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Anaerobic Biodegradation of Pyrene by *Paracoccus denitrificans* Under Various Nitrate/Nitrite-Reducing Conditions

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Abstract As a polycyclic aromatic hydrocarbon (PAH), pyrene is one of hazardous persistent organic pollutants in the aquatic environment. The aim of this study was to investigate the influence of denitrifying conditions on pyrene degradation in a pure culture. With a strain isolated from petrol-contaminated river sediment, treatments of pyrene biodegradations were set up using various ratios of nitrate to nitrite (NO_3^-/NO_2^-). Results showed that various NO_3^-/NO_2^- conditions significantly influenced the anaerobic pyrene degradation efficiency. Nitrite could induce the complete denitrification process so that NO_2^- acted as a key factor to promote high degradation efficiency. The low N treatment of NO_3^- and NO_2^- concentrations made the denitrifying-pyrene-degradation process more effective. Additionally, high C/N value stimulated high degradation rates. High concentrations of NO_3^- and NO_2^- as well as toxic intermediate product accumulation might inhibit the bacterial growth and biodegradation process. The information from this study should be useful to design bioremediation strategies of PAH.

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1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) discharged from various activities, such as shipping, petroleum spills, and surface runoff, are persistently hazardous organic pollutants to the aquatic environment (Zakaria et al. 2002). Because of their low aqueous solubility, low volatility, and high affinity for solid particles, PAHs have always been found in sediments, particularly near urban and industrialized regions (Gonul and Kucuksezgin 2012; Mirza et al. 2012). Consumption of seafood collected from PAH-contaminated sites may affect human health adversely (Moon et al. 2010). As the result of their toxicity, carcinogenicity, and mutagenic characteristics, several members of PAHs have been placed on the priority list of pollutant in many countries (Wild and Jones 1995; Kuang et al. 2011).

Biodegradation is a viable, ecological-friendly, and inexpensive technology for both ex situ and in situ remediation of PAH-contaminated sites under anaerobic and aerobic conditions (Venkata Mohan et al. 2006; Perelo 2010). In the anaerobic bioremediation process, microbes obtain energy by transferring electrons from organic matter to alternative electron acceptors, such as NO_3^-, ferric ion, and sulfate. Among these alternative electron acceptors, NO_3^- has the

highest efficiency of energy yield during reduction, which is pretty common in the freshwater environment (Burgin and Hamilton 2007). Several studies have identified that NO_3^- can be used by bacteria to biodegrade PAHs. Mihelcic and Luthy (1988) first showed that low molecular weight PAHs were degraded under denitrifying conditions. Then, their coworkers demonstrated that three- and four-ring PAHs could also be degraded by denitrifiers and the anaerobic degradation rates might be comparable with aerobic degradation rates when cell densities were similar (McNally et al. 1998). Chang et al. (2001, 2002, 2003, 2008) reported that PAH degradation rates under a nitrate-reducing condition were greater than those under sulfate-reducing and methanogenic conditions in river sediments, while the opposite results were obtained in soil, sludge, and mangrove sediment. Effects of substrate interactions on PAH degradation are also observed under nitrate-reducing conditions. PAH degradation can be stimulated by other PAHs (McNally et al. 1999) and different electron donors (Chang et al. 2008). In addition, impact factors on PAH degradation, such as pH, temperature, and NO_3^- and PAH concentrations, have been studied (Lu et al. 2011). In the process of NO_3^- as electron acceptor, NO_3^- is reduced to NO_2^-. The produced NO_2^- becomes a potential electron acceptor, whereas it can also be an inhibitor of respiratory oxidase activity (Rothery et al. 1987). However, less attention has been paid to PAH biodegradation using both NO_3^- and NO_2^- as electron acceptors.

The ratio of carbon to nitrogen sources (C/N) is another important factor for the denitrifying process. Her and Huang (1995) reported that reaching complete denitrification using the aromatic carbon source required a higher C/N than that using nonaromatic carbon source. Dou et al. (2009) showed that higher aromatic carbon sources led to higher degradation rates with NO_3^- as electron acceptor. Nevertheless, no information is available about the influence of C/N on PAH biodegradation with NO_3^- and NO_2^- as electron acceptors.

The objective of this study was to investigate the effects of various NO_3^-/NO_2^- on nitrate-reducing degradation of PAH (pyrene) using a pure culture isolated from PAH-contaminated river sediments. The use of pure culture was to better understand the microorganism responsible for anaerobic PAH biodegradation (Rockne et al. 2000). The C/N was also considered as an important impact factor on pyrene degradation coupling with denitrification.

2 Materials and Methods

2.1 Organism Isolation and Identification

The pure culture, strain M-1, was isolated from sediments collected from the Huadi join within Pearl River, Guangzhou, China, where there was a high level of PAHs and an active denitrifier population (Huang et al. 2011). The culture was enriched as a pyrene-degrading NO_3^- reducer. The enrichment was initiated at 28 °C in an anaerobic mineral medium (Dou et al. 2009) with 30 mg L^{-1} pyrene (Sigma-Aldrich, Shanghai, China) as the electron donor and 2.0 g L^{-1} $NaNO_3$ as the electron acceptor. After 2 weeks, the culture was transferred (10 %*v*/*v*) into a fresh medium amended with pyrene (30 mg L^{-1}) and $NaNO_3$ (2.0 g L^{-1}). After seven times of transfers (one time per week) in the medium, the enriched culture was inoculated into anaerobic agar dilution series of the medium amended with 2 % noble agar. Colonies of strain M-1 were isolated after 1 week of the incubation. In order to prepare the pyrene-containing medium (for enrichment and isolation degrading strain, and following degradation test), pyrene was added to the serum bottles and plates as concentrated pyrene solution in acetone. Once the acetone/pyrene solution was added to the serum bottles, the bottles were shaken making the pyrene distribute evenly around the bottom and sides of the bottles. The acetone was allowed to evaporate completely in sterile environment, leaving pyrene coated onto the insides of the bottles. Then, a mineral medium was added to prepare the pyrene suspensions. For agarose plates, the acetone/pyrene mixture was spread using a sterile coating rod and the acetone was also allowed to evaporate completely before use. Pyrene evenly crystallized on the surface of the agarose and did not interfere with subsequent plate spreading.

Total DNA was extracted from strain M-1 with Fast DNA spin kit (Bio 101, Qbiogene Inc., CA, USA) as described in the manufacturer's instructions. The following primer sets were used for PCR amplification: forward primer BSF8/20 (5′- AGAGTTTGATCCT GGCTCAG −3′) and the reverse primer BSR1541/20 (5′- GGTTACCTTGTTACGACTT −3′). The PCR

mixture contained 50 ng of extracted DNA, 1 μL of 5 mM concentrations of each primer, 25 μL of PCR mix (TaKaRa Bio Inc., Shiga, Japan), then replenish with ddH_2O to 50 μL. The PCR amplification was performed in a total volume of 50 μL in 0.2 mL reaction tubes using PCR reactor (T-gradient, Biometra, USA) as follows: 5 min at 94 °C, 30 cycles (30 s at 94 °C, 30 s at 55 °C, 45 s at 72 °C), and 7 min at 72 °C. The presence of PCR products was confirmed by 1 % agarose gel electrophoresis then sequenced. A comparison of the sequence to those in GenBank using the Blast alignment tool showed that strain M-1 (GenBank ID: JX295970) was closely related to *Paracoccus denitrificans* PD1222 (100 % 16S rRNA sequence similarity) phylogenetically and was a member of the *Paracoccus* in the α-subclass of the *Proteobacteria*. An important characteristic of *P. denitrificans* is its ability to reduce NO_3^- to N_2 single-handedly under anaerobic growth conditions (Baker et al. 1998). *P. denitrificans* is a metabolically versatile strain, which is able to obtain energy both from degrading organic compounds and from converting inorganic compounds chemolithotrophically, such as sulfur oxidation (Baker et al. 1998).

Table 1 Treatments for pyrene biodegradation in denitrifying condition

Treatment	Pyrene (mg L^{-1})	NO_3^- (mmol L^{-1})	NO_2^- (mmol L^{-1})	Inoculum
A1	30	2	2	+
A2	30	5	2	+
A3	30	8	2	+
B1	30	2	5	+
B2	30	5	5	+
B3	30	8	5	+
C1	30	2	8	+
C2	30	5	8	+
C3	30	8	8	+
CK1	30	0	0	+
CK2	30	5	5	–
CK3	30	5	0	+
CK4	30	0	5	+
P1	10	10	0	+
P2	20	10	0	+
P4	40	10	0	+
P5	50	10	0	+

2.2 Experimental Setup

To investigate the influence of denitrifying conditions on pyrene degradation in solution, treatments included different initial NO_3^- and NO_2^- concentrations with a fixed pyrene initial concentration (30 mg L^{-1}) (Table 1). Specifically, treatments A1, A2, and A3 were amended by 2, 5, and 8 mmol L^{-1} NO_3^- with 2 mmol L^{-1} NO_2^-, respectively. Treatments B1, B2, and B3 were amended by 2, 5, and 8 mmol L^{-1} NO_3^- with 5 mmol L^{-1} NO_2^-, respectively. Treatments C1, C2, and C3 were amended by 2, 5, and 8 mmol L^{-1} NO_3^- with 8 mmol L^{-1} NO_2^-, respectively. Four control treatments were N free (CK1), inoculum free (CK2), NO_3^- amended only (CK3), and NO_2^- amended only (CK4). The treatment groups of A, B, C, and CK were operated for 12 days. Additionally, treatments P1, P2, P4, and P5 were amended with initial pyrene concentrations of 10, 20, 40, and 50 mg L^{-1}, respectively, and with sufficient NO_3^- (10 mmol L^{-1}), and the treatments were operated for 25 days. The culturing experiments were preformed in 40-mL serum vials amended with 20 mL solution under a 20-mL headspace in an incubator (28 °C) darkly. The inoculum size was 10 %*v*/*v* of anaerobically grown cells (final concentration, 3.9×10^5 cells mL^{-1}). Experiments of each treatment were performed in triplicate.

2.3 Chemical Analysis and Viable Counts

Pyrene concentrations were extracted and determined according to the methods of US EPA 3550C-2007 and US EPA 8270D-2007 using a gas chromatograph/mass spectrometer detector (Shimadzu GC/MS QP 2010). The injection and detector port temperatures were 270 and 200 °C, respectively, and the oven temperature was ramped at 40 °C min^{-1} from 150 to 300 °C. The detection limit for pyrene was 0.002 mg L^{-1}. The most possible intermediate products and its degradation character were estimated by the software bundled in GC/MS (GCMS solution 2.50) and EPI suite 4.1 (US EPA). Concentrations of NO_3^- and NO_2^- were measured with ion chromatography (Metrohm 882, Metrohm AG, Herisau, Switzerland). The pH values were determined by pH meter (Mettler Toledo FE20). Nitrous oxide (N_2O) and carbon dioxide (CO_2) concentrations of the headspace were measured by a gas chromatography (Shimadzu GC 2014, Japan) equipped with a flame ionization detector and a stainless steel column

packed with Porapak-Q. The column and detector temperatures were 70 and 370 °C, respectively. A 5-mL sample of the headspace gas was withdrawn from each serum vial using a gastight syringe and manually injected into the GC. The upper detection limit of CO_2 in the system was 1 % (*v*/*v*) of injected gas (about 8.2 μmol). 4′,6-Diamidino-2-phenylindole (DAPI) direct count was used to measure viable counts in culturing. DAPI was added to a final concentration of 10 μg mL^{-1} and incubated for 30 min at 24 °C. A subsample (2 mL) was filtered on a 0.2-μm black polycarbonate filter (Nucleopore, Whatman, USA) and dried for 5 min. The DAPI-stained cells were visualized in ultraviolet light using epifluorescence microscopy (OPTEC BK-FL). At least 20 random fields were enumerated for each sample. Counting was only on bacteria with clearly blue light.

2.4 Reaction Stoichiometry

The stoichiometry of electron transformation was determined from the ratio of electrons consumed from NO_3^- reduction and NO_2^- reduction to electrons produced from pyrene degradation at the end of the experiment. The theoretical stoichiometry was calculated assuming complete oxidation of the pyrene to CO_2 with NO_3^- and NO_2^- reduction, as shown in the following half-reaction equations:

$$C_{16}H_{10} + 32H_2O \rightarrow 16CO_2 + 74H^+ + 74e^- \quad (1)$$

$$NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O \quad (2)$$

$$2NO_2^- + 6H^+ + 4e^- \rightarrow N_2O + 3H_2O \quad (3)$$

$$N_2O + 2H^+ + 2e^- \rightarrow N_2 + H_2O \quad (4)$$

3 Results

3.1 Degradation Efficiencies in Various Treatments

Pyrene degradation efficiencies in the various treatments are shown in Fig. 1. Only 2.0 % degradation efficiency was observed in the inoculum-free treatment (CK2). With inoculation but without NO_3^- and NO_2^- as electron acceptors, the degradation efficiency of CK1 was also low (4.0 %). On the contrary, degradation efficiencies of treatments of CK3 and CK4, which were inoculated and amended with NO_3^- or NO_2^-, were 54 and 51 %, respectively. For the various NO_3^-/NO_2^- treatments (A, B, and C groups), degradation efficiencies of the low N amended group were higher (group A, 75 % in average) than those of the higher N amended groups (47 and 35 % for groups B and C, respectively). In the low N amended group (A), lower concentration of NO_3^- resulted in the higher degradation efficiency. Comparing with NO_3^- only treatment (CK3), the NO_3^-+NO_2^- treatment (A2) reached higher degradation efficiency (Fig. 1).

For treatments P1 to P5, degradation efficiencies decreased from 86 to 52 % with the initial pyrene concentrations from 10 to 50 mg L^{-1} (Fig. 1). If the degradation process was divided into two periods, during the first period (days 0–10), the degradation rates and degradation efficiencies of the higher pyrene treatments (P4 and P5) were higher than those of the low pyrene treatments (P1 and P2) (Table 2). However, in the second period (days 11–25), the degradation rates of the high pyrene treatments sharply decreased and little of pyrene was degraded (5.6 and 0.56 %), while the low pyrene treatments kept significant degradation rates and degradation efficiencies.

The amount of CO_2 produced within the initial 5 to 6 days of incubation was over 8.2 μmol, accounting for 20 % of the initial carbon amendment. The intermediate product was estimated to be 2,2′-methylenebis[6-(1,1-dimethylethyl)-4-methyl-phenol (CAS number 119-47-1), which was not biodegradable in the anaerobic condition.

3.2 Nitrogen Variation and Electron Transformation in Pyrene Degradation

Table 3 lists the amounts of NO_3^- reduction, NO_2^- reduction, N_2O accumulation, electrons produced by pyrene consumption, and electrons consumed by NO_3^- and NO_2^- reductions of treatments groups A, B, C and CK at the end of incubation (12 days). The amount of NO_3^- reduction in CK3 was 86.7 μmol (72.6 %).

The amount of NO_2^- reduction of CK4 was 30.5 μmol (29.6 %), while CK3 resulted in a large

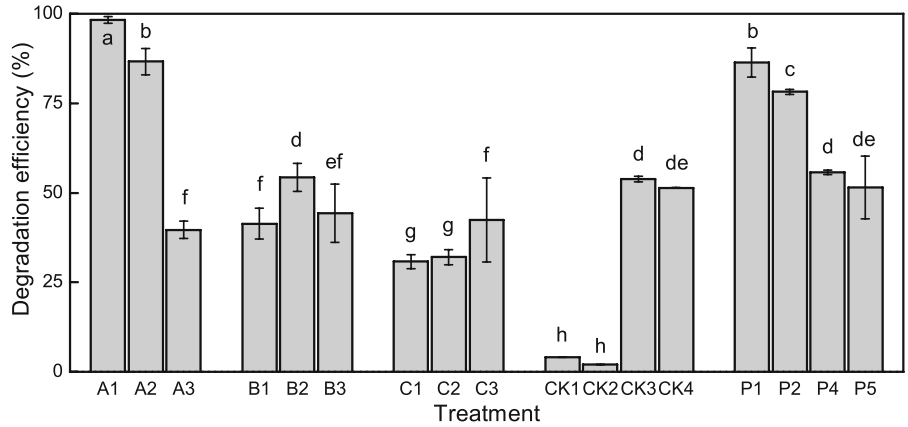

Fig. 1 Pyrene degradation efficiencies of various treatments at the end of incubation. Treatment groups A, B, C, and CK were amended with 30 mg L^{-1} of pyrene and an incubation period of 12 days. *A1*, *A2*, and *A3* represent treatments with 2, 5, and 8 mmol L^{-1} of NO_3^-, respectively, and 2 mmol L^{-1} of NO_2^-; *B1*, *B2*, and *B3* represent treatments with 2, 5, and 8 mmol L^{-1} of NO_3^-, respectively, and 5 mmol L^{-1} of NO_2^-; *C1*, *C2*, and *C3* represent treatments with 2, 5, and 8 mmol L^{-1} of NO_3^-, respectively, and 8 mmol L^{-1} of NO_2^-; *CK1* represents N-free treatment; *CK2* represents treatment with 5 mmol L^{-1} of NO_3^- and 5 mmol L^{-1} of NO_2^- but no inoculums; and *CK3* and *CK4* represent treatments with 5 mmol L^{-1} of NO_3^- and 5 mmol L^{-1} of NO_2^-, respectively. Treatment group P was amended with 10 mmol L^{-1} of NO_3^- and an incubation period of 25 days. *P1*, *P2*, *P4*, and *P5* represent treatments with 10, 20, 40, and 50 mg L^{-1} of pyrene, respectively

amount of NO_2^- accumulation (67.9 %). The treatments with relative higher NO_3^- (A3 and B3) resulted in NO_2^- accumulation.

The amount of N_2O accumulation was quite small compared to NO_3^- and NO_2^- reductions. The amount of N_2O accumulation of the treatment with NO_3^- as the single electron acceptor (CK3) was much higher than that of the treatment with NO_2^- as the single electron acceptor (CK4).

According to Eqs. 1, 2, 3 and 4, the amount of electron transformation during the pyrene degradation and N reduction was calculated (Table 3). For treatment groups A, B, and C, the ratios of electrons consumed to electrons produced were greater than 1.0, while the higher N concentration treatments resulted in higher ratios with the correlation coefficient (R)=0.791 (p<0.05). The ratios of treatments with NO_3^- as the single electron acceptor (CK3) and with NO_2^- as the single electron acceptor (CK4) were 3.26 and 0.89, respectively.

3.3 Viable Counts and pH Variation During Pyrene Degradation

In treatment groups A, B, and C, pH values decreased from 7.6 to 6.9 approximately during the 12-day operation period. Viable counts in all treatments increased from the inoculum concentration (3.9×10^5 cells mL^{-1}) to about 10^7 cells mL^{-1} in day 6 then dramatically decreased in day 12 (Fig. 2). In addition, the maximum viable counts in day 6 decreased with the increase of NO_3^- (groups A to C) or NO_2^- (groups 1 to 3). The correlation coefficient between initial N (NO_3^-+ NO_2^-) concentrations and maximum viable counts in day 6 was −0.814 (p<0.01).

Table 2 Degradation rates in treatments with different pyrene concentration and 10 mmol L^{-1} of NO_3^-

Treatments	Degradation rates (μmol L^{-1} day^{-1})		Degradation efficiency (%)	
	Days 0–10	Days 11–25	Days 0–10	Days 11–25
P1[a]	1.22	1.63	28.8	57.6
P2	3.58	2.05	42.1	36.1
P4	8.53	0.63	50.1	5.58
P5	10.8	0.08	50.9	0.56

[a]P1, P2, P4, and P5 represent the treatments with 10, 20, 40, and 50 mg L^{-1} of pyrene, respectively

Table 3 The amounts of NO_3^- reduction, NO_2^- reduction, N_2O accumulation, electrons produced by pyrene consumption, and electrons consumed by NO_3^- and NO_2^- reductions of different treatments (all amended with 30 mg L^{-1} of pyrene) at the end of incubation (12 days)

Treatment	Pyrene consumed (μmol)	E-produced (μmol)	NO_3^- reduction (μmol)	NO_2^- reduction (μmol)	N_2O accumulation (μmol)	E-consumed (μmol)	Ratio of E-consumed to E-produced
A1	2.44	181	34.5 (86.3 %)	15.0 (49.3 %)	0.001	218	1.20
A2	2.48	184	36.4 (45.1 %)	7.8 (27.4 %)	0.017	197	1.08
A3	1.19	88.0	33.4 (24.4 %)	−1.55 (−2.45 %)	0.047	163	1.86
B1	0.98	72.9	21.7 (66.3 %)	16.3 (20.0 %)	0.004	157	2.16
B2	1.66	123	58.5 (53.1 %)	43.3 (39.5 %)	0.003	422	3.44
B3	1.04	77.2	24.4 (15.2 %)	−2.20 (−2.22 %)	0.021	117	1.52
C1	0.72	53.6	15.8 (32.9 %)	39.1 (23.0 %)	0.018	196	3.66
C2	0.76	56.2	44.0 (36.0 %)	56.1 (29.7 %)	0.124	387	6.90
C3	1.68	124	84.7 (45.7 %)	86.6 (50.1 %)	0.001	683	5.50
CK3	1.23	91.1	86.7 (72.6 %)	−67.9 (−)	0.172	296	3.26.
CK4	1.54	114	1.96 (−)	30.5 (29.6 %)	0.002	101	0.88

E-produced represents electrons produced by pyrene consumption; E-consumed represents electrons consumed by NO_3^- or NO_2^- reduction. A1, A2, and A3 represent treatments with 2, 5, and 8 mmol L^{-1} of NO_3^-, respectively, and 2 mmol L^{-1} of NO_2^-; B1, B2, and B3 represent treatments with 2, 5, and 8 mmol L^{-1} of NO_3^-, respectively, and 5 mmol L^{-1} of NO_2^-; C1, C2, and C3 represent treatments with 2, 5, and 8 mmol L^{-1} of NO_3^-, respectively, and 8 mmol L^{-1} of NO_2^-; and CK3 and CK4 represent treatments with 5 mmol L^{-1} of NO_3^- and 5 mmol L^{-1} of NO_2^-, respectively. The negative values indicate NO_2^- accumulation

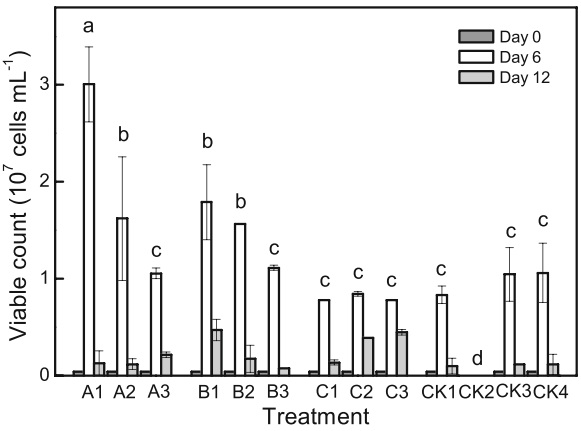

Fig. 2 Viable counts of various treatments during pyrene degradation. The treatments were amended with 30 mg L^{-1} of pyrene and an incubation period of 12 days. *A1*, *A2*, and *A3* represent treatments with 2, 5, and 8 mmol L^{-1} of NO_3^-, respectively, and 2 mmol L^{-1} of NO_2^-; *B1*, *B2*, and *B3* represent treatments with 2, 5, and 8 mmol L^{-1} of NO_3^-, respectively, and 5 mmol L^{-1} of NO_2^-; *C1*, *C2*, and *C3* represent treatments with 2, 5, and 8 mmol L^{-1} of NO_3^-, respectively, and 8 mmol L^{-1} of NO_2^-; *CK1* represents N-free treatment; *CK2* represents treatment with 5 mmol L^{-1} of NO_3^- and 5 mmol L^{-1} of NO_2^- but no inoculums; and *CK3* and *CK4* represent treatments with 5 mmol L^{-1} of NO_3^- and 5 mmol L^{-1} of NO_2^-, respectively

4 Discussion

4.1 Influence of Denitrifying on Pyrene Degradation Efficiency

Results from the control treatments (CK1, CK2, CK3, and CK4) clearly showed that strain M-1 could degrade pyrene through the denitrifying process (Fig. 1). Generally, in the denitrification process, the activities of denitrification enzymes can vary among individual organisms in different conditions (Zumft 1997). In this study, the obvious NO_2^- accumulation in treatment CK3 (using NO_3^- as sole electron acceptor) indicated that the bacterial reduction of NO_3^- to NO_2^- in the beginning stage was dominant compared to NO_2^- reduction. Similar results were observed from the anaerobic degradation of ethylbenzene by denitrifying bacteria that NO_2^- first accumulated then reduced (Rabus and Widdel 1995). Dou et al. (2009) also reported that NO_2^- accumulated during naphthalene degradation under a NO_3^- reduction condition. These results might attribute that the high NO_3^- reductase activity (induced by high NO_3^- concentrations in vitro) limited the NO_2^- reductase so that growing cells excreted NO_2^- (Carlson and Ingraham 1983). More

evidences of NO_2^- accumulation could be observed from the treatments with relatively high NO_3^- concentrations (A3 and B3) (Table 3). Nevertheless, strain M-1 still could use NO_2^- as a respiratory electron acceptor to degrade pyrene (CK4, Table 3). There was no NO_2^- accumulation in most of the treatments added with NO_2^- initially, except for the treatments of relative high NO_3^- added (A3 and B3) (Table 3). Nitrite can act as a strong inducer of NO_2^- reductase (Körner and Zumft 1989) and subsequent nitric oxide and N_2O reductases (Zumft 1997). Little N_2O accumulation in the treatments with relatively high NO_2^- concentrations was also the evidence (Table 3).

Similar degradation efficiencies were obtained from treatments CK3 and CK4 (Fig. 1), while the electrons consumed by NO_3^- reduction (CK3, 86.7 μmol) were higher than by NO_2^- reduction (CK4, 30.5 μmol) (Table 3). Actually, each electron consumed by the N-oxides reduction should yield equal amount of energy (Koike and Hattori 1975). However, with a relatively high NO_3^- concentration, the denitrification process is inhibited before the NO_2^- reduction step, in which 2 mol electrons are transferred by 1 mol NO_3^- reduced to NO_2^- (Eq. 2). In the condition of NO_2^- amended, reductases for NO_2^- reduction and the subsequent denitrifying steps were induced before high NO_3^- reductase activity was present. This nitrite-inducement lead to the complete denitrification process, in which 3 mol electrons are transferred by 1 mol NO_2^- reduced to N_2 (Eqs. 3 and 4). Therefore, NO_2^- treatments can effectively increase the PAH degradation efficiency through the inducing of complete reduction from nitrite to molecular nitrogen.

Nevertheless, high NO_2^- concentrations should decrease the bacterial activity (Rothery et al. 1987). With initially higher NO_2^- concentrations, the degradation efficiencies of treatment groups B and C were lower than those of group A. On the other hand, NO_3^- reduction rates are always faster than NO_2^- reduction rates (Almeida et al. 1995; Sung et al. 2002). In the condition of relatively high NO_3^- concentrations added, NO_2^- reduction would be limited (Carlson and Ingraham 1983) and even lead to NO_2^- accumulation (A3 and B3) (Table 3). As the results, with low level NO_2^- treatment in group A, the degradation efficiency decreased with the increase of initial NO_3^- concentrations (Fig. 1).

4.2 Influence of C/N on Pyrene Degradation

The C/N is an important factor for pyrene degradation because microorganisms require appropriate amount of carbon and nitrogen sources as nutrients for cellular metabolism and successful growth (Bamforth and Singleton 2005). Actually, at the beginning (from 0 to 10 days), the degradation rates in the high pyrene treatments (P4 and P5) were higher than those in the low pyrene treatments (P1 and P2) (Table 2). The low degradation efficiencies in P4 and P5 were attributable to the toxic intermediate product accumulation, which inhibited the degradation rates in the later period (days 11 to 25). The viable counts in all the treatments dramatically decreased in day 12 (Fig. 2), which suggested that certain inhibitor might be generated during the anaerobic degradation. Previous studies demonstrated that some breakdown metabolites, such as oxy-PAHs, PAH ketones, quinines, and coumarins, were more toxic and persistent to microbes than their corresponding parents (Bamforth and Singleton 2005). In this study, the most probable intermediate product was 2,2′-methylenebis[6-(1,1-dimethylethyl)-4-methyl-phenol, which might be toxic to organisms or inhibitory to organisms activities. As the result, higher initial pyrene concentrations led to more toxic intermediate product generation that the degradation rates dropped to nearly 0 μmol L^{-1} day^{-1} in P4 and P5 treatments after day 10 (Table 2). On the contrary, in the low pyrene treatments (P1 and P2), the degradation rates kept to be significant during the incubation period and eventually high degradation efficiencies were obtained at the end of experiments (Table 2, Fig. 1). Consequently, a phenomenon

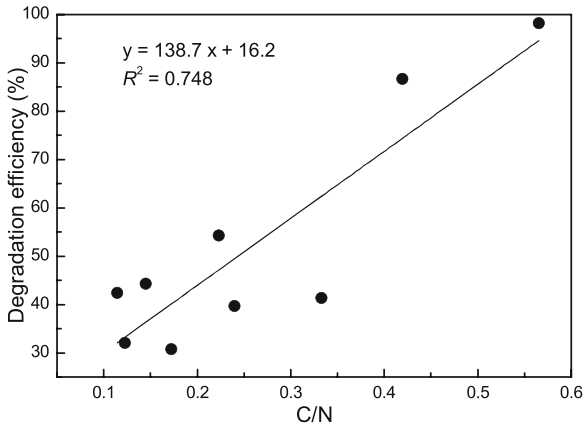

Fig. 3 Relationship between pyrene degradation efficiency and C/N values

that the degradation efficiency decreased with increase of initial pyrene concentrations was observed in the whole experiment period (Fig. 1).

Atagana et al. (2003) suggested that the best C/N value (*w*/*w*) for degrading creosote was higher (25) than the predicted one (5). In this study, since strain M-1 could use both NO_3^- and NO_2^- as electron acceptors, the C/N was defined as a molar ratio of pyrene-C to (NO_3^--N+NO_2^--N). As shown in Fig. 3, the degradation efficiencies increased linearly with the C/N values (in the range of 0.1 to 0.6) with a coefficient of determination (R^2)=0.748. The increase of degradation efficiencies with the C/N values might be related to the availability of carbon source for organisms. Compared with the nonaromatic carbon source, it is more difficult to assimilate the aromatic carbon source into microbial biomass (Her and Huang 1995). Consequently, higher concentrations of pyrene should be beneficial to cell growth then increase microbial activity. As evidenced in Table 2, the higher pyrene amended treatments (P4 and P5) had higher degradation rates before toxic intermediate product accumulated. On the other hand, high concentration of NO_2^- and NO_3^- inhibited the maximum growth of strain M-1 during the culture (Fig. 2). High N concentrations can be toxic to the microbial population (Knowles 1982; Carlson and Ingraham 1983; Atagana et al. 2003). Therefore, it should be a useful strategy to keep relatively high C/N values in pyrene biodegradation under the denitrifying condition.

4.3 Products and Electron Transformation in Pyrene Degradation

In the process of pyrene degradation of the low pyrene treatments (P1 and P2), CO_2 was produced and degradation rates were continuously high (Table 2), which indicated that pyrene could be mineralized as the sole carbon source. However, the high pyrene treatments (P4 and P5) inhibited the degradation process in the later period probably due to intermediate product accumulation.

Most of NO_3^- and NO_2^- were reduced to N_2 with little N_2O accumulation (Table 3), suggesting that strain M-1 was a denitrifier with the ability of performing the complete denitrification. No evidence showed that pyrene would inhibit a certain step of denitrification in the NO_2^- added treatments, which was different from Guo et al. (2011). However, the electrons of denitrification consumed were not all from the pyrene degradation, especially in the high N treatments (B and C), in which the ratios of electron consumption to production were higher than 1.0 (Table 3). One possible explanation was that reduced sulfur (S^{2-}), from the Na_2S added into the medium initially, might be used in the process of nitrate-driven sulfur oxidation, which was demonstrated in river sediments (Burgin and Hamilton 2008; Yang et al. 2012). Another explanation might be related to the endogenous denitrification (Her and Huang 1995), which needs further study. Therefore, over the N demand for bacterial growth, lower N treatments (with the ratio of electron consumption to production close to one, Table 3) led to more effective process of denitrifying-pyrene-degradation.

This study demonstrated that a *P. denitrificans* strain M-1 isolated from petroleum-contaminated sediment could degrade pyrene under denitrification condition. Various NO_3^-/NO_2^- conditions influenced the pyrene degradation efficiency significantly. In the anaerobic pyrene-degrading process, NO_2^- could induce the complete denitrification process and act a key factor to promote pyrene degradation. High N concentrations and toxic intermediate product accumulation might inhibit the bacterial growth and biodegradation process. The results suggest that controlling high C/N values and low NO_2^- and NO_3^- concentration treatments should be useful strategies to obtain higher PAH degradation efficiencies under the denitrifying condition.

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