured Gram-positive PAH-RHD α genotypes may be the important group of pyrene degraders in soils, and two pyrene catabolic pathways, targeted by *nidA* and *nidA3*, might potentially contribute to in situ biodegradation of pyrene. Moreover, the soil environment was indicated to significantly affect the response of functional microbial populations and biological process to pyrene spiking. These findings provide new insights into in situ pyrene biodegradation in soils and help to identify functional microbes from different soils.

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