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Substrate interactions during anaerobic biodegradation of BTEX by the mixed cultures under nitrate reducing conditions

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Abstract

The enriched BTEX-degrading bacteria were used to investigate the substrate interactions during anaerobic biodegradation of all the possible BTEX binary combinations. Beneficial and detrimental substrate interactions were observed in comprehensive mixtures of benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene and *p*-xylene. The amendment of toluene or ethylbenzene could stimulate benzene degradation. Lower concentrations of *m*-xylene would enhance the degradation of benzene, whereas degradation of benzene was inhibited with higher concentrations of *m*-xylene. The simultaneous presence of toluene and ethylbenzene could stimulate the degradation of each other. The addition of toluene stimulated *o*-xylene degradation, whereas the amendment of ethylbenzene inhibited the degradation of *o*-xylene. Lower concentrations of toluene or ethylbenzene would enhance the degradation of *m*-xylene and *p*-xylene, whereas higher concentrations of toluene or ethylbenzene had a slight inhibitory effect on *m*-xylene and *p*-xylene degradation. The amendment of benzene, *m*-xylene or *p*-xylene would inhibit the degradation of other BTEX compounds. When the concentration of BTEX mixtures was over 150 mg/l, the degradation of benzene, *o*-xylene, *m*-xylene and *p*-xylene was severely inhibited.

Keywords: BTEX; Anaerobic biodegradation; Substrate interactions; Nitrate reduction

1. Introduction

Benzene, toluene, ethylbenzene and xylenes (BTEX) are important contaminants present in soil and groundwater, which usually originate from the accidental leakage of underground storage tanks containing gasoline and jet fuel. Due to their relatively higher water solubility, BTEX compounds always migrate far away from polluted sources and contaminate drinking water supplies. BTEX compounds are of increasing interest because they can produce neurological impairment, and especially benzene can additionally cause hematological effects, which may ultimately lead to aplastic anemia and acute myelogenous leukemia [1]. Clean-up of the BTEX-contaminated soil and groundwater is desirable in order to avoid public health hazards. Bioremediation, expected to be an economical, energy efficient and environmentally sound approach to other remediation processes such as chemical or physical ones, has been developed as a soil and groundwater clean-up technique. Aerobic bioremediation of BTEX compounds generally exhibits faster BTEX degradation rates than anaerobic degradation [2]. However, aerobic strategies are not universally applicable, and anaerobic bioremediation might be more appropriate to clean up some gasoline-contaminated sites. With regard to the fact that gasoline-contaminated sites typically involve a complex mixture of BTEX, it is important to understand the potential stimulation or inhibition interactions caused by the simultaneous presence of multiple BTEX compounds.

Under aerobic conditions, many substrate interactions have been observed during biodegradation of BTEX combinations. Abuhamed et al. observed that the inhibition effect of toluene on benzene was higher than the inhibition effect of benzene on toluene [3]. Prenafeta-Boldú et al. reported that *p*-xylene was not degraded in BTEX mixtures, while in combination with toluene appeared to be mineralized by the fungus *Cladophialophora* sp. Strain T1 [4]. Chang and his colleagues investigated the substrate interactions of BTEX by a mixed culture isolated from

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a gasoline-contaminated site, and demonstrated that the simultaneous presence of benzene and toluene were degraded with a slight inhibitory effect on each other, ethylbenzene was the most potent inhibitor of BTEX degradation, the presence of *p*-xylene inhibited the degradation of benzene, toluene, and ethylbenzene, whereas the presence of either benzene or toluene enhanced the degradation of ethylbenzene and the xylenes [5]. Reardon et al. found that toluene significantly inhibited the biodegradation rate of benzene by *Pseudomonas putida* F1 [6]. Deeb and Alvarez-Cohen observed that benzene and toluene degradation rates were slightly enhanced by the presence of *o*-xylene, whereas the presence of toluene, benzene or ethylbenzene had a negative effect on *o*-xylene degradation [7]. Alvarez and Vogel reported that the degradation of benzene and *p*-xylene was enhanced by the presence of toluene in *Pseudomonas* sp. incubations, whereas the degradation of benzene and toluene was inhibited by the presence of *p*-xylene [8]. This brief overview revealed that there was no general rule for predicting the interactions for BTEX mixtures. What is more, the research in the literature focused on the substrate interactions in BTEX mixtures under anaerobic conditions is still very limited. Da Silva and Alvarez reported that benzene removal was hindered by the presence of toluene [9]. Meckenstock et al. observed that the strain TRM1 degraded only toluene and was not affected by xylene, they also showed that the strain OX39 degraded xylene and was inhibited by toluene [10]. Barbaro et al. noticed the competitive utilization between toluene, ethylbenzene and the xylene isomers under nitrate reducing conditions [11]. Phelps and Young observed the inhibition effect of toluene on BTEX degradation [12]. However, to our knowledge, no previously reported work focusing on substrate interactions has included all the possible binary combinations between the six BTEX substrates.

We previously examined the anaerobic degradation potential of BTEX substrates under nitrate and sulfate reducing conditions by the mixed bacteria, and found that all the BTEX compounds could be biodegraded efficiently [13]. Due to the substrate interactions that occur in the biodegradation of BTEX combinations, it is expected that the biodegradation patterns of individual substrate are different from BTEX combinations. However, whether the isolated bacteria have the degradation ability of BTEX mixtures and what are the substrate interactions during anaerobic biodegradation of the BTEX combinations are still unsolved. Answers to such questions could improve anaerobic bioremediation of BTEX-contaminated sites in the future. Therefore, the detailed research is conducted to study these substrate interactions in the degradation of BTEX combinations by the enriched mixed bacteria. This paper presents results from anaerobic biodegradation experiments with benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene and *p*-xylene as sole substrate, as binary combinations and as six mixtures.

2. Materials and methods

2.1. The enriched mixed bacteria

Anaerobic mixed bacteria were enriched from gasoline-contaminated soil, the procedure of the enrichment was discussed previously [13]. The enriched mixed bacteria were similar to *Pseudomonas aureofaciens*, *Microbacterium Lactuim* and *Bacillus cereus* as identified by standard morphological, physiological and biochemical plate and tube tests using the criteria and procedures described in Bergey's Manual of Systematic Bacteriology [14].

2.2. Experimental design

A series of experiments with all the possible binary combinations between the six BTEX substrates with different initial concentrations of approximately 10, 25 and 50 mg/l were investigated. At the same time, the degradation performances during the simultaneous presence of the six BTEX mixtures with different initial concentrations of approximately 30, 60, 90, 150 and 300 mg/l were studied (corresponding to these values the concentration of each substrate was 5, 10, 15, 25 and 50 mg/l, respectively). Control experiments with the individual compounds of 25 mg/l were run in parallel. In addition, in order to account for abiotic BTEX degradation, the sterile treatments and the microcosms containing no electron acceptor or no microorganisms were also prepared. The sterile samples were established by autoclaving at 121 °C for 3 h.

Anaerobic biodegradation experiments were performed using 50-ml serum bottles, and the bottles were sealed with polytetrafluoroethylene-lined butyl rubber stoppers and aluminum caps. The mineral medium was purged for 3.5 h using pure nitrogen gas. The constituents of the mineral medium were as below: $NaNO_3$ (1.5 g/l), 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 $Na_2S{\cdot}9H_2O$, vitamin (1%, v/v) and trace solutions (1%, v/v) were also added to the mineral medium. 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 $ZnCl_2$ [15,16]. 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 [17]. The pH of the mineral medium was between 6.8 and 7.2. 33 ml of the minimal medium were poured in serum bottle, and then 2 ml of the enriched mixed consortia were added. Prior to inoculating the bacteria, the flask contained the enriched mixed bacteria was homogenized so that the cell materials were distributed evenly throughout the liquor, and this procedure ensured that samples taken from the active flask were representative of the total flask contents. The BTEX substrates were added to each mineral medium to a final concentration as the experimental design. All the microcosms were prepared in an anaerobic glove-box which was filled with pure nitrogen gas. The maintenance of anaerobic conditions was examined by a preliminary experiment. The microcosms were incubated at 20 °C in darkness. Samples were periodically collected to measure the concentrations of BTEX, 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%.

2.3. Chemical and microbiological analysis

BTEX concentrations were analyzed by a model GC-14B gas chromatograph equipped with a capillary column (ULBON HR-1 0.25 mm × 30 m), with a flame ionization detector (FID) (Shimadzu Corp., Japan). Injector, detector and column temperature was hold at 150, 150 and 100 °C, respectively. Nitrogen gas 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, Ca, USA), using an Iopac ASI4 (4 mm × 250 mm) 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.

The total cell counts were analyzed by DNA intercalating dye 4′,6-diamidine-2′-phenylindol-dihydrochloride (DAPI, Sigma) staining [18]. 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

3.1. Effect of BTEX compounds on benzene degradation

For a constant benzene initial concentration of approximately 25 mg/l, its degradation was studied with simultaneous presence of toluene, ethylbenzene, *o*-xylene, *m*-xylene or *p*-xylene, respectively. The results of benzene degradation with simultaneous presence of toluene, *o*-xylene or *m*-xylene were shown in Fig. 1(a)–(c), respectively (the data with ethylbenzene and *p*-xylene were not shown in this paper). It could be found from Fig. 1(a) that the degradation of benzene was stimulated by the amendment of 10–50 mg/l toluene. Furthermore, the bacterial growth was stimulated when toluene was co-existed. At the end point of the experiments, the bacterial densities were $1.1 \times 10^{10} \pm 5 \times 10^{8}$ cells/l with simultaneous presence of benzene and 54.3 mg/l toluene. The same stimulation effect was observed with the addition of ethylbenzene, the bacterial densities were $1.0 \times 10^{10} \pm 4 \times 10^{8}$ cells/l with simultaneous presence of benzene and 56.6 mg/l ethylbenzene. In contrast, as could be seen from Fig. 1(b) that *o*-xylene had an inhibition effect on the degradation of benzene, and the inhibition effect was stronger with higher concen-

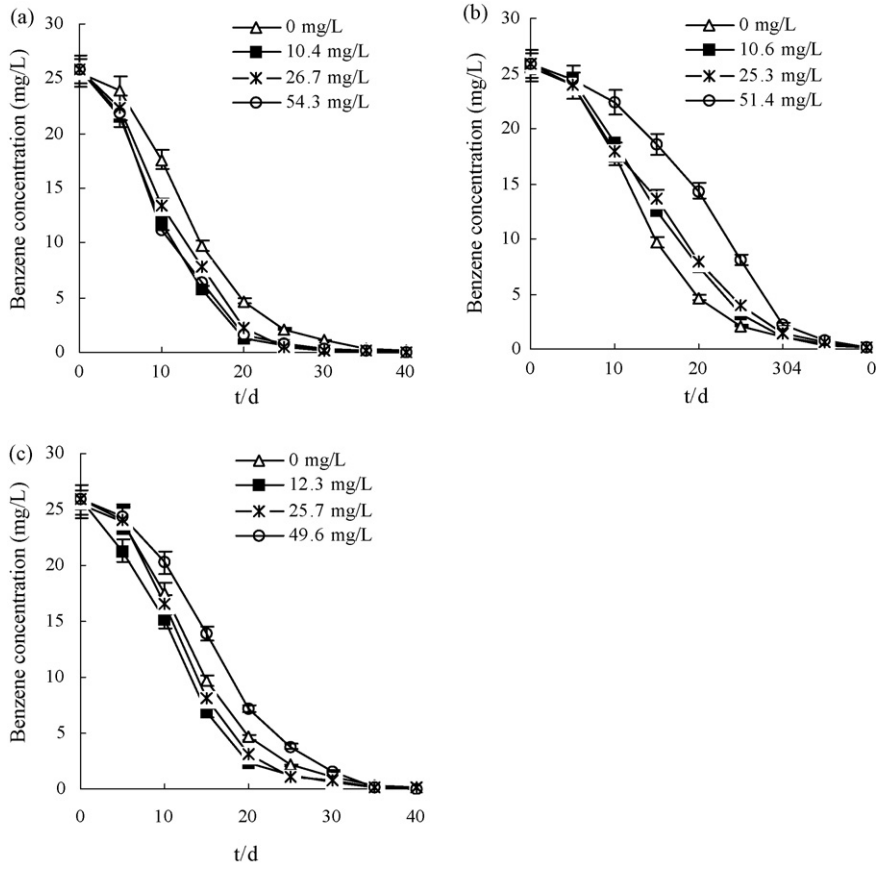

Fig. 1. Biodegradation of benzene with the amendment of different concentrations of other BTEX compounds. (a) Toluene; (b) *o*-xylene; (c) *m*-xylene. The error bars represent the standard deviations of the three parallel experiments.

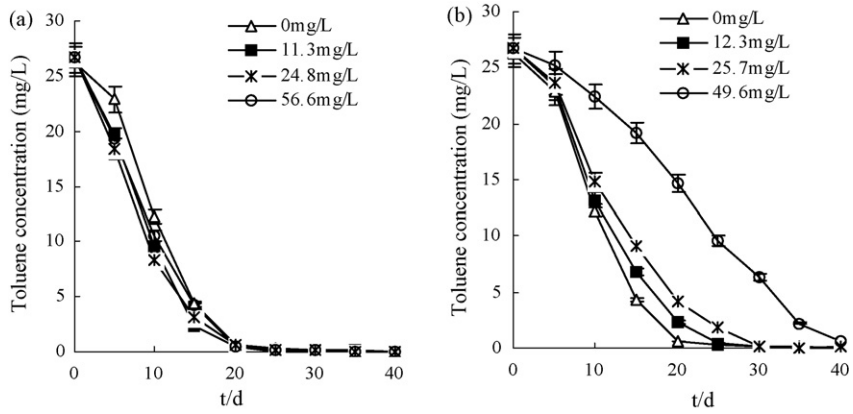

Fig. 2. Biodegradation of toluene with the amendment of different concentrations of other BTEX compounds. (a) Ethylbenzene and (b) *m*-xylene. The error bars represent the standard deviations of the three parallel experiments.

trations of *o*-xylene. At the end point of degradation, the bacterial densities were $7.4 \times 10^9 \pm 3 \times 10^8$ cells/l with benzene alone, but $4.4 \times 10^9 \pm 2 \times 10^8$ cells/l with simultaneous presence of benzene and 51.4 mg/l *o*-xylene. The same phenomenon was observed with the addition of different concentrations of *p*-xylene, but the inhibitory effect of *p*-xylene on benzene degradation was slightly less than that of *o*-xylene. From Fig. 1(c) it could be concluded that when the concentration of *m*-xylene was below 25 mg/l, *m*-xylene enhanced the degradation of benzene, however, when the concentration of *m*-xylene was over 50 mg/l, *m*-xylene had a slight inhibitory effect on the degradation of benzene.

3.2. Effect of BTEX compounds on toluene degradation

The degradation of toluene with different concentrations of ethylbenzene or *m*-xylene was shown in Fig. 2(a) and (b), respectively (the data with other BTEX compounds were not shown). From Fig. 2(a) it could be concluded that the presence of ethylbenzene exerted slightly positive effect on the degradation of toluene. At the same time, the simultaneous presence of ethylbenzene could stimulate bacterial growth. The bacterial densities were $1.5 \times 10^{10} \pm 6 \times 10^8$ cells/l with toluene alone, and $1.9 \times 10^{10} \pm 7 \times 10^8$ cells/l with simultaneous presence of toluene and 56.6 mg/l ethylbenzene. From Fig. 2(b) it could be found that *m*-xylene had an inhibitory effect on toluene degradation in binary mixtures, furthermore the inhibitory effect was stronger with higher concentrations. For example, with the amendment of 49.6 mg/l *m*-xylene, the complete toluene degradation was inhibited by over 20 days compared to the presence of toluene alone. The same inhibition effects were observed when toluene was co-existed with benzene, *o*-xylene or *p*-xylene. The data of total cell counts showed that compared to the presence of toluene alone, the amendment of benzene, *o*-xylene, *m*-xylene or *p*-xylene inhibited the bacterial growth.

3.3. Effect of BTEX compounds on ethylbenzene degradation

Fig. 3(a) and (b) revealed the substrate interactions during ethylbenzene degradation with the amendment of toluene or *p*-xylene, respectively (the data with other BTEX compounds were not shown). From the profiles of Fig. 3(a) it could be inferred that the presence of toluene had a slight stimulation effect on the degradation of ethylbenzene.

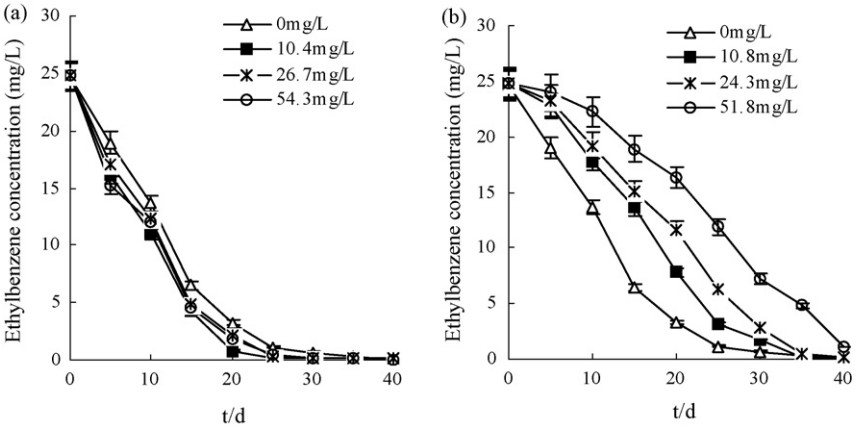

Fig. 3. Biodegradation of ethylbenzene with the amendment of different concentrations of other BTEX compounds. (a) Toluene and (b) *p*-xylene. The error bars represent the standard deviations of the three parallel experiments.

The beneficial effect was also supported by the fortuitous growth of biomass. For example, the bacterial densities were $2.4 \times 10^{10} \pm 8 \times 10^{8}$ cells/l with simultaneous presence of 54.3 mg/l toluene and ethylbenzene, in comparison, the bacterial densities were $1.3 \times 10^{10} \pm 4 \times 10^{8}$ cells/l with ethylbenzene alone. Comparing Fig. 2(a) with Fig. 3(a) it could be concluded that the simultaneous presence of toluene and ethylbenzene could stimulate the degradation of each other. From Fig. 3(b) it could be found that the amendment of *p*-xylene inhibited the degradation of ethylbenzene, and the complete degradation of ethylbenzene was inhibited by over 15 days in the presence of 51.8 mg/l *p*-xylene. Other data that was not listed in this paper showed that benzene, *o*-xylene or *m*-xylene had a slight inhibitory effect on ethylbenzene degradation. Among all the BTEX compounds, the effect of *p*-xylene on ethylbenzene degradation was the strongest.

3.4. Effect of BTEX compounds on xylenes degradation

To elucidate the effect of benzene, toluene and ethylbenzene on xylenes degradation, a series of experiments containing these substrates was conducted, some of the results were illustrated in Figs. 4–6. As depicted in Fig. 4(a), the presence of toluene could enhance the degradation of *o*-xylene. At the same time, the simultaneous presence of toluene could stimulate bacterial growth compared to the presence of *o*-xylene alone. The bacterial densities were $1.4 \times 10^{10} \pm 6 \times 10^{8}$ cells/l with simultaneous presence of 54.3 mg/l toluene and *o*-xylene, compared to this, the bacterial densities were $9.1 \times 10^{9} \pm 4 \times 10^{8}$ cells/l with *o*-xylene alone. Fig. 4(b) shows that the degradation of *o*-xylene was inhibited with the amendment of different concentrations of *m*-xylene ranging from 10 to 50 mg/l. The effect of benzene, ethylbenzene or *p*-xylene on *o*-xylene degradation was the same to the effect of *m*-xylene. Figs. 5(a) and 6(a) indicated that benzene inhibited the degradation of *m*-xylene and *p*-xylene. Figs. 5(b) and 6(b) illustrated that when toluene concentration was below 10 mg/l, toluene had a stimulatory effect on the degradation of *m*-xylene and *p*-xylene, however, when toluene concentration was over 25 mg/l, toluene had an inhibitory effect on *m*-xylene and *p*-xylene degradation. The same effect was observed when using ethylbenzene as the co-existed substrate with *m*-xylene or *p*-xylene.

3.5. Degradation of BTEX mixtures with different initial concentrations

Biodegradation experiments with six BTEX mixtures were carried out at initial concentrations ranging from 30 to 300 mg/l, and the results were shown in Fig. 7. Fig. 7(a)–(d) shows a typical profile of complete degradation without a lag phase for all the BTEX compounds when the initial concentrations of BTEX mixtures were below 150 mg/l. Fig. 7(e) shows that when the concentration of BTEX mixtures reached a value of 300 mg/l, toluene and ethylbenzene could be degraded nearly without a lag phase, whereas benzene, *o*-xylene, *m*-xylene and *p*-xylene were degraded with a lag phase of 20–30 days. The results of the experiments indicated that with lower initial concentrations of six BTEX mixtures there was no apparent negative effect on the degradation of these substrates, while higher concentrations of BTEX mixtures inhibited the degradation of the compounds, especially for benzene, *o*-xylene, *m*-xylene and *p*-xylene.

4. Discussion

In the case of the enriched mixed bacteria, the stimulation effect of BTEX degradation in mixtures was observed for benzene, *o*-xylene, *m*-xylene and *p*-xylene when either of them was present in binary mixtures with toluene or ethylbenzene. Whereas, Meckenstock et al. found that growth and *o*-xylene degradation of strain OX39 were inhibited by toluene when toluene concentration was as low as 4.2 mg/l [10]. The competitive utilization phenomena between toluene, ethylbenzene and the xylene isomers under nitrate reducing conditions were also observed by other researchers [11,12]. The stimulating effect observed in this study may be resulted from the fortuitous growth of biomass in the presence of toluene or ethylbenzene. At the end of *o*-xylene degradation, the bacterial densities with *o*-xylene plus 54.3 mg/l toluene were 53% higher than with *o*-xylene alone. The fortuitous growth of biomass may be due to both toluene and ethylbenzene were easily acceptable carbon

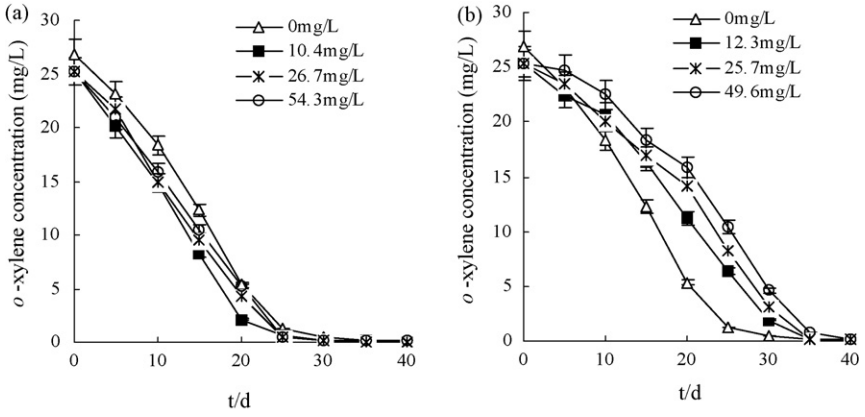

Fig. 4. Biodegradation of *o*-xylene with the amendment of different concentrations of other BTEX compounds. (a) Toluene and (b) *m*-xylene. The error bars represent the standard deviations of the three parallel experiments.

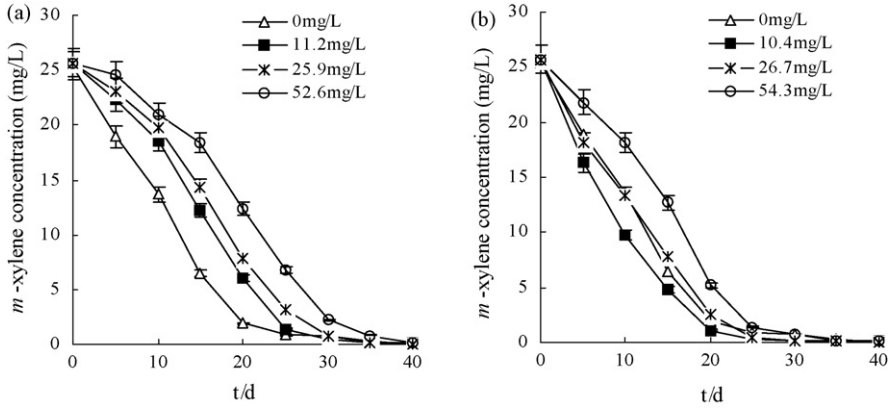

Fig. 5. Biodegradation of *m*-xylene with the amendment of different concentrations of other BTEX compounds. (a) Benzene and (b) toluene. The error bars represent the standard deviations of the three parallel experiments.

sources for the enriched mixed bacteria compared to other BTEX compounds [13,16]. Of course, another possibility through the induction of enzymes seemed also existed.

On the other hand, the amendment of benzene, *o*-xylene or *p*-xylene with any of the other BTEX compounds in binary mixtures had a negative effect on their degradation, although the degradation of these substrates was not entirely inhibited. However, Meckenstock et al. observed that toluene degradation by strain TRM1 was not inhibited by *o*-xylene when *o*-xylene concentration was up to 53 mg/l [10]. The inhibitory effect observed in this research may be due to the high concentration of BTEX combinations toxic to the mixed bacteria, this phenomenon had also been found in previous study and resulted in lower degradation rates of BTEX [13]. Among all the BTEX compounds the most potent inhibitor of BTEX degradation was *p*-xylene. Moreover, the inhibitory effects of *p*-xylene on toluene and ethylbenzene degradation were much more pronounced than on other BTEX compounds. One possible reason may be due to the competitive metabolism in which *p*-xylene inhibited the utilization of toluene and ethylbenzene because of competition for the active binding site of an enzyme. Xylene degradation could interfere with the very similar toluene degradation pathway because the addition of fumarate to the methyl group was observed as the initial reaction for toluene and xylene degradation pathways [19–22]. Morasch and Meckenstock identified 4-methylbenzylsuccinic acid and 4-methylphenylitaconic acid in supernatants of cultures and elucidated the pathway of *p*-xylene degradation, and indicated that degradation of *p*-xylene was initiated by fumarate addition to one of the methyl groups [19]. Evans et al. postulated that there was an anaerobic degradation pathway that started with the oxidative addition of acetylcoenzyme-A to toluene, yielding (2-methylbenzyl)-succinic acid and (2-methylbenzyl)-fumaric acid [20]. Biegert et al. [21] and Beller and Spormann [22] conducted in vitro studies with denitrying bacteria and suggested that the first step of anaerobic toluene degradation in these species was the addition of the methyl carbon of toluene to the double bond of fumarate to form benzylsuccinate. This implied that one of the consecutive enzymes in the pathway exhibited the substrate specificity, and the same enzyme systems seemed to be involved in the reaction of anaerobic toluene and xylenes degradation.

In addition, other effects in BTEX mixtures were found when studying the effect of toluene or ethylbenzene on the degradation of *m*-xylene and *p*-xylene, and the effect of *m*-xylene on the degradation of benzene. These effects were mainly including the positive effect with the simultaneous presence of lower

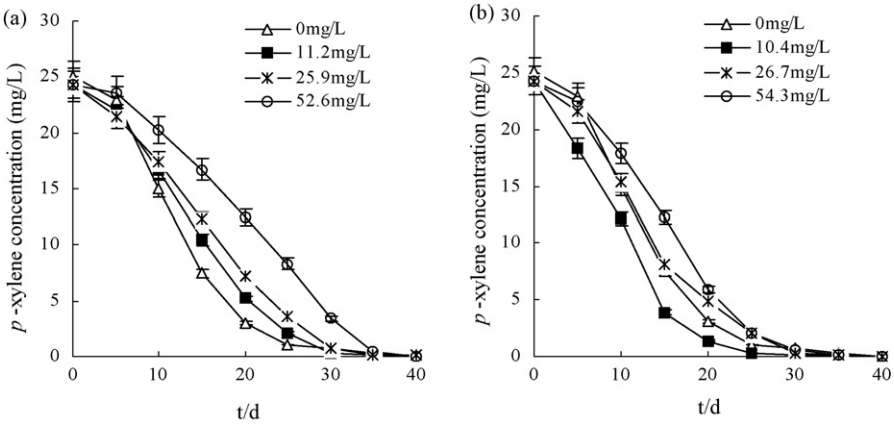

Fig. 6. Biodegradation of *p*-xylene with the amendment of different concentrations of other BTEX compounds. (a) Benzene and (b) toluene. The error bars represent the standard deviations of the three parallel experiments.

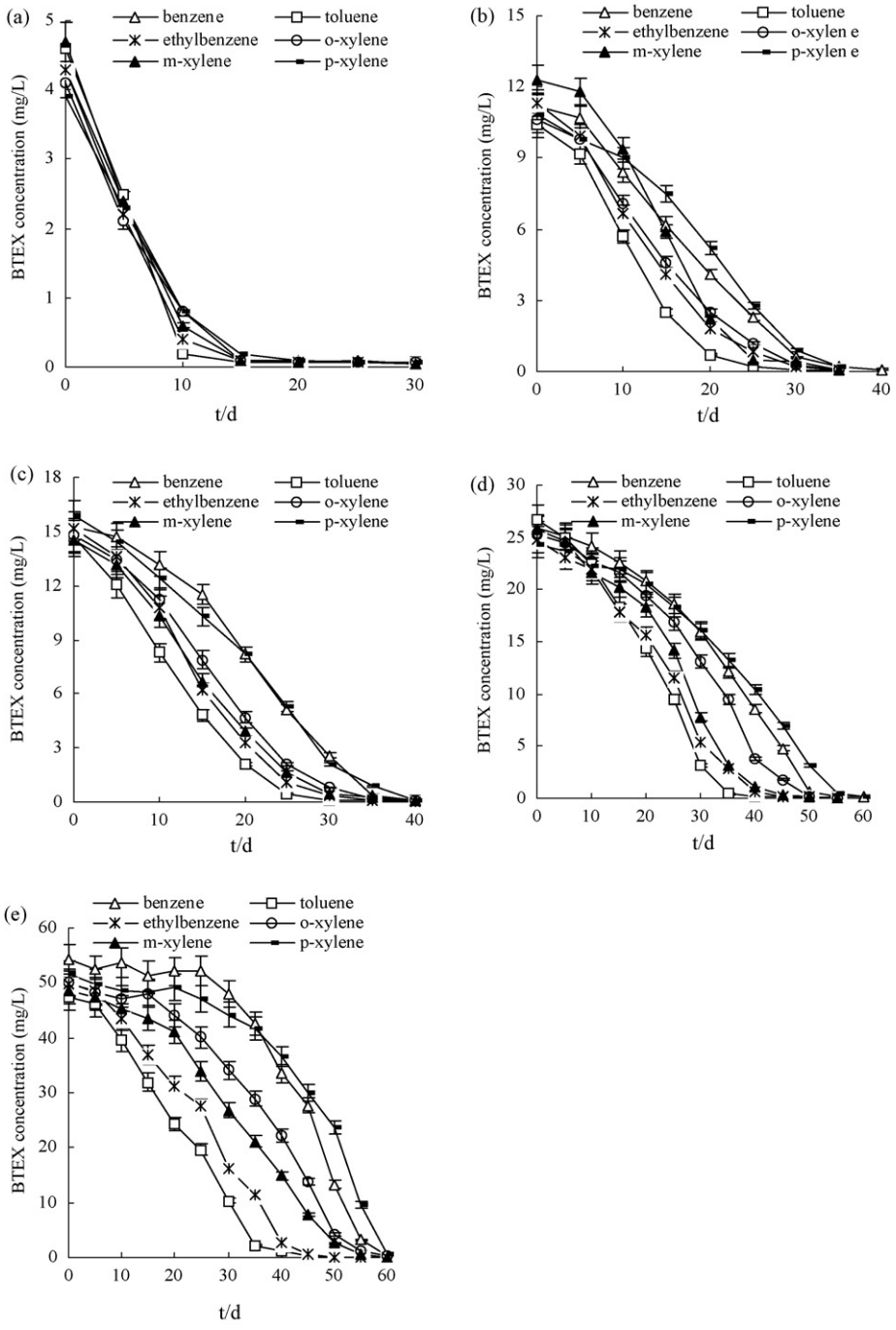

Fig. 7. Biodegradation curve of BTEX mixtures under different initial concentrations of (a) 30 mg/l; (b) 60 mg/l; (c) 90 mg/l; (d) 150 mg/l; (e) 300 mg/l. The error bars represent the standard deviations of the three parallel experiments.

concentrations of toluene, ethylbenzene or *m*-xylene, and the negative effect with the simultaneous presence of higher concentrations. Just as discussed above, the presence of toluene or ethylbenzene may lead to higher biomass production. Hence, higher degradation rates of *m*-xylene and *p*-xylene with simultaneous presence of toluene or ethylbenzene could be obtained because the effect of inhibition was offset by the fortuitous cell growth. However, when the concentration of toluene or ethylbenzene was increased, the toxic inhibition effect on *m*-xylene and *p*-xylene degradation outweighed the enhancement effect on bacterial growth, which led to the decrease of *m*-xylene and *p*-xylene degradation rate. It was an interesting observation that lower concentrations of *m*-xylene could stimulate the degradation of benzene, but it was difficult to explain from the available information, especially because *m*-xylene was also difficult to be biodegraded [13], the possibility through enhancing growth of biomass or induction of enzymes was unlikely existed.

Under the condition of simultaneous degradation of 25 mg/l benzene and 25 mg/l toluene mixtures, the concentrations of nitrate and nitrite were analyzed during the whole incubation

Table 1
Biodegradation rates of BTEX under different concentrations of BTEX mixtures and single substrate (mg/l/d)

Substrate	The initial concentration of six BTEX mixtures					The initial concentration of single BTEX			
	30 mg/l	60 mg/l	90 mg/l	150 mg/l	300 mg/l	5 mg/l	10 mg/l	25 mg/l	50 mg/l
Benzene	0.35 ± 0.03	0.36 ± 0.04	0.42 ± 0.04	0.47 ± 0.03	0.93 ± 0.06	0.26 ± 0.02	0.46 ± 0.05	0.76 ± 0.06	1.46 ± 0.05
Toluene	0.44 ± 0.04	0.49 ± 0.04	0.63 ± 0.05	0.78 ± 0.05	1.29 ± 0.07	0.34 ± 0.03	0.62 ± 0.05	1.04 ± 0.06	1.67 ± 0.07
Ethylbenzene	0.39 ± 0.05	0.48 ± 0.03	0.60 ± 0.04	0.63 ± 0.03	1.18 ± 0.05	0.32 ± 0.02	0.57 ± 0.03	0.97 ± 0.05	1.59 ± 0.06
o-Xylene	0.33 ± 0.03	0.41 ± 0.04	0.51 ± 0.03	0.52 ± 0.04	0.89 ± 0.05	0.28 ± 0.02	0.51 ± 0.04	0.84 ± 0.05	1.54 ± 0.07
m-Xylene	0.41 ± 0.04	0.51 ± 0.04	0.53 ± 0.03	0.62 ± 0.05	0.92 ± 0.04	0.29 ± 0.03	0.53 ± 0.03	0.89 ± 0.06	1.49 ± 0.05
p-Xylene	0.31 ± 0.02	0.33 ± 0.03	0.46 ± 0.04	0.42 ± 0.03	0.85 ± 0.06	0.25 ± 0.02	0.46 ± 0.05	0.79 ± 0.05	1.39 ± 0.06

time. The accumulation of nitrite was found during the reduction of nitrate, at the same time, the amount of nitrite produced was less than nitrate consumed. Therefore, it could be concluded that only some amounts of the nitrate were completely transferred to nitrogen gas. The reduction of 1 mol of NO_3^- to NO_2^- needs 2 mol electrons, and the reduction of 1 mol of NO_3^- to N_2 needs 5 mol electrons. The oxidation of 1 mol of benzene and toluene to carbon dioxide yielded 30 and 36 mol electrons, respectively. The electron balance between the amount theoretically derived from the amount of organic substrates consumed and the amount of electrons required for nitrate reduced to nitrite or nitrogen gas was close to 1.0. This supported the theoretical stoichiometry and the hypothesis that benzene and toluene were mineralized to carbon dioxide and water, and the corresponding stiochiometric equations were as follows:

$$C_6H_6 + 15NO_3^- \rightarrow 6CO_2 + 15NO_2^- + 3H_2O \quad (1)$$

$$C_6H_6 + 6NO_3^- + 6H^+ \rightarrow 6CO_2 + 3N_2 + 6H_2O \quad (2)$$

$$C_7H_8 + 18NO_3^- \rightarrow 7CO_2 + 18NO_2^- + 4H_2O \quad (3)$$

$$C_7H_8 + 7.2NO_3^- + 7.2H^+ \rightarrow 7CO_2 + 3.6N_2 + 7.6H_2O \quad (4)$$

To quantify the effect of BTEX mixtures on the degradation of each substrate, the average degradation rate of each BTEX substrate was calculated according to the variation of BTEX concentration between starting concentration and the end of approximately 0.5 mg/l, the results were listed in Table 1.

From Table 1 it could be found that, when the single substrate initial concentration was below 5 mg/l, the degradation rates of each BTEX substrate were higher with BTEX mixtures than with benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene or *p*-xylene alone. However, when the initial single substrate concentration was over 10 mg/l, each BTEX substrate degradation rates with BTEX combinations were lower than the degradation rates of individual BTEX systems. Under lower initial substrate concentration of 5 mg/l, the degradation rates in single substrate systems were lower due to the scarcity of carbon and energy source for bacterial growth [13]. Whereas the concentration of the mixtures was nearly six times higher than that of the single substrate systems, therefore there were much more substrates to satisfy the bacterial growth, which resulted in relatively higher degradation rates in BTEX mixtures systems. For example, the bacterial densities were $1.2 \times 10^{10} \pm 6 \times 10^8$ cells/l when the concentration of the mixtures was 30 mg/l. However, the bacterial densities were only $4.5 \times 10^9 \pm 2 \times 10^8$ cells/l when the concentration of the toluene was 5 mg/l. In contrast, when the initial single substrate concentration was 15 mg/l, the concentration of the mixtures was 90 mg/l and was toxic to the mixed bacteria, which resulted in relatively lower degradation rates compared to single substrate systems. Heipieper et al. stated that the main target for the toxicity of organic solvents to bacterial and eukaryotic cells was the cell membrane [23,24]. Sikkema et al. argued that the toxic effects of organic solvents were independent from the structural features of the molecules but rather were strongly related to their ability to accumulate in the membrane [25]. Therefore, the accumulation of the BTEX compounds in membranes may be another possibility to inhibit the bacterial growth, because these compounds accumulated in membranes can cause an increase in membrane fluidity which leads to an aspecific permeabilization [23,25].

Inspection of Table 1, it could also be found that the sum of degradation rates of the six BTEX substrates in mixtures was higher than the degradation rate of the single substrate when initial concentration was in the same level. For example, when the initial concentration of the mixtures was 30 mg/l, the sum of the six BTEX compounds degradation rates was 2.2 mg/l/d, which was two to three times higher than the degradation rate of single substrate with the initial concentration of 25 mg/l; under the condition of initial mixtures concentration 60 mg/l, the sum of degradation rates was 1.5–2 times higher than the degradation rate of single substrate with the initial concentration of 50 mg/l. The bacterial consortium contained bacteria similar to *P. aureofaciens*, *M. Lactuim* and *B. cereus* as identified using the methods described in Bergey's Manual of Systematic Bacteriology [14]. From these results, it could be inferred that different BTEX substrates were degraded by different bacteria.

5. Conclusions

The results of this research suggested that substrate interactions among BTEX mixtures were complicated, which were mainly including (1) the presence of one BTEX compound (e.g. toluene) would stimulate the degradation of the other BTEX substrates; (2) the presence of one BTEX compound (e.g. benzene, *p*-xylene) would inhibit the degradation of the other BTEX substrates; (3) the presence of lower BTEX concentrations had a stimulated effect on other BTEX compounds, whereas higher concentrations had an inhibited effect. The clear understanding of these substrate interactions would be beneficial to a better approach for remediation of BTEX-contaminated soil or ground-

water within a complex mixture such as gasoline. Experiments studying the effect of gasoline mixtures on the mineralization of BTEX would be conducted in our laboratory.

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