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ScienceDirect
Journal of Environmental Sciences 19(2007) 1049–1054
JOURNAL OF ENVIRONMENTAL SCIENCES
ISSN 1001-0742
CN 11-2629/X
www.jesc.ac.cn

Anaerobic biodegradation of benzene series compounds by mixed cultures based on optional electronic acceptors

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Received 29 November 2006; revised 16 January 2007; accepted 29 January 2007

Abstract

A series of batch experiments were performed using mixed bacterial consortia to investigate biodegradation performance of benzene, toluene, ethylbenzene and three xylene isomers (BTEX) under nitrate, sulfate and ferric iron reducing conditions. The results showed that toluene, ethylbenzene, *m*-xylene and *o*-xylene could be degraded independently by the mixed cultures coupled to nitrate, sulfate and ferric iron reduction. Under ferric iron reducing conditions the biodegradation of benzene and *p*-xylene could be occurred only in the presence of other alkylbenzenes. Alkylbenzenes can serve as the primary substrates to stimulate the transformation of benzene and *p*-xylene under anaerobic conditions. Benzene and *p*-xylene are more toxic than toluene and ethylbenzene, under the three terminal electron acceptors conditions, the degradation rates decreased with toluene > ethylbenzene > *m*-xylene > *o*-xylene> benzene > *p*-xylene. Nitrate was a more favorable electron acceptor compared to sulfate and ferric iron. The ratio between sulfate consumed and the loss of benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene, *p*-xylene was 4.44, 4.51, 4.42, 4.32, 4.37 and 4.23, respectively; the ratio between nitrate consumed and the loss of these substrates was 7.53, 6.24, 6.49, 7.28, 7.81, 7.61, respectively; the ratio between the consumption of ferric iron and the loss of toluene, ethylbenzene, *o*-xylene, *m*-xylene was 17.99, 18.04, 18.07, 17.97, respectively.

Key words: benzene, toluene, ethylbenzene and three xylene isomers (BTEX); anaerobic biodegradation; nitrate reduction; sulfate reduction; ferric iron reduction

Introduction

Mono-aromatic hydrocarbons such as benzene, toluene, ethylbenzene and three isomers of xylene (BTEX) are the main constituents of gasoline, diesel fuel, creosote and the waste product of coal gasification. Due to leakage of underground storage tanks and pipelines, accidental spills at production wells and accidents during transport, these compounds have become the most frequently encountered soil and groundwater contaminants. BTEX often have the greatest environmental impact due to their high water solubility and mobility allowing them to migrate through groundwater systems (Coates *et al*., 2002). BTEX contamination is especially problematic when the underlying aquifer serves as a domestic water source. Not only can BTEX produce an undesirable and unpalatable odor, but also these compounds are toxic.

Among all remediation technologies for treating BTEX-contaminated groundwater and soil, bioremediation appears to be an economical, energy efficient and environmentally sound approach. In general, aerobic biodegradation is considered much faster than anaerobic processes (Chiang *et al*., 1989), in certain cases, it has been successfully applied in bioremediation process. However, in subsurface soil and groundwater environments, oxygen may be limiting because aerobic microorganisms consumed 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 under anaerobic conditions (Lovley, 1997). Field and laboratory microcosm studies have shown that anaerobes can degrade BTEX using various electron acceptors, for example nitrate (Burlanl and Edwards, 1999; Coates *et al*., 2001), Fe^{3+} (Lovley *et al*., 1996), sulfate (Coates *et al*., 1996) and manganese (Villatoro-Monzón *et al*., 2003).

Significant advances have been made towards understanding the genetic and biochemical bases of BTEX biodegradation (Beller and Spormann, 1998; Rabus and Heider, 1998; Leutwein and Heider, 1999). However, little attention has been given to the differences in the ability to degrade BTEX by the same enriched bacteria under different electron acceptor conditions. Therefore to help develop anaerobic bioremediated clean-up technologies, systematic studies are required for a mechanistic understanding of the complex interplay on degrading BTEX under variable

Project supported by the National Basic Research Program (973) of China (No. 2006CB403400). *Corresponding author.
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electron acceptor conditions. Furthermore, information regarding the rates and effectiveness of individual substrates for *in situ* 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 a periodically submerged soil. In this work, it was shown that BTEX could be degraded under strictly anaerobic conditions in the presence of nitrate, sulfate and ferric iron.

1 Materials and methods

1.1 Mixed bacterial consortium isolation conditions

Anaerobic BTEX-degrading bacteria were enriched from sediments of a BTEX contaminated soil under nitrate, sulfate and ferric iron reducing conditions in a mineral medium in 250-ml glass bottles. Enrichment bottles were sealed with Teflon-lined Mininert screw caps and incubated at 20°C in an anaerobic chamber that containing pure nitrogen gas. BTEX and electron acceptors were amended twice per month to avoid substrates depletion. After 7 months, the mixed bacterial consortia capable of oxidizing BTEX were obtained.

1.2 Experimental set-up

Microcosms were prepared to determine the ability of the isolated cultures to degrade BTEX. Seven groups of experiments were conducted with benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene, *p*-xylene and the mixtures of them as a substrate, respectively. Each group included three different electron acceptors: nitrate, sulfate and ferric iron. In order to account for abiotic BTEX degradation, controls containing no electron acceptor, no microorganisms were run in parallel. In addition, microcosms without BTEX substrates were also prepared. Table 1 summarizes the experimental design. Transfers were done by adding 1 ml of mixed cultures to 9 ml of a minimal medium in sterile 20-ml serum bottles. The mineral medium contained the following constituents: NH_4Cl (1.0 g/L), KH_2PO_4 (1.0 g/L), $MgCl_2$ (0.1 g/L), $CaCl_2{\cdot}2H_2O$ (0.05 g/L). In addition, 0.1% of $Na_2S{\cdot}9H_2O$ (2.5%, w/v), vitamin (1%, v/v) and trace solutions (1%, v/v) were also added. BTEX and electron acceptors were added to each microcosm to a final concentration as the experimental design. All the microcosms were prepared in an anaerobic glovebox which was full of pure nitrogen gas, and the serum bottle was sealed with a composite stopper. The microcosms were incubated at 25°C in darkness. Samples were periodically collected to measure the concentrations of BTEX, nitrate, nitrite, sulfate and ferric iron. All the experiments were conducted in triplicate. Each data represent the mean of three measurements, and the standard deviation is less than 10%.

1.3 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 were 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. Ferrous and ferric iron were analyzed by the 1,10-phenanthroline method at a wavelength of 510 nm (Standard methods).

2 Results and discussion

2.1 BTEX degradation in microcosms in control experiments

In the absence of any terminal electron acceptor and in the abiotic control experiments, the loss of BTEX was negligible (between 3.4% and 7.3% of its initial concentration) over a period of 65 d, showing that volatile losses were relatively minor. Similarly, no electron acceptor depletion was occurred in abiotic or substrate-unamended incubations.

2.2 Anaerobic degradation of BTEX individually under various electron acceptor reduction conditions

Figure 1 shows the results of anaerobic biodegradation of BTEX individually under nitrate, sulfate and ferric iron reducing conditions. As shown in Figs.1a and 1f, benzene and *p*-xylene could be degraded under nitrate and sulfate

Table 1 Experimental set-up used for degradation studies

Substrate	With 1-ml cultures				Without cultures		
	NO_3^- *	SO_4^{2-} *	Fe^{3+} *	Without electron acceptor	NO_3^- *	SO_4^{2-} *	Fe^{3+} *
Benzene (mg/L)	25.5	26.1	26.7	26.7	25.5	26.1	26.7
Toluene (mg/L)	26.3	24.9	25.9	25.9	26.3	24.9	25.9
Ethylbenzene (mg/L)	24.7	25.7	24.3	26.3	24.7	25.7	24.3
o-Xylene (mg/L)	26.9	24.8	25.6	25.6	26.9	24.8	25.6
m-Xylene (mg/L)	25.4	24.1	26.2	26.2	25.4	24.1	26.2
p-Xylene (mg/L)	25.1	26.6	24.9	24.9	25.1	26.6	24.9
BTEX mixtures (mg/L)	25.9	25.9	25.9	–	–	–	–
Without substrate	+	+	+	–	–	–	–

* Initial electronic acceptor concentrations are 247.6 mg/L for NO_3^-, 152.6 mg/L for SO_4^{2-}, and 622.4 mg/L for Fe^{3+}, respectively.
–: Without control experiment; +: with control experiment.

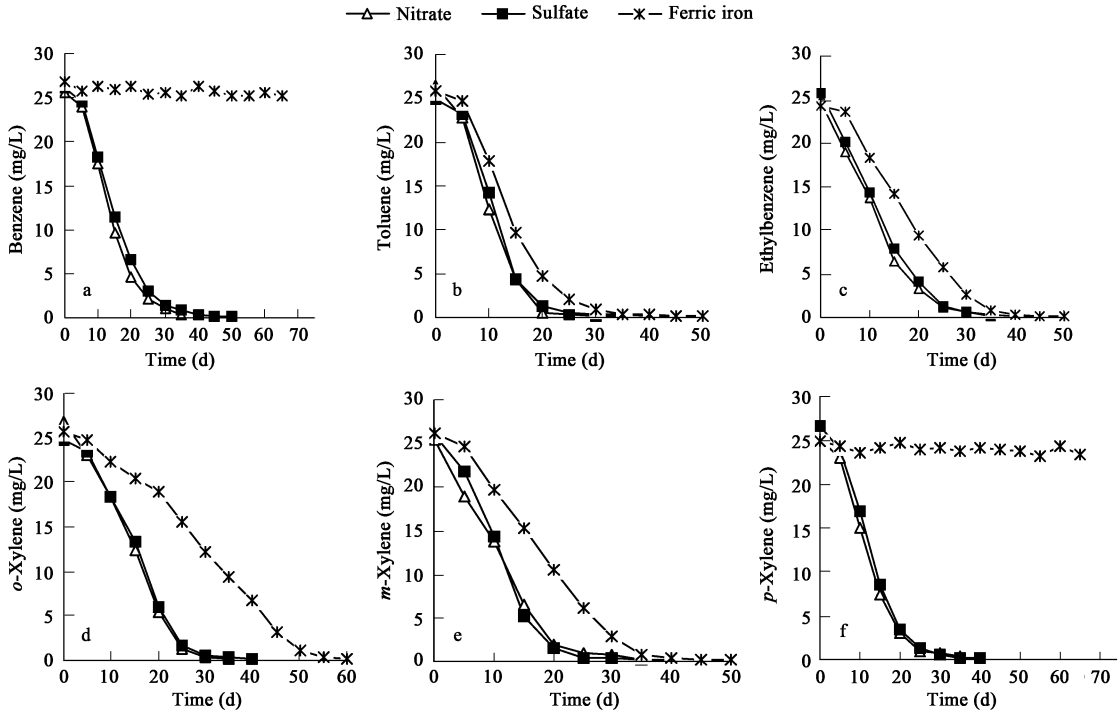

Fig. 1 Anaerobic biodegradation curves of BTEX at various electron acceptor reducing conditions. (a) benzene; (b) toluene; (c) ethylbenzene; (d) *o*-xylene; (e) *m*-xylene; (f) *p*-xylene.

reducing conditions, however, insignificant disappearance of them was observed when using ferric iron as electron acceptor during two-month incubation. The data in Figs.1b, 1c, 1d and 1e demonstrated that toluene, ethylbenzene, *o*-xylene, *m*-xylene could be served as electron donors and carbon sources for the enriched cultures under a variety of redox conditions, and were decreased to non-detectable levels within a period of 60 d. The results of the experiments showed that BTEX anaerobic degraders could potentially remediate a site contaminated with BTEX using a wide variety of terminal electron acceptors.

2.3 Variation curve of nitrate, nitrite and sulfate concentrations during incubation

Figure 2 presents data on the variation of nitrate, nitrite and sulfate when the substrate was benzene, similar results were also observed with toluene, ethylbenzene, *o*-xylene, *m*-xylene and *p*-xylene as substrates (data not shown). Compared between Figs.1a and 2, it could be found that nitrate removal went hand-in-hand with benzene degradation, which indicates that benzene degradation is coupled to nitrate reduction and is due to biological process. At the same time, nitrite was detected as the intermediate compound. While under the condition of not amending the enriched cultures or not adding organic substrate, the nitrate concentration was not changed during the whole incubations, the reason may be that the reduction of nitrate could not be occurred for lack of the biodegradation cultures or the carbon sources. The same phenomena were observed under the condition of sulfate reducing condition, from Fig.1a and Fig.2 it could be found that there was a good relationship between BTEX degradation and the reduction of sulfate.

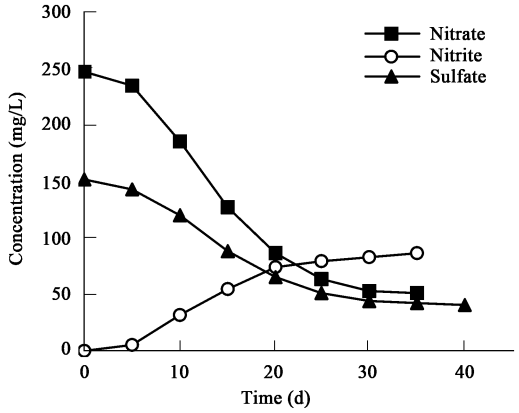

Fig. 2 Curve of nitrate, nitrite and sulfate during anaerobic biodegradation of benzene.

2.4 Variation curve of ferric iron concentrations during incubation

Figure 3 illustrates the variation dynamics of ferric iron concentrations under different substrates conditions. Compared between Fig.1 and Fig.3 it could be concluded there was a close relationship between ferric iron reduction and the degradation of BTEX. No ferric iron reduction was occurred when the organic substrate was benzene or *p*-xylene, this was corresponding to the phenomenon that neither benzene nor *p*-xylene could be degraded which was illustrated in Fig.1. One plausible explanation for this observation is that the inoculation time of 7 months was not enough to enrich the benzene and *p*-xylene degrading bacterium due to their special molar configuration.

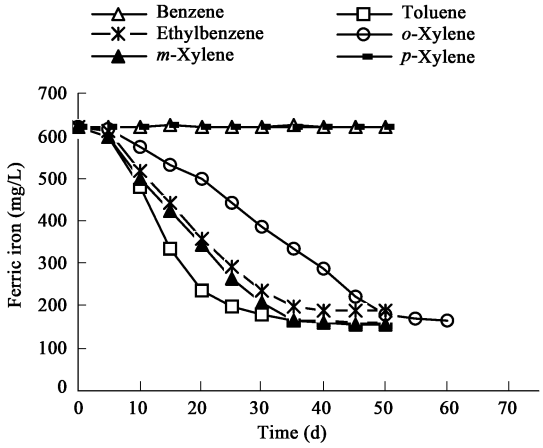

Fig. 3 Curve of Fe^{3+} during anaerobic biodegradation of BTEX.

2.5 Anaerobic degradation of BTEX mixtures under various electron acceptor conditions

The biodegradation of BTEX mixtures under nitrate, sulfate and ferric iron reduction conditions was conducted, respectively. Fig.4 presents the data on the degradation of BTEX mixtures during the whole incubation.

As shown in Fig.4, BTEX mixtures could be degraded by the enriched cultures under various electron acceptor reduction conditions. Although no much more experiments about the metabolic aspects were performed in this study, the independent removal of BTEX supports the fact there was no apparent inhibition in the degradation of benzene, toluene, ethylbenzene, *m*-xylene, *o*-xylene, and *p*-xylene. This finding supports the application of the system for treatment of areas contaminated with BTEX. According to Alvarez and Vogel (1991), individual compounds can stimulate the degradation of others through the induction of enzymes or can act as a primary substrate stimulating microbial growth and thereby favoring the co-metabolism of another compound. From Fig.4c, it could be found that under ferric iron reduction condition benzene and *p*-xylene could be degraded in the presence of other substrates, while insignificant degradation was observed under ferric iron reducing conditions compared to sterile controls without other substrates, which indicates that alkylbenzenes can serve as the primary substrates to stimulate the transformation of benzene and *p*-xylene under anaerobic conditions. Consequently, it could be concluded that the transformation of benzene and *p*-xylene with ferric iron proceeded via co-metabolism.

2.6 Characteristics of the anaerobic biodegradation rates

The biodegradation rate of each organic substrate under various electron accepting conditions was calculated and is shown in Table 2.

Table 2 Degradation rates of BTEX under various electron acceptor conditions (mg/(L·d))

Substrates	NO_3^-	SO_4^{2-}	Fe^{3+}
Benzene	0.86	0.82	/
Toluene	1.04	1.01	0.95
Ethylbenzene	0.97	0.87	0.74
o-Xylene	0.84	0.79	0.41
m-Xylene	0.89	0.84	0.81
p-Xylene	0.79	0.76	/

As can be seen from Table 2, the rates of BTEX disappearance in terms of various terminal electron acceptors were as the following order: nitrate > sulfate > ferric iron. Therefore, nitrate was a more favorable electron acceptor compared to sulfate and ferric iron.

Under the three 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 is the most favorable compound for anaerobic degradation by the enriched cultures, which is consistent with other reports comparing the biodegradation of various BTEX compounds (Heider *et al*., 1998). Anaerobic biodegradation of benzene and *p*-xylene was also achieved when either nitrate or sulfate was provided as a terminal electron acceptor, but at a lower rate compared to toluene and ethylbenzene. It appears that benzene and *p*-xylene are more toxic than toluene and ethylbenzene, this finding is generally in good agreement with other studies reported in the literature (Mikesell *et al*., 1994; Langenhoff *et al*., 1996; Mallakin and Ward, 1996).

2.7 Stoichiometry between the consumption of electron acceptors and BTEX

Under the assumption of no cell growth, the theoretical stiochiometric equations for anaerobic oxidation of BTEX to carbon dioxide with nitrate, sulfate and ferric iron as the

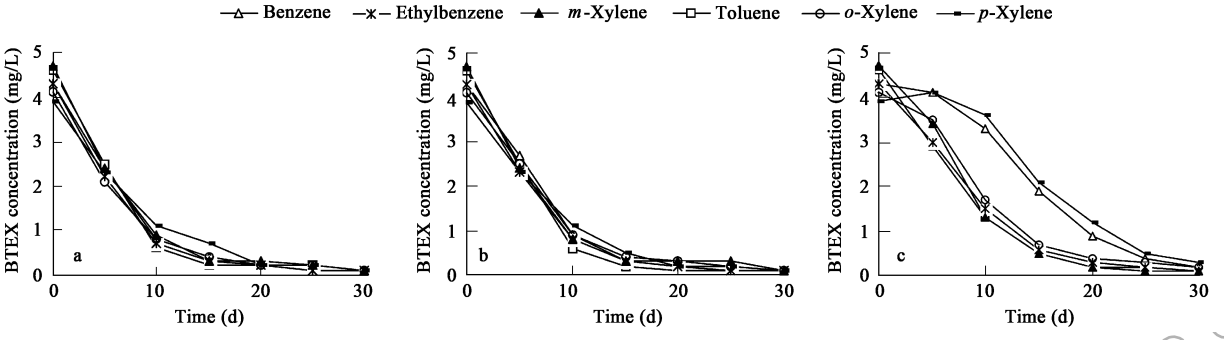

Fig. 4 Curve of biodegradation of BTEX under different electron acceptor reducing conditions. (a) nitrate; (b) sulfate; (c) ferric iron.

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

Substrates	NO_3^-		SO_4^{2-}		Fe^{3+}	
	Theoretical	Measured	Theoretical	Measured	Theoretical	Measured
Benzene	4.77	7.53	4.62	4.44	21.54	/
Toluene	4.85	6.24	4.70	4.51	21.91	17.99
Ethylbenzene	4.91	6.49	4.75	4.42	22.18	18.04
o-Xylene	4.91	7.28	4.75	4.32	22.18	18.07
m-Xylene	4.91	7.81	4.75	4.37	22.18	17.97
p-Xylene	4.91	7.61	4.75	4.23	22.18	/

electron acceptors were as follows:

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

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

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

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

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

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

$$C_6H_6+18H_2O + 30Fe^{3+} \longrightarrow 6HCO_3^-+30Fe^{2+}+36H^+ \quad (7)$$

$$C_7H_8+21H_2O + 36Fe^{3+} \longrightarrow 7HCO_3^-+36Fe^{2+}+43H^+ \quad (8)$$

$$C_8H_{10}+24H_2O + 42Fe^{3+} \longrightarrow 8HCO_3^-+42Fe^{2+}+50H^+ \quad (9)$$

According to these equations, the theoretical ratios of electron acceptor consumption and BTEX degradation could be calculated and the results are listed in Table 3. The measured ratios could be calculated based on the variation of the concentrations of electron acceptor and BTEX between each sampling intervals during the incubation, and the results are also shown in Table 3.

From Table 3, it could be observed that under nitrate reduction condition, the measured ratios were higher than the theoretical ratios, the reason for which is that the theoretical ratio is calculated by assuming nitrate is ultimately reduced to nitrogen gas, in fact nitrate was not completely transferred to N_2, but part of them was accumulated as nitrite, and the equations were as following:

$$C_6H_6+15NO_3^- \longrightarrow 6CO_2+15NO_2^-+3H_2O \quad (10)$$

$$C_7H_8+18NO_3^- \longrightarrow 7CO_2+18NO_2^-+4H_2O \quad (11)$$

$$C_8H_{10}+21NO_3^- \longrightarrow 8CO_2+21NO_2^-+5H_2O \quad (12)$$

On the basis of the above presumed stoichiometric equations, the theoretical ratios between the consumption of nitrate and C_6H_6, C_7H_8, C_8H_{10} were 11.92, 12.13 and 12.28, respectively, which was higher than the corresponding measured values. From Fig.2 it could also be found that the accumulation of nitrite was occurred, at the same time the production of nitrite was lower than the consumption of nitrate, which indicated that part of the nitrite was reduced to nitrogen gas.

Under the condition of sulfate reduction, the relative error between the theoretical and the calculated ratios were less than 10%, supports the theoretical stoichiometry and hypothesis that BTEX are being mineralized to carbon dioxide and water.

However, when ferric iron was a terminal electron acceptor, the calculated ratios were lower than the theoretical ratios, the reason for which may be that BTEX is not completely transformed to carbon dioxide by the mixed cultures during the whole incubations, which results in ferric iron consumptions were less than predicted by the theoretical equations. Furthermore, another possible reason in part is that during this experiment the anaerobic oxidations of BTEX were also coupled to cultures growth and converted into cell materials.

3 Conclusions

Toluene, ethylbenzene, *m*-xylene and *o*-xylene could be degraded independently by the mixed cultures coupled to nitrate, sulfate and ferric iron reduction. Under ferric iron reducing conditions the degradation of benzene and *p*-xylene could be occurred only in the presence of other alkylbenzenes. Benzene and *p*-xylene are more toxic than toluene and ethylbenzene, under the three terminal electron acceptors conditions, the degradation rates decreased with toluene > ethylbenzene > *m*-xylene > *o*-xylene> benzene > *p*-xylene. Nitrate was a more favorable electron acceptor compared to sulfate and ferric iron. The measured ratios of nitrate reduction to BTEX degradation were between the theoretical ratios that were calculated by assuming nitrate reduced to nitrogen gas and nitrite; the measured ratios of sulfate reduction to BTEX degradation were nearly the same to theoretical ones; the measured ratios of ferric iron reduction to BTEX degradation were slightly lower than the theoretical ones.

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