Effect of pyrene on denitrification activity and abundance and composition of denitrifying community in an agricultural soil

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ABSTRACT

Toxicity of pyrene on the denitrifiers was studied by spiking an agricultural soil with pyrene to a series of concentrations (0–500 mg kg⁻¹) followed by dose–response and dynamic incubation experiments. Results showed a positive correlation between potential denitrification activity and copy numbers of denitrifying functional genes (*nirK*, *nirS* and *nosZ*), and were both negatively correlated with pyrene concentrations. Based on the comparison of EC₅₀ values, denitrifiers harboring *nirK*, *nirS* or *nosZ* gene were more sensitive than denitrification activity, and denitrifiers harboring *nirS* gene were more sensitive than that harboring *nirK* or *nosZ* genes. Seven days after spiking with EC₅₀ concentration of pyrene, denitrifiers diversity decreased and community composition changed in comparison with the control. Phylogenetic analyses of three genes showed that the addition of pyrene increased the proportion of *Bradyrhizobiaceae*, *Rhodospirillales*, *Burkholderiales* and *Pseudomonadales*. Some species belonging to these groups were reported to be able to degrade PAHs.

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) have been identified as carcinogenic, mutagenic, and teratogenic (Perera, 1997). As a result, the US Environmental Protection Agency (US EPA) and the European Community placed PAHs in the priority pollutant list (Wild and Jones, 1995). Unfortunately, PAHs pollutant streams that enter the environment have not been sufficiently stemmed; indeed their intensity continues to increase, mainly due to the rising demand for fossil fuels (Conte et al., 2001). Being both chemically very stable while exhibiting a high hydrophobicity ensures that PAHs are effectively adsorbed onto soil particles and in particular soil organic matter (Means et al., 1980). With the continued build-up of PAHs in soil, the resulting impacts on soil ecosystem health are of great concern, with several studies having been carried out to evaluate the toxic effects of PAHs on dehydrogenase and urease activities, soil respiration (Miles and Doucette, 2001; Klimkowicz-Pawlas and Maliszewska-Kordybach, 2003) and soil bacteria in relation to nitrogen (N) cycle (e.g. nitrifying bacteria) (Maliszewska-Kordybach et al., 2007).

Denitrification is a crucial microbial process in the N cycle in which oxidized N compounds (NO₃⁻, NO₂⁻) are reduced into

gaseous products (NO, N₂O, and N₂). N₂O is the potent greenhouse gas that can cause ozone depletion in the stratosphere. Soils are considered to be the major source of N₂O emission contributing 65% of the total global emission, with 6.0 Tg N₂O–N yr⁻¹ from natural soils and 4.2 Tg N₂O–N yr⁻¹ from agricultural soils (Intergovernmental Panel on Climate Change, 2001). Studies have shown that denitrifying communities are affected by soil conditions including temperature, moisture, pH and substrate (e.g. NO₃⁻, organic carbon) concentration (Braker et al., 2010; Henderson et al., 2010; Liu et al., 2010; Stres et al., 2008; Lalissee-Grundmann et al., 1988; Renner and Becker, 1970). High exposures to metals such as cadmium, copper, zinc and silver can also be detrimental to denitrifying communities (Holtan-Hartwig et al., 2002; McKenney and Vriesacker, 1985; Throbäck et al., 2007). In addition, the selective inhibition of different steps in the denitrification process in soil could aggravate the ecological consequences of pollution. It has been shown that the reduction of NO₂⁻ appeared to be more sensitive than the reduction of NO₃⁻ (Bollag and Barabasz, 1979; McKenney and Vriesacker, 1985), which would result in the accumulation of NO₂⁻ to toxic levels, and a selective inhibition of NO reductase or N₂O reductase by heavy metals enhanced NO or N₂O emission from soils (Holtan-Hartwig et al., 2002). With the increase of PAHs in soil, ecotoxicity of PAHs on denitrification in soil deserves more attention. It is therefore of ecological significance to understand the responses of denitrifying communities and their functions in different phases of

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denitrification processes in soil to PAHs stress, and to identify sensitive and resistant organisms.

A complete denitrification process involves a nitrate reductase encoded by the *narG*, *narH* or *napA* gene, a nitrite reductase encoded by the *nirK* or *nirS* gene, a nitric oxide reductase encoded by the *qnorB* or *cnorB* gene and a nitrous oxide reductase encoded by the *nosZ* gene. Most denitrifiers belong to a wide range of subclasses of *Proteobacteria*, while there are still some closely related to *Archaea*, *halophilic* and *hyperthermophilic* branches as well as *mitochondria* of certain fungi (Zumft, 1997). The common difference between true denitrifiers and other microorganisms with nitrate-reducing ability is that the true denitrifiers have either a copper-containing enzyme encoded by *nirK* or a cytochrome *cd1* enzyme encoded by *nirS* (Braker et al., 2000; Zumft, 1997). Therefore, the *nirK* and *nirS* genes have frequently been used as gene markers to analyze denitrifying community. Although some denitrifiers lack this nitrous oxide reductase enzyme, the *nosZ* gene could be used as a target for different populations of the denitrifying bacteria capable of nitrous oxide reduction (Throbäck et al., 2004). Since not all denitrifiers have the complete suite of denitrification enzymes (Zumft, 1997), two or more functional genes are often used as molecular markers for this microbial group. In most studies, the analyses of the molecular ecology of the denitrifying community were based on *nirK*, *nirS* and *nosZ* genes (Braker et al., 2000; Throbäck et al., 2004). The activity of reductases and the expression of functional genes involved in denitrification process varies among individual strains (Zumft, 1997), and community composition is thus considered to be able to influence functionality at both community and ecosystem levels (Braker et al., 2010). Nevertheless, some studies have reported correlations between abundance and function as well as between structure and function of denitrifying communities (Bremer et al., 2009; Hallin et al., 2009; Rich et al., 2003) while others not (Dandie et al., 2008; Miller et al., 2008; Wertz et al., 2009). Therefore, it is important to determine the correlations among changes in function, abundance and composition of the denitrifying community in an agricultural soil in response to PAHs stress.

The objective of this study was therefore to assess the toxicity of pyrene on denitrification activity and the abundance, diversity and composition of the denitrifying community. In order to make comprehensive assessment, acute toxicity and dynamic toxicity were tested. The acute toxicity was determined through a dose–response test of denitrification activity and abundance of denitrifiers against pyrene. The dynamic toxicity was determined during a 7-day course after pyrene perturbation by measuring N_2O accumulation and reduction as well as the diversity and community composition of the denitrifiers.

2. Material and methods

2.1. Soil sampling and characteristics

The soil sample was collected from the surface (0–20 cm in depth) of an agricultural field located in Beiye Chang (39°44'N, 116°25'E), Daxing district, Beijing, in September 2008. *Brassica rapa pekinensis*, *Raphanus sativus* and *Brassica oleracea* var. *botrytis* were grown in this field. This field has been regularly fertilized with organic manure and ammonium nitrate for more than twenty years. After visible thick roots and leaves were removed, the fresh soil sample was passed through a 2 mm sieve-mesh, and then stored at 4 °C until further experiments were conducted. Part of the soil sample was air-dried and passed through a 0.45 mm sieve for chemical analyses.

Soil pH was determined by a pH meter (FE20, METTLER TOLEDO, Switzerland) at a soil: water ratio of 1: 2.5 (w/v). Nitrate and ammonium were determined by an automated Continuous Flow Analyzer (SAN++, SKALAR, Netherlands). Total organic carbon was determined by Element Analyzer (Vario EL III, Elementar, USA). We measured 16 PAHs which were listed as priority pollutants by US EPA in triplicate by GC–MS (6890N/5975C, Agilent, USA) according to EPA method 8270. Characteristics of the soil sample were as follows: pH, 7.69; NO_3^- -N, 384 mg kg⁻¹; NH_4^+ -N, 772 mg kg⁻¹; organic carbon, 19.9 g kg⁻¹; pyrene, 59.7 µg kg⁻¹; Σ PAHs, 1.03 mg kg⁻¹.

2.2. Spiking of soil with pyrene

Crystalline pyrene (L08162, Alfa Aesar, Lancaster, USA) was dissolved in acetone and uniformly sprayed onto soil to produce sub-samples with pyrene levels of 0 (pyrene-free, with acetone only), 20, 50, 100, 200, 400, 700, 1000, 2000 and 5000 mg kg⁻¹ dry weight soil (Brinch et al., 2002; Peng et al., 2010). These sub-samples were homogenized by continuous hand shaking in 1000 mL glass bottles and the bottles were closed for 5 min to let the solvent disperse, followed by storage at 25 °C in the dark for 16 h. When acetone was evaporated off, sub-samples were mixed with non-spiked soil at the ratio of 1:9 and shaken thoroughly, generating final pyrene levels of 0 (non-spiked control), 2, 5, 10, 20, 40, 70, 100, 200 and 500 mg kg⁻¹ dry weight soil (denoted as Pr2, Pr5, Pr10, Pr20, Pr40, Pr70, Pr100, Pr200 and Pr500, respectively). The pyrene-spiked samples were continuously shaken by hand to ensure homogeneous distribution of pyrene in the soil. Triplicate samples of each treatment were collected and stored at –80 °C for DNA extraction and quantitative PCR, and other used for acute and dynamic incubation experiments.

2.3. Potential denitrification activity

Potential denitrification activity (PDA) was determined by using the acetylene (C_2H_2) inhibition technique (Smith and Tiedje, 1979; Schinner et al., 1996). In this study, we modified the method by omitting any extra nitrate and carbon since the content of NO_3^- -N and organic carbon was already high (384 mg kg⁻¹ and 19.9 g kg⁻¹, respectively) due to excessive fertilization. It was reported that the optimum concentrations for NO_3^- -N should not exceed 50 mg kg⁻¹, and an excess of NO_3^- -N (>100 mg kg⁻¹ dry soil) might inhibit N_2O production (Lalisse-Grundmann et al., 1988; Renner and Becker, 1970).

Quadruplicates of pyrene-spiked soil (15 g fresh soil) were weighed into 120-mL serum bottles capped with butyl rubber stoppers. The moistures of all spiked samples were adjusted to 50% (w/w, absolute water content) with deionized water to make nitrate dissolve in water and thereby generating anaerobic conditions. All spiked samples were made anoxic by four cycles of evacuation and filling with N_2 (1.5 atm), while shaken continuously and thoroughly. A final over-pressure in the bottles was released by piercing a 0.5 mm (inner diameter) needle through the rubber septum. To avoid O_2 to enter during this venting, the needle was mounted on a 5 mL plastic syringe (without piston) filled with 1 mL distilled water. Then C_2H_2 was added to the headspace (injection and subsequent release of over-pressure, final concentration 10% v/v) to inhibit N_2O reduction. All bottles were incubated at 25 °C in the dark for 24 h. Gas samples (1 mL) were withdrawn from bottles by glass precision gas syringes and were injected into N_2 -filled (1 atm) 29.0 mL vials capped with butyl rubber septa. These diluted gas samples were then analyzed for N_2O by a gas chromatograph (SP3410, Beijing Analytical Instrument Factory) connected to an ECD detector as described previously by Pang et al. (2009), and the amount of N_2O in the headspace and PDA were calculated as indicated by Schinner et al. (1996).

2.4. Time-course of N_2O accumulation and reduction

Dynamics of N_2O accumulation and reduction were determined by anaerobic incubation with and without C_2H_2 (Yoshinari et al., 1977). Non-spiked control soil and soil treated with EC₅₀ concentration of pyrene (concentration of pyrene in soil causing 50% inhibition of denitrification activity; denoted as EC50 treatment) were prepared for the dynamic experiment. Fifteen grams of soil (fresh weight) was transferred to a 120-mL serum bottle capped with butyl rubber stoppers, with eight replicates for each treatment. Soil moisture, oxygen-exhaust and soil incubation conditions were as the same as for the PDA experiment. Four of eight replicates in each treatment were randomly selected and injected with 10% C_2H_2 (final concentration 10% v/v) to inhibit the N_2O reduction. The moment of the injection of C_2H_2 was regarded as the start of incubation. All bottles were then incubated at 25 °C in the dark for 7 days. The N_2O concentration of each replicate was analyzed at intervals of 24 h. Triplicate soil samples of each treatment (control and EC50, without C_2H_2) were collected at the end of incubation (on the 7th day) and stored at –80 °C before DNA extraction.

2.5. DNA extraction

The DNA was extracted from 0.5 g soil (stored at –80 °C) with the Fast DNA SPIN kit for soil (BIO101) following the manufacturer's instructions. The recovered DNA was eluted in 10 mM Tris–HCl buffer (pH 7.5). The purity and the quantity of the DNA were determined by UV–Vis Spectrophotometer (ND-1000, NanoDrop, USA) at 260 and 280 nm. 260/280 ratios were found to be all above 1.8. The DNA solution was stored at –20 °C.

The DNA extraction of the soils spiked with 0–500 mg kg⁻¹ of pyrene were destined to real-time quantitative PCR, and DNA from the EC50 experiment to clone libraries of functional genes.

2.6. Real-time quantitative PCR

Quantification of *nirK*, *nirS* and *nosZ* genes was performed on a iCycler iQ™5 Thermocycler (BioRad, USA). The 25-µL reaction mixtures contained 12.5 µL of SYBR

Green Premix ExTaq (TaKaRa, Japan), 0.5 μL of each 20 μM primer, 0.25 μL 25 mM BSA and 1 μL of template DNA. Thermal cycling conditions for *nirK* and *nirS* genes were as follows: pre-incubation at 94 $^{\circ}\text{C}$ for 2 min, 40 cycles consisting of denaturation at 94 $^{\circ}\text{C}$ for 30 s, annealing at 58 $^{\circ}\text{C}$ for 40 s, extension at 72 $^{\circ}\text{C}$ for 30 s, and fluorescence was read during each cycle at 83 $^{\circ}\text{C}$ for 10 s, followed by melting curve analysis at 58–94 $^{\circ}\text{C}$ (0.5 $^{\circ}\text{C}$ per reading), 10-s hold. Thermal cycling conditions for *nosZ* gene were similar to that of *nirK* and *nirS* genes except for the annealing temperature starting at 58 $^{\circ}\text{C}$ for the initial 5 cycles with touchdown of -1°C by cycle followed by 53 $^{\circ}\text{C}$ for 35 cycles, and melting curve analysis at 53–94 $^{\circ}\text{C}$ (0.5 $^{\circ}\text{C}$ per reading).

The standard curves for the quantitative PCR were established using *nirK*, *nirS* and *nosZ* gene fragments cloned into plasmid pGEM-T Easy Vector (3015 bp, Promega, Madison, USA). *nirK*, *nirS* and *nosZ* gene fragments were amplified with the primer pairs 1F and 5R (Braker et al., 1998), R3cd and cd3aF (Michotey et al., 2000), Z-F and 1622R (Throbäck et al., 2004), respectively. The amplicons were gel-purified using the Gel Clean-up System (Promega, Madison, USA) and cloned using the pGEM-T Easy cloning kit according to the manufacturer's instructions. Plasmids were transformed into *Escherichia coli* JM109 competent cells. Plasmid DNA was extracted using a Qiagen Plasmid Mini Kit (Qiagen Nordic) and plasmid concentration was determined by using a NanoDrop ND-1000 spectrophotometer. Plasmid DNA was diluted in ten-fold series to generate standard curves. All quantitative PCR reactions including unknown samples and standard curves were performed in triplicate and no template control (NTC) treatments were included in all runs.

2.7. Cloning, sequencing and phylogenetic analysis

For the construction of a clone library, DNA extracts from three replicated soil samples were mixed and served as the template of PCR. Three primer sets (1F/5R, R3cd/cd3aF, Z-F/1622R) as described in above were used in the PCR amplification of *nirK*, *nirS* and *nosZ* gene fragments. The 25- μL reaction mixtures contained 1 μL of template DNA, 0.5 μL of each 20 μM primer, 2.5 μL of $10\times$ buffer (Mg^{2+} plus), 2 μL of 10 mM dNTPs mixture (2.5 mM of each), 0.25 μL of 25 mM bovine serum albumin (BSA), and 1.25 units of Taq DNA polymerase (Takara). Thermal cycling conditions were the same as those of quantitative PCR. PCR products of the correct size were purified and then ligated to pGEM-T Easy Vector and transformed into *Escherichia coli* JM109 competent cells. The bacteria were transferred into LB agar (containing ampicillin, X-Gal and IPTG) plates. After incubation overnight at 37 $^{\circ}\text{C}$, white colonies (putative positive clones) were picked as correct inserts. More than fifty white clones were selected for each treatment. In total, three separate clone libraries (*NirK* clone library, *NirS* clone library and *NosZ* clone library) were constructed for control and EC50 treatments, respectively. The insert size in each clone was examined by vector-targeted PCR (primers T7 and SP6). The clones with correct insert size were used for sequencing analyses in SinoGenoMax Co., Ltd (Beijing, China).

Vector and primer sequences were removed using DNASTAR Lasergene 7.1. The gene fragment sequences were compared with available sequences in the GenBank database using the NCBI BLASTx program (<http://ncbi.nlm.nih.gov/blast>). Reference sequences were retrieved from NCBI RefSeq and GenBank. These retrieved nucleotide sequences were aligned with reference sequences using MEGA4 (Tamura et al., 2007). Phylogenetic analysis was performed using a neighbor-joining algorithm and distance calculation by MEGA4. Sequences that were 95% or more identical were considered as a unique operational taxonomic unit (OTU) (Katuyama et al., 2008).

The nucleotide sequences of *nirS*, *nirK* and *nosZ* gene fragments determined in this study were submitted in GenBank database under the following accession numbers: *nirK*, HQ221373–HQ221409; *nirS*, HQ221410–HQ221435; and *nosZ*, HQ221436–HQ221463.

2.8. Data analyses

Ecotoxicity of pyrene for denitrification was described by EC_{50} values which were calculated on the basis of Logistic functions regression models assessing the relationships of pyrene concentration–PDA and pyrene concentration–copy numbers of *nirK*, *nirS* and *nosZ* genes by using SigmaPlot5 (Jandel Corporation, CA, USA) (Throbäck et al., 2007) and were expressed in milligrams of pyrene per kilogram of dry soil. The amounts of *nirK*, *nirS*, and *nosZ* in the soil were calculated on an oven-dry weight (105 $^{\circ}\text{C}$) basis.

Diversity indices, Shannon–Weiner (H'), and Simpson's Reciprocal ($1/D$) were calculated by using the following formulas: $H' = -\sum(p_i)(\log_2 p_i)$, $D = 1/(1 - \sum p_i^2)$. Here, p_i is the proportion of clones in OTU i relative to the total clone numbers (Yoshida et al., 2009).

One-way ANOVA was performed to determine significant variations at the level of $P < 0.05$ by using SPSS (11.0).

3. Results

3.1. Potential denitrification activity

Potential denitrification activity (PDA) was expressed in terms of N_2O concentration in the serum bottle headspace (Fig. 1). PDA

Fig. 1. Potential denitrification activity (PDA, presented as $\text{mg N kg}^{-1} \text{ dry soil h}^{-1}$) under different pyrene concentrations. The data are means of quadruplicate measurements. Bars represent standard deviation. The fitting curve and the mean EC_{50} value (59.74 mg kg^{-1}) were obtained by applying “Four Parameter Logistic Curve” ($y = \min + (\max - \min)/(1 + (x/\text{EC}_{50})^{\text{Hillslope}})$) ($r^2 = 0.97$, $P < 0.0001$) to the relation of pyrene concentration and PDA.

decreased with increasing pyrene concentrations, and was the lowest ($0.16 \text{ mg N kg}^{-1} \text{ h}^{-1}$) in the Pr500 treatment. PDA in spiked soil was not significantly lower than the control until pyrene concentration was as high as 70 mg kg^{-1} . The mean EC_{50} value of pyrene for PDA was 59.7 mg kg^{-1} ($r^2 = 0.97$, $P < 0.0001$).

3.2. Dynamics of N_2O accumulation and reduction

One day after pyrene perturbation, N_2O accumulation (NA) and N_2O reduction rate (NR) in the EC_{50} treatment were 213% and 55.5% of those in the control treatment (Fig. 2). However, NA of the EC_{50} treatment increased to 438% times of the control treatment on the 4th day and then decreased to 162% of the control treatment on the 7th day. NR of the EC_{50} treatment increased to 96.3% of the control treatment on the 3rd day. From the 3rd day to the 7th day, NRs of the two treatments were comparatively stable, and the ratio of NR of the EC_{50} treatment to that of the control treatment fluctuated within the range of 96.3–98.6%. Meanwhile, the soil pyrene

Fig. 2. Time-course of N_2O production and N_2O reduction ratio (reduced N_2O /total N_2O) of the control and EC_{50} treatments with and without 10% C_2H_2 during anaerobic incubation. The data are means of quadruplicate measurements. Bars represent standard deviation.

concentration of the EC50 treatment declined from 59.7 mg kg⁻¹ on the 1st day to 33.4 mg kg⁻¹ on the 7th day.

3.3. Abundance of *nirK*, *nirS* and *nosZ* genes

The copy numbers of *nirK*, *nirS* and *nosZ* genes in response to pyrene concentrations are shown in Fig. 3. The copy numbers of *nirK* gene (around 10⁷) in the control and spiked treatments were an order of magnitude lower than those of the *nirS* and *nosZ* genes (around 10⁸) in respective treatments. The copy numbers of the three genes generally decreased with the increase of pyrene concentrations with the exception that the copy numbers of *nirK* gene in the Pr2 (4.40 × 10⁷ copies g⁻¹ dry soil), Pr10 (4.24 × 10⁷ copies g⁻¹ dry soil) and Pr20 (4.02 × 10⁷ copies g⁻¹ dry soil) treatments were significantly higher than the non-spiked control (3.66 × 10⁷ copies g⁻¹ dry soil). The EC50 values of pyrene for copy numbers of *nirK*, *nirS* and *nosZ* genes were 39.3, 17.7 and 31.5 mg kg⁻¹ ($r^2 > 0.85$ and $P < 0.01$, figures not shown), respectively. The linear regression ($r^2 > 0.74$, $P < 0.01$) showed that PDA was positively correlated with copy numbers of the three genes (Fig. 4).

3.4. Diversity of the denitrifying community

Diversity of the denitrifying community was evaluated by Shannon–Weiner index and Simpson's Reciprocal index based on *NirK*, *NirS* and *NosZ* clone libraries in the control and EC50 treatments (Table 1). A total of 93 clones and 37 OTUs for *nirK* gene, 89 clones and 26 OTUs for *nirS* gene as well as 97 clones and 31 OTUs for *nosZ* gene were obtained from the control and the EC50 treatments. Both Shannon index and Simpson index of the three genes in the control treatment were higher than in the EC50 treatment.

3.5. Phylogenetic trees of *nirK*, *nirS* and *nosZ* genes

Phylogenetic trees based on the *NirK*, *NirS* and *NosZ* nucleic acid sequences (480 bp, 371–386 bp and 387–420 bp, respectively) are shown in Figs. 5–7, respectively. The clones of the three genes obtained in this study spread throughout the trees and affiliated to *Proteobacteria*. The phylogenetic tree of the *nirK* gene was divided into five major clusters (cluster I–IV). The clones belonging to clusters I, II and III were closely related to *Rhizobiaceae*, *Bradyrhizobiaceae*

Fig. 4. The copy numbers of the *nirK*, *nirS* and *nosZ* genes in non-spiked control and spiked treatments (Pr2–Pr500). The copy numbers are expressed relative to denitrification activity.

and *Phyllobacteriaceae*, respectively, affiliated to *Alphaproteobacteria*. The clones belonging to IV were closely related to *Alcaligenaceae*, affiliated to *Betaproteobacteria*.

The *NirS* tree was divided into six clusters (cluster I–VI). The *NirS* clones in clusters II and III were primarily related to *NirS* of *Rhodobacteraceae* and *Bradyrhizobiaceae*, respectively, affiliated to *Alphaproteobacteria*. The *NirS* clones in cluster IV were relatively closely related to *NirS* of *Rhodocyclaceae*, affiliated to *Betaproteobacteria*; *NirS* clones in cluster V were relatively closely related to *NirS* of *Pseudomonadaceae*, affiliated to *Gammaproteobacteria*, while those in clusters I and cluster VI were relatively distantly related to *NirS* of known denitrifying bacteria. BLAST homology search also showed that the clone sequences in clusters I and VI did not match *NirS* from any denitrifiers isolates, although some of them showed high similarities to uncultured deduced *NirS* clones from environmental samples.

The *NosZ* tree was divided into seven clusters (cluster I–VII). *NosZ* clones in clusters I, II, III and IV were relatively closely related to *NosZ* of *Brucellaceae*, *Rhizobiaceae*, *Bradyrhizobiaceae* and *Rhodospirillaceae*, respectively, belonging to *Alphaproteobacteria*. *NosZ* clones in clusters V, VI and VII were closely related to *NosZ* of *Alcaligenaceae*, *Burkholderiaceae* and *Oxalobacteraceae*, respectively, belonging to *Betaproteobacteria*.

3.6. Shifts of *NirK*, *NirS* and *NosZ* diversities

Relative abundance of *NirK*, *NirS* and *NosZ* clones of the EC50 treatment was different from that of the control treatment (Fig. 8).

Table 1
Diversity indices of *nirK*, *nirS* and *nosZ* clones in the control and EC50 treatments.

Gene	Name of treatments	No. of clones	No. of OTUs (5% cut)	Shannon–Wiener Index (H')	Simpson's Reciprocal Index (1/D)
<i>nirK</i>	Control	46	22	2.78	15.92
	EC50	47	21	2.62	11.88
	Total	93	37	—	—
<i>nirS</i>	Control	47	22	2.75	14.04
	EC50	41	15	2.4	10.65
	Total	88	26	—	—
<i>nosZ</i>	Control	48	18	2.51	10.07
	EC50	49	18	2.26	5.88
	Total	97	31	—	—

Fig. 3. The copy numbers of the *nirK*, *nirS* and *nosZ* genes in non-spiked control and spiked treatments (Pr2–Pr500). The quantitative PCR was run in triplicate for each treatment, and average values and the standard deviations are shown.

Fig. 5. Neighbor-joining tree based on NirK nucleic acid sequences. Clones with >95% sequence similarity were considered to be the same OTU. Sequences with the names starting with CK-nirK and EC50-nirK were obtained from the control treatment and EC50 treatment, respectively. NirK sequences of the reference known denitrifying bacteria are indicated by each accession number in parentheses. Bootstrap values (%) were generated from 1000 replicates, and the values > 50% are shown. A number in parentheses represents the total number of clones in each OTU.

In the EC50 treatment, the proportions of the NirK clones belonging to cluster I and cluster III decreased by 12.6% and 4.95%, respectively, compared to the control treatment, while those belonging to clusters II and IV related to *Bradyrhizobiaceae* and *Alcaligenaceae* increased by 14.4% and 4.21%, respectively.

The proportions of the NirS clones belonging to clusters II, III and VI decreased by 12.1%, 1.82% and 3.63%, respectively, compared with the control treatment. The NirS clones belonging to cluster IV related to *Rhodocyclaceae* were detected in the control treatment but not in the EC50 treatment. A notable change

Fig. 6. Neighbor-joining tree based on NirS nucleic acid sequences. Clones with >95% sequence similarity were considered to be the same OTU. Sequences with the names starting with CK-nirS and EC50-nirS were obtained from the control treatment and EC50 treatment, respectively. NirS sequences of the reference known denitrifying bacteria are indicated by each accession number in parentheses. Bootstrap values (%) were generated from 1000 replicates, and the values > 50% are shown. A number in parentheses represents the total number of clones in each OTU.

was that the proportion of the clones belonging to cluster I (unknown branch) increased 25.1% in the EC50 treatment. In addition, the proportion of the NirS clones related to *Pseudomonadaceae* slightly increased with the addition of pyrene.

In the EC50 treatment, the proportion of NosZ clones belonging to the cluster I decreased by 4.29%, while those belonging to cluster IV related to *Rhodospirillaceae* increased by 2.00%, and those belonging to V and VII affiliated to *Burkholderiales* increased by 2.95%, compared to the control treatment, respectively. The NosZ clones in cluster III related to *Bradyrhizobiaceae* which were detected in the control treatment but not in the EC50 treatment.

4. Discussion

4.1. Potential denitrification activity and dynamic of N_2O

Our results showed that PDA generally decreased with increasing pyrene concentrations. This was consistent with the results obtained by Contreras-Ramos et al. (2009) that the addition of PAHs to the sludge-amended soil reduced the N_2O emission rate. Maliszewska-Kordybach et al. (2007) tested EC₅₀ values of phenanthrene for nitrification potential in 50 different soils and found that the EC₅₀ values were within the range of 146–1670 mg kg⁻¹. In our study, the mean EC₅₀ value of pyrene for PDA was lower than this range. The lower EC₅₀ of pyrene than phenanthrene could be mostly attributed to the fact that the toxicity of phenanthrene with three benzene rings was lower than that of pyrene with four benzene rings and that phenanthrene was easier to be degraded than pyrene.

In the time-course experiment, the N_2O accumulation of the EC50 treatment was higher than that of the control treatment. Holtan-Hartwig et al. (2002) also found that N_2O accumulated to a higher level in the heavy metal-spiked soil than in the non-spiked soil. This was likely because N_2O reductase was inhibited more readily by pyrene than NO reductase and the selective inhibition resulted in the accumulation of N_2O (Holtan-Hartwig et al., 2002). The N_2O accumulation and N_2O reduction in the EC50 treatment gradually diminished, and was close to the level of the control treatment during the 7-day incubation. Meanwhile, pyrene concentration of the EC50 treatment declined during the 7-day incubation. Previous studies showed that PAHs could be degraded under denitrifying conditions by using nitrate as electron acceptors, a mechanism named co-metabolism (Ambrosoli et al., 2005; Coates et al., 1997; McNally et al., 1998; Rockne and Strand, 2001; Rockne et al., 2000). Pollutant degradation and transformation to more volatile or less toxic forms were commonly used strategies for bacteria to reduce the toxicity of their immediate surroundings (Ford, 1993). In our study, most of denitrifiers were closely related to the orders of *Rhizobiales*, *Rhodobacterales*, *Burkholderiales* and *Pseudomonadales* through phylogenetic analyses, and it has been reported that some species in these orders (Keum et al., 2006; Johnson et al., 2004; Zhang et al., 2004; Guo et al., 2008; Balashova et al., 1999), and a great number of other bacterial and fungal species in soil (Haritash and Kaushik, 2009) could degrade PAHs through co-metabolism. Degradation as well as aging of PAHs could alleviate stress and promote the recovery of denitrification enzyme activity (Maliszewska-Kordybach, 2005).

Fig. 7. Neighbor-joining tree based on NosZ nucleic acid sequences. Clones with >95% sequence similarity were considered to be the same OTU. Sequences with the names starting with CK-nosZ and EC50-nosZ were obtained from the control treatment and EC50 treatment, respectively. NosZ sequences of the reference known denitrifying bacteria are indicated by each accession number in parentheses. Bootstrap values (%) were generated from 1000 replicates, and the values > 50% are shown. A number in parentheses represents the total number of clones in each OTU.

4.2. Abundance of the denitrifying community

Our results showed that the copy numbers of the *nirK* gene were around 10^7 , while those of *nirS* and *nosZ* genes were as high as 10^8 , indicating that *nirS* and *nosZ* genes were dominant in the present agricultural soil. Some studies, however, showed much lower copy numbers (10^6) for the *nirS* gene and similar copy numbers for the *nirK* gene (10^7) in paddy soil (Yoshida et al., 2009, 2010). The niche differentiation or abundance difference in the *nirS*- and *nirK*-type denitrifiers depended on the habitat selection (Enwall et al., 2010) and nutrient concentration. The *nirS* population was more adapted to soils with comparatively high concentrations of organic nutrients (Cole et al., 2004) and NO_3^- -N (Bárta et al., 2010). Due to fertilization, the present soil was high in organic carbon (19.9 g kg^{-1}) and in NO_3^- -N (384 mg kg^{-1}), and high soil fertility might be responsible for the high copy numbers of the *nirS* gene.

Our study clearly showed that PDA was positively related to the copy numbers of *nirK*, *nirS* and *nosZ* genes, and high concentration of pyrene addition decreased both PDA and the abundance of denitrifiers harboring the three genes. Previous studies showed that denitrification enzyme activity was correlated with the copy numbers of *nirK* gene (Throbäck et al., 2007) and *nosZ* gene (Hallin et al., 2009), suggesting that denitrifiers harboring these functional genes played an important role in the denitrification process, and

Fig. 8. Temporal shifts in *NirK*, *NirS* and *NosZ* clone distributions. *NirK*, *NirS* and *NosZ* clones were grouped into four, six and seven clusters, respectively.

the abundance of the denitrifying community could predict the corresponding process (Hallin et al., 2009).

The mean EC₅₀ values of pyrene for copy numbers of *nirK*, *nirS* and *nosZ* genes were lower than that of pyrene for *PDA*, indicating that denitrifiers harboring *nirK*, *nirS* or *nosZ* gene was more sensitive to pyrene than *PDA*, and denitrifiers harboring *nirS* gene was more sensitive to pyrene than that harboring *nirK* or *nosZ* gene. Previous studies suggested that soil microorganisms, being in intimate contact with the soil environment, could be considered as the best indicators of soil pollution because they might be responsive to contaminants and react rapidly to soil perturbation (Maliszewska-Kordybach et al., 2007). Considering that *nirK* and *nirS* genes carry out nitric oxide reduction process whereas *nosZ* gene implements nitrous oxide reduction, our study further demonstrated that microbial functions and abundance of microorganisms carrying out different denitrification processes were different in sensitivity to pyrene contamination.

4.3. Shifts in diversity and composition of the denitrifying community

In our study, both Shannon index and Simpson index of *nirK*, *nirS* and *nosZ* genes decreased with the addition of pyrene, indicating that pyrene reduced denitrifiers diversity. It has been reported that the addition of heavy metals or organic pollutants could decrease microbial diversity (Shannon index and richness) (Bamborough and Cummings, 2009; Kozdrój and van Elsas, 2001). The two diversity indices for *nirK* gene in our study were similar to those reported in forest soils and a paddy soil (Katuyama et al., 2008; Yoshida et al., 2009), and those of *nosZ* gene were similar to those reported in meadow and turfgrass soil (Dell et al., 2010; Stres et al., 2004; Zhang et al., 2006). However, the diversity indices for *nirS* gene in our study were lower than those in a flooded paddy field reported by Yoshida et al. (2009). There are substantial evidences that the diversity and composition of soil bacterial communities can be influenced by a wide range of biotic and abiotic factors including vegetation, trophic status, salinity, soil pH and heavy metals (McArthur et al., 1988; Lefranc et al., 2005; Lozupone and Knight, 2007; Fierer and Jackson, 2006; Gans et al., 2005).

Based on the *nirK* clone library analysis, the majority of clones from clusters I, II and III were related to *Rhizobiales* (*Rhizobiaceae*, *Bradyrhizobiaceae* and *Phyllobacteriaceae*), and few clones in cluster IV were related to *Burkholderiales* (*Alcaligenaceae*), similar to the previous studies (Bremer et al., 2007; Saito et al., 2008; Yoshida et al., 2009). However, this did not mean that these *nirK*-harboring denitrifiers belonged to *Rhizobiales* or *Burkholderiales*, since the *nirK* phylogeny was incompatible with the 16S rDNA phylogeny (Heylen et al., 2006; Jones et al., 2008; Philippot et al., 2002; Yoshida et al., 2009). Our results showed that over 80% of *NirS* clones distributed in clusters I and VI (unknown branch), cluster II (*Rhodobacterales*). The *NirS* clones in clusters I and VI were distantly related to known denitrifiers, and some of our clones in the two clusters were dissimilar to the uncultured *NirS* clone sequences (e.g. AM419603.1 and EF615461.1), indicating that they were unique to our soil. However, Saito et al. (2008) reported that *NirS* clones related to *Burkholderiales* and *Rhodocyclales* dominated in the rice paddy soil (Saito et al., 2008). A possible reason was that plant communities or the rhizosphere and soil factors could affect the structure and activity of the denitrifying community (Kowalchuk et al., 2002; Philippot et al., 2002). Based on the *nosZ* clone library analysis, the overwhelming majority of clones (cluster I–III, and cluster V–VII) were related to *Rhizobiales* (*Brucellaceae*, *Rhizobiaceae* and *Bradyrhizobiaceae*) and *Burkholderiales* (*Alcaligenaceae*, *Burkholderiaceae* and *Oxalobacteraceae*) and only few clones

in cluster IV were related to *Rhodospirillales* (*Rhodospirillaceae*), and the *NosZ*-based denitrifiers composition from our studied soil was similar to that from other farmland soils (Enwall et al., 2005; Horn et al., 2006).

Our study clearly demonstrated the temporal shifts in community composition of the *nirS*-, *nirK*- and *nosZ*-harboring denitrifiers in soil after the addition of pyrene. Previous studies also showed that ammonium, fertilization, plant species, sampling time, water logging and temperature all temporarily altered the structure of *nirK*-harboring denitrifying community (Avrahami et al., 2002; Braker et al., 2010; Bremer et al., 2007; Wolsing and Priemé, 2004; Yoshida et al., 2009). The genetic shifts of the denitrifying community due to PAHs perturbation could be attributed to the inhibition of sensitive and the development of tolerant species (Leahy and Colwell, 1990; Ford, 1993; Whittaker, 1975). In our study, the development of tolerant species could be reflected by the higher proportion of *Bradyrhizobiaceae*, *Rhodospirillales*, *Burkholderiales* and *Pseudomonadales* in the EC₅₀ treatment, and it has been reported that *Afipia broomeae*, *Burkholderia* sp. DBT1, *Achromobacter* sp. NCW, *Thalassospira xianhensis* sp. nov. and *Pseudomonas putida* belonging to the above four groups were isolated from contaminated soils, wastewater, sludge and marine, and these species were able to degrade PAHs and other hydrocarbons (Bodour et al., 2003; Guo et al., 2008; Zhao et al., 2010; Balashova et al., 1999).

5. Conclusion

Our results clearly showed that pyrene reduced denitrification activity as well as the abundance, diversity and composition of the denitrifying community. The abundance of the denitrifying community harboring *nirK*, *nirS* or *nosZ* gene is more sensitive to pyrene than potential denitrification activity. Our study further demonstrated that the abundance of denitrifiers carrying out different processes were different in sensitivity to pyrene. Pyrene addition increased the proportions of four groups including *Bradyrhizobiaceae*, *Rhodospirillales*, *Burkholderiales* and *Pseudomonadales*, with each containing some species that can degrade PAHs.

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