Journal of Hazardous Materials 165 (2009) 325–331

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

# Anaerobic degradation of naphthalene by the mixed bacteria under nitrate reducing conditions

Junfeng Dou[a,*], Xiang Liu[b], Aizhong Ding[a]

[a] College of Water Sciences, Beijing Normal University, Beijing 100875, PR China
[b] Department of Environmental Science and Engineering, Tsinghua University, Beijing 100084, PR China

ARTICLE INFO

*Article history:*
Received 15 June 2008
Received in revised form 1 October 2008
Accepted 1 October 2008
Available online 9 October 2008

*Keywords:*
Polycyclic aromatic hydrocarbons (PAHs)
Naphthalene
Nitrate reduction
Anaerobic biodegradation
Mixed bacteria

ABSTRACT

Mixed bacteria were enriched from soil samples contaminated with polycyclic aromatic hydrocarbons (PAHs). The anaerobic degradation characteristics by the enriched bacteria with different initial naphthalene concentrations were investigated under nitrate reducing conditions. The results showed that the mixed bacteria could degrade nearly all the naphthalene over the incubations of 25 days when the initial naphthalene concentration was below 30 mg/L. The degradation rates of naphthalene increased with increasing initial concentrations. A high naphthalene concentration of 30 mg/L did not inhibit neither on the bacterial growth nor on the naphthalene degradation ability. The accumulation of nitrite was occurred during the reduction of nitrate, and a nitrite concentration of 50 mg/L had no inhibition effect on the degradation of naphthalene. The calculation of electron balances revealed that most of the naphthalene was oxidized whereas a small proportion was used for cell synthesis.



## 1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) originating from coal and petroleum are organic compounds containing two or more aromatic rings, which are known to have toxic, mutagenic and carcinogenic properties [1]. The US Environmental Protection Agency (USEPA) has listed 16 typical PAHs as priority pollutants [2]. Many industrial areas have been highly contaminated by PAHs due to accidental leakage of jet fuel and spillage during the transportation, and PAHs have become widespread environmental pollutants [3]. The fate of PAHs in soils and sediments is of a great environmental concern in recent years. Biodegradation is expected to be an economical and energy efficient approach in comparison to other remediation processes such as chemical or physical ones. At the same time, the efficacy of bioremediation still remains a critical point [4].

Under aerobic conditions, PAHs degradation has been studied extensively. Numerous aerobic bacteria that use PAHs as sources of carbon and energy in the presence of oxygen have been isolated, and the associated degradation pathway has been elucidated [5–10]. In general, aerobic biodegradation is considered as much faster than anaerobic processes, especially for unsubstituted PAHs, such as naphthalene [11]. However, aerobic processes are not universally applicable because the contaminated sites are frequently rendered anaerobic as a result of indigenous microorganisms consuming the available molecular oxygen faster than it can be replenished. Therefore, in these sites anaerobic degradation of aromatic hydrocarbons may be the determining mechanism and depends on the activity of bacteria capable of metabolizing hydrocarbons [12].

Until the late 1980s, it was assumed that the microorganisms required molecular oxygen to degrade PAHs, and that PAHs in anaerobic conditions were recalcitrant to biodegradation. Since then, significant advances have been made towards understanding the bacterial characteristic and biochemical bases of anaerobic PAHs degradation under nitrate, $Fe^{3+}$, and sulfate reducing conditions [13–17]. Mihelcic and co-workers were the first researchers that have shown the degradation of lower molecular weight PAHs under nitrate reducing conditions, and they observed that naphthalene could be reduced from 3 mg/L to non-detectable levels by the enriched microorganisms [18–20]. Then several laboratories have documented the biodegradation of naphthalene under nitrate reducing conditions [21,22]. Recently, several anaerobic naphthalene degrading cultures have been purified and many catabolic pathways have been elucidated with nitrate or sulfate as the electron acceptor [23–28]. Zhang and Young first reported the biochemical pathway for anaerobic naphthalene degradation which involved an initial carboxylation step to 2-naphthoic acid (2-NA) [29], then 2-NA was sequentially reduced starting at the unsubstituted ring through a series of five hydrogenation reactions [25,30]. Safinowski and Meckenstock proposed the methylation as a novel general mechanism of activation reactions in anaerobic

* Corresponding author. Tel.: +86 10 58802739; fax: +86 10 58802739.
*E-mail address:* doujf@bnu.edu.cn (J. Dou).

0304-3894/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jhazmat.2008.10.002

degradation of unsubstituted aromatic hydrocarbons [28]. Safinowski and Meckenstock reported the cometabolic transformation of different PAHs by the naphthalene degrading culture of N47 [27]. In addition, a number of pure cultures able to metabolize fluoranthene [6,31,32] and pyrene [6,33,34] have also been reported. Both Eriksson et al. and Hayes et al. found that anaerobic PAHs degraders could be enriched from the environments without PAHs contamination [35,36]. In addition, McNally et al. drew an attracting conclusion that anaerobic degradation rates under denitrifying conditions were nearly equal to aerobic degradation rates when cell densities were similar [37].

To develop anaerobic bioremediation technologies, systematic studies are required for understanding the degradation performance of PAHs under anaerobic conditions. The rate and extent of PAHs biodegradation are affected by physical, chemical, biological or environmental factors [38,39]. Higher concentrations of PAHs would limit the degradation of the substrates, and the bacterial growth became limited with an increase in PAHs concentrations [40,41]. Al-Bashir et al. found that substrate-dependent kinetics were dominant once the aqueous phase concentration of PAHs dropped below the saturation level [42]. However, information regarding the rates and effectiveness of the substrate for remediation of PAHs contaminated sites is still essential. In the present study, naphthalene is selected as a target compound, which is composed of two fused aromatic rings, has long been used in enrichment cultures to isolate PAHs catabolizing bacteria from contaminated soils. The aim of this study was to investigate the degradation performance of naphthalene under nitrate reducing conditions in the presence of the mixed bacteria enriched from gasoline contaminated soil.

## 2. Materials and methods

### 2.1. Chemicals and materials

Naphthalene was obtained from Sigma –Aldrich with a specified purity of 99%. Deionized water from a Milli-Q purification system (Millipore, USA) was used in preparing samples. The polymer resins of Amberlite-XAD2 (Sigma–Aldrich Co., USA) were washed with deionized water several times to remove inorganic impurities, followed by acetone to remove organic impurities, and then acetone was thoroughly evaporated at 70 °C. The mineral salts medium using in this study contained the following constituents: $NaNO_3$ (2.0 g/L), $NH_4Cl$ (1 g/L), $KH_2PO_4$ (1 g/L), $MgCl_2$ (0.1 g/L) and $CaCl_2{\cdot}2H_2O$ (0.05 mg/L). The medium was supplemented with 0.1% of $Na_2S{\cdot}9H_2O$, vitamin solution (1% [vol/vol]) and trace elements solution (1% [vol/vol]). Each liter of trace elements solution contained 30 mg of $CoCl_2{\cdot}6H_2O$, 0.15 mg of $CuCl_2$, 5.7 mg of $H_3BO_3$, 20 mg of $MnCl_2{\cdot}4H_2O$, 2.5 mg of $Na_2MoO_4{\cdot}2H_2O$, 1.5 mg of $NiCl_2{\cdot}2H_2O$, and 2.1 mg of $ZnCl_2$ [43]. Each liter of the vitamin solution contained 20 mg of biotin, 20 mg of folic acid, 50 mg of riboflavin, 50 mg of thiamine, 50 mg of nicotinic acid, 50 mg of pantothenic acid, 1 mg of cyanocobalamin, 50 mg of *p*-aminobenzoic acid, and 50 mg of thiotic acid [29]. The pH of the medium was between 6.8 and 7.2. The gasoline contaminated soil used as the material for enriching the mixed bacteria was collected from the Zihe riverbed in Shandong Province, PR China. This site has been heavily contaminated with PAHs and other components of gasoline for more than 30 years [44].

Anaerobic biodegradation experiments were performed using 50-ml serum bottles, and the bottles were sealed with Teflon coated rubber stoppers (Alltech Associates, Inc.) and aluminum caps. All the experiments were conducted in an anaerobic glovebox which was filled with pure nitrogen gas.

### 2.2. Mixed bacteria isolating conditions

For enrichment of anaerobic naphthalene degrading bacteria, 120 g soils were initially mixed with 100 ml of the mineral medium. In addition, approximately 20 mg naphthalene was added to the medium as solid crystals. The incubation was performed at 20 °C for 10 months in the soil container that contained pure nitrogen gas.

Naphthalene was added to 150-ml glass bottle with a syringe as a 0.1% solution in acetone (8 ml per bottle). Time was given for the acetone to evaporate completely to avoid toxicity or utilization of acetone as the carbon source. 80 ml mineral medium and 0.6 g of Amberlite-XAD2 were added to the 150-ml glass bottle. Then 0.5 g of the incubated soil was added. After 20–25 days, transfers were done by adding 10 ml of the soil solutions to 90 ml of the fresh medium amendment with naphthalene and 0.6 g of Amberlite-XAD2. After it was transferred four times, 10 ml of soil solutions was added to 90 ml of the fresh medium amendment with naphthalene only. After it was transferred three times again, the mixed bacterial consortia capable of anaerobic oxidizing naphthalene were obtained.

### 2.3. Experimental set-up

A series of microcosms were prepared to investigate the ability of the enriched bacteria to degrade naphthalene. Six different initial concentrations of approximately 5, 10, 15, 20, 25 and 30 mg/L were designed. Due to the evaporation of naphthalene, there was equilibrium between the naphthalene in gas and liquid phase. Actually, the initial concentrations were 3.7, 9.1, 12.8, 18.4, 23.2 and 28.7 mg/L, respectively. In order to account for abiotic naphthalene degradation, control experiments with naphthalene concentration of 15 mg/L containing no electron acceptor or no microorganisms were run in parallel. At the same time, the sterile treatments were conducted. The sterile samples were established by autoclaving at 121 °C for 3 h, followed by the addition of mercuric chloride (400 mg/L). In addition, microcosms with electron acceptor and microorganisms but without naphthalene were also prepared.

To prepare the microcosms, naphthalene was initially coated onto the insides of the serum bottles by acetone delivery. Aliquots of the acetone/naphthalene mixtures were added to the serum bottles and distributed evenly around the bottom and sides of the bottles. The volume of the aliquots was varied depending on the target naphthalene concentration as the experimental design. The acetone was allowed to evaporate completely, leaving naphthalene adsorbed on the insides of the bottles. 33 ml of the minimal medium was poured in serum bottle. The compositions of minimal medium were the same to those used for enriching experiments except for the nitrate concentration of about 400 mg/L. The mineral medium was purged for 3.5 h using pure nitrogen gas. Then 2 ml of the enriched mixed bacteria solutions were added to the serum bottles to get the initial cell densities of $1 \times 10^7$ cells/ml.

The microcosms were incubated at 20 °C in darkness. Samples were periodically collected to measure the concentrations of naphthalene, nitrate and nitrite. All the experiments were conducted in triplicate. Each data represented the mean of three measurements, and the standard deviation was less than 10%.

### 2.4. Chemical and microbiological analysis

Naphthalene was analyzed using a Shimadzu LC-10AD HPLC equipped with a UV–visible detector. A 20 μl injection was separated on a 250 × 4.8 Aichrom C18 column. The wavelength used for detection was 254 nm. The mobile phase was a mixture of water/methanol (15:85, v/v), with a flow rate of 1.0 ml/min [45].

Nitrate and nitrite were analyzed by ion chromatography (Dionex DX100, Sunnyvale, CA, USA), using an Iopac ASI4 (4 mm × 250 mm) analytical column, the eluent was $Na_2CO_3$–$NaHCO_3$ (3.5 mmol/L and 1.0 mmol/L), and the flow rate was 1.2 ml/min [46].

The total cell counts were analyzed by DNA intercalating dye 4′,6-diamidine-2′-phenylindol-dihydrochloride (DAPI, Sigma) staining [47]. For that, suspensions of the samples were concentrated onto 0.2 μm pore-size black polycarbonate membrane filters (Nucleopore, Whatman, USA). Filters were washed with sterilized deionized water and air-dried in the dark. In order to fix the cells, the filters were dipped into a 4% formaldehyde solution for 2–3 h. Then filters were stained with DAPI dye (1 μg/ml) for 15 min in the dark. DAPI-stained cells were identified and enumerated under an epifluorescent microscope equipped with ultraviolet (UV) excitation filter set (Nikon UV-2A, UV excitation at 330–500 nm, dichroic mirror 400 nm, longpass >420 nm, Kawasaki, Japan). The procedure was performed by DAPI staining in at least three independent experiments.

## 3. Results and discussion

### 3.1. Anaerobic degradation of naphthalene in control experiments

The disappearance of naphthalene under different conditions of without electron acceptor, without microorganisms, with microorganisms and nitrate, and the sterile treatments was shown in Fig. 1. As can be seen from Fig. 1, when naphthalene was added at 15 mg/L initially, the naphthalene concentration was detected at about 13 mg/L at day 0. This loss was probably because part of naphthalene was irreversibly bound to the inside walls of the serum bottles or the stoppers. Another reason may be that part of naphthalene was evaporated into headspace of the bottle due to the evaporation of naphthalene. It could also be observed that naphthalene concentration decreased from 13 to below 0.1 mg/L with the simultaneous presence of nitrate and the enriched bacteria after 20 days incubation. However, under the condition of without microorganisms or without electron acceptor or the sterile treatments, there was no significant change in the naphthalene levels and the concentration of naphthalene was stable at 13 mg/L over the whole incubations

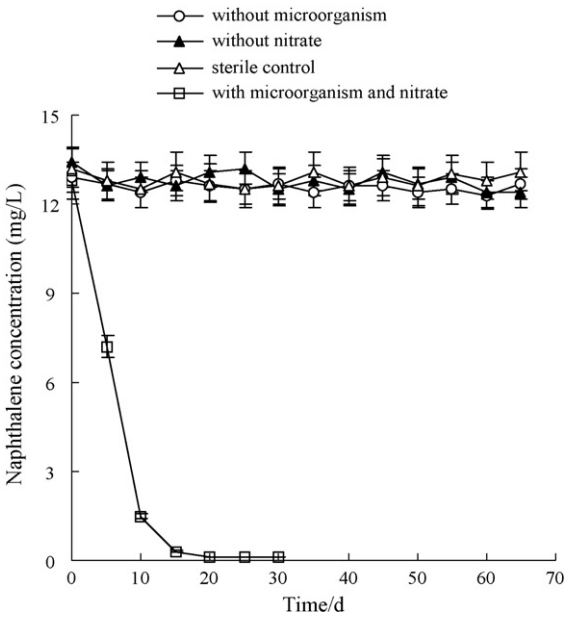


**Fig. 1.** The disappearance of naphthalene under different conditions.

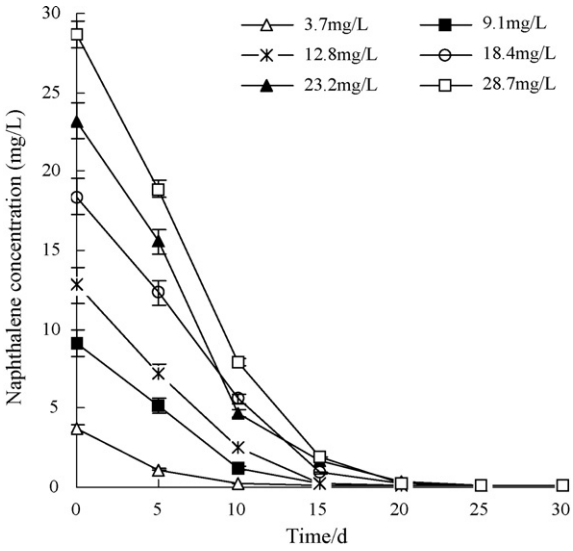


**Fig. 2.** Anaerobic degradation profiles of different naphthalene initial concentrations.

of 65 days. All the results of the control experiments indicated that no significant loss of naphthalene occurred due to abiotic process, and the disappearance of naphthalene with the enriched bacteria and nitrate was due to biological process.

### 3.2. Anaerobic naphthalene degradation characteristics with various initial concentrations

Naphthalene degradation by the enriched mixed bacteria was evaluated in microcosms with different initial naphthalene concentrations. The results of these anaerobic degradation experiments were presented in Fig. 2.

The data in Fig. 2 demonstrated that the enriched mixed bacteria could degrade naphthalene without a lag upon inoculating. Naphthalene could be decreased to non-detectable levels within a period of 25 days incubation when the initial concentration was below 30 mg/L, which implied that naphthalene could serve as electron donor and carbon source for the enriched bacteria. Although significant naphthalene removal was observed for all the different initial

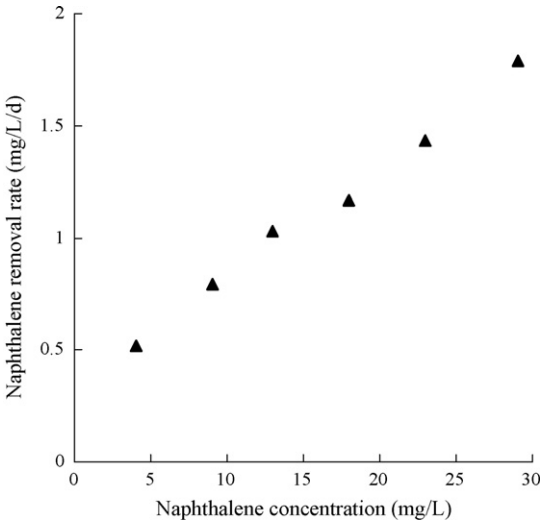


**Fig. 3.** Relationship between biodegradation rates and naphthalene initial concentrations.

concentrations, naphthalene degradation rates were different. The biodegradation rates at each initial substrate concentration were calculated according to the variation of naphthalene concentration between starting concentration and the end of approximately 0.5 mg/L, and the results were shown in Fig. 3.

From the profile of Fig. 3, it could be observed that naphthalene degradation rates were increased from 0.5 to 1.8 mg/L/d, and there was seemingly no toxic inhibition effect on the enriched bacteria when the concentration of naphthalene was below 30 mg/L. One possible explanation for the higher degradation rates with higher initial concentrations is that naphthalene could act as a carbon and energy source and thereby higher initial naphthalene concentrations could promote the growth of the enriched bacteria, which resulted in faster naphthalene degradation. Mihelcic and Luthy obtained the naphthalene degradation rates of 0.12–0.20 mg/L/d under nitrate reducing conditions in soil–water systems [19]. Al-Bashir et al. observed the naphthalene degradation rate of 1.8 mg/L/d in flooded soil under denitrifying conditions, this value was nearly the same to the maximum rate obtained in this study [21]. While Bregnard et al. demonstrated that anaerobic degradation rate of naphthalene was very low under nitrate reducing conditions, and it was only 0.01 mg/L/d [48]. Thus, it can be considered that the enriched mixed bacteria obtained in this study were more efficient to degrade naphthalene under nitrate reducing conditions, and could potentially remediate a site contaminated with naphthalene using nitrate as terminal electron acceptors.

### 3.3. Variation curve of nitrate and nitrite concentrations during incubation

Monitoring the loss of nitrate could provide direct information that naphthalene biodegradation was dependent on the presence of nitrate, and could also demonstrate the relationship between naphthalene biodegradation and nitrate reduction. Fig. 4 showed the loss of nitrate and the accumulation of nitrite under various initial naphthalene concentrations.

Fig. 4 showed that the nitrate concentration decreased over time in experiments of simultaneous amended with the enriched mixed bacteria and naphthalene. As can be seen from Fig. 4, nitrite was accumulated during the reduction of nitrate. Because nitrite was a product of denitrification, the presence of nitrite coupled with the loss of nitrate supported the conclusion that naphthalene degradation was coupled to nitrate reduction. The enriched bacteria could utilize naphthalene without a significant lag phase, as also indicated by decreasing nitrate concentrations immediately after inoculating. Compared between Figs. 2 and 4, it could be safely

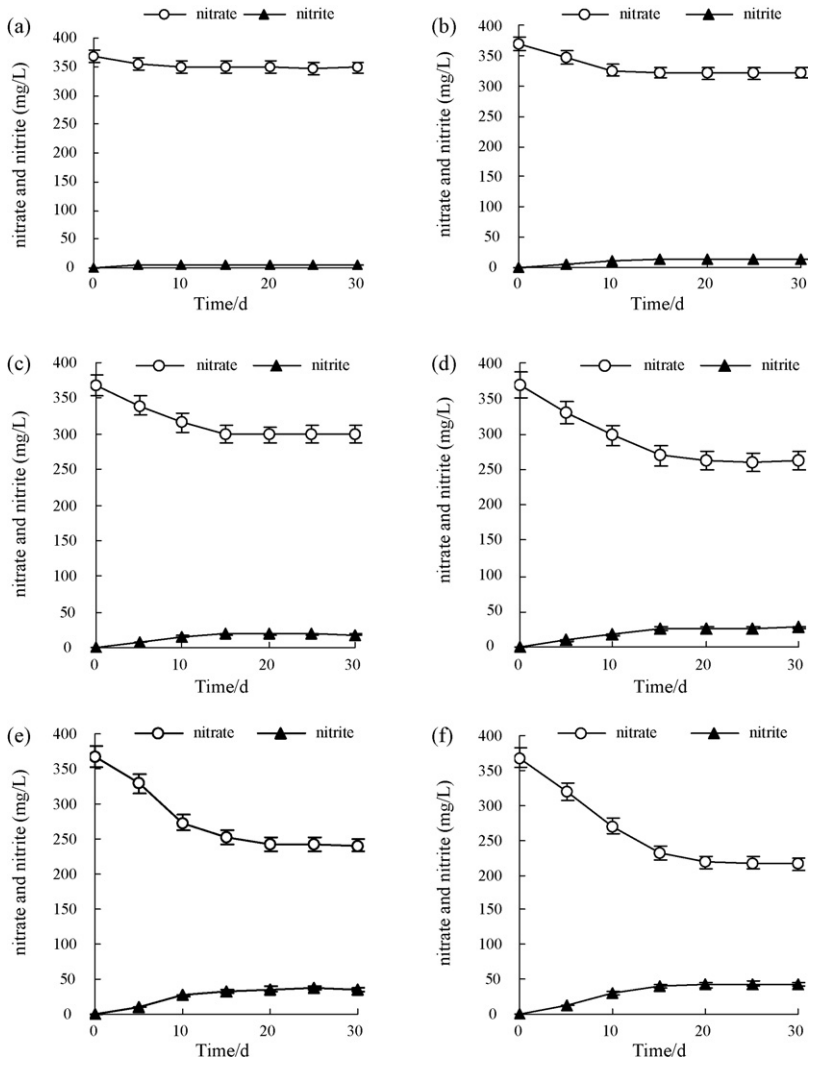


**Fig. 4.** Variation curve of nitrate and nitrite during degradation of different initial concentrations of naphthalene: (a) initial concentration of 3.7 mg/L, (b) initial concentration of 9.1 mg/L, (c) initial concentration of 12.8 mg/L, (d) initial concentration of 18.4 mg/L, (e) initial concentration of 23.2 mg/L and (f) initial concentration of 28.7 mg/L.

**Table 1**
The measured ratios between nitrate and naphthalene consumption.

| Initial naphthalene concentration (mg/L) | Measured ratios between nitrate and naphthalene consumption | Cell numbers ($\times 10^7$ cell /ml) |
|---|---|---|
| 3.7 | 11.53 ± 0.63 | 1.3 ± 0.1 |
| 9.1 | 10.71 ± 0.72 | 1.9 ± 0.1 |
| 12.8 | 11.22 ± 0.78 | 2.1 ± 0.2 |
| 18.4 | 12.02 ± 0.59 | 2.9 ± 0.2 |
| 23.2 | 11.41 ± 0.81 | 3.2 ± 0.2 |
| 28.7 | 11.17 ± 0.62 | 4.1 ± 0.3 |

concluded that nitrate reduction went hand-in-hand with naphthalene degradation, which indicated that naphthalene degradation was coupled to nitrate reduction and was due to biological process. On the contrary, under the condition of not adding the enriched cultures or not amending naphthalene substrate, neither the loss of nitrate nor the accumulation of nitrite was observed over the whole incubations, which was due to the fact that the reduction of nitrate could not be occurred when there was no the biodegradation bacteria or the carbon source. It was interesting to find that the accumulation of nitrite did not inhibit the degradation of naphthalene even if the concentration of nitrite was over 50 mg/L. The reason may be that the isolating procedure helped the mixed bacteria adapt themselves to the higher nitrite concentration conditions when they were enriched from the contaminated soils. Rocken and Strand found that nitrite was only detected transiently, but the production of $N_2O$ was observed in their experiments [49].

### 3.4. Stoichiometry between nitrate consumption and naphthalene degradation

The half reaction for complete anaerobic oxidation of naphthalene to carbon dioxide with nitrate as the electron acceptors could be stated as following:

$$C_{10}H_8 + 20H_2O \rightarrow 10CO_2 + 48H^+ + 48e^- \quad (1)$$

$$NO_3^- + 6H^+ + 5e^- \rightarrow 0.5N_2 + 6H_2O \quad (2)$$

$$NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O \quad (3)$$

Based on Eqs. (1) and (2), the theoretical molar ratio of nitrate consumption to naphthalene degradation was 9.6. According to Eqs. (1) and (3), the theoretical molar ratio of nitrate consumption to naphthalene degradation was 24. The measured ratios could be calculated based on the variation of nitrate and naphthalene over the whole incubations, and the calculated results according to the experimental data were illustrated in Table 1.

From Table 1 it could be observed that 10.71–12.02 mol nitrate was consumed when 1 mol of naphthalene was degraded, which was higher than the theoretical value of 9.6 that was expected assuming the complete reduction of nitrate to nitrogen gas with complete naphthalene mineralization, but was lower than the theoretical value of 24 that was calculated according to the assumption that nitrate was only reduced to nitrite. The reason for this phenomenon was that nitrate was reduced to nitrite, but only part of the nitrite was further reduced to nitrogen gas. From Fig. 4 it could also be observed that the accumulation of nitrite was lower than the consumption of nitrate, which supported the fact that only part of the nitrite was reduced to nitrogen gas.

According to Eqs. (1)–(3), it could be found that the reduction 1 mol of $NO_3^-$ to $NO_2^-$ needs 2 mol electrons, and the reduction 1 mol of $NO_3^-$ to $N_2$ needs 5 mol electrons, while the complete oxidation of 1 mol of naphthalene to carbon dioxide yields 48 mol electrons. The electron balances of naphthalene consumption and nitrate reduction were shown in Table 2. As can be seen from Table 2, the amount of electrons consumed for nitrate reduced to nitrite or nitrogen gas was 88.3–92.5% of that expected based on theoretical calculations using the amount of naphthalene consumed for the complete oxidation to carbon dioxide. This deviation from theory may be due to partial utilization of the naphthalene for cell synthesis, and/or due to a lack of complete naphthalene mineralization.

### 3.5. Variation of bacterial densities during incubation

The numbers of DAPI-stained cells at the end of the incubation were shown in Table 1. From Table 1 it could be observed that cell numbers of the enriched bacteria rose significantly at the end of incubation, and increased with increasing the initial concentrations, which implied that the growth of the mixed bacteria coincided with the loss of naphthalene.

Bacterial cell counts were converted to total biomass weight using a factor of $1.72 \times 10^{-10}$ mg/bacterial cell [50]. The mass of microbes grown can be obtained subtracting the initial value from

**Table 2**
Electron balance of naphthalene consumption and nitrate reduction.

| Initial naphthalene concentration (mg/L) | Amount of naphthalene consumed (μmol) | Amount of electrons produced from naphthalene consumed (μmol) | Amount of $NO_3^-$ reduced to $NO_2^-$ (μmol) | Amount of $NO_3^-$ reduced to $N_2$ (μmol) | Amount of electrons consumed by $NO_3^-$ reduction (μmol) | Ratio of electrons consumed to produced (%) |
|---|---|---|---|---|---|---|
| 3.7 | 0.98 | 47.25 | 4.34 | 7.01 | 43.72 | 92.5 |
| 9.1 | 2.46 | 118.12 | 10.12 | 16.81 | 104.28 | 88.3 |
| 12.8 | 3.47 | 166.69 | 14.00 | 24.95 | 152.76 | 91.6 |
| 18.4 | 5.00 | 240.19 | 20.85 | 34.70 | 215.20 | 89.6 |
| 23.2 | 6.32 | 303.19 | 26.86 | 44.95 | 278.46 | 91.8 |
| 28.7 | 7.82 | 375.38 | 32.64 | 53.79 | 334.21 | 89.0 |

**Table 3**
Comparison of the mass of microbes grown between the measured and expected values.

| Initial naphthalene concentration (mg/L) | Mass of total naphthalene used (μg) | Measured mass of microbes grown (μg) | Microbial yield | Mass of naphthalene used for microbes grown (μg) | Expected mass of microbes grown (μg) | Relative error between measured and expected mass of microbes (%) |
|---|---|---|---|---|---|---|
| 3.7 | 126.0 | 18.1 ± 6.0 | 0.14 | 9.40 | 16.6 | 8.6 |
| 9.1 | 315.0 | 54.2 ± 6.0 | 0.17 | 36.9 | 65.2 | −18.4 |
| 12.8 | 444.5 | 66.2 ± 12.0 | 0.15 | 37.2 | 65.6 | 0.9 |
| 18.4 | 640.5 | 114.4 ± 12.0 | 0.18 | 66.6 | 117.6 | −2.8 |
| 23.2 | 808.5 | 132.4 ± 12.0 | 0.16 | 66.0 | 116.5 | 12.8 |
| 28.7 | 1001.0 | 186.6 ± 18.1 | 0.19 | 109.7 | 193.7 | −3.7 |

the final value, and the results were listed in Table 3. The microbial yield was calculated based on the mass of microbes grown and the mass of the naphthalene utilized, and the results were also shown in Table 3. The microbial yield was between 0.14 and 0.19, which suggested that the enriched mixed bacteria could growth well using naphthalene as carbon source under nitrate reducing conditions.

As stated in the above section, if there was no bacterial growth and all the naphthalene was oxidized to $CO_2$ and $H_2O$, the electron balances could not be gained, and the amount of electrons consumed was only 88.3–92.5% of the amount of electrons theoretically produced. Therefore, the value of approximately 7.5–11.7% of the naphthalene was used for bacterial growth which could be hypothesized. In addition, since naphthalene was the only available carbon source in the microcosms, according to McCarty [51], the stoichiometric reaction for cell synthesis could be stated as the following equation:

$$C_{10}H_8 + \ldots \rightarrow 2C_5H_7O_2N + \ldots \quad (4)$$

Where, $C_5H_7O_2N$ was the composition of biomass. Based on Eq. (4), the biomass production could be evaluated using the hypothetical amount of naphthalene used for cell synthesis, and the results were listed in Table 3. Based on the data in Table 3, it could be observed that there was no significant difference between the actual and expected mass of microbes, which showed that the hypothesis of about 7.5–11.7% of the naphthalene used for bacterial growth seemed reasonable. Therefore, it could be concluded that approximately 88.3–92.5% of naphthalene was completely mineralized to $CO_2$, which was just as shown in Eq. (1).

## 4. Conclusions

The enriched mixed bacteria were capable of biodegrading naphthalene efficiently and without a lag phase under nitrate reducing conditions. The naphthalene biodegradation ability of the enriched bacteria was not inhibited by a high concentration of 30 mg/L. The enriched mixed bacteria could growth well using naphthalene as carbon source, and about 7.5–11.7% of the naphthalene was used for bacterial growth. The amount of electrons derived from naphthalene consumed for denitrification was nearly equal to the amount of electrons required for nitrate reduced to nitrite or nitrogen gas. The results obtained in the present study suggested that anaerobic degradation of naphthalene by the enriched mixed bacteria appeared to be a feasible method to remediate naphthalene contaminated sites. Further study on the feasibility of the enriched bacteria under field conditions is required to promote the remediation of PAHs contaminated soil under nitrate reducing conditions.

## Acknowledgements

The Project Sponsored by the Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry, and by grants from the National 973 Program Project, PR China (no. 2006CB403400).

## References

[1] L.H. Keith, W.A. Telliard, Priority pollutants, I-a perspective view, Environ. Sci. Technol. 13 (1979) 416–423.
[2] R.A. Kanaly, S. Harayama, Biodegradation of high molecular weight polycyclic aromatic hydrocarbons by bacteria, J. Bacteriol. 182 (2000) 2059–2067.
[3] H.H. Tabak, J.M. Lazorchak, L. Lei, A.P. Khodadoust, J.E. Antia, R. Bagchi, M.T. Suidan, Studies on bioremediation of polycyclic aromatic hydrocarbon-contaminated sediments: bioavailability, biodegradability, and toxicity issues, Environ. Toxicol. Chem. 22 (2003) 473–482.
[4] S.V. Mohan, T. Kisa, T. Ohkuma, A.K. Robert, Y. Shimizu, Bioremediation technologies for treatment of PAHs-contaminated soil and strategies to enhance process efficiency, Rev. Environ. Sci. Biotechnol. 5 (2006) 347–374.
[5] I. Kelley, J.P. Freemanand, C.E. Cerniglia, Identification of metabolites from degradation of naphthalene by a *Mycobacterium* sp., Biodegradation 1 (1990) 283–290.
[6] B. Boldrin, A. Thiem, C. Fritsche, Degradation of phenanthrene, fluorene, fluoranthene, and pyrene by a *Mycobacterium* sp., Appl. Environ. Microbiol. 59 (1993) 1927–1930.
[7] M. Uyttebroek, J.J. Ortega-Calvo, P. Breugelmans, D. Springael, Comparison of mineralization of solid-sorbed phenanthrene by polycyclic aromatic hydrocarbon (PAH)-degrading *Mycobacterium* spp. and *Sphingomonas* spp., Appl. Microbiol. Biotechnol. 72 (2006) 829–836.
[8] D.J. Vacca, W.F. Bleam, W.J. Hickey, Isolation of soil bacteria adapted to degrade humic acid-sorbed phenanthrene, Appl. Environ. Microbiol. 71 (2005) 3797–3805.
[9] S.J. Kim, O. Kweon, R.C. Jones, J.P. Freeman, R.D. Edmondson, C.E. Cerniglia, Complete and integrated pyrene degradation pathway in mycobacterium vanbaalenii PYR-1 based on systems biology, J. Bacteriol. 189 (2007) 464–472.
[10] O. Kweon, S.J. Kim, R.C. Jones, J.P. Freeman, M.D. Adjei, R.D. Edmondson, C.E. Cerniglia, A polyomic approach to elucidate the fluoranthene-degradative pathway in mycobacterium vanbaalenii PYR-1, J. Bacteriol. 189 (2007) 4635–4647.
[11] C.E. Cerniglia, Biodegradation of polycyclic aromatic hydrocarbons, Biodegradation 3 (1992) 351–368.
[12] J.A. Cunningham, H. Rahme, G.D. Hopkins, C. Lebron, M. Reinhard, Enhanced in situ bioremediation of BTEX-contaminated groundwater by combined injection of nitrate and sulfate, Environ. Sci. Technol. 35 (2001) 1663–1670.
[13] J.D. Coates, R.T. Anderson, J.C. Woodward, E.J.P. Phillips, D.R. Lovley, Anaerobic hydrocarbon degradation in petroleum-contaminated harbor sediments under sulfate-reducing and artificially imposed iron-reducing conditions, Environ. Sci. Technol. 30 (1996) 2784–2789.
[14] J.D. Coates, J.C. Woodward, J. Allen, P. Philip, D.R. Lovley, Anaerobic degradation of polycyclicaromatic hydrocarbons and alkanes in petroleum-contaminated marineharbor sediments, Appl. Environ. Microbiol. 63 (1997) 3589–3593.
[15] B.R. Sharak Genthner, G.T. Townsend, S.E. Lantz, J.G. Mueller, Persistence of polycyclic aromatic hydrocarbon components of creosote under anaerobic enrichment conditions, Arch. Environ. Contam. Toxicol. 32 (1997) 99–105.
[16] M. Safinowski, C. Griebler, R.U. Meckenstock, Anaerobic cometabolic transformation of polycyclic and heterocyclic aromatic hydrocarbons, evidence from laboratory and field studies, Environ. Sci. Technol. 40 (2006) 4165–4173.
[17] M.M. Rothermich, L.A. Hayes, D.R. Lovley, Anaerobic, sulfate-dependent degradation of polycyclic aromatic hydrocarbons in petroleum-contaminated harbor sediment, Environ. Sci. Technol. 36 (2002) 4811–4817.
[18] J.R. Mihelcic, R.G. Luthy, Microbial degradation of acenaphthene and naphthalene under denitrification conditions in soil–water systems, Appl. Environ. Microbiol. 54 (1988) 1188–1198.
[19] J.R. Mihelcic, R.G. Luthy, Degradation of polycyclic aromatic hydrocarbon compounds under various redox conditions in soil-water systems, Appl. Environ. Microbiol. 54 (1988) 1182–1187.
[20] D.L. McNally, J.R. Mihelcic, D.R. Lueking, Polycyclic aromatic hydrocarbon degrading microorganisms in in Great Lakes sediments, J. Great Lakes Res. 24 (1998) 392–403.
[21] B. Al-Bashir, T. Cseh, R. Leduc, R. Samson, Eject of soil/contaminant interactions on the biodegradationof naphthalene in £ooded soil under denitrifying conditions, Appl. Microbiol. Biotechnol. 34 (1990) 414–419.
[22] J. Flyvbjerg, E. Arvin, B.K. Jensen, S.K. Olsen, Microbial degradation of phenols and aromatic hydrocarbons in creosote contaminated groundwater under nitrate-reducing conditions, J. Contam. Hydrol. 12 (1993) 133–150.
[23] E. Annweiler, W. Michaelis, R.U. Meckenstock, Identical ring cleavage products during anaerobic degradation of naphthalene, 2-methylnaphthalene, and tetraline indicate a new metabolic pathway, Appl. Environ. Microbiol. 68 (2002) 852–858.
[24] A. Galushko, D. Min, B. Schink, F. Widdel, Anaerobic degradation of naphthalene by a pure culture of a novel type of marine sulphate-reducing bacterium, Environ. Microbiol. 1 (1999) 415–420.
[25] R.U. Meckenstock, E. Annweiler, W. Michaelis, H.H. Richnow, B. Schink, Anaerobic naphthalene degradation by a sulfate-reducing enrichment culture, Appl. Environ. Microbiol. 66 (2000) 2743–2747.
[26] K.J. Rockne, J.C. Chee-Sanford, R.A. Sanford, B.P. Hedlund, J.T. Staley, S.E. Strand, Anaerobic naphthalene degradation by microbial pure cultures under nitrate-reducing conditions, Appl. Environ. Microbiol. 66 (2000) 1595–1601.
[27] M. Safinowski, R.U. Meckenstock, Enzyme reactions in anaerobic 2-methylnaphthalene degradation by the sulfate-reducing enrichment culture N 47, FEMS Microbiol. Lett. 240 (2004) 99–104.
[28] M. Safinowski, R.U. Meckenstock, Methylation is the initial reaction in anaerobic naphthalene degradation by a sulfate-reducing enrichment culture, Environ. Microbiol. 8 (2006) 347–352.
[29] X.M. Zhang, L.Y. Young, Carboxylation as an initial reaction in the anaerobic metabolism of naphthalene and phenanthrene by sulfidogenic consortia, Appl. Environ. Microbiol. 63 (1997) 4759–4764.
[30] X.M. Zhang, E.R. Sullivan, L.Y. Young, Evidence for aromatic ring reduction in the biodegradation pathway of carboxylated naphthalene by a sulfate reducing consortium, Biodegradation 11 (2000) 117–124.
[31] M. Bouchez, D. Blanchet, J.P. Vandecasteele, Degradation of polycyclic aromatic hydrocarbons by pure strains and by defined strain associations: inhibition

phenomena and cometabolism, Appl. Microbiol. Biotechnol. 43 (1995) 156–164.
[32] J.G. Mueller, P.J. Chapman, B.O. Blattmann, P.H. Pritchard, Isolation and characterization of a fluoranthene-utilizing strain of Pseudomonas paucimobilis, Appl. Environ. Microbiol. 56 (1990) 1079–1086.
[33] S.A. Churchill, J.P. Harper, P.F. Churchill, Isolation and characterization of a Mycobacterium species capable of degrading three- and four-ring aromatic and aliphatic hydrocarbons, Appl. Environ. Microbiol. 65 (1999) 549–552.
[34] D. Dean-Ross, C.E. Cerniglia, Degradation of pyrene by Mycobacterium flavescens, Appl. Microbiol. Biotechnol. 46 (1996) 307–312.
[35] M. Eriksson, E. Sodersten, Z. Yu, G. Dalhammar, M.W. Mohn, Degradation of polycyclic aromatic hydrocarbons at low temperature under aerobic and nitrate-reducing conditions in enrichment cultures from northern soils, Appl. Environ. Microbiol. 69 (2003) 275–284.
[36] L.A. Hayes, K.P. Nevin, D.R. Lovley, Role of exposure on anaerobic degradation ofnaphthalene and phenanthrene in marine harbor sediments, Org. Geochem. 30 (1999) 937–945.
[37] D.L. McNally, J.R. Mihelcic, D.R. Lueking, Biodegradation of three- and four-ring polycyclic aromatic hydrocarbons under aerobic and denitrifying conditions, Environ. Sci. Technol. 32 (1998) 2633–2639.
[38] J.G. Leahy, R.R. Colwell, Microbial degradation of hydrocarbons in the environment, Microbiol. Rev. 54 (1990) 305–315.
[39] J. Chen, M.H. Wong, Y.S. Wong, N.F.Y. Tam, Multi-factors on biodegradation kinetics of polycyclic aromatic hydrocarbons (PAHs) by Sphingomonas sp. a bacterial strain isolated from mangrove sediment, Marine Pollut. Bull. 57 (2008) 695–702.
[40] A.L. Juhasz, M.L. Britz, G.A. Stanley, Evaluation of a creosote-based medium for the growth and preparation of a PAH-degrading bacterial community for bioaugmentation, J. Ind. Microbiol. Biotech. 24 (2000) 272–284.
[41] M. Bouchez, D. Blanchet, J.P. Vandecasteele, Degradation of polycyclic aromatic-hydrocarbons by pure strains and by defined strain associations – inhibition phenomena and cometabolism, Appl. Microbiol. Biotechnol. 43 (1995) 156–164.
[42] B. Al-Bashir, T. Cseh, R. Leduc, R. Samson, Effect of soil/contaminant interaction on the biodegradation of naphthalene in flooded soil under denitrifying conditions, Appl. Microbiol. Biotechnol. 34 (1990) 414–419.
[43] Z. Hu, J. Dou, X. Liu, X. Zheng, D. Deng, Anaerobic biodegradation of benzene series compounds by mixed cultures based on optional electronic acceptors, J. Environ. Sci. 19 (2007) 1049–1054.
[44] X. Zhu, J. Liu, J. Zhu, Y. Chen, Characteristics of distribution and transport of petroleum contaminants in fracture-karst water in Zibo Area, Shandong Province, China, Sci. China Ser. D: Earth Sci. 43 (2000) 141–150.
[45] J. Yang, X. Liu, T. Long, G. Yu, Influence of nonionic surfactant on the solubilization and biodegradation of phenanthrene, J. Environ. Sci. 15 (2003) 859–862.
[46] J. Dou, X. Liu, Z. Hu, D. Deng, Anaerobic BTEX biodegradation linked to nitrate and sulfate reduction, J. Hazard. Mater. 151 (2008) 720–729.
[47] K.G. Porter, Y.S. Feig, The use of DAPI for identifying and counting aquatic microflora, Limnol. Oceanogr. 25 (1980) 943–948.
[48] T.P.A. Bregnard, P. Hohener, A. Haner, J. Zeyer, Degradation of weathered diesel fuel by microorganisms from a contaminated aquifer in aerobic and anaerobic microcosms, Environ. Toxicol. Chem. 15 (1996) 299–307.
[49] K.J. Rockne, S.E. Strand, Anaerobic biodegradatrion of naphthalene, phenanthrene, and biphenyl by a denitrifying enruchment culture, Water Res. 35 (2001) 291–299.
[50] D.L. Balkwill, F.R. Leach, J.T. Wilson, J.F. McNabb, D.C. White, Equivalence of microbial biomass measures based on membrane lipid and cell wall components, adenosine triphosphate, and direct counts in subsurface aquifer sediments, Microb. Ecol. 16 (1988) 73–84.
[51] P.L. McCarty, Stoichiometry of biological reactions, Prog. Water Technol. 7 (1975) 157–172.