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Anaerobic BTEX degradation in soil bioaugmented with mixed consortia under nitrate reducing conditions

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Abstract

Different concentrations of BTEX, including benzene, toluene, ethylbenzene, and three xylene isomers, were added into soil samples to investigate the anaerobic degradation potential by the augmented BTEX-adapted consortia under nitrate reducing conditions. All the BTEX substrates could be anaerobically biodegraded to non-detectable levels within 70 d when the initial concentrations were below 100 mg/kg in soil. Toluene was degraded faster than any other BTEX compounds, and the high-to-low order of degradation rates were toluene > ethylbenzene > *m*-xylene > *o*-xylene > benzene > *p*-xylene. Nitrite was accumulated with nitrate reduction, but the accumulation of nitrite had no inhibitory effect on the degradation of BTEX throughout the whole incubation. Indigenous bacteria in the soil could enhance the BTEX biodegradation ability of the enriched mixed bacteria. When the six BTEX compounds were simultaneously present in soil, there was no apparent inhibitory effect on their degradation with lower initial concentrations. Alternatively, benzene, *o*-xylene, and *p*-xylene degradation were inhibited with higher initial concentrations of 300 mg/kg. Higher BTEX biodegradation rates were observed in soil samples with the addition of sodium acetate compared to the presence of a single BTEX substrate, and the hypothesis of primary-substrate stimulation or cometabolic enhancement of BTEX biodegradation seems likely.

Key words: benzene; toluene; ethylbenzene; xylene; BTEX; anaerobic biodegradation; nitrate reduction; soil; bioaugmentation; cometabolism

Introduction

Benzene, toluene, ethylbenzene, and three xylene isomers (BTEX) are important contaminants present in soil, which usually originate from the accidental leakage of underground storage tanks containing gasoline and jet fuel or spillage during transportation. Due to their relatively higher water solubility, BTEX always migrate from soil to groundwater systems and contaminate drinking water supplies far from their source. BTEX compounds have raised increasing concern because they can produce neurological impairment; especially benzene can additionally cause hematological effects, which may ultimately lead to aplastic anemia and development of acute myelogenous leukemia (ATSDR, 2004). Remediation of the BTEX contaminated soil is desirable to avoid public health hazards.

Some technologies for treating BTEX-contaminated soil have been provided, which include physical, chemical, and biological methods. Chemical remediation methods, especially, advanced oxidation processes such as Fenton's reagent (Fe^{2+}/H_2O_2) and Fenton-like reagent (Fe^{3+}/H_2O_2), are known to be effective in removing BTEX from contaminated soil (Kang and Hua, 2005; Matera *et al*., 2006; Watts *et al*., 2000). Among all remediation technologies for clearing BTEX-contaminated soil, bioremediation seems to be an economical and energy efficient approach (Shim *et al*., 2002). Microorganisms are able to degrade BTEX under aerobic and anaerobic conditions (Corseuil *et al*., 1998; Deeb and Alvarez-Cohen, 1999; Langenhoff *et al*., 1996; Schreiber and Bahr, 2002). Under aerobic conditions, BTEX compounds are easily biodegraded (Deeb and Alvarez-Cohen, 2000). Numerous aerobic bacteria have been isolated that use BTEX as sources of carbon and energy in the presence of oxygen, and the associated degradation pathway has been elucidated (Alfreider and Vogt, 2007; Fahy *et al*., 2006). In general, aerobic biodegradation is considered to have a broader catabolic range and to be much faster than anaerobic processes (Chiang *et al*., 1989; Corseuil *et al*., 1998; Ruiz-Aguilar *et al*., 2002). However, aerobic processes are not universally applicable because hydrocarbon contaminated soils are frequently rendered anaerobic as a result of indigenous microorganisms consuming the available molecular oxygen faster than it can be replenished. The majority of the hydrocarbon contaminants are degraded within anaerobic zones, implying that they are being biodegraded by anaerobic bacteria.

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Therefore, in these sites anaerobic degradation of aromatic hydrocarbons might be the determining mechanism and depend on the activity of bacteria capable of metabolizing hydrocarbons (Cunningham *et al*., 2001; Lovley, 1997). BTEX are reported to be degraded and often completely oxidized to CO_2 under denitrifying conditions (Ball and Reinhard, 1996; Coates *et al.*, 2001; Schreiber and Bahr, 2002; Szykowny and Keasling, 1997).

Bioaugmentation, the addition of specialized microorganisms to enhance the biodegradation efficiency of contaminants in soil, has proved to be a feasible and economic method compared with other treatment techniques, and has received increasing attention in recent years (Vogel, 1996). The authors in a previous work examined the anaerobic degradation potential of BTEX in liquid mineral media under nitrate and sulfate reducing conditions by the mixed bacterial consortia that were enriched from gasoline contaminated soil. They suggested that all the BTEX compounds could be anaerobically biodegraded efficiently (Dou *et al*., 2008). The questions whether the isolated bacteria have the degradation ability of BTEX in soil and whether the simultaneous presence of BTEX mixtures, or the easily biodegraded organic carbon in soil have an influence on BTEX degradation are still unsolved. Answers to such questions could improve bioremediation BTEX contaminated soils in the future.

In this work, known concentrations of benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene, and *p*-xylene were added to soil samples. This was done to measure the effects of the following factors on BTEX degradation under nitrate reducing conditions: initial concentrations of BTEX in soil, the presence of the BTEX individually or simultaneously, and the addition of sodium acetate.

1 Materials and methods

1.1 Soil characterization

Soil samples were collected from the top layer (10–20 cm) of the uncontaminated grounds in Tsinghua University garden, and they were sieved through a 2-mm mesh screen to remove roots, stems, and other debris. The BTEX concentrations in the soils were below detection limit. The characteristics of the soil were analyzed and presented in Table 1.

1.2 Mixed bacterial consortium isolation conditions

For enrichment of anaerobic BTEX-degrading bacteria, 160 g of soil from gasoline contaminated sites were initially mixed with 100 ml of the mineral medium. The composition of the medium used was as follows: $NaNO_3$ (3 g/L), Na_2SO_4 (3 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 g/L). The medium was supplemented with 100 μl each of benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene, and *p*-xylene. The incubation was performed at 20°C for 5 months in an anaerobic chamber that contained pure nitrogen gas, and all the media and solutions were prepared under anaerobic conditions. Then, 0.5 g of the incubated soils were added to 100-ml glass bottle, which contained 80 ml mineral medium, and the medium contained the following constituents: NH_4Cl (1 g/L), KH_2PO_4 (1 g/L), $MgCl_2$ (0.1 g/L), $CaCl_2{\cdot}2H_2O$ (0.05 mg/L), $NaNO_3$ (1.5 g/L), and Na_2SO_4 (1.5 g/L). The medium was supplemented with 0.1% of $Na_2S{\cdot}9H_2O$, vitamin (1%, *V*/*V*), and trace elements solutions (1%, *V*/*V*). Each liter of trace salts stock 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 $ZnCl_2$ (Kazumi *et al*., 1996; Hu *et al*., 2007). In addition, 2 μl each of benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene, and *p*-xylene were added as pure stock with a 10-μl syringe. After 7–9 d, transfers were done by adding 1 ml of mixed cultures to 9 ml of the above medium in sterile 20-ml serum bottles. After it was transferred 8 times every 7–9 d, the mixed bacterial consortium capable of anaerobic degradation of BTEX was obtained. For maintenance, the enriched mixed bacteria were transferred every 6–8 weeks, and then were stored at 4°C.

Table 1 Characteristics of the soil used in this study

Parameter	Value
Sand (0.05–2.0 mm) (% dw)	25.4
Silt (0.002–0.05 mm) (% dw)	42.7
Clay (< 0.002 mm) (% dw)	31.9
Total soil carbon (% dw)	1.78
Soil organic carbon (% dw)	1.33
Total P (mgP/kg soil)	11.3
NH_4^+-N (mgN/kg soil)	8.9
NO_3^--N (mgN/kg soil)	19.3
Soil pH (in reagent grade water)	7.2

1.3 BTEX degradation in soil

Anaerobic biodegradation experiments were performed using 50-ml serum bottles containing 2 ml of the enriched mixed consortia, 20 g of soil, and 30 ml of mineral medium. The initial concentration of inoculated bacteria was approximately 1×10^8 cells/g soil. The constituents of the mineral medium were as follows: NH_4Cl (1.0 g/L), KH_2PO_4 (1.0 g/L), $MgCl_2{\cdot}5H_2O$ (0.1 g/L), and $CaCl_2{\cdot}2H_2O$ (0.05 g/L). In addition, 0.1% of $Na_2S{\cdot}9H_2O$, vitamin (1%, *V*/*V*) and trace solutions (1%, *V*/*V*) were also added to the mineral medium. The pH of the mineral medium was between 6.8 and 7.2.

Under nitrate reducing conditions, a series of experiments were conducted with benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene, and *p*-xylene as substrates. For each substrate, four different initial concentrations of approximately 25, 50, 100, and 150 mg/kg were used. Then, the following factors were used to investigate their effects on BTEX biodegradation: treatment with sodium acetate as an electron donor; the simultaneous presence of different initial concentrations of BTEX mixtures. To compare the effects of abiotic or biotic BTEX degradation in soil samples, control experiments with or without the enriched mixed consortia in the sterile soils were run in parallel. These sterile soil samples were established by autoclaving at 121°C for 3 h. To detect the biodegradation of BTEX due to indigenous microorganisms, non-sterile

soil samples without the enriched mixed consortia were also prepared.

All the experiments were conducted in an anaerobic glove box filled with pure nitrogen gas, and the serum bottle was sealed with a composite stopper. The microcosms were incubated at 20°C in darkness. The maintenance of anaerobic conditions was examined by a preliminary experiment. Samples were periodically collected to measure the concentrations of BTEX, nitrate, and nitrite. All the soil samples and the controls were prepared in triplicate, and each data represented the mean of three measurements.

1.4 Chemical analysis

BTEX concentrations were analyzed by a gas chromatograph (Shimadzu GC-14B, Shimadzu Corp., Japan) equipped with a capillary column (ULBON HR-1 0.25 mm × 30 m, Shimadzu Corp., Japan) and with a flame ionization detector (FID). Injector, detector, and column temperature were held at 150, 150, and 100°C, respectively. Nitrogen served as carrier gas, and oxygen and hydrogen served as fuel gas for the FID.

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

2 Results and discussion

2.1 Results of control experiments

As a preliminary examination, resazurin was added to the serum bottles to check the status of the anaerobic condition, the results showed that the color disappeared within 3 d, which suggested that the soil samples were maintained under anaerobic conditions. Fig.1 shows the disappearance of toluene in soil under the conditions of sterile soil without addition of microorganisms, non-sterile soil without addition of microorganisms, sterile soil added with microorganisms, and non-sterile soil added with microorganisms (data of other substrates were similar to toluene and not shown in this article).

For the soil samples after a 50-d incubation, remaining toluene amounts were as follows (Fig.1): (1) in the sterile soil and without the BTEX adapted microorganisms (94.9% to 99.2%); (2) non-sterile soil and without the BTEX adapted consortia (90.8% to 98.7%); (3) sterile soil and with the BTEX adapted microorganisms (undetectable to 1.7%); (4) non-sterile soil and added with the BTEX adapted consortia (undetectable to 1.1%). No significant difference was observed between the sterile control soil and the non-sterile control soil when the BTEX adapted microorganisms were not added, which indicated that indigenous bacteria in the BTEX uncontaminated soil did not have the ability to anaerobically biodegrade BTEX. However, from Fig.1 it was found that when the enriched cultures were added the removal rates were higher using the non-sterile soil compared to the sterile soil. The reason for this phenomenon might be that indigenous bacteria in

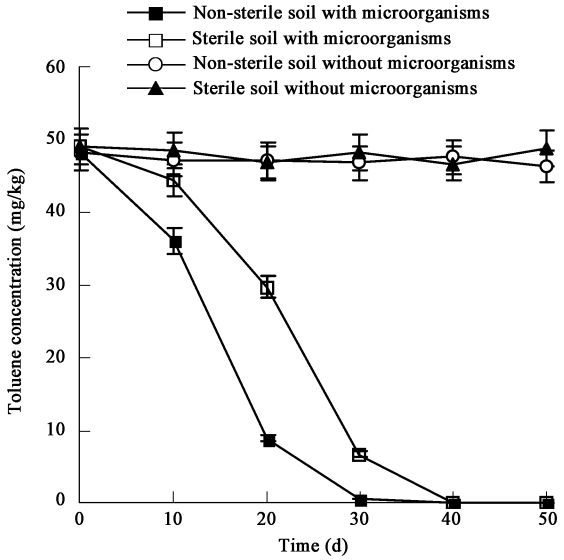

Fig. 1 Biodegradation curve of toluene under different soil matrix conditions. The error bars represent the standard deviations of the three parallel experiments.

the soil could make use of the transformation products formed from BTEX degradation as substrates (Da Silva *et al*., 2004; Vezzulli *et al*., 2004), and thus stimulate the biodegradation of BTEX. The control experimental results strongly support that BTEX degradation in soil is due to microbial action, and that such action is enhanced by bioaugmentation with the BTEX-adapted consortia.

2.2 Degradation of BTEX with various initial concentrations in soil

The anaerobic biodegradation of BTEX substrates with various initial concentrations in soil augmented with the enriched mixed bacteria was studied, and the results are shown in Fig.2. It can be clearly seen that all the BTEX substrates could be anaerobically biodegraded by the augmented microorganisms to non-detectable levels within 70 d when the initial concentrations were below 100 mg/kg in soil. Especially for toluene and ethylbenzene, they were degraded very quickly, with more than 98% removed in less than 50 d when the initial concentration was below 150 mg/kg. The same phenomena were also observed in our previous experiments that were conducted in liquid medium (Dou *et al*., 2008). Although the experiments on the feasibility of the enriched mixed bacteria in field conditions were not much conducted in this study, the effective removal of BTEX from soil suggested that the enriched mixed bacteria could potentially remediate BTEX contaminated soil using nitrate as terminal electron acceptor.

2.3 Changes in the concentrations of nitrate and nitrite

Figure 3 shows the changes of nitrate and nitrite concentrations in soil solutions with different initial benzene concentrations. Similar results were also observed with other BTEX substrates (data not shown).

The data in Fig.3a showed that in the absence of BTEX substrates only small amount of nitrate was reduced, owing

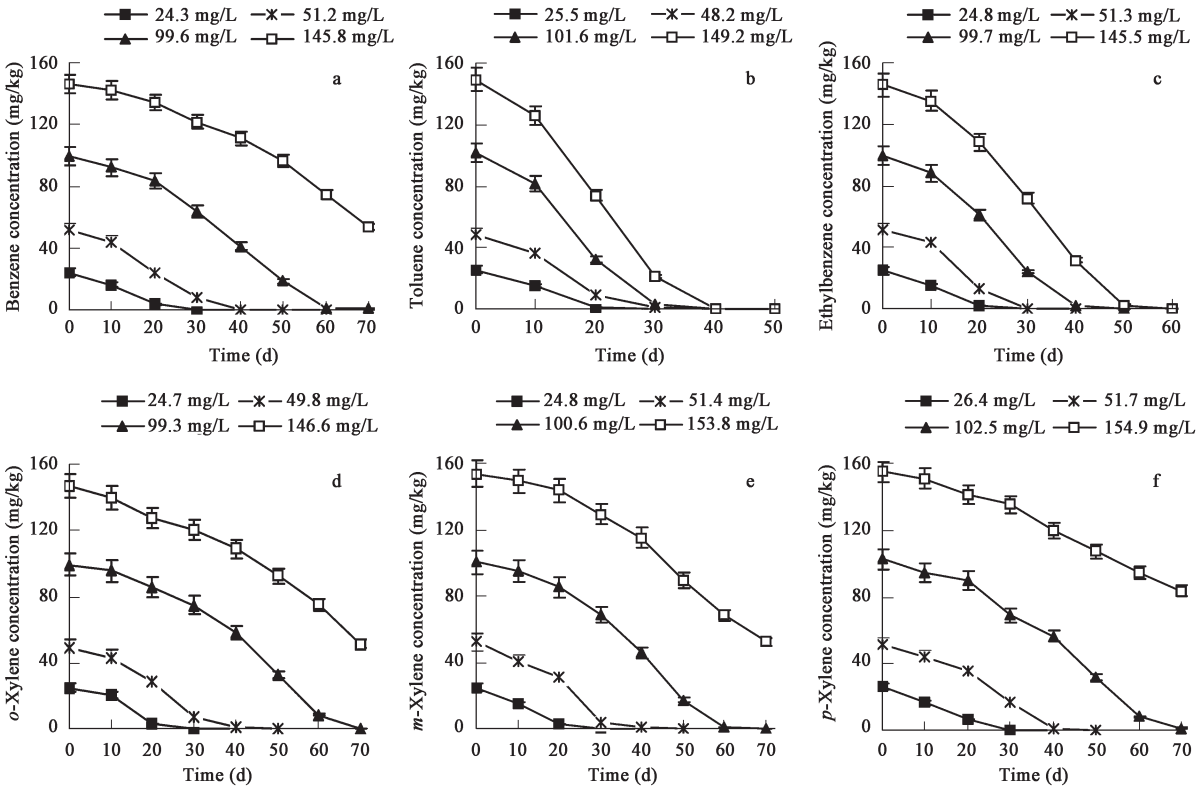

Fig. 2 Anaerobic biodegradation curves of BTEX in soil under different initial concentrations. (a) benzene; (b) toluene; (c) ethylbenzene; (d) *o*-xylene; (e) *m*-xylene; (f) *p*-xylene.

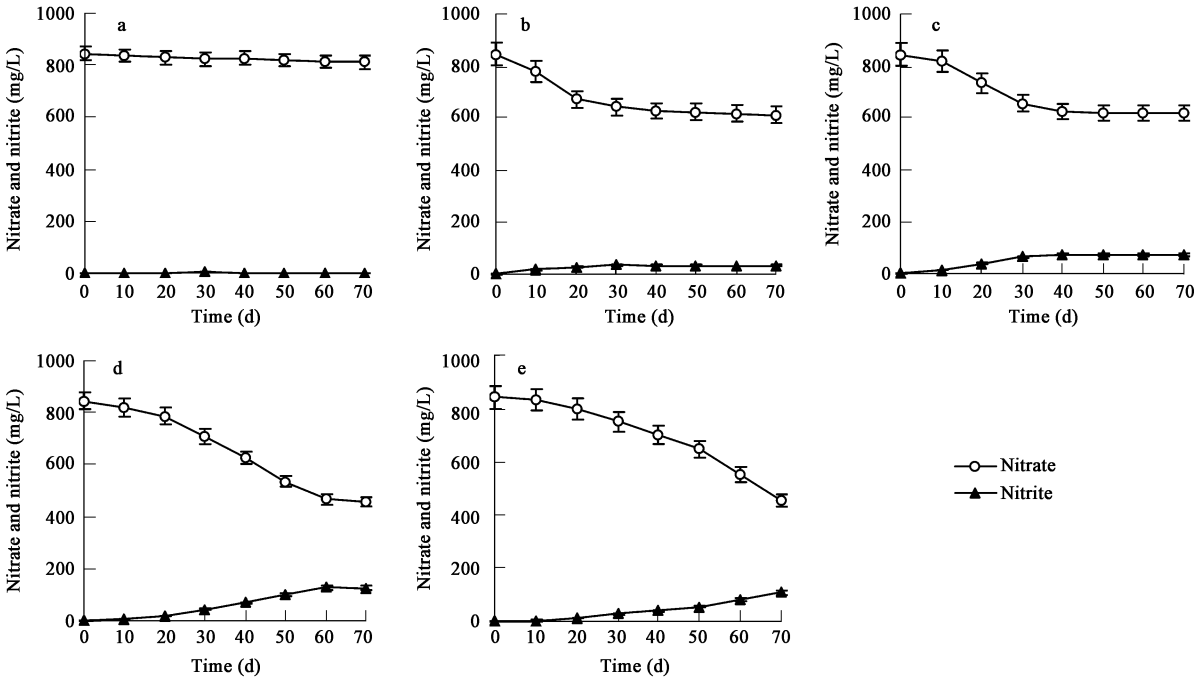

Fig. 3 Changes in the concentrations of nitrate and nitrite in the soil solution during anaerobic biodegradation of benzene. Initial benzene concentration: (a) 0 mg/kg; (b) 25 mg/kg; (c) 50 mg/kg; (d) 100 mg/kg; (e) 150 mg/kg.

to the activity of indigenous bacteria in soil (Gogoi *et al*., 2003). In addition, the results of the control experiments showed that no significant loss of nitrate was observed during the whole incubations under the condition of not inoculating the mixed bacteria. This is likely because that the reduction of nitrate was very slow by the indigenous bacteria in soil and the enriched bacteria could not reduce nitrate for lack of BTEX substrates (Hu *et al*., 2007). By comparing the results of Figs.2a and 3, it could be seen that nitrate reduction went hand-in-hand with benzene degradation, which indicated that benzene degradation was coupled to nitrate reduction and was attributable to

the activity of the inoculated mixed bacteria (Dou *et al*., 2008). Meanwhile, nitrite was detected as the intermediate compound. Nitrite was accumulated during the reduction of nitrate, but the inhibitory effect on the degradation of BTEX was not observed throughout the incubation even if the concentration of nitrite was over 120 mg/L (Fig.3). However, Burland and Edwards (1999) observed that nitrite partially inhibited the microorganisms responsible for benzene degradation, and resulted in a slower rate of benzene degradation.

2.4 Characteristics of the biodegradation rates

According to the data in Fig.2, the biodegradation rates at each initial substrate concentration could be calculated, and the results are listed in Fig.4.

It was found that in two of the examined substrates, namely toluene and ethylbenzene, the degradation rates increased with increasing the initial concentration within the range used in this study. In every experiment, toluene degraded faster than any other BTEX compounds. Toluene showed the highest rate of degradation and was the most favorable BTEX substrate for anaerobic degradation by the enriched bacteria, which was in agreement with other reports comparing the biodegradation of various BTEX compounds (Heider *et al*., 1998; Lovley, 1997). However, for benzene, *o*-xylene, *m*-xylene and *p*-xylene, the degradation rates increased with increasing the substrate concentration and reached maximum values at the substrate concentration of 100 mg/kg in soil. The degradation rates then decreased with the increase of substrate concentration, which indicated that the higher initial concentration of these substrates would be toxic to the augmented cultures and would inhibit the degradation ability. The high-to-low order of degradation rates for the soil samples were observed as toluene > ethylbenzene > *m*-xylene > *o*-xylene > benzene > *p*-xylene, which was similar to the order obtained in the previous experiments that were conducted in liquid medium (Dou *et al*., 2008). It seemed that benzene and *p*-xylene were more toxic than other BTEX compounds, especially *p*-xylene, whose special structure made it difficult to be biodegraded initiated by fumarate addition to the methyl group analogously to toluene, *o*-xylene, and *m*-xylene degradation (Boll *et al*., 2002). This finding was in good agreement with other studies reported in the literature (Da Silva and Alvarez, 2004; Kao and Borden, 1997; Langenhoff *et al*., 1996). By comparing the results of this study with our previous research (Dou *et al*., 2008), it could be concluded that BTEX degraded faster in soil than in liquid medium. One reason for this conclusion might be that soil provides a solid phase for better enriched mixed bacterial growth. Another possibility is that indigenous bacteria in soil enhance the degradation ability of the augmented bacteria, or there is collaboration between the indigenous bacteria and the augmented microorganisms (Essaid *et al*., 2003).

2.5 Anaerobic degradation of BTEX mixtures in soil

The biodegradation of BTEX mixtures with different initial concentrations in soil under nitrate reduction conditions was conducted, and the results are presented in Fig.5. When the initial concentration of BTEX mixtures in soil was 30 mg/kg, namely, the concentration of each substrate was approximately 5 mg/kg, all of the BTEX compounds could be degraded simultaneously to undetectable level within 10 d. Furthermore, the degradation rates for all the substrates were nearly in the same level as the presence of the single BTEX substrate. When the initial concentrations were increased up to 90 and 150 mg/kg, the BTEX substrates could be degraded completely after a period of 40 and 50 d, respectively. However, benzene and *p*-xylene showed a relatively slower degradation rate. When the BTEX mixtures concentration reached a value at 300 mg/kg, toluene, ethylbenzene, and *m*-xylene could be degraded to an undetectable level after 50 d. Whereas the degradation rates of benzene, *o*-xylene, and *p*-xylene were slow, benzene, *o*-xylene, and *p*-xylene were degraded with a lag phase of 20 d compared to toluene and ethylbenzene. For *o*-xylene, approximately 78% was degraded after 50 d degradation, whereas for benzene and *p*-xylene, only 31% and 48% was degraded throughout the experimental period. These results indicated that with lower initial concentrations of BTEX mixtures there was no apparent inhibitory effect on the degradation of these substrates. Alternatively, higher concentrations of BTEX mixtures inhibited degradation of these substrates, especially for benzene, *o*-xylene, and *p*-xylene (Prenafeta-Boldú *et al*., 2002).

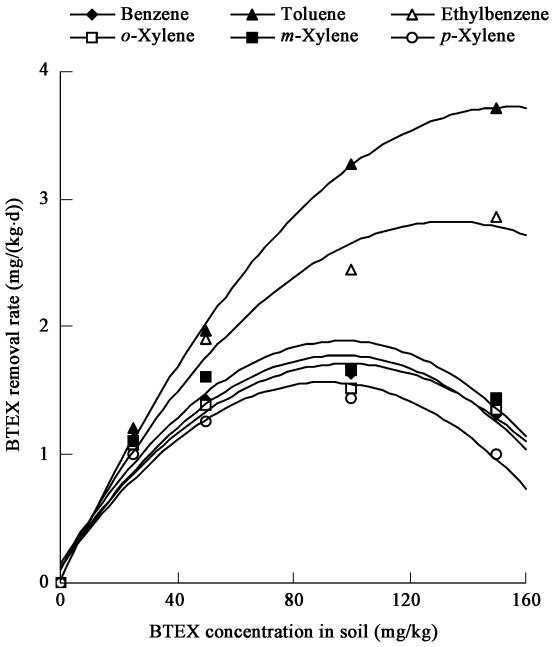

Fig. 4 Relationship between biodegradation rates and initial concentration of BTEX in soil.

2.6 Anaerobic degradation of BTEX in the presence of sodium acetate

Figure 6 presents the effects of sodium acetate (NaAc) on benzene degradation in soil (data on other BTEX compounds were similar to that on benzene and were not shown in this article). From Fig.6, it can be concluded

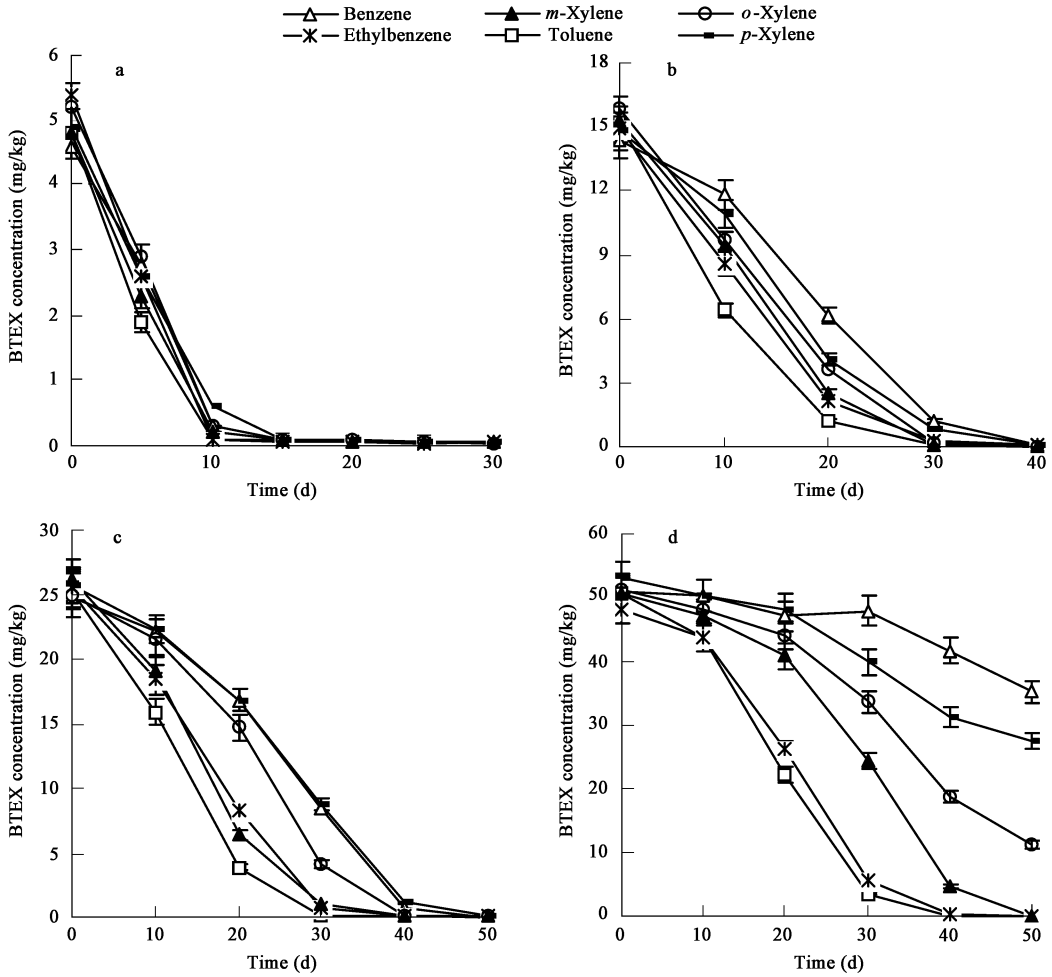

Fig. 5 Biodegradation curve of BTEX mixtures in soil under different initial concentrations. Initial BTEX mixtures concentration: (a) 30 mg/kg; (b) 90 mg/kg; (c) 150 mg/kg; (d) 300 mg/kg.

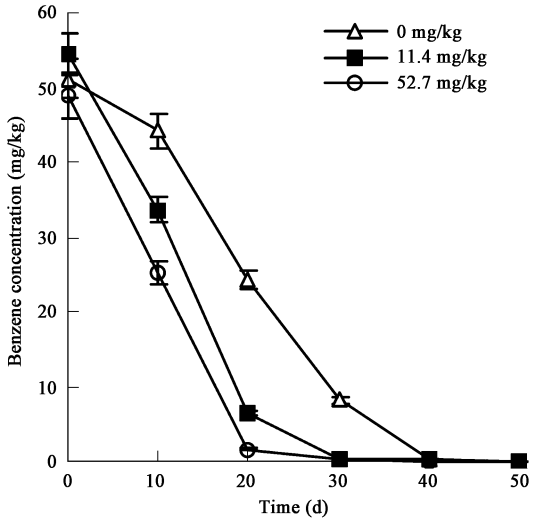

Fig. 6 Biodegradation curve of benzene under different NaAc concentrations in soil.

that benzene degradation was enhanced with the addition of NaAc compared to the control. This conclusion can be extrapolated to other BTEX compounds according to the experimental results in this study. Alvarez and Vogel (1991) reported that some substrates could enhance the degradation of others through the induction of enzymes or could act as a primary substrate stimulating microbial growth, thereby favoring the cometabolism of another compound. One possible explanation for the enhanced degradation rates in the presence of NaAc is that the easily available carbon source acts as a primary substrate promoting the growth of the enriched bacteria and thereby resulting in faster degradation of BTEX compounds. This thus favors the cometabolism of BTEX substrates. Cometabolism was an important technique for the enhancement of BTEX compounds because utilization of sodium acetate by the inoculated bacteria can directly and efficiently provide energy required for the synthesis of the new proteins. It is also possible that the stimulation of the degradation rates is through the induction of degradation enzymes by the presence of sodium acetate.

3 Conclusions

The enriched anaerobic BTEX-adapted mixed consortia were capable of biodegrading benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene, and *p*-xylene efficiently in soil under nitrate reducing conditions. Indigenous bacteria in the soil could stimulate the BTEX anaerobic biodegradation when the soil samples were augmented with the

enriched mixed bacteria. Toluene degraded faster than any other BTEX compounds in soil, and the degradation rates decreased with toluene > ethylbenzene > *m*-xylene > *o*-xylene> benzene > *p*-xylene. With lower initial concentrations of BTEX mixtures there was no apparent inhibitory effect on the degradation of these substrates, whereas higher initial concentrations of BTEX mixtures inhibited their degradation, especially of benzene, *o*-xylene, and *p*-xylene. BTEX degradation was enhanced when sodium acetate was simultaneously present in soil. The results obtained in the present study suggested that the degradation of BTEX in the soil augmented with enriched anaerobic BTEX-adapted mixed consortia appeared to be a feasible method to remediate BTEX contaminated soil. Further study on the feasibility of the enriched bacteria under field conditions is required to promote the remediation of BTEX contaminated soil under anaerobic conditions.

Acknowledgements

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