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Anaerobic BTEX biodegradation linked to nitrate and sulfate reduction

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Abstract

Effective anaerobic BTEX biodegradation was obtained under nitrate and sulfate reducing conditions by the mixed bacterial consortium that were enriched from gasoline contaminated soil. Under the conditions of using nitrate or sulfate as reducing acceptor, the degradation rates of the six tested substrates decreased with toluene > ethylbenzene > *m*-xylene > *o*-xylene > benzene > *p*-xylene. The higher concentrations of BTEX were toxic to the mixed cultures and led to reduce the degradation rates of BTEX. Benzene and *p*-xylene were more toxic than toluene and ethylbenzene. Nitrate was a more favorable electron acceptor compared to sulfate. The measured ratios between the amount of nitrate consumed and the amount of benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene, *p*-xylene degraded were 9.47, 9.26, 11.14, 12.46, 13.36 and 13.02, respectively. The measured ratios between sulfate reduction and BTEX degradation were 3.51, 4.33, 4.89, 4.81, 4.86 and 4.76, respectively, which were nearly the same to theoretical ones, and the relative error between the measured and calculated ratios was less than 10%.

Keywords: BTEX; Anaerobic biodegradation; Nitrate reduction; Sulfate reduction

1. Introduction

Leakage of gasoline and jet fuel from underground fuel storage vessels or spillage during the transportation can lead to the contamination of soil and groundwater with monoaromatic hydrocarbons (benzene, toluene, ethylbenzene and three isomers of xylene, or collectively referred to BTEX). Due to their relatively high water solubility, BTEX will migrate through groundwater systems and contaminate drinking water supplies far from their source. The fate of BTEX in contaminated soil and groundwater has been the focus of study in recent years because of their high toxicity and carcinogenicity [1].

The BTEX compounds are relatively stable due to the aromaticity of their benzene ring and the lack of reactive functional groups. Among all remediation technologies for treating BTEX-contaminated groundwater and soil, bioremediation, the use of microbial degradation processes to reduce contaminant concentrations, appears to be an economical, energy efficient and environmentally sound approach. Under aerobic conditions, all of the BTEX compounds are rapidly biodegraded [2]. However, groundwater and soil environments 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 mechanisms and depend on the activity of bacteria capable of metabolizing hydrocarbons [3,4]. With few exceptions, all BTEX compounds have been shown to be degraded by microorganisms using various electron acceptors, for example nitrate [4–6], sulfate [4,7,8], Fe^{3+} [8,9] and manganese [9]. Among all the BTEX, benzene was difficult to be biodegraded, for example, some studies demonstrated that benzene was recalcitrant during the reduction of nitrate [10–12].

Significant advances have been made towards understanding the genetic and biochemical bases of BTEX biodegradation [13–15]. Many pure cultures of anaerobic organisms that can degrade BTEX have been described [11,16,17], and many catabolic pathways have been elucidated [18,19]. However, little attention has been given to the degradation characteristic of BTEX by the same enriched bacteria under different electron acceptor conditions. Furthermore, to help develop anaerobic bioremediated clean-up technologies, systematic studies are required for understanding the degradation performance of

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Table 1
Experimental design of the degradation tests using nitrate as electron acceptor

Substrate	With 1 mL cultures								Without cultures
	Nitrate (0 mg/L)	Nitrate (100 mg/L)	Nitrate (100 mg/L)	Nitrate (250 mg/L)	Nitrate (500 mg/L)	Nitrate (1000 mg/L)	Nitrate (1000 mg/L)	Nitrate (1000 mg/L)	Nitrate (250 mg/L)
Benzene (mg/L)	25	0	10	25	50	80	100	150	25
Toluene (mg/L)	25	0	10	25	50	80	100	150	25
Ethylbenzene (mg/L)	25	0	10	25	50	80	100	150	25
o-Xylene (mg/L)	25	0	10	25	50	80	100	150	25
m-Xylene (mg/L)	25	0	10	25	50	80	100	150	25
p-Xylene (mg/L)	25	0	10	25	50	80	100	150	25

BTEX under variable electron acceptor conditions. In addition, information regarding the rates and effectiveness of individual substrates for remediation of BTEX contaminated sites is essential.

The aim of this study was to investigate the degradation performance of BTEX under various electron acceptor reducing conditions in the presence of a mixed cultures enriched from gasoline contaminated soil. In this work, it was shown that BTEX could be degraded under anaerobic conditions in the presence of nitrate and sulfate.

2. Materials and methods

2.1. Mixed bacterial consortium isolation conditions

For enrichment of anaerobic BTEX-degrading bacteria, 160 g soils from gasoline contaminated sites were initially mixed with 100 mL of the mineral medium. The composition of the medium used was the following: $NaNO_3$ (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), $CaCl_2{\cdot}2H_2O$ (0.05 g/L). The medium was supplemented with 100 μL each component of benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene, *p*-xylene. The incubation was performed at 20 °C for 5 months in an anaerobic chamber that containing pure dinitrogen 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), Na_2SO_4 (1.5 g/L). The medium was supplemented with 0.1% of $Na_2S{\cdot}9H_2O$, vitamin (1% [vol/vol]) and trace solutions (1% [vol/vol]). In addition, 2 μL each component of benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene, *p*-xylene were added as pure stock with a 10 μL syringe. After 7–9 days, 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, the mixed bacterial consortia capable of anaerobic oxidizing BTEX were obtained. For maintenance, the enriched mixed bacteria were stored at 4 °C, and transferred every 6–8 weeks.

2.2. Experimental set-up

Microcosms were prepared to determine the ability of the enriched cultures to anaerobic degrade BTEX. Two groups of experiments were conducted with nitrate and sulfate as electron acceptor, respectively. Each group included six different substrates: benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene and *p*-xylene. For each substrate, six different initial concentrations of approximately 10, 25, 50, 80, 100, and 150 mg/L were used. In order to account for abiotic BTEX degradation, control experiments containing no electron acceptor, no microorganisms were run in parallel. In addition, microcosms with electron acceptor and microorganisms but without BTEX substrates were also prepared. Table 1 and Table 2 summarized the experimental design.

Anaerobic degradation experiments were done in serum bottles with the volume of 50 mL. The mineral medium was purged for 3.5 h using pure dinitrogen gas, the constituents of the mineral medium were as below: NH_4Cl (1.0 g/L), KH_2PO_4 (1.0 g/L), $Mg\,Cl_2$ (0.1 g/L), $CaCl_2{\cdot}2H_2O$ (0.05 g/L). In addition, 0.1% of Na_2S9H_2O, vitamin (1% [vol/vol]) and trace solutions (1% [vol/vol]) were also added to the mineral medium. The pH of the medium was between 6.8 and 7.2. 33 mL of the minimal medium were poured in serum bottle, and then 2 mL of

Table 2
Experimental design of the degradation tests using sulfate as electron acceptor

Substrate	With 1 mL cultures								Without cultures
	Sulfate (0 mg/L)	Sulfate (150 mg/L)	Sulfate (150 mg/L)	Sulfate (150 mg/L)	Sulfate (500 mg/L)	Sulfate (500 mg/L)	Sulfate (800 mg/L)	Sulfate (800 mg/L)	Sulfate (150 mg/L)
Benzene (mg/L)	25	0	10	25	50	80	100	150	25
Toluene (mg/L)	25	0	10	25	50	80	100	150	25
Ethylbenzene (mg/L)	25	0	10	25	50	80	100	150	25
o-Xylene (mg/L)	25	0	10	25	50	80	100	150	25
m-Xylene (mg/L)	25	0	10	25	50	80	100	150	25
p-Xylene (mg/L)	25	0	10	25	50	80	100	150	25

the mixed cultures were added to get the initial concentration of inoculated bacteria of 5×10^7 cells/mL. The electron acceptors (NO_3^- or SO_4^{2-}) and BTEX were added to each mineral medium to a final concentration as the experimental design. All the microcosms were prepared in an anaerobic glovebox which was filled with pure dinitrogen gas, and the serum bottle was sealed with a composite stopper. The microcosms were incubated at 20 °C in darkness. Samples were periodically collected to measure the concentrations of BTEX, nitrate, nitrite, sulfate and sulfide. All the experiments were conducted in triplicate. Each data represented the mean of three measurements, and the standard deviation was less than 10%.

2.3. Analytical methodology

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

Nitrate, nitrite and sulfate were analyzed by ion chromatography (Dionex DX100), using an Iopac ASI4 (4 mm × 250 mm) analytical column, the eluent was Na_2CO_3–$NaHCO_3$ (3.5 mmol/L, 1.0 mmol/L), and the flow rate was 1.2 ml/min. Sulfide was determined by the methylene blue formation reaction in a colorimetric microassay in a spectrophotometer at 665 nm.

3. Results and discussion

3.1. BTEX degradation in microcosms in control experiments

Fig. 1 showed the disappearance of toluene under the conditions of without electron acceptor, without microorganisms, with microorganisms and nitrate, with microorganisms and sulfate (data of other substrates were similar to toluene and not shown in this paper). As can be seen, toluene concentration decreased from 25 mg/l to below 0.2 mg/L was observed in the presence of nitrate (or sulfate) and microorganisms. While for the control experiments without microorganisms or without electron acceptor, the toluene concentration only decreased from 24.9 to 23.2 and 23.0 mg/L over the first 10 days, respectively, while after 10 days, there was no further degradation of toluene even after more than 57 days of incubation. This decrease was probably due to some of the toluene being irreversibly adsorbed to the inside walls of the microcosm bottles. All the results of the control experiments showed that the BTEX loss was less than 10 % of the initial concentration after 67 days of incubation.

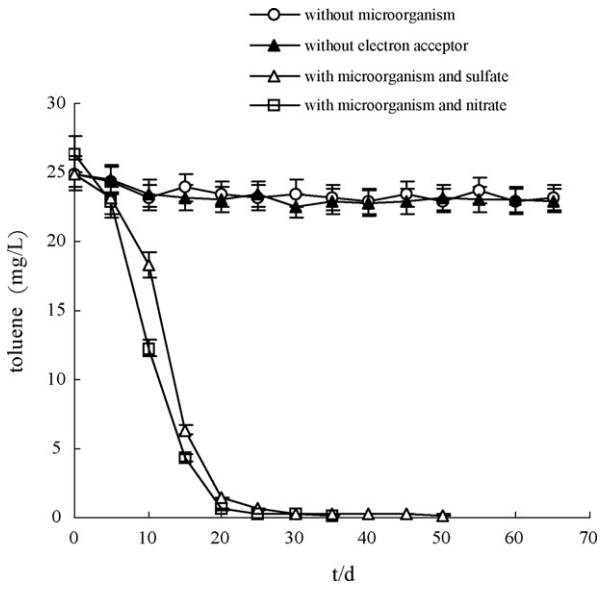

Fig. 1. Control experiment results in the presence of the substrate of toluene.

3.2. Degradation curves of BTEX under various initial concentration conditions

Figs. 2 and 3 showed the results of anaerobic biodegradation of BTEX substrates with various initial concentrations under nitrate and sulfate reducing conditions, respectively. The data in Figs. 2 and 3 demonstrated all the substrates used in this study could be served as electron donors and carbon sources for the enriched cultures, and could be decreased to non-detectable levels within a period of 60 days incubation when the initial concentration was below 100 mg/L. The results of the experiments showed that BTEX anaerobic degraders could potentially remediate a site contaminated with BTEX using nitrate and sulfate as terminal electron acceptors.

3.3. Variation curve of nitrate, nitrite, sulfate and sulfide concentrations during incubation

Fig. 4 showed the variation of nitrate and nitrite concentrations, Fig. 5 showed the variation of sulfate and sulfide concentrations under various benzene initial concentrations. Similar results were also observed with toluene, ethylbenzene, *o*-xylene, *m*-xylene and *p*-xylene as substrates (data not shown).

Compared between Figs. 2(a) and 4, it could be concluded that nitrate reduction went hand-in-hand with benzene degradation, which indicated that benzene degradation was coupled to nitrate reduction and was due to biological process. At the same time, nitrite was detected as the intermediate compound. While under the condition of not adding the enriched cultures or not amending BTEX substrate, no loss of nitrate was observed during the whole incubations, which was due to the fact that the reduction of nitrate could not be occurred for lack of the biodegradation cultures or the carbon sources. As Fig. 4 showed, nitrite was accumulated during the reduction of nitrate. It was interesting to find that the accumulation of nitrite did not inhibit the degradation of BTEX even if the concentration of nitrite was over 350 mg/L. However, Burland and Edwards [20] observed that nitrite partially inhibited the microorganisms responsible for benzene degradation, and resulted in a slower rate of benzene degradation.

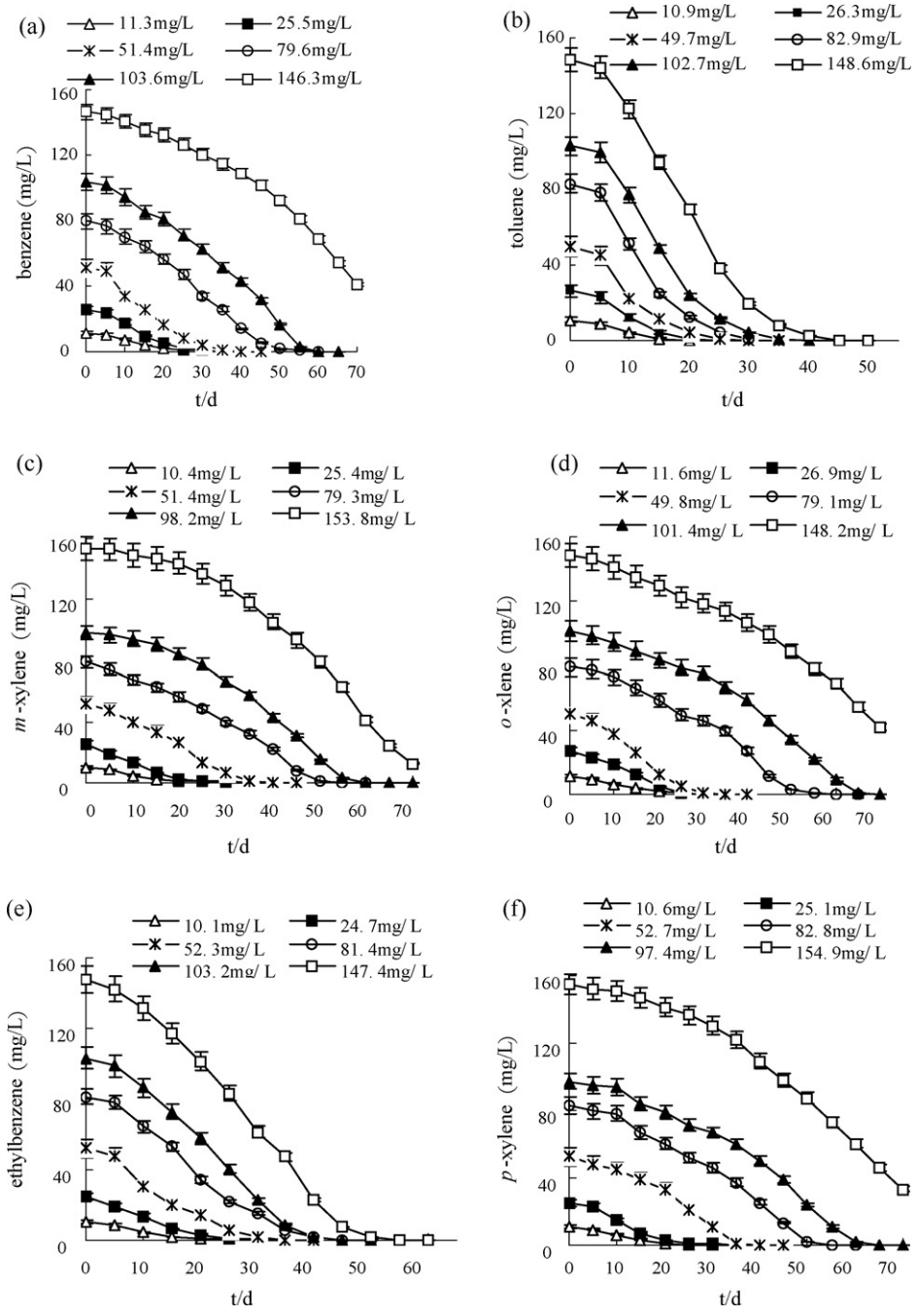

Fig. 2. Anaerobic biodegradation curves of BTEX using nitrate as electron acceptor. (a) Benzene, (b) toluene, (c) ethylbenzene, (d) *o*-xylene, (e) *m*-xylene, (f) *p*-xylene.

The same phenomena were observed under the condition of sulfate reducing condition, the reduction of sulfate did not occur when BTEX was not available or in sterile controls that did contain BTEX. Inspection of Fig. 5 it could be concluded that sulfide produced was lower than sulfate consumed, the reason may be that some of the sulfide were in the form of H_2S, and which could not be analyzed in the solution. From Figs. 3(a) and 5 it could be found that there was a good relationship between benzene degradation and the reduction of sulfate, and it could also be concluded that the accumulating of sulfide in the medium was not toxic to the enriched bacteria, the reason for which may be that the mixed bacteria were enriched from the medium that contained sulfide about 130 mg/L.

3.4. Characteristics of the biodegradation rates

The biodegradation rates at each initial substrate concentration were calculated under nitrate and sulfate reducing conditions and were shown in Fig. 6.

As can be seen from Fig. 6, under both nitrate and sulfate reducing conditions, (1) for benzene, *o*-xylene and *p*-xylene,

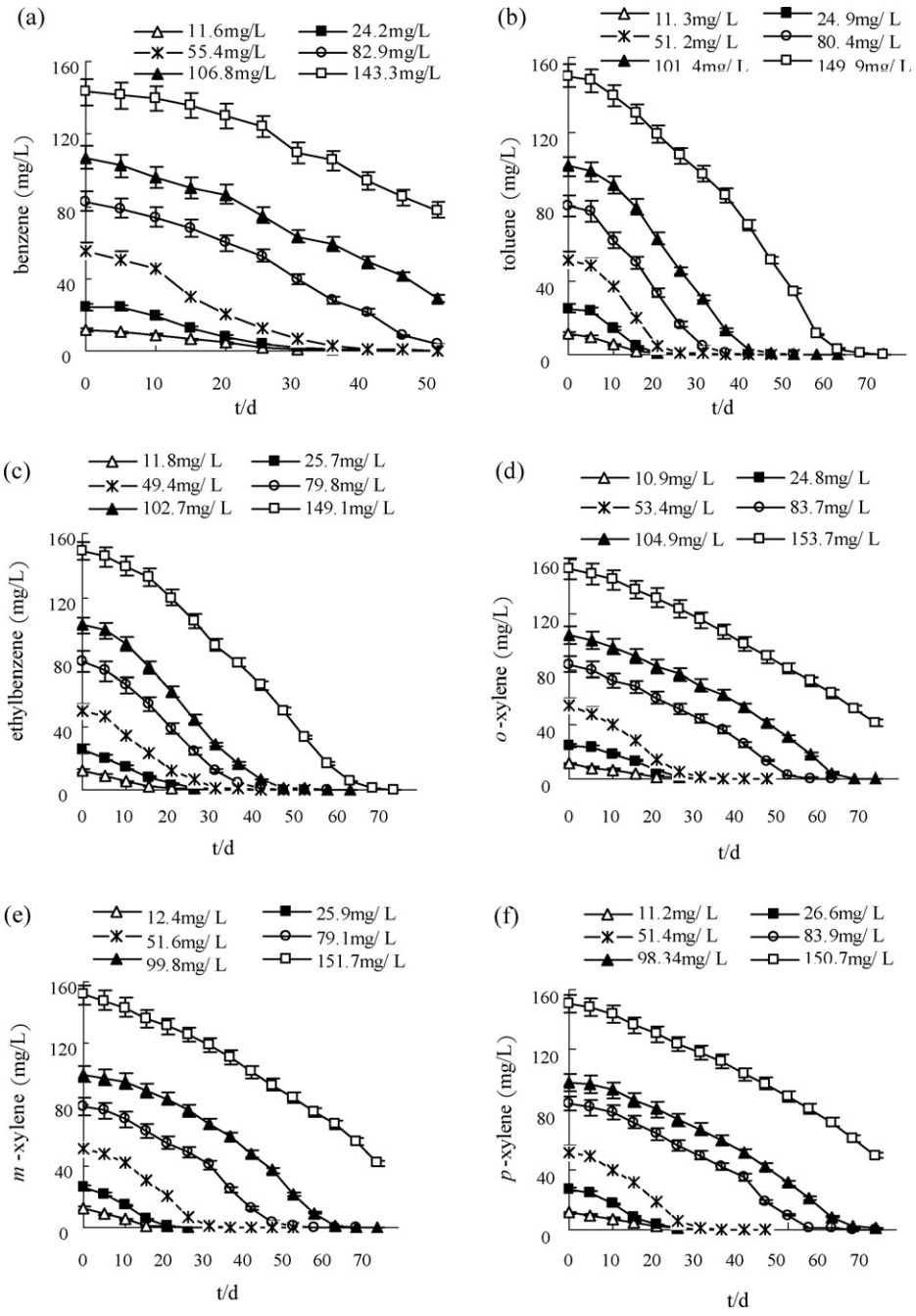

Fig. 3. Anaerobic biodegradation curves of BTEX using sulfate as electron acceptor. (a) Benzene, (b) toluene, (c) ethylbenzene, (d) *o*-xylene, (e) *m*-xylene, (f) *p*-xylene.

the degradation rates increased with increasing the substrate concentration and reached maximum values at the substrate concentration of 50 mg/L, and then decreased with the increase of substrate concentration, indicating substrate inhibition; (2) for *m*-xylene, the mixed cultures decreased the degradation rate when the substrate concentration was more than 80 mg/L; (3) for toluene and ethylbenzene, the degradation rates increased with increasing substrate concentration and then decreased after reaching a maximum value at 100 mg/L. The degradation rate of BTEX reached a maximum value when the initial concentrations were higher than a level, which indicated the higher initial concentrations of the substrates would be toxic to the cultures and inhibit the degradation ability.

Under both of the terminal electron acceptors conditions, the high-to-low degradation rates of the six tested substrates were in the order of toluene > ethylbenzene > *m*-xylene > *o*-xylene > benzene > *p*-xylene. Toluene showed the highest rate of degradation and was the most favorable BTEX hydrocarbon for anaerobic degradation by the enriched cultures, which was consistent with other reports comparing the biodegradation of various BTEX compounds [3,21]. Anaerobic biodegradation of benzene and *p*-xylene was also achieved when either

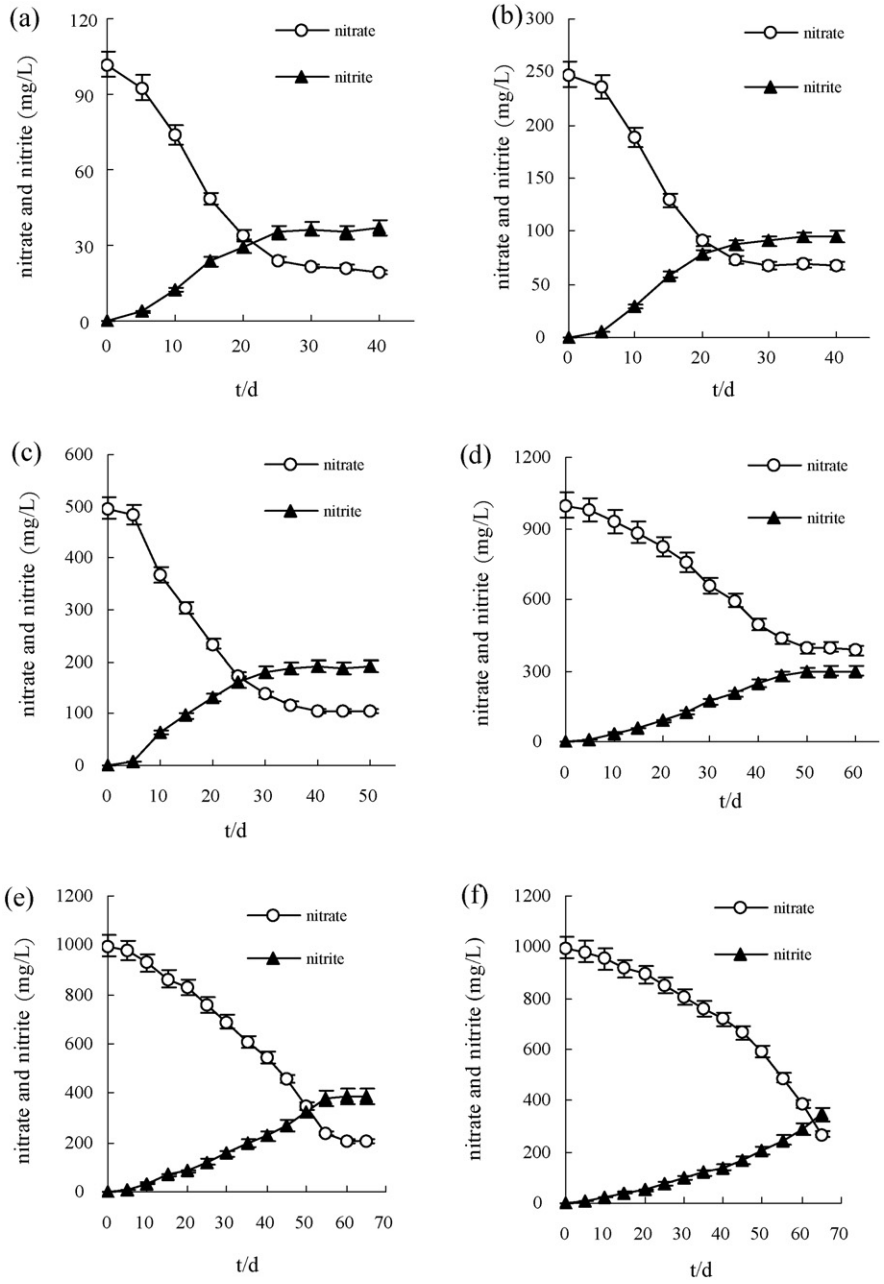

Fig. 4. Variation curve of nitrate and nitrite during anaerobic biodegradation of benzene. (a) Initial benzene concentration of 10 mg/L, (b) initial benzene concentration of 25 mg/L, (c) initial benzene concentration of 50 mg/L, (d) initial benzene concentration of 80 mg/L, (e) initial benzene concentration of 100 mg/L, (f) initial benzene concentration of 150 mg/L.

nitrate or sulfate was provided as a terminal electron acceptor, but at relatively lower rates compared to toluene and ethylbenzene. It appeared that benzene and *p*-xylene were more toxic than toluene and ethylbenzene, this finding was generally in good agreement with other studies reported in the literature [12,22,23].

From Fig. 6 it could be observed that the rates of BTEX disappearance in terms of nitrate reduction were higher compared to sulfate. According to thermodynamic principles, the higher energy yielding reaction would be occurred when utilizing nitrate as electron acceptor compared to sulfate, so microbes utilized nitrate first, followed sulfate [24]. Therefore, nitrate was a more favorable electron acceptor than sulfate for the mixed cultures used in this study.

3.5. Stoichiometry between the consumption of electron acceptors and BTEX degradation

Given that there was no cell growth, the theoretical stiochiometric equations for anaerobic oxidation of BTEX to carbon dioxide with nitrate and sulfate as the electron acceptors could be stated:

$$C_6H_6 + 6NO_3^- + 6H^+ \rightarrow 6CO_2 + 3N_2 + 6H_2O \quad (1)$$

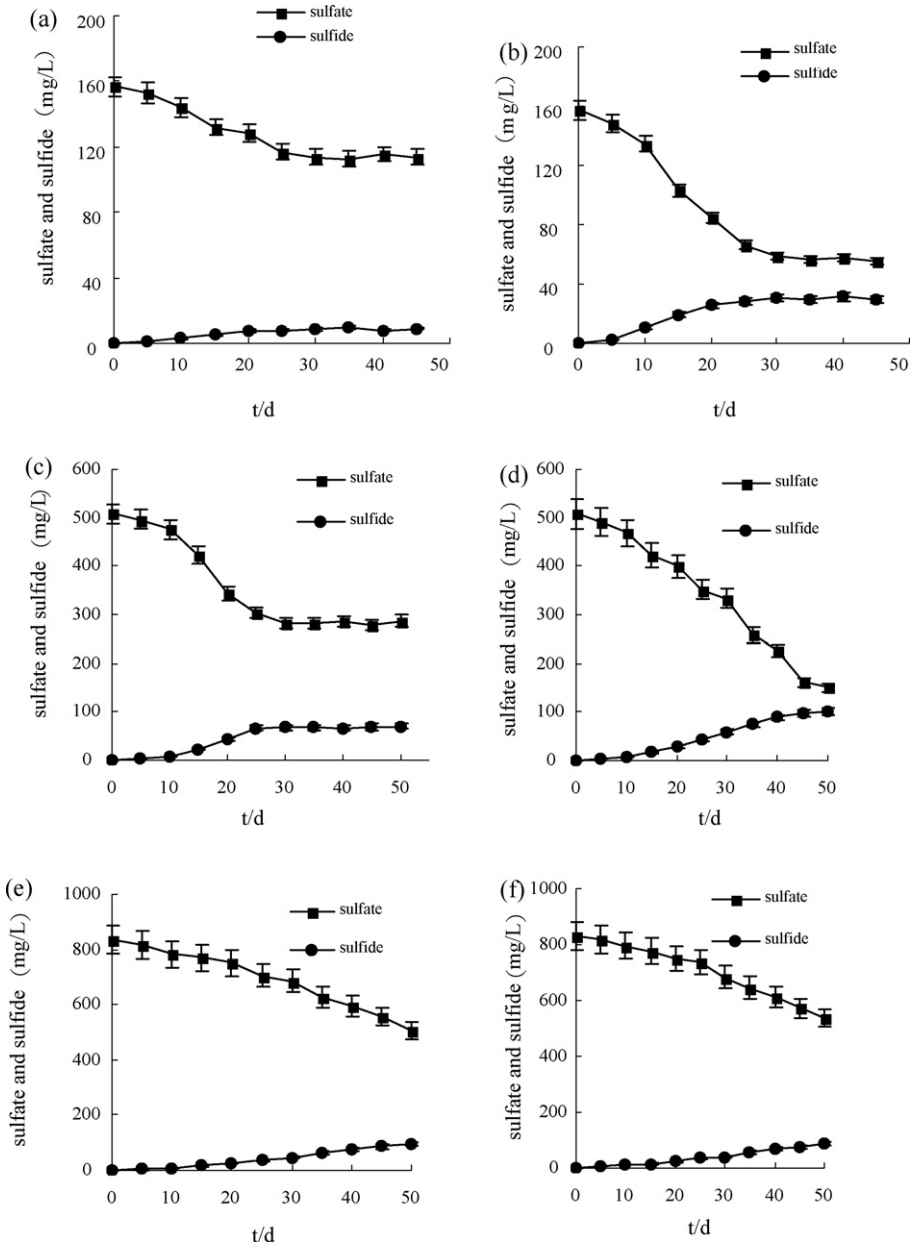

Fig. 5. Variation curve of sulfate and sulfide during anaerobic biodegradation of benzene. (a) Initial benzene concentration of 10 mg/L, (b) initial benzene concentration of 25 mg/L, (c) initial benzene concentration of 50 mg/L, (d) initial benzene concentration of 80 mg/L, (e) initial benzene concentration of 100 mg/L, (f) initial benzene concentration of 150 mg/L.

$$C_7H_8 + 7.2NO_3^- + 7.2H^+ \rightarrow 7CO_2 + 3.6N_2 + 7.6H_2O \quad (2)$$

$$C_8H_{10} + 8.4NO_3^- + 8.4H^+ \rightarrow 8CO_2 + 4.2N_2 + 9.2H_2O \quad (3)$$

$$C_6H_6 + 3.75SO_4^{2-} \rightarrow 6CO_2 + 3.75S^{2-} + 3H_2O \quad (4)$$

$$C_7H_8 + 4.5SO_4^{2-} \rightarrow 7CO_2 + 4.5S^{2-} + 4H_2O \quad (5)$$

$$C_8H_{10} + 5.25SO_4^{2-} \rightarrow 8CO_2 + 5.25S^{2-} + 5H_2O \quad (6)$$

Based on Eqs. (1)–(6), the theoretical ratios of electron acceptor consumption to BTEX degradation could be obtained, and they were listed in Table 3. The measured ratios could be calculated based on the variation of electron acceptor and BTEX between each sampling intervals during the incubation, and the calculated results were illustrated in Table 3.

Table 3
Theoretical and measured ratios between electron acceptors and BTEX consumption

Substrates	NO_3^-		SO_4^{2-}	
	Theoretical ratios	Measured ratios	Theoretical ratios	Measured ratios
Benzene	6	9.47 ± 0.45	3.75	3.51 ± 0.26
Toluene	7.2	9.26 ± 0.42	4.50	4.33 ± 0.32
Ethylbenzene	8.4	11.14 ± 0.46	5.25	4.89 ± 0.36
o-Xylene	8.4	12.46 ± 0.76	5.25	4.81 ± 0.24
m-Xylene	8.4	13.36 ± 0.53	5.25	4.86 ± 0.33
p-Xylene	8.4	13.02 ± 0.38	5.25	4.76 ± 0.31

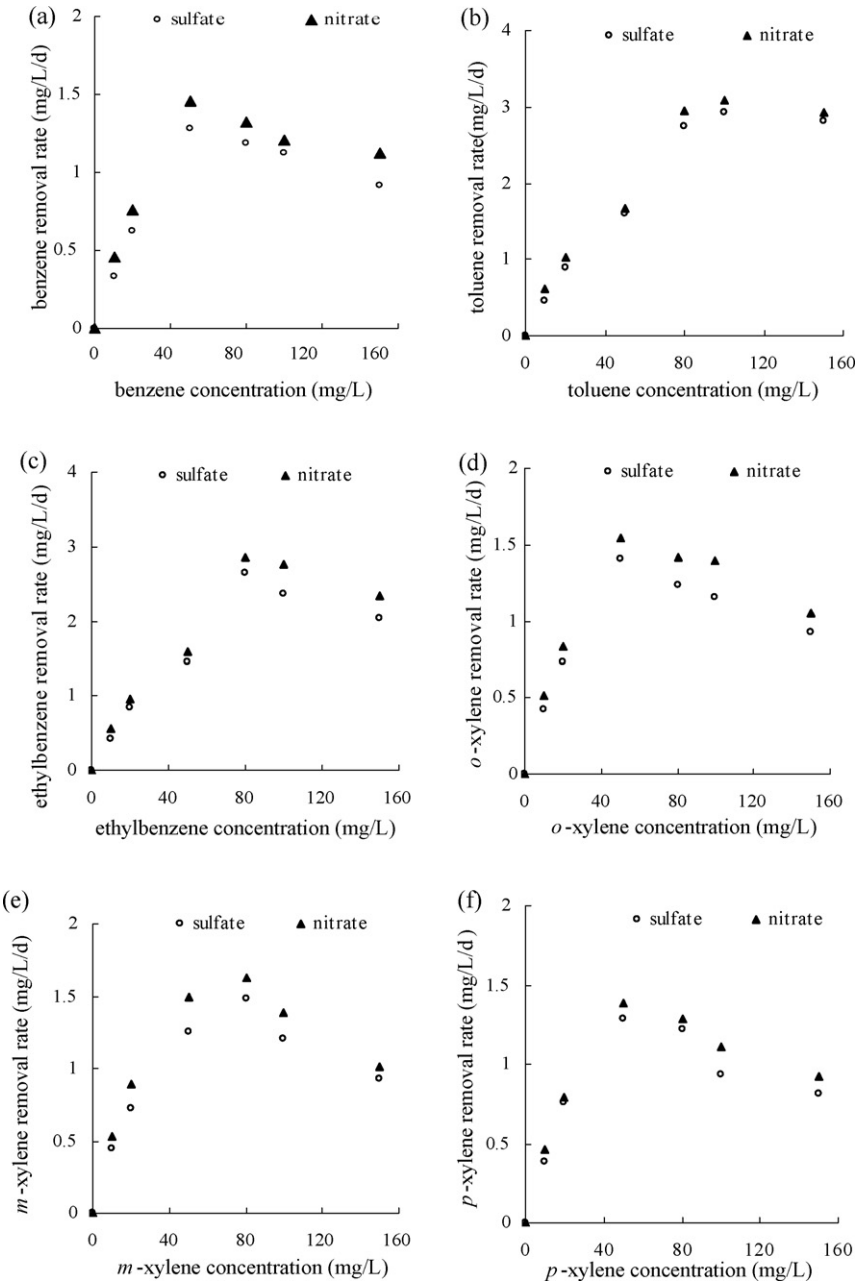

Fig. 6. Relationship between biodegradation rates and concentration of BTEX under various electron acceptor conditions. (a) Benzene, (b) toluene, (c) ethylbenzene, (d) *o*-xylene, (e) *m*-xylene, (f) *p*-xylene.

The data in Table 3 showed that, under nitrate reducing condition, the measured ratios between electron acceptors and BTEX consumption were higher than the theoretical ones. The reason for this phenomenon was that the theoretical ratios were calculated according to the assumption that nitrate was ultimately reduced to dinitrogen gas, in fact it was not in this case. From Fig. 4, it could be concluded that the accumulation of nitrite was occurred, which supported the fact that nitrate was not completely transferred to dinitrogen gas, and the corresponding stiochiometric equations were as following:

$$C_6H_6 + 15NO_3^- \rightarrow 6CO_2 + 15NO_2^- + 3H_2O \quad (7)$$

$$C_7H_8 + 18NO_3^- \rightarrow 7CO_2 + 18NO_2^- + 4H_2O \quad (8)$$

$$C_8H_{10} + 21NO_3^- \rightarrow 8CO_2 + 21NO_2^- + 5H_2O \quad (9)$$

Based on the Eqs. (7)–(9), the theoretical ratios between the consumption of nitrate and the degradation of C_6H_6, C_7H_8, C_8H_{10} were 15, 18 and 21, respectively, which was higher than the corresponding measured values. The reason for this phenomenon was that part of the nitrite was further reduced to dinitrogen gas. From Fig. 4 it could also be observed that the production of nitrite was lower than the consumption of nitrate, which supported the fact that part of the nitrite was reduced to dinitrogen gas.

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

Table 4
Electron balance of BTEX consumption and nitrate reduction

Substrates	Amount of substrate consumed (μmol)	Amount of electrons from substrate 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)
Benzene	11.39	341.79	74.98	28.42	292.03
Toluene	9.94	357.73	60.20	33.55	288.17
Ethylbenzene	8.14	341.71	50.17	37.62	288.43
o-Xylene	8.86	371.94	80.11	32.23	321.39
m-Xylene	8.36	351.00	85.40	20.80	274.82
p-Xylene	8.26	346.84	78.09	25.88	285.57

Table 5
Electron balance of BTEX consumption and sulfate reduction

Substrates	Amount of substrate consumed (μmol)	Amount of electrons from substrate consumed (μmol)	Amount of SO_4^{2-} reduced to S^{2-} (μmol)	Amount of electrons consumed by SO_4^{2-} reduction (μmol)
Benzene	10.76	322.94	38.05	304.37
Toluene	9.40	338.42	41.75	334.03
Ethylbenzene	8.43	354.19	40.45	323.61
o-Xylene	8.12	341.01	40.71	325.69
m-Xylene	8.50	357.10	41.53	332.28
p-Xylene	8.72	366.25	41.93	335.40

electrons. The oxidation 1 mol of benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene and *p*-xylen yield 30 mol, 36 mol, 42 mol, 42 mol, 42 mol and 42 mol electrons, respectively. The electron balances of BTEX consumption and nitrate reduction under the condition of initial BTEX concentration of 25 mg/L were shown in Table 4. As can be seen from Table 4, the amount of electrons that can be theoretically derived from the amount of BTEX consumed was 16 to 28% greater than the amount of electrons required for nitrate reduced to nitrite or dinitrogen gas. This deviation could be explained by partial utilization of the BTEX for cell synthesis.

The reduction 1 mol of SO_4^{2-} to S^{2-} needs 8 mol electrons. The electron balances of BTEX consumption and sulfate reduction under the condition of initial BTEX concentration of 25 mg/L were shown in Table 5. As shown in Table 5, the amount of electrons that can be theoretically derived from the amount of BTEX consumed was 1–9% greater than the amount of electrons required for sulfate reduction. At the same time, inspection of Table 3 indicated that, under sulfate reducing condition, the measured ratios were slightly lower than the theoretical ratios, and the relationships between the measured and theoretical ratios for benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene and *p*-xylene were 96, 96, 93, 91, 92 and 89%, respectively. One possible explanation for this may be that in this experiment the anaerobic oxidations of BTEX were also coupled to cultures growth and converted into cell materials. The fact that the relative error between the theoretical and the calculated ratios was less than 10%, supported the theoretical stoichiometry and the hypothesis that BTEX were mineralized to carbon dioxide and water.

4. Conclusions

Effective anaerobic BTEX biodegradation was observed under nitrate and sulfate reducing conditions by the mixed bacterial consortium derived from the gasoline contaminated soil. Under both nitrate and sulfate as the terminal electron acceptors conditions, benzene and *p*-xylene were more toxic than toluene and ethylbenzene, the degradation rates decreased with toluene > ethylbenzene > *m*-xylene > *o*-xylene > benzene > *p*-xylene. Nitrate was a more favorable electron acceptor compared to sulfate. The measured ratios of the amount of nitrate consumed to the amount of BTEX degraded were between the theoretical ratios that were calculated by the assumption that nitrate were reduced to nitrogen gas and nitrite. The measured ratios between sulfate reduction and BTEX degradation were nearly the same to theoretical ones, the relative error between the measured and calculated ratios was less than 10%.

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