Dynamic changes in functional gene copy numbers and microbial communities during degradation of pyrene in soils

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nidA gene and fast-growing PAH-degrading *Mycobacterium* can serve as indicators for pyrene contamination.

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ABSTRACT

This study investigates the dynamics of pyrene degradation rates, microbial communities, and functional gene copy numbers during the incubation of pyrene-spiked soils. Spiking pyrene to the soil was found to have negligible effects on the bacterial community present. Our results demonstrated that there was a significant difference in *nidA* gene copy numbers between sampling dates in QZ soil. *Mycobacterium* 16S rDNA clone libraries showed that more than 90% *mycobacteria* detected were closely related to fast-growing PAH-degrading *Mycobacterium* in pyrene-spiked soil, while other sequences related to slow-growing *Mycobacterium* were only detected in the control soil. It is suggested that *nidA* gene copy number and fast-growing PAH-degrading *Mycobacterium* could be used as indicators to predict pyrene contamination and its degradation activity in soils.

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1. Introduction

The environmental fate of PAH is of concern because of their mutagenicity, ecotoxicity, and carcinogenic potential of high molecular weight PAH. Microbial degradation is the major process involved in the decontamination of surface soil (Sims et al., 1990). Pyrene arising from incomplete combustion of hydrocarbons is a widespread pollutant in the soil environment, and, it is one of the most predominant PAH in the environment. Thus, a deeper understanding of the microbial degradation pyrene will facilitate better ways to migrate and remediate PAH pollution.

Many bacteria using pyrene as the sole carbon source have been isolated from various environments. The majority of them are Gram-positive. The fast-growing *Mycobacterium* spp (Miller et al., 2004; Leys et al., 2005), which play a key role in the degradation of high molecular weight PAH, have received particular attention. In addition, some pyrene-degrading γ -Proteobacteria, such as enteric bacterium *Leclercia adecarboxylata*, has also been isolated (Eriksson et al., 2002; Sarma et al., 2004). Uncultivated bacteria including β - and γ -Proteobacteria, identified by DNA-based stable isotopic

probing, are also primary pyrene degraders (Singleton et al., 2006). However, knowledge of pyrene-degrading bacterial populations is still limited, and mainly originates from studies of cultured bacteria. The characterization of indigenous pyrene-degrading microbial populations is therefore necessary for a better understanding of natural biodegradation processes in and for the successful application of bioremediation technologies.

The initial step of PAH metabolism commonly occurs via the incorporation of molecular oxygen into the aromatic nucleus by a multi-component aromatic ring-hydroxylating-dioxygenase (RHD) enzyme system forming cis-dihydrodiol. RHD is composed of a large α subunit and small β subunits (Mason and Cammack, 1992). Functional markers encoding key enzymes of characteristic metabolic pathways can be used to specifically target functional guilds of microorganisms (Lueders and Friedrich, 2003). The most extensively characterized group of pyrene dioxygenase genes, a highly conserved group called *nidAB* genes, has been identified in certain *Mycobacterium* species which have been characterized for their ability to degrade high molecular weight PAH (Heitkamp et al., 1988; Khan et al., 2001; Kim et al., 2006). The biochemical pathways of pyrene degradation by *Mycobacterium* have also been identified (Liang et al., 2006; Kim et al., 2007). The genes encoding the α and β subunits of dioxygenases have been sequenced in many strains (Brezna et al., 2003; Sho et al., 2004; Kim et al., 2007). Lake

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sediment studies showed that copy numbers of *nidA* gene were positively related to pyrene mineralization and *nidA* gene can serve as a biomarker for pyrene degradation (DeBruyn et al., 2007). However, little is known about the relationship between *nidA* gene and pyrene degradation in background soils that were not already contaminated by PAHs.

Numerous studies have focused on microbial dynamics in contaminated water, soil and sediments (Cheung and Kinkle, 2005; DeBruyn et al., 2009; DeBruyn and Sayler, 2009), but few have evaluated the impact of introducing pyrene to microbial communities in background soil. This study aimed to investigate the dynamics of the bacterial community in soils freshly-spiked with pyrene by T-RFLP analysis. This would expand the knowledge of functional marker genes for pyrene degradation from the soil environment. In order to test the hypothesis that *nidA* gene are enriched in pyrene-spiked soil and functionally related to the degradation of pyrene, quantitative real-time PCR (qPCR) was used to monitor the change of *nidA* gene copy numbers. To our knowledge, this study is the first to evaluate the relationship between pyrene-biodegradation potential and *nidA* gene copy numbers in pyrene-spiked to background soil.

2. Materials and methods

2.1. Soils selection and characterization

Surface soils were collected to a depth of 20 cm from a site in Quanzhou (QZ) (25°08.414N; 118°48.384E) and a site in Sanming (SM) (26°18.236N; 117°30.817E) which were background soils. Soil samples were air-dried and passed <0.45 mm sieve-mesh and stored in the dark at 4 °C. Soil class and texture were determined by Malvern Mastersizer 2000 (Malvern Instruments, England). Soil pH was determined in a 1:1 soil: water mixture. Total organic C content was determined by Shimadzu Toc-Vcph analyzer (USA). PAHs were analyzed in triplicate by GC–MS (Agilent 7890A/5975, USA) according to EPA method 8270. The soil characteristics are given in Table 1.

2.2. Pyrene incubation experiment

In order to re-activate the soil microorganisms, dry soil samples (500 g) in duplicates were placed in separate glass beakers, moistened with sterile distilled water to the level of 40% of water holding capacity and incubated at room temperature (25 °C) for a period of two weeks (Klimkowicz-Pawlas and Maliszewska-Kordybach, 2003). The soils were then spiked with 1000 mg L⁻¹ solution of pyrene in acetone to a final concentration at 50 mg kg⁻¹, following the recommendations of Brinch et al. (2002) with some adaptations. Briefly, a sub-sample of 10% was spiked with the pyrene solution 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. The moisture content of the samples was adjusted again to 40% of water holding capacity. 10 g spiked soil was placed into 50 ml glass bottles which were sealed with sterile parafilm. Un-spiked soils were amended with acetone at the amount corresponding to those used in PAH-spiked soils. All the samples were incubated in the dark at 25 °C. Soil moisture content was kept at the constant level by periodically weighing the samples and adding water as necessary. Triplicate samples were collected on day 0, 7, 14, 21, 28, 60, were frozen immediately, and stored at –20 °C until analyses. The same procedures were used for the abiotic controls except the soils were autoclaved for three consecutive days before spiking with pyrene solution (Park and Crowley, 2006).

Table 1

Properties of soils from Sanming (SM) and Quanzhou (QZ) in China.

Properties	SM	QZ
Soil texture	Sand	Sand
Sand (%)	69	82
Silt (%)	30	18
Clay (%)	1	0
TOC (%)	1.17	0.88
pH	4.99	5.84
Total PAH (ng g ⁻¹)	69	94
Pyrene (ng g ⁻¹)	16	13

2.3. Pyrene quantification

Analysis of pyrene followed the procedure described earlier (Maliszewska-Kordybach et al., 2007). At the time of extraction, 100 mg kg⁻¹ of fluoranthene-d10 was used as substitute. Soil samples (1 g) were Soxhlet-extracted with acetone and dichloromethane (1:1) for 48 h followed by cleaning up on SiO₂, Na₂SO₄ and Al₂O₃ micro-columns. And 100 mg kg⁻¹ of phenanthrene-d10 was used as internal standard to the extractant. 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) with the following conditions: Helium was used as a carrier gas (constant velocity of 30 cm s⁻¹) with a split-less injection system at 250 °C. The GC oven was programmed as follows: 50 °C for 1 min, followed by a 10 °C min⁻¹ ramp to 200 °C and then with a ramp of 8 °C min⁻¹ to final temperature of 280 °C and hold for 10 min. Mass spectrometer detection was using the selected ion monitoring system. The data were the average of three replicates.

2.4. DNA extraction and qPCR analysis

Triplicate bottles from each treatment were destructively sampled at various time points. The soil samples were stored frozen at –20 °C until DNA extraction. DNA was extracted from 0.5 g of soil (dry weight) with a FastDNA Spin kit for soil (BIO101) as described by the manufacturer with minor modifications (Dionisi et al., 2004).

Target groups and primer sequences for all qPCR analyses are summarized in Table 2. Total *Bacteria* were quantified using primers (BACT1369F and PROK1492R) and methods as described by Suzuki et al. (2000). Amplification conditions were 30 s 94 °C, 15 s 95 °C, 1 min 56 °C, in a reaction volume of 25 µl, containing 12.5 µl Premix Ex taq™ (Takara), 1 µl of each 10 µM primer (Takara), 0.2 µl 25 mM BSA and 2 µl DNA (in 1:10 dilution of original extracts) as templates. A qPCR assay targeting fast-growing *Mycobacterium* 16S rDNA genes and pyrene dioxygenase gene *nidA* (*Mycobacterium*) were also quantified as described previously (DeBruyn and Sayler, 2009). The annealing temperature was 57 °C and 59 °C, respectively. Amplification reactions were described as above. All the real-time PCR reactions were performed on an iQ™5 thermocycler (BioRad, USA).

The bacterial standard template DNA was generated by PCR with *Bacteria*- (27f/1492r) specific primers. The PCR products were cloned into pGEM-T Easy vector (Promega, USA). Plasmids were transformed into *Escherichia coli* JM109 cells (Takara). These clones were sequenced and used as an internal standard. For generation of the *Mycobacterium* and *nidA* gene standard, *Mycobacterium* sp. 6PY1 (supported by John C. Willison) used as the template was amplified with *Mycobacterium* (*MycF*/*MycR*) and *nidA* (*nidA-F*/*nidA-R*) specific primers and cloned as above. All quantitative PCR reactions were performed in triplicate. Ten-fold serial dilutions of a known copy number of the plasmid DNA were used to perform qPCR assays in triplicate to generate standard curves.

Table 2

Quantitative Real-time PCR primers, PCR primers in this study.

Target group	Probes and Primers	Sequences
<i>Bacteria</i> (16S rDNA)	BACT1369F	5'-CGGTGAATACGTTTCYCGG (Suzuki et al., 2000)
	PROK1492R	5'-GGWTACCTTGTACGACTT (Suzuki et al., 2000)
	Probe (TM1389F)	5'-FAM-CITGTACACACCGCCGTC-TAMRA (Suzuki et al., 2000)
<i>nidA</i>	NidA-F	5'-TTCCCGAGTACGAGGGATAC (DeBruyn et al., 2007)
	NidA-R	5'-TCACGTTGATGAACGACAAA (DeBruyn et al., 2007)
	Probe	5'-FAM-TCCTACCCGTCGCCGTACA-BHQ1 (DeBruyn et al., 2007)
<i>Mycobacterium</i> (16S rDNA)	MycoF	5'-CCTTATGTCCAGGGCTTCA (DeBruyn and Sayler, 2009)
	MycoR	5'-TAGCGACTCCGACTTCACG (DeBruyn and Sayler, 2009)
	Probe	5'-FAM-TTGAAAGGATTCTCCACCTCA-BHQ1 (DeBruyn and Sayler, 2009)
<i>Mycobacterium</i> (16S rDNA)	Myco66f	5'-CATGCAAGTCGAACGAAAA (Leys et al., 2005)
	Myco600r	5'-TGTGAGTTTTCACGAACA (Leys et al., 2005)
<i>Bacteria</i> (16S rDNA)	27f	5'-AGAGTTTGATCMTGGCTCAG (Weisburg et al., 1991)
	1492r	5'-TACGGYTACCTTGTACGACT (Weisburg et al., 1991)

2.5. Bacterial community analysis by T-RFLP analysis

PCR amplification and T-RFLP analysis of bacterial 16S rDNA fragments followed the protocols described previously (Lueders and Friedrich, 2000; Lu et al., 2006). Briefly, the amplification used 27f and 1492r (Weisburg et al., 1991) and 5' end of the 27f primer was labeled with 6-carboxyfluorescein. The 50 μ l reaction mixture contained 1 μ l of DNA template (in 1:10 dilution of original extracts), 5 μ l of $10\times$ buffer, 3.6 μ l of 25 mM $MgCl_2$, 1 μ l of a 10 mM concentration of the dNTP, 2 μ l of 10 mg L^{-1} bovine serum albumin, 1.6 μ l of each primer (10 μ M) and 2.5 U of *Taq* DNA polymerase (Takara). The thermal profile for amplification was as described previously (Rui et al., 2009). The 6-carboxyfluorescein-labeled PCR products were purified using an agarose gel DNA extraction kit (Takara) and digested at 37 °C overnight by *Hae*III (Fermentas, Canada), and the DNA fragments were size separated using a 3130xl Genetic Analyzer (Applied Biosystems) (Peng et al., 2008).

2.6. *Mycobacterium* 16S clone library construction and sequencing

Two clone libraries were constructed. One was for QZ 50 mg kg^{-1} pyrene-treated soil (QT) on day 60, the other was for QZ control soil (QC) on day 60. The PCR amplification used the primers Myco66F/Myco600R (Leys et al., 2005) as described in Table 2. A touchdown PCR protocol was used as previously. The amplification conditions were 95 °C 6 min, then 20 cycles of denaturing at 95 °C 30 s, annealing at 55 °C 0.5/cycle for 45 s and extension at 72 °C 45 s, followed by 15 cycles with an annealing temperature at 45 °C, finally extension at 72 °C for 6 min. PCR products were purified and ligated into the pMD19-T easy vector (Promega, USA) according to the manufacturer's instructions. Plasmids were transformed as described above, and more than eighty five clones were randomly selected and sequenced for each clone library. The sequences were submitted to the Genbank database to search for similarity with other *Mycobacterium* sequences using the Blast alignment tool (NCBI homepage, <http://www.ncbi.nlm.nih.gov/BLAST/>). Sequences were assembled, aligned, and analyzed with MEGA4 software (Tamura et al., 2007). The phylogenetic tree was constructed using the neighbor-joining method. The sequences obtained in this study have been deposited in the EMBL nucleotide sequence database, accession numbers FN690762–FN690936.

3. Results

3.1. Pyrene degradation

Pyrene degradation rates in the two soils were monitored separately following the addition of 50 mg kg^{-1} pyrene. The concentration of pyrene was measured at different incubation times. In QZ soil spiked with pyrene, degradation was noticeable after 7 days, but not statistically significant, and the dissipation of pyrene was significant at the end of the experiment (day 60) and decreased to 9.76 mg kg^{-1} (Fig. 1). In contrast to the pyrene treatment soil, there was little pyrene disappearance observed in the QZ sterilized soil. For SM soil, no significant pyrene

disappearance was observed in both treatments (Fig. 1). The average extraction efficiency was approximately 60% based on the recovery of a pyrene spike.

3.2. Analysis of pyrene catabolic genes

For both soils, the gene copy number of total bacteria ranged from 6×10^9 to 4×10^{10} copies g^{-1} dry weight soil in pyrene added soil. There was no significant difference between pyrene treatment and the two control soils (Fig. 2A and B) with the exception of the QZ soil, the bacterial 16S rDNA copy numbers in pyrene treatment were lower than the control (Fig. 2A). The gene copy number of total *Mycobacterium* 16S rDNA ranged from 1.04×10^9 to 8.63×10^9 copies g^{-1} of dry weight soil. Statistical analysis showed the data from the quantification of bacterial and *Mycobacterium* 16S rDNA were not significantly different between sampling dates ($p > 0.05$). For QZ soil, *Mycobacterium* 16S rDNA copies were higher in pyrene added soil than in the control soil (Fig. 2C). However, for SM soil, the gene copy number of *Mycobacterium* 16S rDNA was detected at levels just above quantification in the control soil, unlike the pyrene added soil (Fig. 2C and D). For QZ soil, between sampling dates of pyrene added soil, there was a significant difference in the abundance of *nidA* gene. The *nidA* gene copy numbers which increased with incubation time ranged from 7.88×10^3 to 3.05×10^6 copies g^{-1} of dry weight soil (Fig. 2E). But for SM soil, no significant differences were observed for *nidA* gene copy numbers in the pyrene added soil (Fig. 2F). In addition, the *nidA* gene copy numbers were not detected (data not shown) in the two control soils, which might be due the fact that the control soils had little pyrene contamination.

3.3. Structure of microbial community

The T-RFLP fingerprints of bacterial communities in soils incubated for different periods after the addition of pyrene were compared with those of the control microcosms. Six fragments (191 bp, 194 bp, 214 bp, 222 bp, 228 bp and 231 bp) were detected as major peaks in the T-RFLP profiles (Fig. 3). T-RFLP patterns showed that there were no substantial changes in bacterial community during the 60-day incubation period, neither in pyrene-spiked nor in the control soil. Only some of the major peaks such as T-RFs 191 bp, 194 bp showed small variations (Fig. 3C and D). In addition, there were no significant changes in bacterial community after the addition of pyrene. However, T-RFLP patterns showed more significant changes in terms of community structure between different soils (Fig. 3).

Detection of *nidA* gene in QZ soil indicated the existence of fast-growing, potentially the PAH-degrading *Mycobacterium* (DeBruyn et al., 2007). To investigate the diversity of *Mycobacterium*, two clone libraries were constructed. One hundred and seventy six clones were successfully sequenced. A neighbor-joining tree generated using Kimura's two-parameter distance method (Kimura, 1980), is shown in Fig. 4. For the clone library of the pyrene treatment, maximum-likelihood analysis indicated that most sequences fell into the PAH-degrading, fast-growing *Mycobacterium* clusters, such as *M. flavescens*, *M. austroafricanum*. For the clone library of the control soil, several sequences fell into *M. gordonae* (Rogall et al., 1990) which is the slower-growing *Mycobacterium* cluster.

4. Discussion

In the present study, background soils were used to examine the diversity of bacterial community structure after the addition of pyrene. We firstly evaluated the influence of pyrene addition on

Fig. 1. Dynamics of pyrene concentration in pyrene-spiked soils. QT, soil QZ being spiked with pyrene; QA, soil QZ being sterilized and spiked with pyrene; ST, soil SM being spiked with pyrene; SA, soil SM being sterilized and spiked with pyrene; data are means $\pm$ SE; $n = 3$.

Fig. 2. Quantification of bacterial 16S rDNA (A, B), *Mycobacterium* 16S rDNA (C, D) pyrene dioxygenase gene *nida* copy numbers (E, F); data are means $\pm$ SE; $n = 3$. Different letters within rows are significantly different ($p < 0.05$) according to one way-ANOVA analysis. QC, QZ control; SC, SM control.

bacterial community using T-RFLP analysis. There was no significant difference in the bacterial community between the pyrene-spiked and the control soil. Our data implicated the inherent resistance of the bacterial community to pyrene concentration of 50 mg kg⁻¹. This was similar to the observations that microbial communities could withstand the Cu or water stress with physiological tolerance (Griffiths et al., 2003; Deng et al., 2009).

Quantification through Real-Time PCR is considered to be an accurate method for estimating the biodegradation potential of a PAH-degrading bacterial consortium in contaminated environments (Cebon et al., 2008). In our experiment, we quantified the bacterial 16S rDNA, *Mycobacterium* 16S rDNA, *nida* gene abundances in two pyrene-spiked soils. 16S rDNA gene was a proxy for bacterial biomass (Kim et al., 2005a). There was little difference in the 16S rDNA gene copy numbers between different treatments, while there was also a decreasing trend observed for 16S rDNA gene

copy numbers with the incubation time in both soils. This indicated that there was no significant population change or bacterial biomass change during the incubation period with pyrene. This is similar to previous study showing that 16S rDNA gene copy numbers were not affected by the addition of naphthalene (Park and Crowley, 2006).

In our study, there was a significant difference in pyrene degradation between the two soils. No significant decrease in SM soil was observed over two months incubation with initial pyrene concentrations of 50 mg kg⁻¹. However, for QZ pyrene-spiked soil, pyrene concentrations began to decrease after 7 days (Fig. 1). This might be related to different soil characteristics. The organic content of SM soil was higher than QZ soils (Table 1). Organic matter is related to bioavailability in terms of a) sorbing PAHs so that they are unavailable to microorganisms through uptake through the cell membranes, and b) also influence the desorption

Fig. 3. Bacterial community structure in QZ control (A), QZ spike-bio (B), SM control (C), and SMspike-bio (D). The figure shows the relative abundance of terminal restriction fragments (TRF) used as a measure of the composition of the bacterial community; data are means $\pm$ SE; $n = 3$.

kinetics in that there is more organic matter for PAHs to diffuse out of. Organic matter content determined the rate of pyrene transfer from soil to microorganisms (Macleod and Semple, 2002). Hence the pyrene degradation rate of SM soil could be slower than QZ soil.

In the samples that were used for DNA analysis, the *nidA* gene copy numbers increased significantly to approximate 500-fold in QZ soil. The dissipation of pyrene was up to 80% after 60-day incubation in QZ soil. *nidA* gene responded to pyrene dissipation significantly and suggested that there might be a direct relationship between gene copy numbers and degradation rates for pyrene. This observation was similar to previous studies that there was a positive relationship between *nidA* gene copy numbers and pyrene

mineralization (Wang et al., 1996; DeBruyn et al., 2007). But *nidA* gene was not detected in all samples of this study, such as control soil. Previous studies also showed that *nidA* gene was not common in clean soils but for PAH historically contaminated soils (Margesin et al., 2003; Hall et al., 2005; DeBruyn et al., 2007; DeBruyn et al., 2009). In addition, soil pH may be another factor influenced the *nidA* gene. Although lower pH may favor pyrene permeate in to the cell and help gene induction, causing increased biodegradation in resting cell, lower pH (below 7.5) did slow down the growth of *Mycobacterium vanbaalenii* PYR-1 and accumulated more pyrene and toxic intermediate in cytosol, burdening cell activity (Kim et al., 2005b). This may explain that *Mycobacterium* 16S rDNA gene

Fig. 4. Phylogenetic relationship of representative *Mycobacterium* 16S rDNA clone sequences generated from QZ samples of pyrene treatment incubations (QTMYC) and control soil (QCMYC). Bootstrap values (for 5000 interactions) over 50% are indicated on branches.

was not detectable in most SM soil (pH 4.99) before spiking with pyrene. After pyrene was added, *Mycobacterium* 16S rDNA gene was detected at much lower level in SM soil than in QZ soil. This might be the reason that the insignificant degradation in SM soil was observed. But the influence of pH in soil needs further investigation.

In this study, the *mycobacteria* 16S rDNA gene was quantified. For QZ soil, copy numbers of *Mycobacterium* 16S rDNA gene in all samples were higher in the pyrene treatment than in the control, indicating *Mycobacterium* was induced to grow and was the major player in pyrene degradation. However, there were no significant

differences in *Mycobacterium* 16S rDNA gene copy numbers between different incubation times. Compared with *nidA* gene copy numbers, copy numbers of *mycobacteria* 16S rDNA gene was higher. This is expected as not all *mycobacteria* carries *nidA* gene. However, as both *Mycobacterium* 16S rDNA copy numbers and *nidA* gene copy numbers were increased by the addition of pyrene, we infer that *nidA* gene was associated with *Mycobacterium*. This is in agreement with the fact that *Mycobacterium* spp. is the primary carriers of *nidA* genotypes in the contaminated environment (DeBruyn et al., 2007; DeBruyn and Sayler, 2009). For most samples of the SM control soil, *Mycobacterium* 16S rDNA gene was not detected (Fig. 2D). This

might be due to the fact that less *mycobacteria* was carrying the *nidA* gene (Debruyne and Sayler, 2009) and might be below the detection limit.

Mycobacterium 16S rDNA clone libraries were constructed. Most sequences closely related to fast-growing PAH-degrading *mycobacteria* and only few sequences related to slow-growing *mycobacteria*. Slow-growing *mycobacteria* was only detected in the control soil. This result was consistent with the finding that no *nidA* gene was detected in the control soil as *nidA*-carrying organisms identified thus far have been fast-growing *mycobacteria* (DeBruyn et al., 2007). Fast-growing PAH-degrading *mycobacteria* carrying *nidA* gene may not be selected without the addition of pyrene. In contrast to the control soil, all sequences were fast-growing PAH-degrading *Mycobacterium* in the pyrene-spiked soil. These observations supported that high diversity of *Mycobacterium* (Cheung and Kinkle, 2001; Leys et al., 2005) occurred in PAH-contaminated soil. It is also further suggested the association of *nidA* gene with fast-growing PAH-degrading *mycobacteria* in pyrene-contaminated soil. These results indicate that *nidA* gene and its abundance may play a key role in pyrene degradation after the addition of pyrene.

In summary, this study highlights the importance of *nidA* gene in pyrene degradation. *Mycobacteria* were prevalent while *nidA* gene was not always detected in pyrene-spiked pristine soils. It is the presence of *nidA*, and not just presence of *mycobacteria*, that is likely a more important causal agent for pyrene degradation in soil.

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