Response of bacterial *pdo1, nah* and *C12O* genes to aged soil PAH pollution in a coke factory area

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Abstract: Soil pollution caused by polycyclic aromatic hydrocarbons (PAHs) is threatening human health and environmental safety. Investigating the relative prevalence of different PAH-degrading genes in PAH-polluted soils and searching for potential bioindicators reflecting the impact of PAH pollution on microbial communities are useful for microbial monitoring, risk evaluation and potential bioremediation of soils polluted by PAHs. In this study, three functional genes, *Pdo1*, *nah* and *C12O*, which might be involved in the degradation of PAHs from a coke factory, were investigated by real-time quantitative PCR (qPCR) and clone library approaches. The results showed that the *pdo1* and *C12O* genes were more abundant than the *nah* gene in the soils. There was a significantly positive relationship between *nah* or *pdo1* gene abundance and PAH content, while there was no correlation between *C12O* gene abundance and PAH content. Analyses of clone libraries showed that all the *pdo1* sequences were grouped into *Mycobacterium*, while all the *nah* sequences were classified into three groups: *Pseudomonas*, *Comamonas* and *Polaromonas*. These results indicated that the abundances of *nah* and *pdo1* genes were positively influenced by levels of PAHs in soil and could be potential microbial indicators reflecting the impact of soil PAH pollution and that *Mycobacteria* were one of the most prevalent PAHs degraders in these PAH polluted soils. Principal component analysis (PCA) and correlation analyses between microbial parameters and environmental factors revealed that total carbon (TC), total nitrogen (TN) and dissolved organic carbon (DOC) had positive effects on the abundances of all PAH-degrading genes. It suggests that increasing TC, TN and DOC inputs could be a useful way to remediate PAH polluted soils.

Key words: Polycyclic aromatic hydrocarbon, bioindicator, qPCR, PAH-degrading gene, persistent organic pollutants, soil pollution.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are recalcitrant toxic pollutants (Couling et al. 2010), of which 16 are listed as priority pollutants by the United States Environmental Protection Agency (USEPA) (Mackay et al. 1992). PAHs are distributed ubiquitously throughout the environment and are generated continuously from incomplete combustion and thermal decomposition processes of organic matter, especially of coal and oil (Johnsen et al. 2005). Human and ecosystem health are threatened by PAHs because of their toxic, mutagenic and carcinogenic properties and high enrichment ratio within living organisms (Johnsen et al. 2005; Peng et al. 2008). PAHs are composed of two or more fused aromatic rings in various structural configurations, characterized by low aqueous solubility, high solid-water distribution ratios and chemical stability. The aqueous solubility and, as a consequence, the bioavailability of PAHs decreases with increasing number of aromatic rings

(Peng et al. 2008). In addition, many studies have demonstrated that the extractability and the bioavailability of PAHs decrease significantly with time in the aging processes (Northcott and Jones 2001; Luo et al. 2012). Most PAHs released into the environment will finally enter into the soil ecosystem, where they will impair human and other organisms through the food chain (Fismes et al. 2002). Therefore, it is of great importance to detect the effect of PAH pollution on certain bacterial populations associated with PAH degradation and seek appropriate bioindicators to reflecte the impact of PAH pollution in aged industrial soils.

Soil microorganisms play a key role in energy flow, nutrient cycling and organic matter turnover including biodegradation of organic pollutants. Although PAHs may undergo chemical oxidation and photolysis, in most cases microbial degradation is still the major process involved in the dissipation of PAHs in the soil (Park et al. 1990). On the other hand, PAHs in the soil exert pressure on various microbes which inhabit it. Microorganisms are sensitive to even low levels of pollutants in soil and rapidly respond to soil pollution, so microbial parameters are usually considered as potential bioindicators of soil pollution (Sun et al. 2012).

Many microorganisms with the capability of degrading different PAHs have been isolated based on culture-dependent techniques (Bastiaens et al. 2000; Juhasz et al. 2000; Chang et al. 2011). However, it is well known that culture-independent approaches allow for a broader recognition of microbial populations responsible for PAH degradation in environments (Baldwin et al. 2003; Jurelevicius et al. 2012). Recently, the abundance and composition of PAH-degrading microbial populations present in PAH-polluted environments has been investigated to some extent through direct extraction of DNA from the environment and analysis of PAH-degrading genes (Singleton et al. 2005; Lillis et al. 2010; Paissé et al. 2012). *Pdo1*, *nah* and *C12O* are three important PAH-degrading genes which have been detected widely from environments polluted by aromatic compounds or bacteria isolated from those environments (Baldwin et al. 2003; Johnsen et al. 2006; Khan et al. 2009; Tuan et al. 2011). The *Pdo1* gene encodes the pyrene dioxygenases associated with degradation of high molecular weight (HMW) PAHs (Johnsen et al. 2006), the *nah* gene encodes naphthalene dioxygenases associcated with degradation of low molecular weight (LMW) PAHs (Baldwin et al. 2003), and the *C12O* gene encodes catechol 1,2-dioxygenase associated with cleavage of the last aromatic ring in the degradative pathway of PAHs (Sei et al. 1999). Johnsen et al. (2006) analyzed *nah*, *phnA* and *pdo1* genes using a PCR assay but only detected the presence of *nah* and *pdo1* in soil microcosms spiked with street dust as a PAH source. Among the two genes detected, the *pdo1* gene was found to play an important role in the degradation of PAHs in the environment. Sei et al. (2004) found that abundances of the *C12O* and *C23O* genes increased significantly in aromatic compound spiked seawater compared to those in the control, whereas community structure of bacterial

16S rDNA tended to be simplified by the spiked aromatic compound. To our knowledge, most of these studies focused on description of PAH-degrading genes based on microcosms spiked with a few PAHs (e.g., Scelza et al. 2007; Li et al. 2012; Meynet et al. 2012), while studies on comparison of genes encoding different PAH-degrading enzymes in field sites polluted by aged PAHs are scant. Therefore, this study aimed to investigate the prevalence of different PAH-degrading genes and the relationship between the PAH-degrading genes and levels of PAHs in soils from a coke factory area. The abundances of *pdo1*, *nah* and *C12O* genes in each soil were determined using real-time quantitative PCR (qPCR) assay, and the community structures of the PAH-degrading genes were analyzed by clone libraries and sequencing.

2. Materials and methods

2.1. Sampling site and soil collection

Soil samples classified as sandy loam were collected from a coke factory area. The coke factory was located in Xintai (35°54′N, 117°43′E), Shandong province of northern China. It started operation in 2007 and can produce six million tons of secondary petroleum coke every year. Forty sampling sites were selected randomly in an area of about 16 hectares around the coking oven. In each site, five soil subsamples were taken from the top 20 cm of the profile and mixed into one composite sample. All samples were placed on ice and transported to the laboratory, homogenized thoroughly after being gently crumbled to pass through a 2-mm sieve and stored at -20°C prior to use. Soil samples taken from the fridge were used directly for measurement of total water contents (TWC) and dissolved organic carbon (DOC) as well as extraction of total DNA. Air-dried soil samples passing through a 100-mesh sieve were used to determine the contents of total carbon (TC) and total nitrogen (TN). Freeze-dried soil samples passing through a 100-mesh sieve were used for extraction of PAHs.

2.2. Analysis of soil properties and PAH contents

TWC of the soil samples was determined through weight loss at 105°C for 24 h. Soil pH was measured in a ratio of soil to water of 1:2.5 using a pH-meter. TC and TN of the soil samples were determined using the Dumas method by an Element Analyser (Vario EL III, Elementar, Hanau, Germany). DOC of the soil samples was measured following the method described by Sparling et al. (1998).

PAHs in the soil samples were extracted using ultrasonic extraction method (USEPA, 2000). Briefly, the PAHs in 1 g of freeze-dried soil, which had been grounded and passed through a 100-mesh sieve, were extracted by 30 ml of dichloromethane-hexane (1:1, v:v) in an ultrasonic bath for three times. The extract was collected by centrifugation and concentrated with a rotary evaporator. The concentrated extract was cleaned up with a

chromatography column filled from bottom to top with 80-mesh neutral silica gel (12 cm, 180°C heated for 12 h), 100-mesh neutral alumina (6 cm, 250°C heated for 12 h), and anhydrous sodium sulfate (1 cm, 450°C heated for 6 h). PAHs were eluted using 70 ml of hexane:dichloromethane (7:3, v:v) after alkanes removed by 15 ml of hexane. The concentrated eluent contain PAH mixture with a rotary evaporator continued to be concentrated to 1 ml through evaporation under a stream of N_2 gas.

PAHs were separated on an Agilent HP7890 Gas Chromatograph equipped with Agilent DB-5MS Column (30 m×0.25 mm×0.25 μm) under a flow of 1 mL min^{-1}. The temperature programme was as follows: 2 min at 80°C, to 120°C at 3°C min^{-1}, to 200°C at 5°C min^{-1}, to 290°C at 7°C min^{-1}, and at 290°C for 15 min. PAH detection was performed with an Agilent 5975C mass spectrometer with selected ion mode using electron impact at 70 eV.

Hexamethylbenzene was used as the internal standard and a PAH Solution Mix consisting of 16 USEPA priority PAHs (AccuStandard, New Haven, CT, USA) as the external standard. PAHs were identified on the basis of their retention times, target and qualifier ions, and quantified by a calibration curve (the ratio of external to internal standard). Phe-d10 was spiked into the soil samples 24 h prior to extracting PAHs to determine the recovery rate of the extraction method used.

2.3. Soil DNA extraction and qPCR assays

Total community DNA was extracted from 0.5 g soil using the MoBio Ultraclean soil DNA isolation kit (MoBio Labroratories, Solana Beach, CA, USA) according to the manufacturer's instructions. The concentration of the extracted DNA was determined using Nanodrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

PAH-degrading genes, *nah*, *pdo1* and *C12O*, were amplified on an iCycler iQ 5 thermocycler (Bio-Rad Laboratories, Hercules, CA, USA). The reaction mixture (25μL) contained 2 μL of template DNA (1-10 ng), 0.5 μL of each of the forward and reverse primers (10 μM) and 12.5 μL of 2× SYBR® Premix Ex Taq™ (TaKaRa Biotechnology, Dalian, China). The primer pairs and amplification conditions are shown in Table 1.

Standard curves for qPCR assays were constructed following the procedure described by Lillis et al. (2010). Briefly, *nah, pdo1* and *C12O* genes were amplified from extracted DNA with the primer pairs listed in Table 1. The PCR products were cloned using the pGEM-T Easy Vector system (Promega Madison, WI, USA). Plasmids from the positive clones with each target gene insert were extracted and sequenced. The copy numbers of the plasmid DNA were calculated after determining the concentration. Serial dilutions were used to generate an external standard curve.

2.4. Construction of clone libraries and phylogenetic analysis

Clone libraries of the *pdo1* and *nah* genes were constructed because they were positively correlated with the total PAH content. The PCR products of the *pdo1* and *nah* genes from eight representative soil samples with different levels of PAHs were pooled and purified using a gel purification kit (Promega, Madison, WI, USA). The clone libraries of the genes were constructed by cloning PCR products using the pGEM-T Easy Vector system (Promega, Madison, WI, USA) according to the manufacturer's instructions, respectively. Eighty white colonies were randomly selected from each clone library, respectively. Inserts of each clone were amplified by PCR using the vector-specific primers T7 (5'-TAATACGACTCACTATAGGG-3') and SP6 (5'-TATTTAGGTGACACTATAG-3') following the method described by Lillis et al. (2010). Then, 50 and 74 positive clones from *pdo1* and *nah* clone libraries were used for sequencing, respectively. Classifications of the *pdo1* and *nah* sequences were identified by aligning with the BLAST search program (http://blast.ncbi.nlm.nih.gov). For each clone library, sequences sharing a 97% nucleotide sequence identity were defined as one operational taxonomic unit (OTU) using MOTHUR software. Coverage of each clone library was computed according to the following equation: C=1-(n/N), where n was the number of OTU containing only one clone and N was the total number of clones examined (Chelius and Triplett, 2001). Only one representative sequence of each OTU was used for phylogenetic analyses. Phylogenetic trees were constructed using the neighbour-joining method with 1000 bootstrap replicates of MEGA version 4.0. Sequences of the *pdo1* and *nah* clones used in phylogenetic tree construction were deposited in the GenBank nucleotide sequence database under accession numbers KF561983 to KF561985 and KC878841 to KC878848, respectively.

2.5. Statistical analyses

One-way analysis of variance (ANOVA) based on Fisher LSD test using SPSS 13.0 was used to assess differences between the abundances of different genes in the same soil. Principal component analysis (PCA) was conducted using CANOCO 4.5 to indicate the relationship between abundances of PAH-degrading genes and environmental factors including soil properties and content of PAHs. Spearman's correlation between various parameters was calculated using STATISTIC 6.0. The probability levels of $P < 0.05$ and $P < 0.01$ were considered to be statistically significant.

3. Results

3.1. Soil basic properties and content of PAHs

Soil basic properties are shown in Table 2. Sample Nos. were designated as No. 1 to 40

respectively from low to high content of PAHs in soils. The soil pH ranged from 4.44 to 7.97. The contents of TN, TC and DOC ranged from 0.46 to 2.63 g kg^{-1}, from 6.15 to120 g kg^{-1} and from 19.3 to 43.8 mg kg^{-1} of dry weight soil, respectively. The 16 USEPA priority PAHs were determined in this study. The recoveries for standard Phe-d10 spiked into the soil samples ranged from 70.0% to 94.5%. The contents of total PAHs varied widely from 1.15 to 761 mg kg^{-1} of dry weight soil (Table 2). The PAH mixture was divided into five groups according to their number of aromatic rings. 3-4 ring PAHs were most common in all the soils, making up from 55.5% to 84.1% of total PAHs (Fig. 1). The proportion of HMW PAHs increased with increasing content of total PAHs in soils, while LMW PAHs showed the opposite trend. Phenanthrene, fluoranthene and pyrene were major compounds in all soils. Naphthalene was also one of the major components of PAHs in the soils polluted slightly by PAHs, where, 5-6 ring PAHs were almost undetectable.

3.2. Abundances of the *pdo1*, *nah* and *C12O* genes

Copy numbers of the genes involved in PAH degradation are shown in Fig. 2. Abundance of the *pdo1* gene in soil samples except sample No. 26 and *C12O* gene in soil samples except sample Nos. 21, 26, 39 and 40 was significantly higher than the *nah* gene ($P < 0.05$). Abundances of the *pdo1* and *C12O* genes were in the ranges of 1.63×10^{6}-1.08×10^{9} and 8.68×10^{6}-7.67×10^{8} copies g^{-1} of dry soil, respectively. However, the *nah* gene had the lowest copy number from 5.10×10^{4} to 1.24×10^{8} copies g^{-1} of dry soil. Abundances of the *pdo1* and *C12O* genes were 2.33-3421 times and 0.54-4000 times higher than the *nah* gene, respectively.

3.3. Diversity of the *pdo1* and *nah* gene sequences in the clone library

The clone library of *pdo1* gene had a coverage of 98.0%. 50 clones obtained from the library were assigned into 3 OTUs. The phylogenetic tree of the *pdo1* gene showed that all of OTUs were closely related to *Mycobacterium* (Fig. 3a). The coverage of *nah* gene clone library was computed to be 97.3%. 74 clone sequences of the library were assigned into 8 OTUs. The phylogenetic tree of the *nah* gene assigned 45.9% of the sequences to *Pseudomonas* of Gammaproteobacteria, 17.6% to *Polaromonas* of Betaproteobacteria, and 36.5% to *Comamonas* of Betaproteobacteria (Fig. 3b).

3.4. Relationship of the PAH-degrading gene abundances and environmental factors

PCA analysis of the PAH-degrading genes and environmental factors including soil properties and content of PAHs is illustrated in Fig. 4. PC1 and PC2 accounted for 67.1% and 27.1% of the total variation. Of the genes involved in PAH degradation, *nah* gene abundance had the closest relationship with the content of total PAHs. However, abundance of the other PAH-degrading genes, *pdo1* and *C12O*, were influenced more significantly by TC, TN and DOC than PAHs. Table 3 showed that abundances of the *nah* and *pdo1* genes significantly

correlated with the content of total PAHs (R = 0.76 and 0.57, respectively, $P < 0.01$). However, the *C12O* gene had no significant relationship with content of total PAHs. All of the PAH-degrading genes were correlated significantly with TC (0.54, 0.48 and 0.48 for the *pdo1*, *nah* and *C12O*, respectively, $P < 0.01$), TN (0.69, 0.42 and 0.44 for the *pdo1*, *nah* and *C12O*, respectively, $P < 0.01$), and DOC (0.64, 0.56 and 0.34 for the *pdo1*, *nah* and *C12O*, respectively, $P < 0.01$ for the *pdo1* and *nah*, $P < 0.05$ for the *C12O*). TWC and pH had no significant correlation with abundances of the *pdo1*, *nah* and *C12O* genes.

4. Discussion

In this study, the collected soils from a coke factory were polluted by mostly 3-4 ring PAHs, especially phenanthrene, fluoranthene and pyrene. This result was similar to the previous report by Lors et al (2010). They analyzed the compositional features of PAH mixture in a coal tar contaminated soil and found that 3- and 4-ring PAHs were primarily and secondly abundant. Moreover, phenanthrene was the most concentrated compound among 3-ring PAHs, and fluoranthene and pyrene were the two most concentrated compounds among 4-ring PAHs. It indicated that soils polluted by the coking industry could be characterized by high proportions of 3- and 4-ring PAHs. Interestingly, the portion of HMW PAH increased with increasing total PAH content while LMW PAHs showed a contrary trend in the examined soils. Some studies and the results here have shown that microbial populations involved in PAH degradation may be enriched by PAH pollution (Laurie and Lloyd-Jones 2000; Niepceron et al. 2013), and it is also well known that HMW PAHs are more resistant to degradation by soil microbes than LMW PAHs (Johnsen et al. 2005; Lors et al. 2010). So a higher proportion of LMW PAHs may be eliminated in seriously polluted soils than in slightly polluted soils, and consequently HMW PAHs tend to accumulate in seriously polluted soils (Johnsen et al. 2006).

To elucidate the relationship between abundances of various PAH-degrading genes and PAH content, the genes of *pdo1*, *nah* and *C12O* which might be responsible for PAH degradation were quantified by qPCR in this study. Abundance of the *C12O* gene in soils was high but had no significant correlation to total PAH content. Catechol 1,2-dioxygenase encoded by the *C12O* gene catalyzes the oxygenative ring cleavage of catechol, which is the intermediate metabolite of many aromatic compounds including PAHs (Da Cunha et al. 2006). So abundance of the *C12O* gene might be influnced by the content of not only total PAH but also many other kinds of aromatic compounds in soil. However, there were very significant positive correlations between the abundances of both *nah* and *pdo1* genes and total PAH content. Naphthalene dioxygenase encoded by the *nah*

gene has a relaxed substrate specificity and catalyzes the dioxygenation of many related 2- and 3-ring PAHs such as naphthalene, phenanthrene, anthracene, acenaphthylene, fluorene and so on to their respective *cis*-dihydrodiols (Resnick et al. 1996). The *pdo1* gene sequences detected showed high levels of similarity with *pdo1* genes identified from *Mycobacterium* which is involved in degradation of 2-, 3- and 4-ring PAHs including naphthalene, phenanthrene, fluoranthene, pyrene, chrysene and benz(a)anthracene as well as even 5-ring PAHs e.g. benz(a)pyrene with the presence of pyrene as co-metabolite (Schneider et al. 1996; Johnsen et al. 2006; Zhang et al. 2006). Thus, it was speculated that more organisms containing the *nah* and *pdo1* genes thrive under the condition of high PAH levels in soils. Similar results have been reported in recent studies (Laurie and Lloyd-Jones 2000; Jin et al. 2012), Laurie and Lloyd-Jones (2000) found that copy numbers of *phnAc* and *nahAc* genes involved in PAH degradation in PAH- and petroleum-polluted soils were significantly greater than those in a pristine soil using competitive PCR. Jin et al. (2012) found that naphthalene dioxygenase (NDO) genes were enhanced by naphthalene addition in laboratory microcosms. Johnsen et al. (2006) also found increased prevalence of *nah* and *pdo1* genes in soil microcosms spiked with street dust as a PAH source. These results indicated that the abundances of *nah* and *pdo1* genes were positively influenced by levels of PAHs and could be potential bioindicators reflecting the impact of PAH pollution on microbial communities in soil.

In all soils examined except sample No. 26, abundance of the *pdo1* gene was significantly higher than that of the *nah* gene. Although the approach based on DNA analysis did not reflect the relative contribution of these genes to degradation of PAHs, it did reveal the higher prevalence of bacterial populations harboring the *pdo1* gene than the *nah* gene. In this study, the clone library of the *pdo1* gene showed that all *pdo1* sequences were grouped into *Mycobacterium*, which was unsurprising as the primer used was designed based on the *pdo1* gene from *Mycobacterium* sp. strain 6PY1 (Krivobok et al. 2003; Johnsen et al. 2006). Johnsen et al. (2007) also found that *Mycobacteria* were dominant members of phenanthrene, pyrene and fluoranthene degraders in soils originating from an asphalt and tar production plant. Thus, it was suggested that *Mycobacteria* may be one of the most prevalent PAH degraders in the soils examined. The dominance of *Mycobacteria* corresponded to the compositional features of PAHs in the examined soils with a high share of 3-4 ring PAHs, especially phenanthrene, fluoranthene and pyrene, which can be utilized and degraded by *Mycobacteria* harboring the *pdo1* gene (Johnsen et al. 2006). Some reports showed that *Mycobacteria* had a competitive advantage over PAH degraders from other bacterial genera, due to physiological adaptation to oligotrophy and low PAH bioavailability (Wick et al. 2002; Wells et al. 2005; Uyttebroek et al. 2006).

The phylogenetic analysis showed that all sequences obtained from the *nah* gene clone library were

classified into one of three groups: *Pseudomonas* of Gammaproteobacteria, *Comamonas* of Betaproteobacteria, and *Polaromonas* of Betaproteobacteria. Moreover, there was a very significant correlation between *nah* gene abundance and PAH content. The close relationship between Gammaproteobacteria and Betaproteobacteria and PAH in soils has been reported in recent studies, especially Gammaproteobacteria has been suggested as a bioindicator of PAH pollution (Lors et al. 2010; Niepceron et al. 2013). Lors et al. (2010) monitored the PAH-degrading bacterial community structures by PCR and clone library in a coal tar contaminated soil in the process of windrow treatment. Gammaproteobacteria were detected throughout the process, while Betaproteobacteria were detected when the accumulated content of 2-, 3- and 4-ring PAHs decreased substantially. Thus the Gammaproteobacteria were suggested as a bioindicator of the potential biodegradation of PAHs in contaminated soil. Niepceron et al. (2013) monitored the abundances of ten bacterial phyla and classes by qPCR and *Pseudomonas* isolates using direct culture in soil microcosms spiked with a mixture of seven PAHs over a 90-day period, and found that Betaproteobacteria and Gammaproteobacteria were more sensitive to the PAH mixture than other bacterial groups. Abundance of the Betaproteobacteria was significantly higher compared to the controls only at 60 days of incubation, while abundances of the Gammaproteobacteria and culturable *Pseudomonas* (affliated to the Gammaproteobacteria) significantly increased over the longer period of incubation in response to the addition of PAH mixture. Thus the Gammaproteobacteria was suggested by Niepceron et al. as a bioindicator to detect the impact of multiple pollution by PAHs on bacterial communities. In this study, the *nah* gene clone library displayed that the abundance levels of Gammaproteobacteria and Betaproteobacteria were 45.9% and 54.1% respectively. Although whichever of two microbial groups is more sensitive can not be determined according to that, it suggests that Gammaproteobacteria and Betaproteobacteria are the sensitive microbial taxa of soil PAH pollution.

PAH-degrading genes could be also influenced by soil characteristics. In this study, the content of TC, TN and DOC were correlated significantly with abundance of the PAH-degrading genes e.g. *pdo1*, *nah* and *C12O*. TC and TN contents reflect organic nutrition in soils, and DOC represents the part of organic nutrition that could be utilized by microorganisms directly and easily (Cook and Allan 1992). So soil microorganisms including PAH-degrading bacteria could thrive along with the increase of TC, TN and DOC in soils (Yang et al. 2011; Zhang et al. 2012). Besides providing the nutrients for microorganisms, DOC may increase the solubility and bioavailability of PAHs (Thiele-Bruhn and Brümmer 2004; Kobayashi et al. 2008). For soils polluted by aged PAHs, the low bioavailability of PAHs was usually the key factor that restricted the degradation of PAHs by microorganisms (Kobayashi et al. 2008; Couling et al. 2010; Larsson et al. 2013). Therefore, it has been

suggested that an effective method for remediation of PAH-polluted soils would be to apply organic substances thus increasing inputs of TC, TN and DOC (Li et al. 2012).

Conclusions

The relationship between abundance of different PAH-degrading genes and content of PAHs in the soils from a coke factory were examined in this study. Abundance of the *nah* and *pdo1* genes were significantly correlated with the soil total PAH content. However, no significant relationship was found between the *C12O* gene abundance and the soil total PAH content. This indicated that abundances of the *nah* and *pdo1* genes may be potential microbial indicators reflecting the impact of PAH pollution on microbial communities in soil. Abundance of the *pdo1* gene was significantly higher than the *nah* gene in great majority of the soil samples. Analyses of clone libraries showed that all *pdo1* sequences were grouped into *Mycobacterium* while all *nah* sequences could be classified into one of three groups: *Pseudomonas*, *Comamonas* and *Polaromonas*. It suggested that *Mycobacteria* were more prevalent than bacterial taxa possessing the *nah* gene in aged-PAH polluted soils. The significant positive correlations between contents of TC, TN and DOC and abundance of all PAH-degrading genes implied that increasing inputs of TC, TN and DOC into soils may be a useful way to remediate the soils polluted by PAHs. These findings provide new insights into microbial monitoring, risk evaluation and potential bioremediation of soils polluted by the coking industry. However, more studies are still needed to confirm these conclusions in other soil types or environments polluted by aromatic compounds in different physicochemical conditions.

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423 Table 1 Primer pairs and amplification conditions used in real-time quantitative PCR (qPCR) assays

Target genes	Amplicon size (bp)	Primer pairs	Sequence (5'-3')	Amplification conditions	Reference
nah	377	NAH-F	CAAAARCACCTGATTYATGG	94°C for 1 min (1 cycle); 94°C for 20 s, 60°C (reduced by 1°C per cycle) for 30 s, 72°C for 30 s (5 cycles); 94°C for 20 s, 55°C for 30 s, 72°C for 30 s, and plate read at 83°C for 15 s (35 cycles)	Baldwin et al. 2003
		NAH-R	AYRCGRGSGACTTCTTTCAA		
pdo1	793	pdo1-f	GTTCTACCTCGACCTCATTGCG	94°C for 5 min (1 cycle); 94°C for 1 min, 60°C (reduced by 0.5°C per cycle) for 1 min, 72°C for 1 min (10 cycles); 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, and plate read at 83°C for 10 s (30 cycles)	Johnsen et al. 2006
		pdo1-r	CTGACCCATGTATTCCAGCC		
C12O	324	C12O_F	CGCGGATTGTNGAYSTNTGGCANGCNAAYAC	95°C for 1 min (1 cycle); 94°C for 20 s, 60°C (reduced by 1°C per cycle) for 30 s, 72°C for 30 s (5 cycles); 94°C for 20 s, 55°C for 30 s, 72°C for 30 s (plate read simultaneously)	Tuan et al. 2011
		C12O_R	GACTCAGGTNGCRWANGCRAARTCRTC		

424 Table 2 Basic properties of the soil samples collected from a coke factory

Sample No.[a]	TWC[b] (%)	pH	TN[c] (g kg^{-1})	TC[d] (g kg^{-1})	DOC[e] (mg kg^{-1})	Total PAHs(mg kg^{-1})
1	11.6	7.88	0.46	6.15	22.7	1.15
2	9.70	7.70	0.55	6.28	22.5	1.53
3	9.40	7.56	0.54	6.29	22.6	1.54
4	15.7	7.54	1.00	13.2	25.4	2.18
5	7.70	7.92	0.47	6.69	24.5	2.24
6	9.70	7.65	0.56	7.08	22.9	2.32
7	15.3	7.61	0.82	9.43	24.9	2.62
8	16.7	7.39	0.80	10.7	27.4	2.75
9	14.9	6.81	0.87	15.3	26.7	3.02
10	15.1	5.87	1.06	10.5	23.1	3.59
11	15.3	7.55	1.05	15.2	24.8	3.61
12	16.2	7.73	0.86	11.8	24.6	3.71
13	15.0	5.87	0.84	9.22	20.5	4.38
14	15.7	6.28	0.74	9.54	26.1	4.53
15	14.3	7.69	0.93	13.3	23.3	5.93
16	20.2	4.44	1.41	52.9	28.5	6.35
17	13.3	6.39	0.97	14.5	31.1	6.92
18	18.5	6.73	1.48	52.7	34.2	6.99
19	10.6	5.87	1.34	26.1	39.7	8.76
20	18.1	7.71	1.70	63.3	34.7	10.2
21	18.4	7.25	0.98	26.7	25.5	10.2
22	17.5	6.57	1.27	57.3	26.3	11.7
23	16.5	6.56	0.98	41.3	25.8	12.6
24	15.4	5.99	1.90	81.0	33.7	12.7
25	16.2	7.66	0.76	18.9	28.7	13.2
26	15.6	7.70	0.80	17.6	19.3	13.4
27	14.6	7.66	0.88	18.7	26.7	13.8
28	13.4	7.71	0.97	24.8	26.6	13.9
29	12.1	6.89	1.77	32.1	33.1	14.1
30	12.5	6.60	1.81	78.8	32.1	14.3
31	14.8	5.22	1.71	28.3	30.5	15.9
32	16.2	7.60	1.25	42.0	37.9	21.2
33	15.3	7.34	1.69	33.4	34.4	22.4
34	9.80	7.70	1.36	40.2	29.2	22.8
35	12.7	7.56	0.92	36.9	24.6	26.1
36	13.1	6.38	2.63	120	42.5	30.1
37	10.6	7.70	1.27	40.7	28.2	30.5
38	14.4	7.97	1.37	44.2	30.4	33.5
39	15.7	7.72	0.87	22.5	28.2	113
40	13.0	7.60	1.65	61.1	43.8	761

425 [a] Sample Nos. were designated as No. 1 to 40 respectively from low to high content of PAHs in soils; [b]TWC
426 represents total water content; [c]TN represents total nitrogen; [d]TC represents total carbon; [e]DOC represents
427 dissolved organic carbon.

Table 3 Correlation coefficients between PAH-degrading gene abundance and environmental factors of the soils from a coke factory

	TWC	pH	TN	TC	DOC	PAHs	*pdo1*	*nah*	*C12O*
TWC	1.00								
pH	**-0.32***	1.00							
TN	0.19	**-0.40***	1.00						
TC	0.19	-0.28	**0.88****	1.00					
DOC	0.06	-0.20	**0.79****	**0.71****	1.00				
PAHs	-0.06	0.12	0.21	0.23	**0.46****	1.00			
pdo1	0.03	-0.16	**0.69****	**0.54****	**0.64****	**0.57****	1.00		
nah	-0.01	0.07	**0.42****	**0.48****	**0.56****	**0.76****	**0.72****	1.00	
C12O	0.20	-0.15	**0.44****	**0.48****	**0.34***	-0.08	0.09	-0.11	1.00

Significant correlations were presented in the form of boldface. * Significance level of $P < 0.05$; ** Significance level of $P < 0.01$.

Fig. 1 Compositional features of PAHs in the soils from a coke factory

Fig. 2 Abundance of different PAH-degrading genes in the soils from a coke factory. Error bars are the SD of n=3

Fig. 3 Phylogenetic trees of *pdo1* a) and *nah* b) gene sequences retrieved from the soils in a coke factory. Designation of the clones included the following information: clone number, nucleotide sequence accession number and amount of clones with similarities above 97% to the listed sequence in the clone library in the parentheses. Numbers on nodes represent bootstrap values. The scale bar represents 0.2% estimated sequence divergence

Fig. 4 The principal component analyses (PCA) of PAH-degrading genes and environmental factors of the soils from a coke factory. The values in the parentheses represent the percentage of total variation explained by each axis

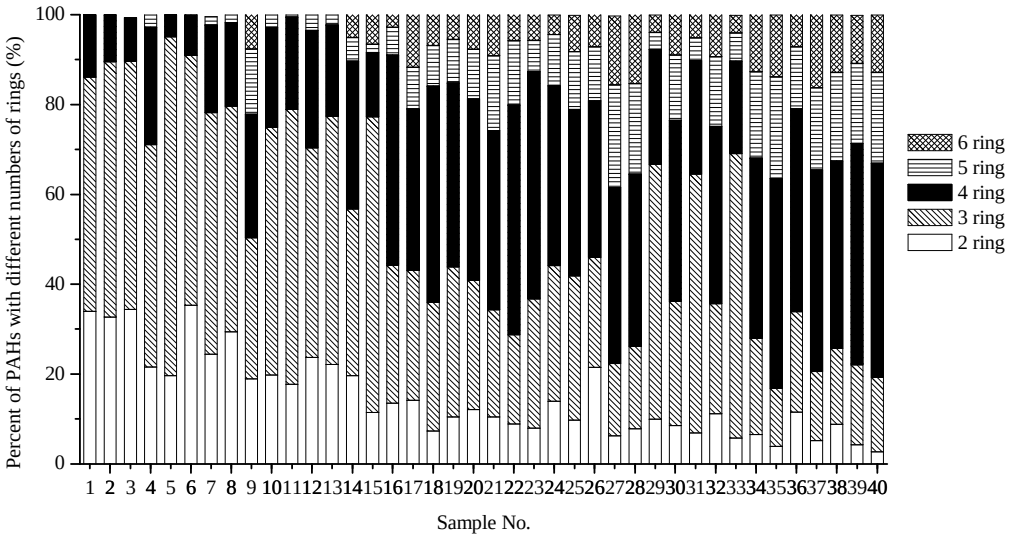
Percent of PAHs with different numbers of rings (%)
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
Sample No.
6 ring
5 ring
4 ring
3 ring
2 ring

Fig. 1

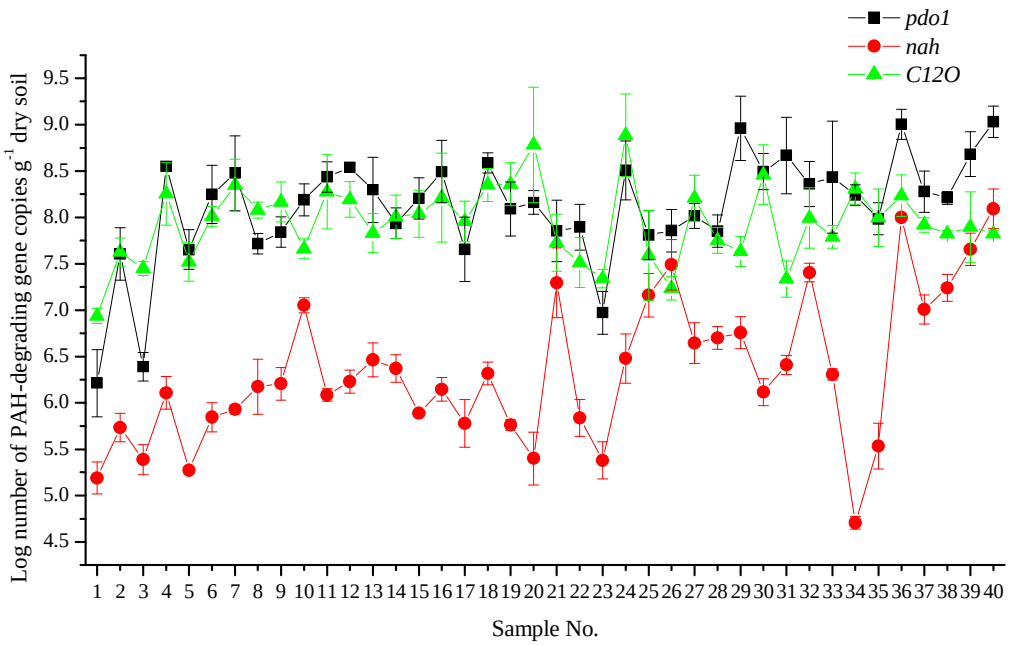
pdo1
nah
C12O
Log number of PAH-degrading gene copies g-1 dry soil
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
Sample No.

Fig. 2

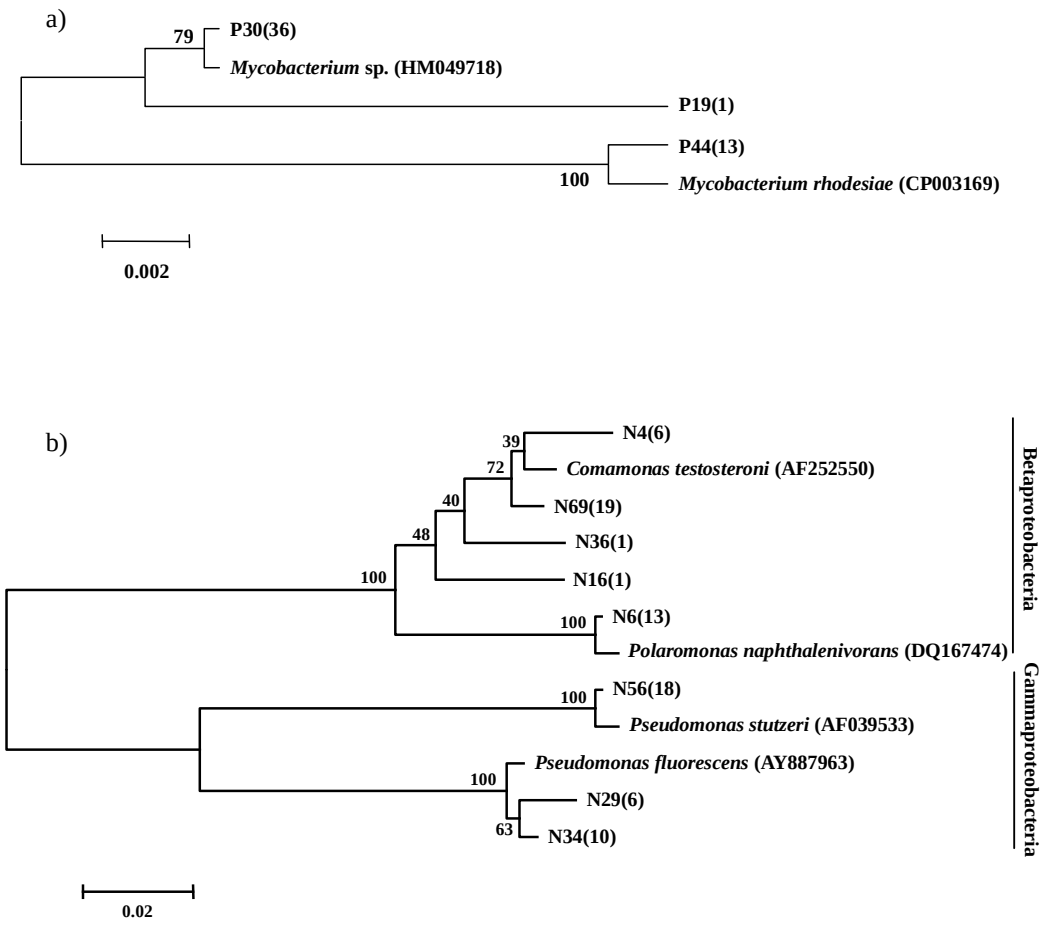
a)
79
P30(36)
Mycobacterium sp. (HM049718)
P19(1)
P44(13)
100
Mycobacterium rhodesiae (CP003169)
0.002
b)
39
N4(6)
72
Comamonas testosteroni (AF252550)
40
N69(19)
48
N36(1)
100
N16(1)
100
N6(13)
Polaromonas naphthalenivorans (DQ167474)
Betaproteobacteria
100
N56(18)
Pseudomonas stutzeri (AF039533)
Pseudomonas fluorescens (AY887963)
100
N29(6)
63
N34(10)
Gammaproteobacteria
0.02

Fig. 3

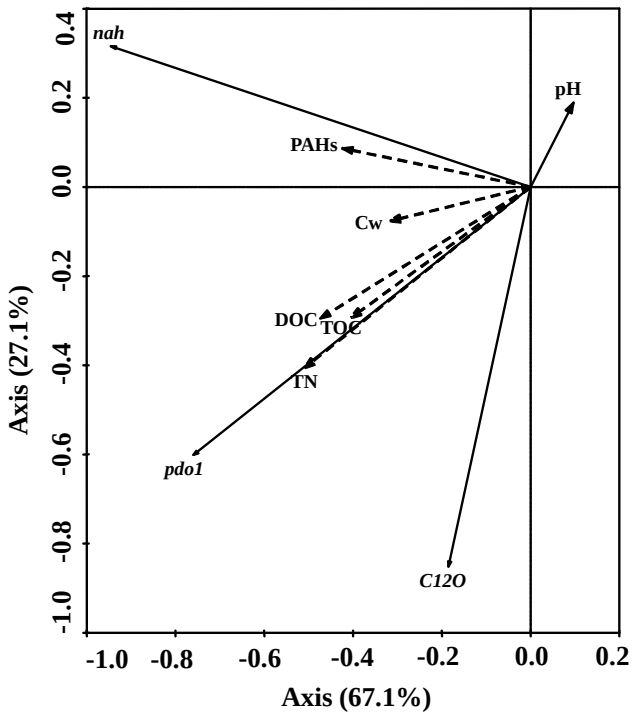
nah
pH
PAHs
Cw
DOC
TOC
TN
pdo1
C12O
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
Axis (27.1%)
-1.0
-0.8
-0.6
-0.4
-0.2
0.0
0.2
Axis (67.1%)

Fig. 4