IN SITU GROUNDWATER REMEDIATION USING

ENRICHER REACTOR-PERMEABLE REACTIVE BIOBARRIER

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Title

In Situ Groundwater Remediation Using

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Permeable reactive biobarrier (PRBB) is a flow-through zone where microorganisms degrade contaminants in groundwater. Discontinuous presence of contaminants in groundwater causes performance loss of a PRBB in removing the target contaminant. A novel enricher reactor (ER) - PRBB system was developed to treat groundwater with contaminants that reappear after an absence period. ER is an offline reactor for enriching contaminant degraders, which were used for augmenting PRBB to maintain its performance after a period of contaminant absence. The ER-PRBB concept was initially applied to remove benzene that reappeared after absence periods of 10 and 25 days. PRBBs without ER augmentation experienced performance losses of up to 15% higher than ER-PRBBs.

The role of inducer compounds in the ER to enrich bacteria that can degrade a mixture of benzene, toluene, ethylbenzene, and xylene (BTEX) was investigated with an objective to minimize the use of toxic chemicals as inducers. Three inducer types were studied: individual BTEX compounds, BTEX mixture, and benzoate (a non toxic and a common intermediate for BTEX biodegradation). Complete BTEX removal was observed for degraders enriched on all three inducer types; however, the removal rates were dependent on the inducer type. Degraders enriched on toluene and BTEX had the highest degradation rates for BTEX of 0.006 to 0.014 day^{-1} and 0.006 to 0.012 day^{-1}, respectively, while degraders enriched on benzoate showed the lowest degradation rates of 0.004 to 0.009 day^{-1}.

The ER-PRBB technique was finally applied to address the performance loss of a PRBB due to inhibition interactions among BTEX, when the mixture reappeared after a

10 day absence period. The ER-PRBBs experienced minimal to no performance loss, while PRBBs without ER augmentation experienced performance losses between 11% and 35%. Presence of ethanol during the BTEX absence period increased the performance loss of PRBB for benzene removal. PRBBs augmented with degraders enriched on toluene alone overcame the inhibition interaction between benzene and toluene indicating that toluene can be used as a single effective inducer in an ER. The ER-PRBB was demonstrated to be a promising remediation technique and has potential for applications to a wide range of organic contaminants.

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CHAPTER 1. GENERAL INTRODUCTION

1.1. Background

The population growth in recent times has increased the demand for safe fresh water. About half of the United States (US) population and more than 20 percent of world population depend on groundwater for drinking and other direct uses. For some parts in the US, groundwater is the sole source of drinking water. Groundwater being a major fresh water resource in the nation and worldwide (about 97% of the world's fresh water), its contamination is a serious environmental concern. While groundwater contamination can occur by both natural processes and human activities, the contamination due to the latter is more predominant.

Major types of the man-made contamination are waste disposal practices such as deep-well injection of liquid wastes, release of toxic chemicals into streams and rivers, accidental spills/leaks from underground storage tanks (UST) and landfills, and improper management of household septic systems. About 50% of the USTs in the US were reported to leak as of 2006. As of 2011, more than 501,000 releases from federally-regulated leaking underground storage tanks (LUST) nationwide have been identified, of which over 88,000 still remain to be cleaned (USEPA 2011). Over 30,000 known abandoned and uncontrolled hazardous waste sites were identified for which cleanup efforts have not begun due to difficulty in identifying the responsible party. Many of these sites experience groundwater contamination by complex mixtures of chlorinated solvents, fuels, metals, and/or radioactive materials. Another source of groundwater contamination is excessive application of fertilizers and pesticides during agricultural practices. The remnants of

nutrients and pesticide chemicals seep through the soil and can quickly reach to shallow groundwater resources.

Once contaminated, groundwater can be difficult and expensive to treat. Some of the most common remediation methods include pump and treat, hydraulic containment, phytoremediation, air sparging, natural attenuation, intrinsic and enhanced bioremediation, and permeable reactive barriers (PRB). In the last 15 years, there has been an explosion of activity directed at the development and implementation of PRBs. A PRB is a reactive treatment zone oriented to intercept and transform or to retain a contaminant through physical, chemical or biological reactions (Figure 1.1).

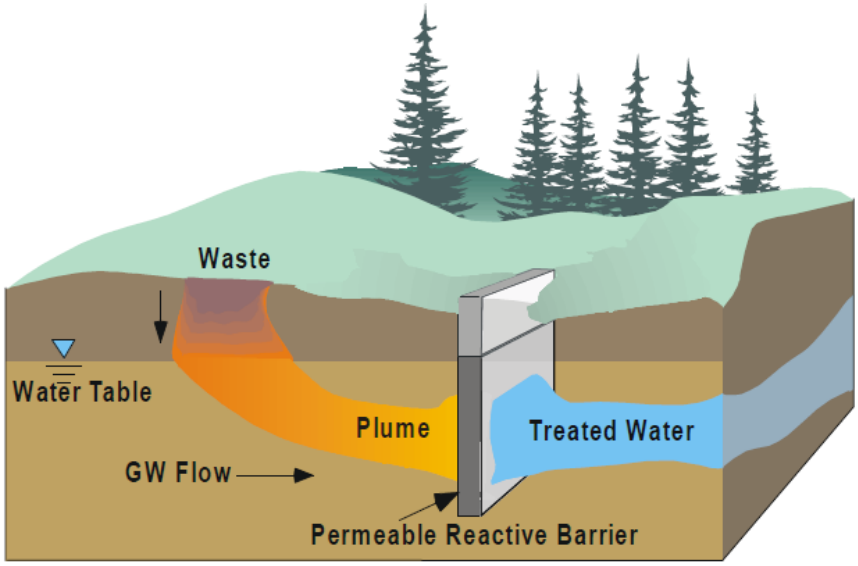


Figure 1.1. Schematic of a permeable reactive barrier (Adapted from Powell et al., 1998)

Degradation of man-made chemicals, particularly carcinogenic and toxic pollutants, in the groundwater by microorganisms (biodegradation) is considered to be eco-friendly. Biodegradation of many of the priority pollutants was found to be feasible in various environments (aerobic, anaerobic or anoxic). Due to advantages over physicochemical treatment such as low operation and maintenance costs, biological treatment is gaining

ground and its application through PRBs, commonly referred to as permeable reactive biobarriers (PRBB), has been widely studied. As contaminated water passes through the PRBB, microorganisms convert the contaminant into innocuous byproducts such as carbon dioxide and water. Different types of biobarriers are currently in use and some of them are permeable mulch walls (also known as biowalls), bioborings, or injection wells. PRBBs have been successfully applied in remediation of groundwater polluted with a wide range of organic as well inorganic contaminants.

1.2. Research Problem Statement

Major limitations of PRBBs are possible loss of capability to biodegrade a contaminant due to 1) periodic absence of a single target compound, 2) periodic absence of a mixture of target compounds, and 3) appearance of an alternate carbon source during the absence period of target compound(s).

The microbial community in a PRBB can include a combination of bacterial culture that can degrade the target contaminant and the culture that cannot. Absence of a target contaminant can cause partial or complete loss of contaminant degraders in number and/or in their biodegradation activity (Mathur et al., 2006; Chong et al., 2008), which can result in performance loss of PRBB when the contaminant reappears. In case of partial loss of contaminant degraders, long lag periods are often required for them to grow to a critical concentration capable of exerting measurable degradation rates (Mathur et al., 2006). In case of complete loss of contaminant degraders, the performance of PRBB may never recover.

Successful PRBB application for a mixture of contaminants is often influenced by many different substrate interactions such as synergistic or antagonistic among individual

contaminants in the mixture (Arvin et al., 1989; Barbaro et al., 1992; Wang and Deshusses, 2007). Synergistic interactions promote the degradation rates of individual contaminants while the antagonistic interactions reduce the degradation rates through various inhibition processes. Absence of one or the entire target contaminants in a mixture (such as benzene, toluene, ethylbenzene, and xylene which are collectively known as BTEX) can lead to a loss of some of the degraders, which are necessary for interspecies interactions for the degradation of contaminant mixture. Partial loss of the degraders can lead to greater substrate interactions (especially inhibition) among co-existing contaminants such as BTEX than prior to the absence period, which can lead to decrease in removal efficiency of a PRBB. No research has been conducted on whether the inhibition interactions among BTEX prevail when BTEX compounds reappear after a period of absence under anaerobic conditions.

In addition to the absence of target contaminant, a PRBB can experience increased performance losses due to the appearance of an alternate carbon source (other than target contaminant or contaminant mixture) during the absence period. The presence of alternate carbon source can either alter the bacterial community promoting the culture that cannot degrade the target contaminant or suppress the enzymatic activity necessary to degrade the target contaminant. Moreover, for mixture of contaminants, the presence of alternate carbon source can lead to increased antagonistic substrate interactions among the contaminant mixture than prior to the absence period.

1.3. Research Goal and Approach

The goal of the research is to develop a successful PRBB system by overcoming the performance losses superimposed by the limitation of absence period. To achieve this

goal, a combined enricher reactor and PRBB (ER-PRBB) system is developed to treat the groundwater with contaminants that co-exist, intermittently present and exhibit substrate interactions. A schematic representation of ER-PRBB system is shown in Figure 1.2. The PRBB is a main bioreactor that treats the contaminated groundwater. ER is an offline aboveground reactor where bacteria are constantly enriched by supplying necessary growth materials and are used to augment the PRBB system to maintain the system performance.

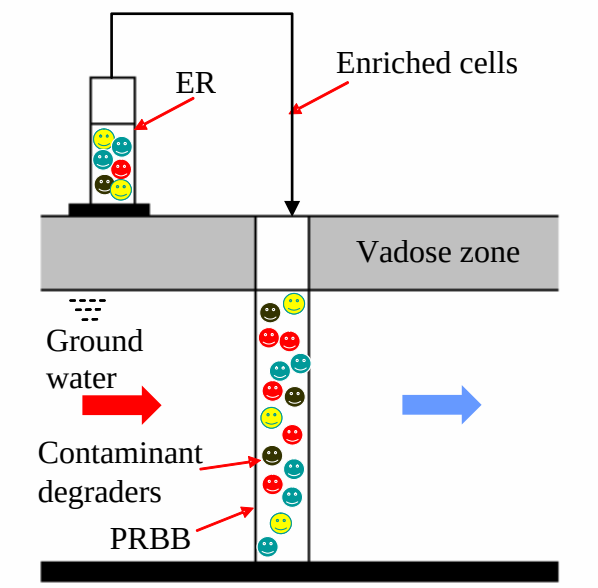


Figure 1.2. A diagram of the enricher reactor-permeable reactive biobarrier (ER-PRBB) concept.

ER is a bioaugmentation technique widely used in biological waste treatment systems, where cells acclimated to target compounds are continuously enriched and are used to augment a main treatment system such as activated sludge. Appropriate growth conditions for culture enrichment such as availability of nutrients and target compounds, and suitable environmental conditions (pH and temperature) are provided in the ERs to induce the desired degradation capability. The use of ER technique in wastewater treatment was introduced by Cardinal and Stenstrom (1991) for the removal of naphthalene

and phenanthrene. This technique was not applied for groundwater remediation. In the present study, the ER-PRBB concept was applied for treating groundwater contaminated with benzene, toluene, ethylbenzene, and xylene isomers (BTEX).

1.4. Objectives

The following objectives and the corresponding hypotheses were developed.

1. To evaluate the removal performance of a PRBB system with and without supply of bacteria from ERs when benzene reappears after a period of absence.

   Hypothesis: Supply of enriched bacteria from an ER will prevent PRBB performance loss during reappearance of benzene in groundwater.

2. To investigate the effects of different carbon sources during the enrichment of microbial consortia on their degradation rates of a mixture of BTEX.

   Hypothesis: Benzoate is a better inducer than individual BTEX compounds or BTEX mixture in enriching bacterial communities capable of degrading BTEX at higher rates.

3. To examine the application of ER-PRBB system to remediate groundwater contaminated with BTEX mixture, when the mixture appears in batches.

   Hypothesis: Supply of enriched bacteria from an ER will minimize antagonistic substrate interactions among BTEX mixture in a PRBB when the mixture reappears after an absence period.

1.5. Dissertation Organization

This dissertation is divided into 6 chapters. This chapter includes background, research problem statement, research goal and approach, objectives and hypotheses, and dissertation organization. A review of pertinent literature is presented in Chapter 2 to

provide an understanding of the topic, as well as to identify the existing gaps that support the need for this research.

Chapter 3 presents the work relevant to the first objective, development and evaluation of an ER-PRBB for remediating groundwater contaminated with a periodically absent contaminant. The work described in Chapter 3 has been published in the proceedings for Water Environment Federation Technical Exhibition and Conference (WEFTEC) 2010 (Kasi et al., 2010) and in *Water Environment Research* (Kasi et al., 2011). Chapter 4 presents the work relevant to the second objective. This chapter is derived from a manuscript entitled "Effect of Carbon Source during Enrichment on BTEX Degradation by Anaerobic Mixed Bacterial Cultures." This manuscript is under review by *Biodegradation* for publication (Kasi et al., 2012). The work relevant to the third objective is presented in Chapter 5. This chapter is derived from a manuscript entitled "A Novel Application of Enricher Reactor - Permeable Reactive Biobarrier for Removing a Mixture of Contaminants with Substrate Interactions." This manuscript will be submitted for publication in *Journal of Hazardous Materials*. Conclusions and recommendations for future work are presented in Chapter 6.

CHAPTER 2. LITERATURE REVIEW

2.1. Permeable Reactive Biobarrier (PRBB)

PRBBs can be classified based on construction type, functionality, and materials inside the biobarrier. To date, the PRBBs based on construction type can be continuous permeable wall, funnel and gate, in-situ reactive vessels, injection system, and hydraulic/pneumatic fracturing. The size of a PRBB is determined based on the desired treatment level, the ground-water velocity through the PRBB, and the residence time required to achieve the target treatment level. Some of the construction based configurations are illustrated in Figure 2.1. Based on functionality, a PRBB can be active or passive. Active biobarriers require substantial operation and maintenance to either frequently deliver the necessary materials to the desired depths or circulate and mix the supplied materials within in the subsurface. Passive biobarriers typically contain reactive materials (e.g., vegetable oils, oxygen releasing compounds) that can last for longer periods of time and maintain the reactive zone. The third type of PRBB classification (which is based on materials inside the barrier) includes permeable mulch walls (also known as biowalls), bioborings, or injection wells. In all these types of PRBBs, target contaminant degrading microbial population is either present from the indigenous population or is established with biostimulation and/or bioaugmentation.

In the biostimulation type of PRBB, the activity and/or number of indigenous contaminant degraders are stimulated by supplying necessary nutrients and environment (Margesin and Schinner, 2001a). Some examples of biostimulation are the addition of macro nutrients such as nitrogen, phosphorus, and carbon, trace minerals, addition of electron acceptors such as oxygen, nitrate, and sulfate, and making appropriate pH

adjustments. The nutrients are typically supplied through addition of fertilizers (Menendez-Vega et al., 2007; Olaniran et al., 2006). Carbon as an electron donor is sometimes supplied from other sources such as peat (Kao and Lei, 2000; Kao et al., 2001), sludge cake and cane molasses (Kao et al., 2003). Electron acceptors such as oxygen are quickly depleted in the subsurface. Hence, time-releasing compounds such as hydrogen peroxide (Menendez-Vega et al., 2007) and calcium peroxide (Kao et al., 2001) were used to supply oxygen in several past studies. In anaerobic conditions, a passive nutrient wall (or briquet) was also used to slowly release nitrate (Kao and Borden, 1997).

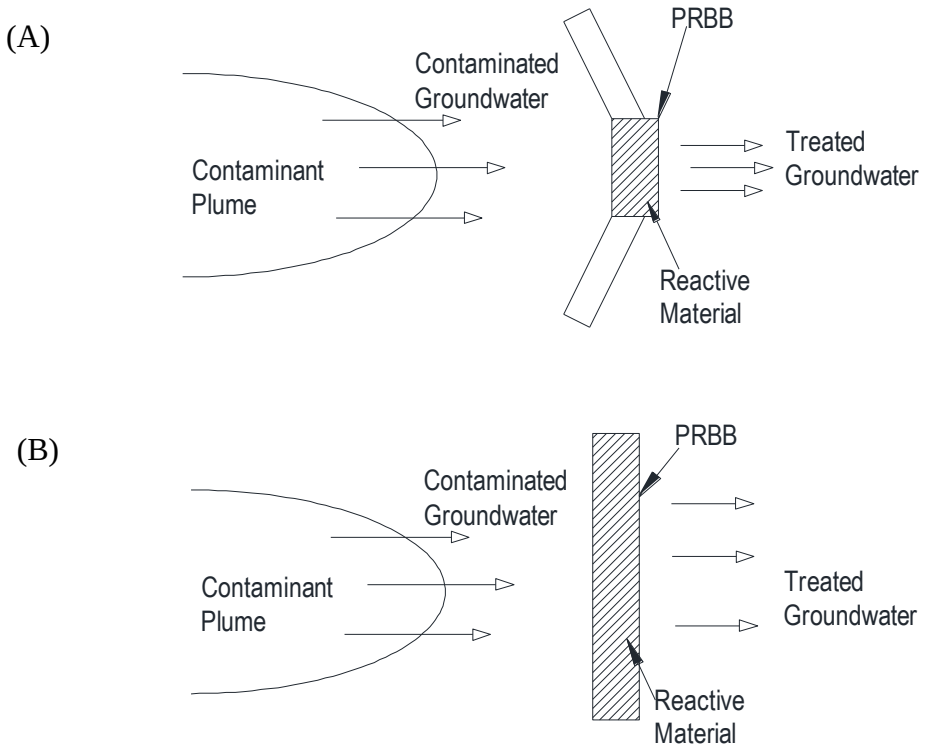


Figure 2.1. PRBB configurations based on construction type: (A) funnel and gate (B) continuous wall.

In the bioaugmentation type of PRBB, the biobarrier is seeded with contaminant-degrading organisms and supplied with necessary nutrients. There are two major reasons that lead to bioaugmentation. The first being the presence of a small indigenous microbial

population that does not have the capability to degrade the target contaminant, and the second is the need to rapidly biodegrade the target contaminant with little or no start-up period. The contaminant-degrading organisms can be indigenous bacteria, pure culture(s), or mixed microbial cultures that are enriched by adapting to the concerned contaminant(s) (Miller et al., 2002). Among these, mixed microbial cultures are often found to be the most effective as they comprise a wide range of degradation mechanisms.

In addition to the type of microbial cultures, the success of bioaugmentation depends on environmental parameters such as humidity, microbial predation and bioavailability. In many situations, to provide necessary conditions for the added organisms, bioaugmentation is combined with biostimulation. For example, Olaniran et al. have supplied nutrients and oxygen to the added microbial pure cultures to degrade dichloroethenes (DCEs) (Olaniran et al., 2006). They found that the bioaugmented microcosms with biostimulation had significantly increased the degradation of DCEs and their intermediates above those observed in the bioaugmented microcosms without biostimulation.

2.2. Groundwater Contaminants

Groundwater contamination by petroleum hydrocarbons is one of the most common current environmental problems. The United States Environmental Protection Agency (USEPA) estimates that millions of liters of gasoline are spilled each year from the underground storage tanks. Gasoline is comprised of a variety of aliphatic and aromatic hydrocarbons, the aromatic portion consisting primarily of benzene, toluene, ethylbenzene, and xylene isomers (collectively known as BTEX compounds). Furthermore, BTEX compounds are among the top 50 chemicals manufactured in the US. In addition to

petroleum industry, they are also employed in the production of other chemicals such as monomers, plasticizers in polymers, and are also widely used as solvents. Aqueous discharge of these industrial effluents can also lead to groundwater contamination by BTEX.

All of the BTEX chemicals can produce neurological impairment, and exposure to benzene can additionally cause acute myelogenous leukemia (Pohl et al., 2003). BTEX compounds are also placed on the priority list of hazardous substances of the 2005 Comprehensive Environmental Response, Compensation and Liability Act. BTEX are relatively more water soluble than other gasoline hydrocarbons and tend to migrate from contaminated soils into nearby aquifers. The USEPA has established maximum contaminant levels (MCL) for these compounds in drinking water: 5 µg/L for benzene, 1 mg/L for toluene, 0.7 mg/L ethylbenzene, and 10 mg/L for total xylenes (which include ortho, meta and para).

2.3. BTEX Physical and Chemical Properties

A summary of various BTEX physical and chemical properties is provided in Table 2.1. All BTEX compounds are relatively very soluble in water than other hydrocarbons in gasoline, with benzene being the most soluble within BTEX. Moreover, relatively low values of octanol/water partitioning coefficient (typical range for organic solvents is between 10^{-3} and 10^{7}) and soil partitioning coefficient (K_{oc}) also indicate that these chemicals can travel long distances in groundwater from the source.

Table 2.1. Physical and Chemical Properties of BTEX. (Adapted from: Zogorski et al., 1997)

Chemical	Physical Description	Molecular Structure	Molecular Weight (g/mol)	Specific Gravity @ 20°C	Boiling Point (^{o}C)	Water Solubility (mg/L)	Log K_{ow}	Vapor Pressure @ 25 ^{o}C (mm Hg)	Log K_{oc}	Henry's Law Constant
Benzene	Colorless to light-yellow liquid with an aromatic odor	C_6H_6	78.11	0.8787	80.1	1780	2.13	95.2	1.5-2.16	0.2219
Toluene	Colorless liquid with a sweet, pungent, benzene-like odor	$C_6H_5CH_3$	92.13	0.8669	110.6	534.8	2.73	28.4	1.56-2.25	0.2428
Ethylbenzene	Colorless liquid with an aromatic odor	$C_6H_5CH_3CH_2$	106.16	0.8670	136.25	161	3.15	9.53	1.98-3.04	0.345
Xylene	Colorless liquid with an aromatic odor	$C_6H_4(CH_3)_2$								
m-Xylene			106.16	0.8842	139.03	146	3.20	8.3	2.04-3.15	0.3139
o-Xylene			106.16	0.8802	144.4	175	3.12	6.6	1.68-1.83	0.0208
p-Xylene			106.17	0.8611	137	156	3.15	8.7	2.05-3.08	0.3139

2.4. Discontinuous Plumes of BTEX in the Groundwater

BTEX are light non-aqueous phase liquids (LNAPL). Discontinuous plumes of LNAPL can occur in ground water. When spilled LNAPLs reach the top of water-table, they form a continuous film on the water table and spread horizontally with water flow. However, when large spills occur, a sufficient head for the LNAPL will be created to displace some of the pore water from the capillary pressure and even from below the original water table. In such cases, hydrostatic equilibrium may be temporarily lost as groundwater tends to mound around the free LNAPL plume. When groundwater seeks to re-establish its equilibrium, it may trap part of the LNAPL plume under the water table (Hamed et al., 2000; Sahloul et al., 2002). These trapped residual LNAPL, also known as isolated ganglia or blobs, act as individual sources and generate discontinuous plumes (Figure 2-2).

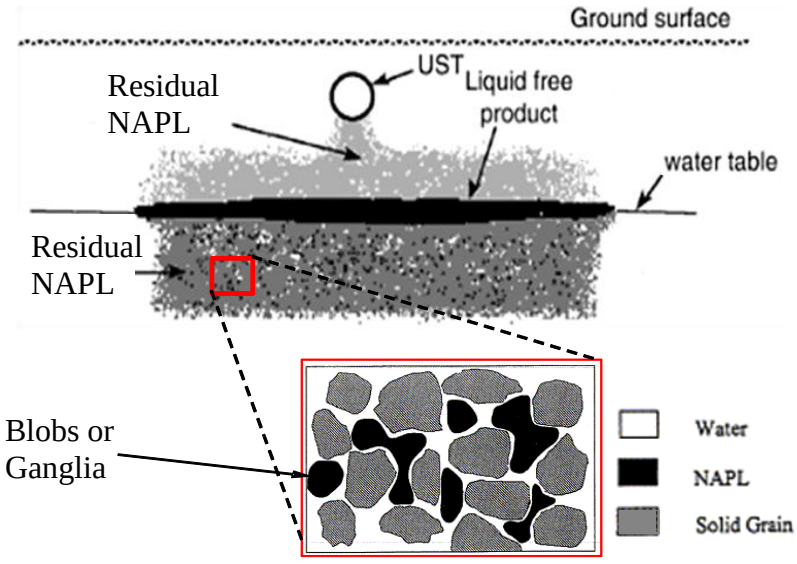


Figure 2.2. Discontinuous plumes of LNAPL in groundwater. (Modified from Sahloul et al., 2002)

Fluctuations in the water table level can also cause LNAPL entrapment in the saturated zone (Figure 2-3b). Groundwater table fluctuations are normal and are affected

by a number of factors that include infiltration, irrigation and pumping. During the drawdown condition of groundwater, a layer of mobile LNAPL follows the water table. As the water table rises during wet conditions (e.g. infiltration), major part of the capillary forces that hold residual LNAPL in the soil pores can be overcome by the forces from the rising groundwater flow, leaving small portions of the LNAPL entrapped in the saturated zone.

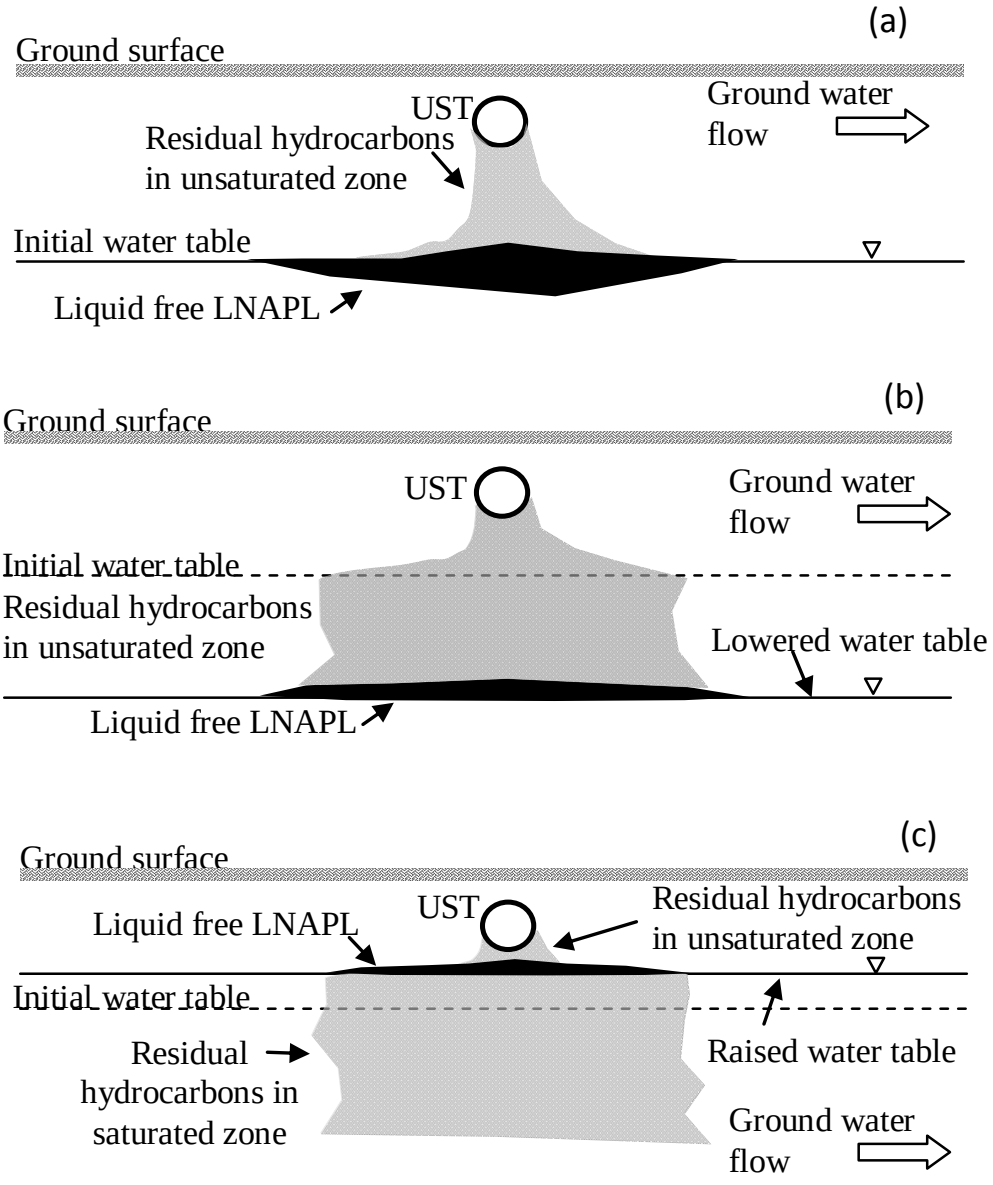


Figure 2.3. Effects of groundwater water table fluctuations on residual LNAPL.

2.5. Biodegradation and Bioremediation of BTEX

BTEX compounds can be biodegraded both aerobically and anaerobically. Aerobic systems typically exhibit a broader catabolic range and faster degradation kinetics than anaerobic systems. However, in the subsurface environment, available oxygen is quickly depleted especially when co-contaminants with high biochemical oxygen demand such as ethanol are present. Furthermore, in aerobic bioremediation, oxygen addition could lead to clogging problems due to the precipitation of metal oxides (Wiesner et al., 1996). In contrast to earlier studies, recent research has reported complete mineralization of individual BTEX compounds in anaerobic conditions.

BTEX degradation has been demonstrated under the major terminal electron accepting processes in anaerobic environments including nitrate, iron, sulfate, and CO_2 (Burland and Edwards, 1999; Chakraborty et al., 2005). In general, anaerobic BTEX degradation was found to occur rather rapidly under denitrifying conditions (Hu et al., 2007). Moreover, past studies showed that BTEX degradation with nitrate as terminal electron acceptor requires relatively less energy for cell synthesis. A summary of energy consumption for different BTEX compounds under various electron acceptor conditions is presented in Table 2.2.

2.5.1. Anaerobic BTEX Metabolic Pathways

Many studies have been conducted on biochemical reactions during anaerobic degradation of BTEX compounds. However, the metabolic pathways and intermediates are not completely elucidated. The observed pathways thus far revealed that anaerobic BTEX degradation uses reductive reactions such as carboxylation and dehydroxylation. In

general, these reactions are initially activated through different pathways specific to a BTEX compound, degrading organism and terminal electron acceptor (TEA) (Figure 2.4).

These initial degradation pathways of BTEX compounds lead to a formation of benzoate or its co-enzyme A (CoA) derivative, benzoyl-CoA, as a central common intermediate. Benzoyl-CoA then undergoes a further ring reduction catalyzed by benzoyl CoA reductase coupled to adenosine triphosphosphate (ATP) hydrolysis (Heider and Fuchs, 1997). Further reduction reactions on benzoyl CoA lead to ring cleavage and formation of acetyl CoA which will be mineralized.

Table 2.2. Anaerobic BTEX biodegradation: redox reactions and associated free energy change.

Redox pathways for BTEX degradation	**Free energy change (ΔG°) (kJ/mol)**
Benzene	
$C_6H_6 + 7.5O_2 + 3H_2O \rightarrow 6H^+ + 6HCO_3^-$	-3066
$C_6H_6 + 6NO_3^- \rightarrow 6HCO_3^- + 3N_2$	-3002
$C_6H_6 + 30FeOOH + 54H^+ \rightarrow 42H_2O + 30Fe^{2+} + 6HCO_3^-$	-1370
$C_6H_6 + 3.75SO_4^{2-} + 3H_2O \rightarrow 2.25H^+ + 6HCO_3^- + 3.75HS^-$	-105
Toluene	
$C_7H_8 + 9O_2 + 3H_2O \rightarrow 7H^+ + 7HCO_3^-$	-3670
$C_7H_8 + 7.2NO_3^- + 0.2H^+ \rightarrow 0.6H_2O + 7HCO_3^- + 3.6N_2$	-3593
$C_7H_8 + 36FeOOH + 65H^+ \rightarrow 51H_2O + 36Fe^{2+} + 7HCO_3^-$	-1635
$C_7H_8 + 4.5SO_4^{2-} + 3H_2O \rightarrow 2.5H^+ + 7HCO_3^- + 4.5HS^-$	-118
Ethylbenzene/xylene	
$C_8H_{10} + 10.5O_2 + 3H_2O \rightarrow 8H^+ + 8HCO_3^-$	-4291/-4307
$C_8H_{10} + 8.4NO_3^- + 0.4H^+ \rightarrow 1.2H_2O + 8HCO_3^- + 4.2N_2$	-4201/-4217
$C_8H_{10} + 42FeOOH + 76H^+ \rightarrow 60H_2O + 42Fe^{2+} + 8HCO_3^-$	-1917/-1933
$C_8H_{10} + 5.2SO_4^{2-} + 3H_2O \rightarrow 2.75H^+ + 8HCO_3^- + 5.25HS^-$	-146/-162

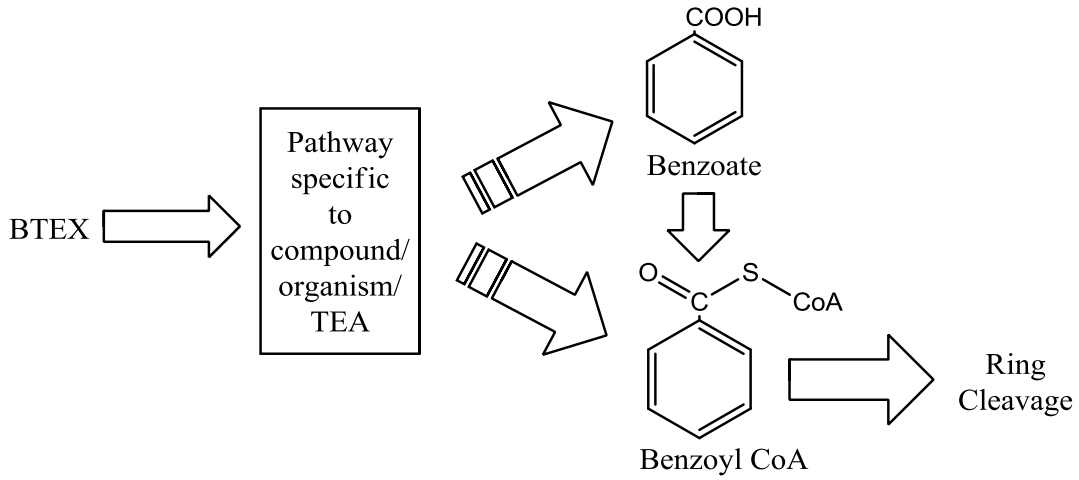


Figure 2.4. Generalized BTEX biodegradation pathways in anaerobic conditions.

The biochemical pathway of anaerobic benzene degradation is currently unknown but several possibilities exist. These include carboxylation, methylation, or hydroxylation with subsequent transformation to the central aromatic intermediate benzoyl-CoA and ring cleavage (Figure 2.5). Possible intermediates in anaerobic degradation of benzene include phenol, toluene, benzoate, cyclohexanone, and propionate.

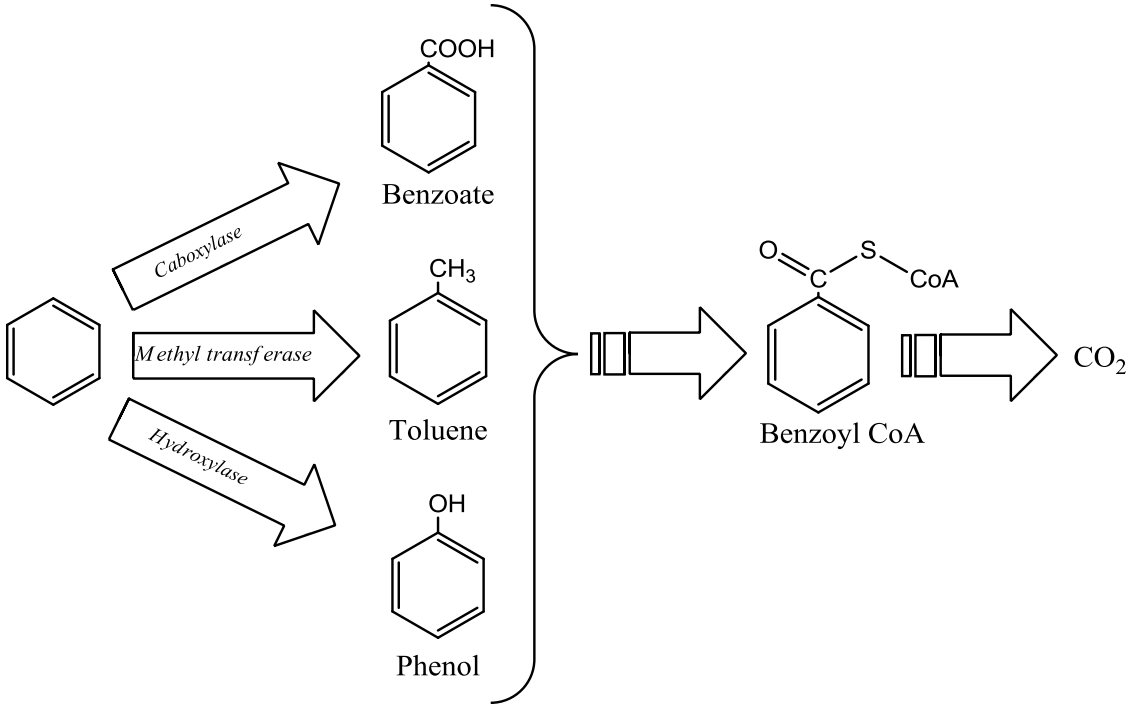


Figure 2.5. Possible benzene denitrification pathways (Ref: Chakraborty and Coates, 2004)

The initial anaerobic degradation of toluene could proceed via different pathways, e.g., (i) via carboxylation to phenylacetate and from there by oxidation via phenylglyoxylate to benzoyl-CoA (Dangel et al., 1991), (ii) via ring hydroxylation to *p*-cresol and by oxidation via 4-hydroxybenzoate to benzoyl-CoA (Rudolphi et al., 1991), or (iii) via phenylpropionate to benzoyl-CoA formation (Evans et al., 1992). However, the most common first step in the catabolism of toluene is reported as the addition of fumarate onto the toluene methyl group to form benzylsuccinate, which is further metabolized to benzoyl-CoA, via a proposed pathway shown in Figure 2.6. Another possible pathway, suggested by Altenschmidt and Fuchs (1992), includes formation of benzyl alcohol as an initial intermediate followed by benzaldehyde, benzoate and finally to benzoyl-CoA.

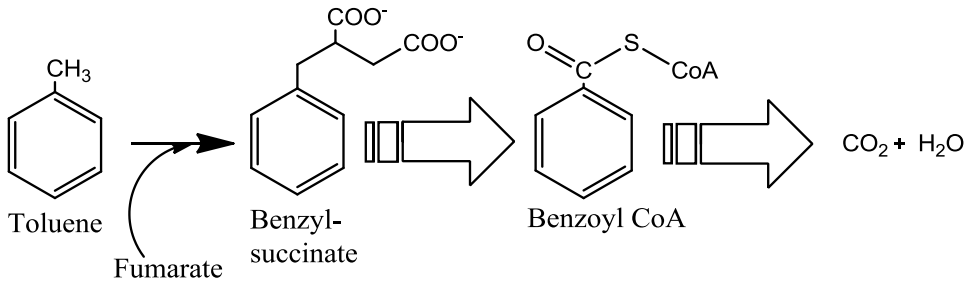


Figure 2.6. Proposed pathway of anaerobic oxidation of toluene to benzoyl-CoA through the formation of benzylsuccinate (Modified from Heider et al., 1998).

The first product of anoxic ethylbenzene oxidation is 1-phenylethanol through dehydrogenation (Figure 2.7). The hydroxyl group of this first intermediate is derived from water (Ball et al., 1996). 1-Phenylethanol is oxidized further to form acetophenone. Ball et al. (1996) proposed that acetophenone may be carboxylated to form benzoyl acetate, which can be activated to benzoyl acetyl coenzyme A (benzoyl acetyl-CoA) and subsequently, a thiolytic cleavage of benzoyl acetyl-CoA would generate acetyl- CoA plus benzoyl-CoA.

Anaerobic degradation of *m*- and *o*- xylenes proceeds, in analogy to that of toluene, via methylbenzylsuccinate (Figure 2.8) to methylbenzoyl-CoA. *p*-Xylene degradation by

denitrifying enriched culture has been identified (Haner et al., 1995), however, the pathways are not yet known.

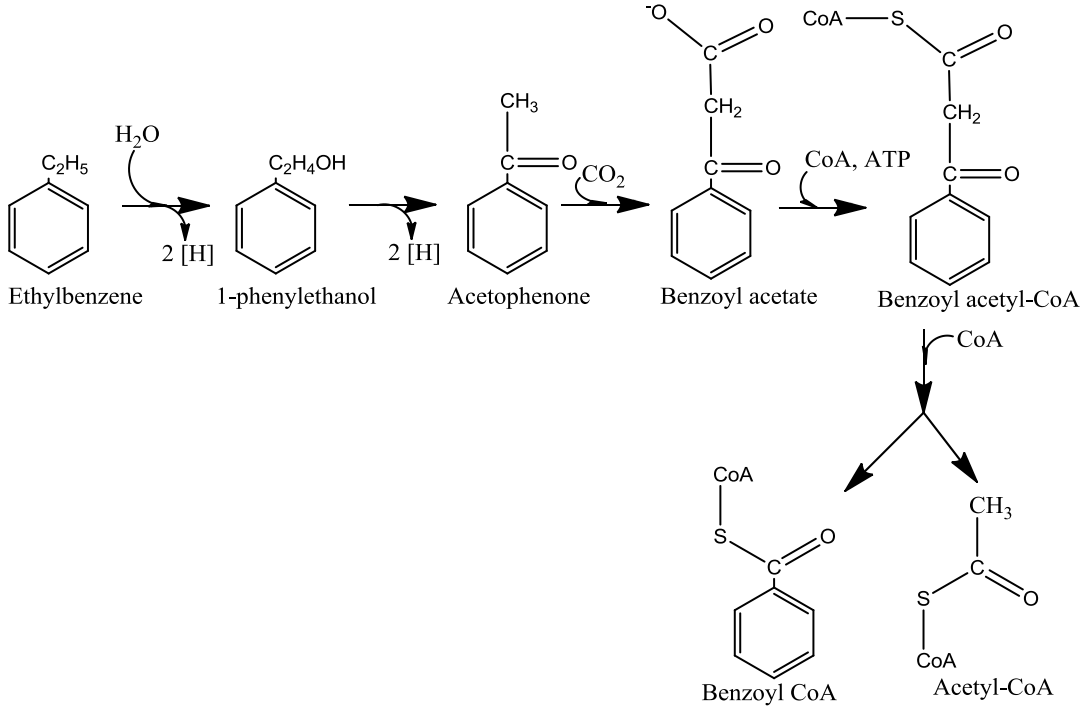


Figure 2.7. Proposed pathway of initial reactions in anoxic ethylbenzene oxidation in strain EB1 (Ref: Ball et al., 1996).

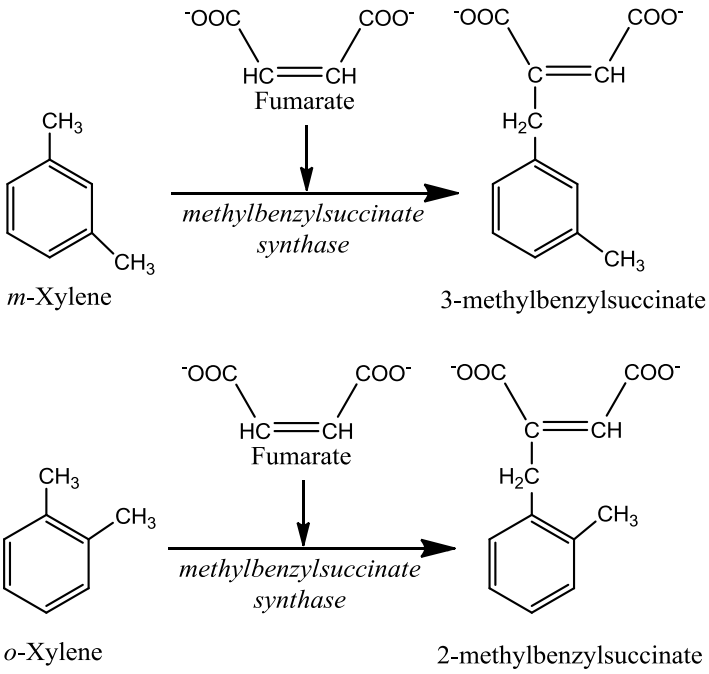


Figure 2.8. Initial anaerobic degradation pathways for *o*- and *m*- xylenes (Ref: Chakraborty and Coates, 2004).

2.5.2. Substrate Interactions Among BTEX During Anaerobic Degradation

Biodegradation of a mixture of compounds by microorganisms is often affected by synergistic and antagonistic substrate interactions. Synergistic interactions can be cometabolism or enhanced biodegradation. In cometabolism, the metabolic transformation of non-growth substrate occurs in the obligate presence of a growth substrate. Enhanced biodegradation represents increased utilization of different substrates for growth.

For antagonistic interactions, the presence of one compound has a negative effect on the biodegradation of another compound. Antagonistic interactions can be preferential degradation, competitive inhibition, non-competitive inhibition and uncompetitive inhibition (Figure 2.9). Preferential degradation, also known as diauxie, is a sequential utilization of substrates in which bacterial community feeds on substrate with the highest energy yield first. In competitive inhibition, a molecule very close in structure to the true substrate binds to the same active site of enzyme, without undergoing a reaction, and effectively reduces the concentration of available degradation enzyme. In non-competitive inhibition, the inhibitor binds at a site other than the active site of enzyme, causes a change in the structure and shape of the enzyme and finally affecting the degradation activity of enzyme for the substrate. Uncompetitive inhibitors bind either to a modified form of enzyme when a substrate is bound or to an enzyme-bound substrate, forming a dead end complex.

Several different substrate interactions have been observed in various studies on biodegradation of BTEX combinations by pure as well as mixed cultures (Arvin et al., 1989; Barbaro et al., 1992; Wang and Deshusses, 2007; Jo et al., 2008). The differences in the substrate interactions are mainly due to catabolic diversity of microbes in different

environmental conditions. Alvarez and Vogel (1991) investigated substrate interactions among benzene, toluene and *p*-xylene during biodegradation by indigenous mixed cultures and two pure cultures (*Pseudomonas* sp. Strain CFS-215 and *Arthrobacter* sp. Strain HCB) in anaerobic conditions. The general trend for all three cultures in their study was that degradation of benzene and toluene was enhanced by the presence of each other and retarded by the presence of *p*-xylene. The degradation of *p*-xylene was enhanced by the presence of toluene for all the pure cultures. The presence of benzene enhanced the *p*-xylene degradation for one culture and inhibited its degradation for the other culture.

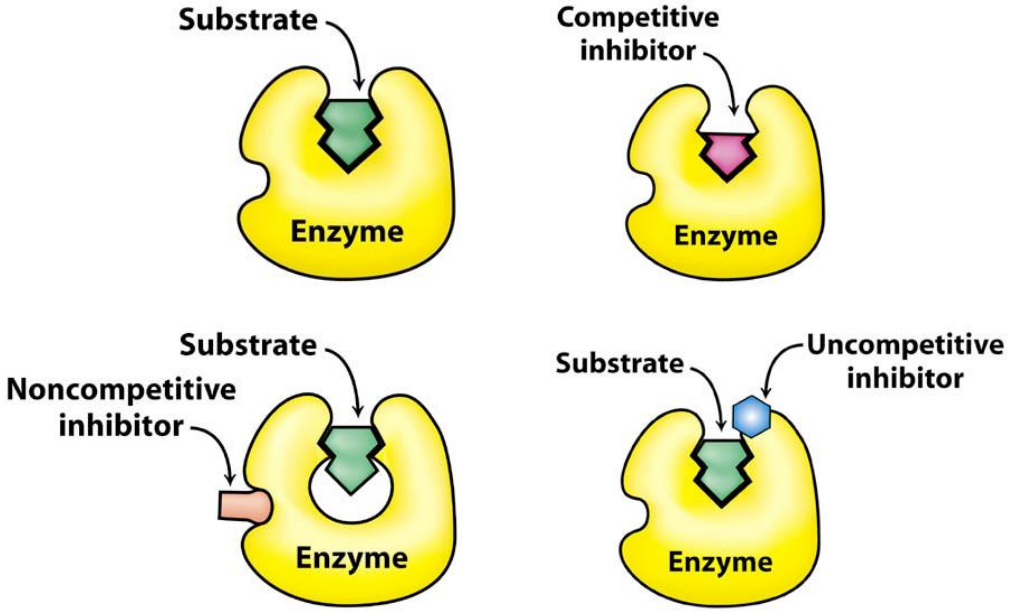


Figure 2.9. Illustration of different types of enzymatic inhibition processes (Adapted from Berg et al., 2006).

As shown earlier in Figure 2.5, anaerobic benzene biodegradation may involve several pathways and the initial enzymatic activities for these pathways usually involve enzymes such as *carboxylase*, *hydroxylase*, or *methyl transferase*. Few studies have also shown that anaerobic toluene degradation can be initiated via carboxylation to phenylacetate or via ring hydroxylation to *p*-cresol, or via phenylpropionate to benzoyl-

CoA formation, in addition to the pathway shown in Figure 2.6. The first two pathways in toluene mineralization utilize the same enzymes as in anaerobic benzene degradation, which can lead to a competition for enzymes between these two compounds when they are present together.

Chang et al. (1993) observed cometabolic degradation of *p*-xylene in the presence of both toluene and benzene for *Pseudomonas fragi* and competitive inhibition for degradation of benzene by the presence of toluene and *p*-xylene for *Pseudomonas fluorescens*. Dou et al. (2008) conducted a study on BTEX compounds as binary combinations on enriched mixed consortia from a gasoline contaminated soil. Stimulation effects on degradation for benzene and xylenes when either of them was present in binary mixtures with toluene or ethylbenzene were observed. The presence of benzene and xylenes had inhibitory effect on degradation of any other BTEX compounds in binary mixtures.

2.5.3. Anaerobic Bioremediation of BTEX Mixtures

Antagonistic substrate interactions are the major issues in bioremediation of BTEX mixtures. Individual bacterial strains were unable to degrade all the BTEX compounds simultaneously. Co-cultures (i.e. more than one bacterial species) were used in various studies for the simultaneous degradation of these compounds. Attaway and Schmidt (2002) showed better removal of all BTEX compounds with a mixture of two *Pseudomonas putida* isolates as compared to individual isolates of the same bacterial cultures. Simultaneous removal of all BTEX compounds except *o*-xylene was observed.

Shim and Yang (2002) have used a fibrous-bed bioreactor with immobilized co-culture of *Pseudomonas putida* and *P. fluorescens* for the removal of BTEX compounds in

oxygen limited conditions. Hydrogen peroxide was used as supplemental oxygen source in the bioreactor. The bioreactor had successfully removed all BTEX compounds simultaneously after a short period of acclimation. The superior performance of co-culture immobilized bioreactor over free cells was attributed to high cell count. The performance of the bioreactor, however, experienced decreased degradation rates due to substrate inhibition effects at BTEX concentrations above 0.5 mg/L. The types of inhibition effects were not discussed in this study.

Choi and Oh (2002) used a combination of several pure bacterial strains to treat benzene, toluene and xylenes (BTX) mixture. The bacterial strains that can degrade each compound in the BTX mixture were identified and combinations of these bacterial strains were used as co-cultures to treat the BTX mixture. Toluene and *m*- and *p*-xylenes were simultaneously removed followed by benzene and then *o*-xylene.

2.6. Bioaugmentation by Enricher Reactors (ER)

When target contaminants are not continuously present and/or environmental conditions are not suitable, biological treatment systems can experience a decrease in performance due to the loss of degradation activity of the bacterial cultures. The decrease in the degradation activity may be due to decrease in the numbers of acclimated bacteria or loss of enzymatic activity for contaminant degradation.

ER is a bioaugmentation technique, used to maintain the acclimated bacteria in the biological treatment systems. In this technique, cells acclimated to target compounds are inoculated in an offline ER and are augmented in the activated sludge system (Figure 2.10). Appropriate growth conditions for culture enrichment such as availability of

nutrients, target compounds and suitable environmental conditions (pH and temperature) are provided in the ERs to induce the desired degradation capability.

The ER technique in wastewater treatment was initially used by Cardinal and Stenstrom for the removal of naphthalene and phenanthrene (Cardinal and Stenstrom, 1991). This concept was subsequently applied successfully in the removal of 1-naphthylamine (Babcock et al., 1992) and pentachlorophenol (Jittawattanarat et al., 2007a), nitrogen (Jittawattanarat et al., 2007b), and pharmaceutical drugs such as cephalexin and cephradine (Saravanane et al., 2001a, b) from wastewater.

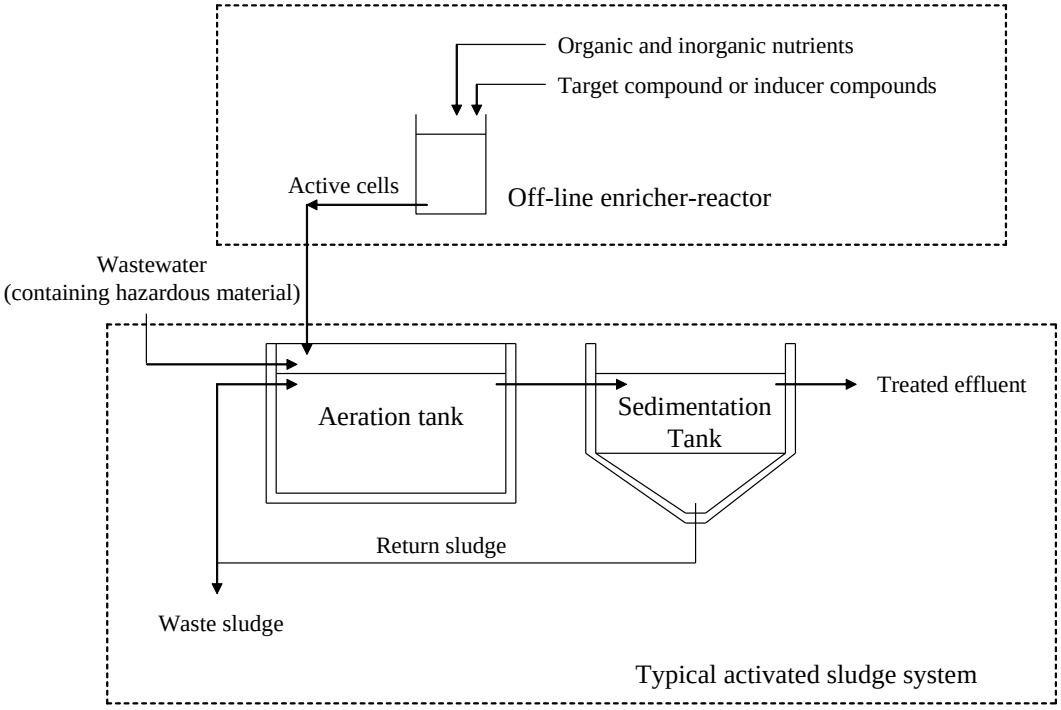


Figure 2.10. Schematic diagram of the enricher-reactor process used in wastewater treatment (Cardinal and Stenstrom, 1991; Babcock et al., 1992)

During the bioaugmentation with ER technique, the acclimated cultures are mixed and wasted through the excess sludge in the activated sludge systems. Jittawattanarat et al. (2007a) developed an immobilized-cell-augmented activated sludge (ICAAS) system (Figure 2.11) based on the ER concept to treat wastewater intermittently contaminated with

pentachlorophenol (PCP). In this process, a portion of the immobilized mixed culture cells that were enriched in an off-line ER, were supplemented to an activated sludge system. Whenever the performance of the activated sludge decreased, the immobilized cells in the aeration tank were returned to the ER for reactivation, while active immobilized cells from the ER were placed in the aeration tank.

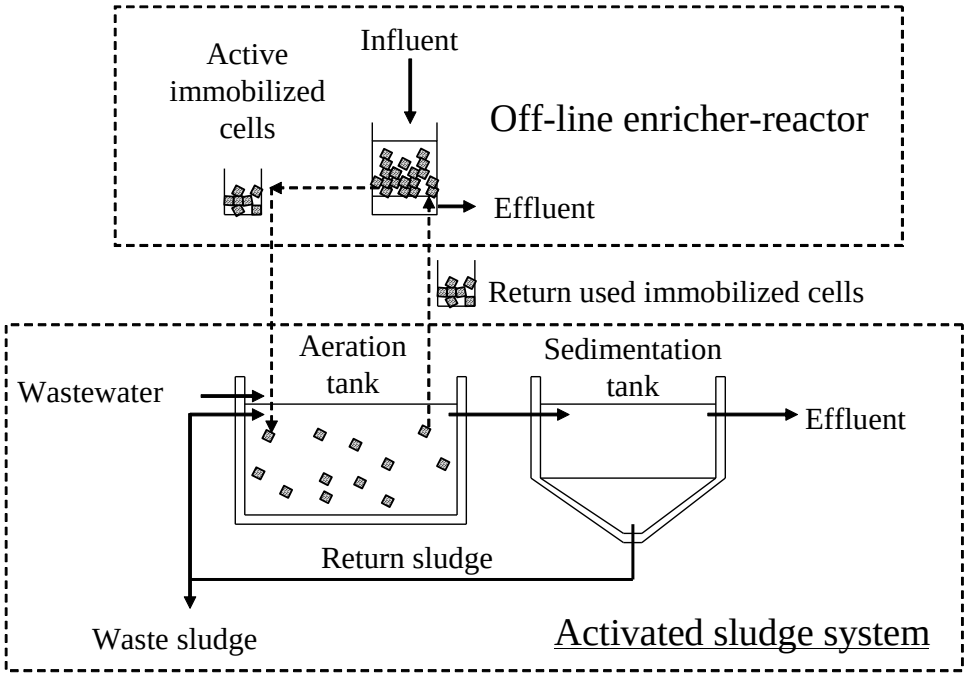


Figure 2.11. Schematic diagram of ICAAS system (Jittawattanarat et al., 2007a)

Providing appropriate growth conditions in an ER is one of the important aspects for its successful performance. Degradation activity of bacterial cultures in the ERs can be maintained by using an inducer compound, which can be the target compound itself or a less hazardous compound. It is possible to maintain the ability of the enriched culture to degrade the target compounds using an intermediate degradation by-product as an inducer compound. Babcock and Stenstrom (1993) have tested 1-acetate-naphthalene, 1-naphthoic acid, 1-naphthalene-sulfonic acid, and gentisic acid, which are intermediate by-products of aerobic 1-naphthylamine (1-NA) degradation, as potential inducer compounds in the ERs.

These intermediates are less-hazardous than 1-naphthylamine. The use of all four inducer compounds retained the degrading capability of the enriched culture for 1-NA degradation, although at reduced rates compared to a culture maintained on 1-NA.

Carbon source during the enrichment has been found to have impact on the degradation ability of microbial species. Rabus and Heider (1998) have tested the degradation ability of denitrifying bacteria, *Azoarcus sp.* strain EbN1, enriched on various carbon source conditions including ethylbenzene, toluene and anaerobic metabolites of ethylbenzene. Profound differences were observed in enzymatic reactions, removal rates, and growth rates. On the contrary, Krieger et al. (1999) observed similar pathways for *Azoarcus* sp. Strain T cells grown on toluene or *m*-xylene under denitrifying conditions. These findings indicate that the effect of carbon source during the enrichment can be species specific. Hence, catabolic activity of a mixed culture bacteria for degrading a target contaminant will greatly differ from one carbon source (or inducer) to another during the enrichment.

2.7. Single Strand Conformation Polymorphism (SSCP)

Typical gel electrophoresis methods use size and length of deoxyribonucleic acid (DNA) molecules to separate and identify the differences. However, bacterial communities with same DNA size and length may only differ in few nucleotide positions, which may be difficult to separate in the normal gel electrophoresis. Single strand conformation polymorphism (SSCP) is a gel electrophoretic method for monitoring these small genetic differences in microbial communities.

The method separates the single-stranded (ss) deoxyribonucleic acid (DNA) molecules of similar length but with sequence diversity. The separation of ssDNA

molecules is achieved under denaturing conditions in a non-denaturing gel. Any small difference in DNA molecules (in nucleotide positions) can result in differences in their secondary or tertiary structures. When the double stranded DNA molecules are separated, the ssDNA molecules undergo conformational changes and take on secondary and tertiary structures due to base pairing between nucleotides within individual strands (Figure 2.12). Hence, a small difference at a particular position in the primary sequence results in a difference in the conformation of the single strand molecules. The electrophoretic mobility of a single-stranded DNA molecule in a non-denaturing gel is highly dependent on its size and structure (or conformation), which results in separation of molecules in a non-denaturing gel at temperatures below 10°C. The method can even detect molecules differing by a single nucleotide, given the changes in their mobility as a consequence of differing conformations. The separated molecules (or bands) in the gels are digitized and further analyzed using various cluster analysis methods.

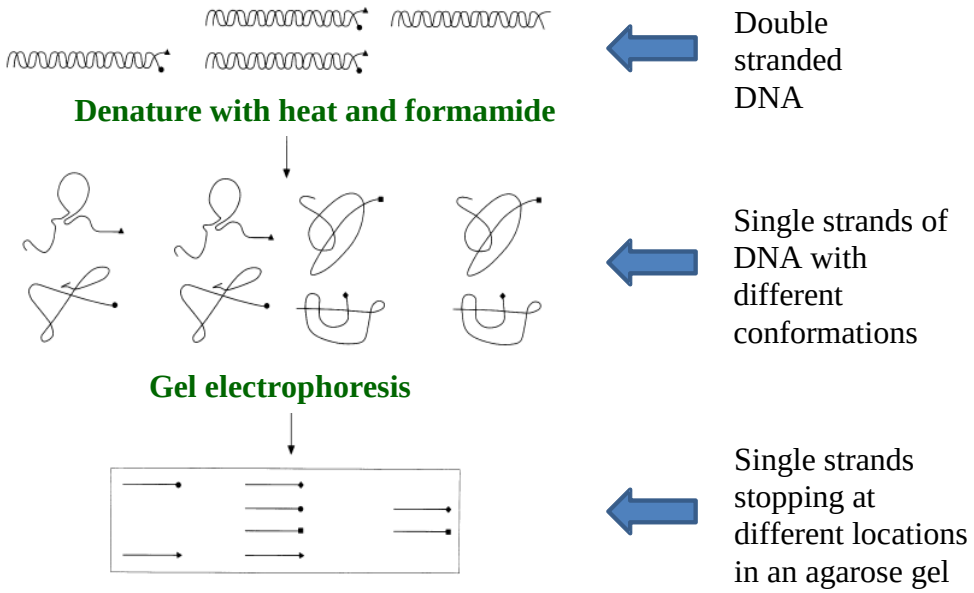


Figure 2.12. SSCP working principle (Modified from Kerr and Curran, 1996)

2.7.1. Cluster Analysis

Cluster analysis is a technique to group objects with greater similarities as compared to the rest in a collection of samples. While there are many clustering algorithms in use in different disciplines, hierarchical clustering is considered to be one of the popular methods in biological applications. In this method, a hierarchical relationship between the groups (or clusters) is developed in a tree fashion, called dendogram. An example dendogram is illustrated in Figure 2.13. The relationships between different clusters are developed by calculating similarities or differences. The similarities are then used in one of the clustering algorithms to generate the dendogram. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) is one of these algorithms, which uses a repetitive process that includes the steps: 1) identifies the minimum distance between any two clusters, 2) combines these two clusters into a pair, 3) calculates the average distance between this pair and all other clusters to form a new matrix, and 4) identifies the closest pair in the new matrix. The process repeats until the last two clusters in the collection are linked.

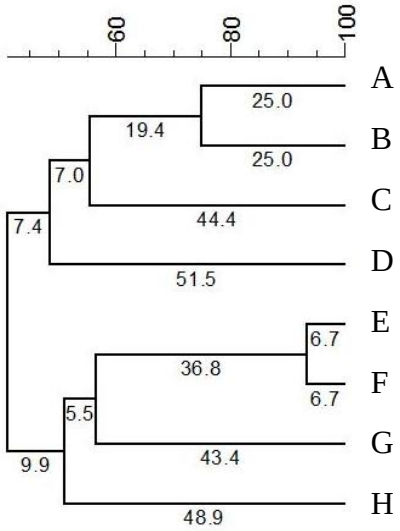


Figure 2.13. An example illustration of dendogram. Letters A, B, C, D, E, F, G and H represent individual clusters. Scale on the top represents the percent similarity and the numbers below the lines represent percent difference.

2.7.2. SSCP and Bioremediation

Several wastewater and bioremediation applications have used SSCP technique to identify bacterial community differences. Leclerc et al. (2001) used the SSCP technique to identify archeal community dynamics in a mixed anaerobic digester. SSCP profiles helped in understanding the digester performance stability in relation to archeal community dynamics. Mehmood et al. (2009) used the SSCP technique to study the microbial community profiling in a four connected on-site aerated lagoon system at a landfill site, which showed that community divergence between lagoons was linked to the nutrient status within each lagoon. Lin et al. (2007) used the SSCP technique to explore the spatial distribution and stability of microbial communities in association with the changes in phenol concentration during a bioremediation process. Structural similarities in microbial communities served as biological indicators to delineate the spatial distribution of contaminants.

CHAPTER 3. GROUNDWATER REMEDIATION USING ENRICHER REACTOR - PERMEABLE REACTIVE BIOBARRIER FOR PERIODICALLY ABSENT CONTAMINANTS

3.1. Introduction

PRBBs are cost effective in situ groundwater remediation systems. A PRBB is a biologically active flow-through zone in which microorganisms convert the contaminants into innocuous products as the groundwater passes through it. The indigenous microorganisms are sometimes inadequate in number and/or their activity is limited because of unsuitable growth conditions. Hence, the microbial population in the PRBBs is enhanced by biostimulation and/or bioaugmentation.

In the biostimulation type of PRBB, the activity and/or number of indigenous contaminant degraders are stimulated by providing necessary nutrients and environment (Margesin and Schinner, 2001a). Biostimulation is not a viable alternative when indigenous contaminant degraders are not present (Vogel, 1996). Also, some degraders are not good candidates for biostimulation because they are slow growers or do not respond to the process efficiently. As a consequence, biostimulation is sometimes not applicable when there is a need for rapid biodegradation of the target contaminant with little or no start-up period. Hence, biobarriers are often inoculated with contaminant-degrading microorganisms. The augmented microorganisms can be indigenous (Otte et al., 1994), pure (Portier et al., 1988; Shin and Crawford, 1995) or mixed cultures (Maxwell and Baqai, 1995; Miller et al., 1995). Among these, mixed microbial cultures are often found to be the most effective as they comprise a wide range of degradation mechanisms (Alvey

and Crowley, 1996). Bioaugmentation combined with biostimulation is also used for enhancing the performance of PRBBs (Olaniran et al., 2006; Stallwood et al., 2005).

Successful performance of the PRBBs requires a continuous maintenance of the microorganisms capable of utilizing the target contaminants, which in turn requires a continuous supply of the necessary nutrients and the target contaminants (Fantroussi and Agathos, 2005). However, in groundwater, the contaminants are often present as discontinuous plumes. In case of non-aqueous phase liquid (NAPL), discontinuous plumes can result from trapped residual NAPL (Hamed et al., 2000; Sahloul et al., 2002). The discontinuity of the plumes results in periodic absence of the contaminants in the PRBB.

The removal performances of the PRBBs are sometimes poor as a result of possible reduction in number of contaminant degraders and/or biodegradation activity induced by the disappearance of target contaminant. Depending on the length of contaminant absence period, the degraders may experience partial or complete loss in numbers and/or their degrading abilities. In case of partial loss of degraders, the performance of a biobarrier may be recovered after a period of time (Mathur et al., 2006), during which, the degraders will grow to a critical concentration capable of exerting measurable degradation. However, during the recovery period, the effluent of biobarrier might not meet the treatment goal. For complete loss of degraders, the degraders may not grow and the removal performance of the PRBBs can never be improved.

ER is an offline reactor used to maintain specific contaminant degraders in biological waste treatment systems. Cells acclimated to target compounds are enriched in ER and are used to augment a main treatment system such as activated sludge. Appropriate growth conditions for culture enrichment such as availability of nutrients and target

compounds, and suitable environmental conditions (pH and temperature) are provided in the ERs to induce the desired degradation capability. ER has been successfully applied in wastewater treatment for the removal of naphthalene and phenanthrene (Cardinal and Stenstrom, 1991), 1-naphthylamine (Babcock et al., 1992), pentachlorophenol (Jittwattanarat et al., 2007a), nitrogen (Jittwattanarat et al., 2007b; Leu and Stenstrom, 2010), and pharmaceutical drugs such as cephalexin and cephradine (Saravanane et al., 2001a; b).

The work presented in this chapter was used to evaluate the use of an ER as a means to maintain the performance of PRBB subjected to contaminants that appear in batches. ER-PRBBs are a novel process and therefore have not been studied. The working principle of an ER-PRBB system is shown in Figure 1.2 in Chapter 1. A laboratory scale setup was used to examine the performance of ER-PRBB system during the reappearance of a contaminant after a period of absence. Benzene was selected as a model contaminant. Benzene is one of the major aromatic compounds in the petroleum hydrocarbons and is known to be carcinogenic (Pohl et al., 2003). The United States Environmental Protection Agency has established a maximum contaminant level of 5 µg/L for benzene in drinking water.

Continuous flow experiments with sand columns, representing PRBBs, which were initially inoculated with anoxic benzene degraders, were run in the presence and absence of benzene. Two different scenarios during the benzene absence period were considered, one scenario with no carbon source and the other with ethanol as a carbon source. The rationale for ethanol use was that it is a common additive to gasoline in many countries and commonly found in groundwater contaminated along with benzene. Removal

performances were compared for columns that did and did not receive benzene degraders from ER during the reappearance of benzene. Total genomic DNA was extracted from bacterial samples collected from the PRBBs. Polymerase chain reaction (PCR) followed by SSCP were conducted to investigate the bacterial community dynamics.

3.2. Materials and Methods

3.2.1. Chemicals

Benzene (99.5% purity) was purchased from Sigma-Aldrich Chemical Co., MO, USA. Mineral salts, vitamins, and trace metals used for bacterial medium preparation (Table 3.1) were purchased from VWR International Co., PA, USA. A benzene standard and a fluorobenzene internal standard for benzene analysis were obtained from VWR International Co., PA, USA.

3.2.2. Cultivation of Benzene Degraders

Benzene degraders were cultivated by acclimating mixed liquor suspended solids from the Moorhead Wastewater Treatment Plant, Moorhead, MN, USA to benzene under anoxic conditions which were created by purging with nitrogen gas. The culture was initially grown with methanol as a carbon source and fed with synthetic groundwater. The synthetic groundwater, hereafter referred to as mineral salt medium (MSM), was prepared according to the composition shown in Table 3.1, which was modified from Edwards et al. (1992). To eliminate the competition from sulfate reducing bacteria with denitrifying bacteria for the same carbon source, all sulfate compounds in MSM used by Edwards et al. (1992) were replaced with chlorides in this research, such as $CaSO_4$ was replaced by $CaCl_2$. The culture was later gradually adapted to benzene by increasing benzene while reducing the methanol in the feed; the total mass of carbon (27.7 mg/L) supplied was kept

constant during the gradual acclimation to benzene. The final concentration of benzene in the synthetic groundwater that the culture was exposed to was 30 mg/L. The acclimated culture was tested for their benzene degradation ability in a 250 mL serum bottle.

Table 3.1. Chemical composition of mineral salt medium (MSM) per liter solution.

Compound	Concentration (g/L)
KH_2PO_4	0.272
K_2HPO_4	0.348
NH_4Cl	0.535
$CaCl_2$	0.07
$FeCl_2$	0.02
$MgCl_2$	0.625
$NaHCO_3$	2.6
KNO_3	0.505
Trace metals	
$ZnCl_2$	0.0002
$NiCl_2$	0.0015
$MnCl_2$	0.002
$CoCl_2$	0.003
Vitamins	
Biotin	0.0002
Folic acid	0.0002
Pyrodoxine HCl	0.001
Riboflavin	0.0005
Thiamine	0.0005
Nicotonic acid	0.0005
Pentothemic acid	0.0005
p-aminobenzoate	0.0005
B_{12}	0.0001

3.2.3. Sand Column Reactors Setup

Glass columns with an internal diameter of 1.5 cm and a length of 30 cm (53 mL total volume) were used as PRBBs. Each column was filled with 75 grams of quartz sand, resulting in a void volume of 20 mL and a free head space volume of 10 mL above the sand. The quartz sand was industrial silica-quartz sand from Le Sueur, MN (Unimin Corporation, CT, USA). The sand was washed with tap water followed by de-ionized (DI)

water and dried at 105°C for 24 hours. The cleaned sand was then combusted at 550°C for 20 min and cooled to room temperature before use.

3.2.4. Tracer Study

Chloride and benzene tracer studies were conducted on two sand columns for determining the flow characteristics. One of the columns was initially saturated with a background solution of 0.01 M $CaCl_2$. After saturation, a 0.33 column pore volume (PV; total column PV = 30 mL) of a 0.05 M $CaCl_2$ solution was pumped in an up-flow mode using a peristaltic pump at 24 hour interval, resulting in a flow rate of 0.42 mL/hr, for a total of 3 PV. An effluent sample was collected at the beginning of every pumping period and measured for electrical conductivity using a YSI conductivity meter (Model 32, YSI Incorporated, OH, USA) as a surrogate for chloride concentration. For the benzene tracer test, a benzene solution of 10 mg/L was pumped through the second column in the same manner as the chloride tracer test. The effluent was also sampled in the same manner as the chloride tracer test and analyzed for benzene. Parameter estimation (velocity, dispersion coefficient, retardation factor and Peclet Number) of the chloride and benzene breakthrough curves (BTC) was performed using CXTFIT 2.0, a one-dimensional transport model developed by United States Department of Agriculture (Toride et al., 1995).

3.2.5. ER Operation and Benzene Degrading Activity Test

The activity of the adapted benzene degrading culture was continuously maintained in the ER by constantly supplying synthetic groundwater containing MSM and benzene. The ER was operated as a sequencing batch reactor (SBR) in a plastic vessel with a working volume of 3 liters. The SBR operation cycle included 30 minutes for filling, 70

hours and 20 minutes for reaction, 1 hour for settling, 10 minutes for drawing. During the filling, the system received synthetic groundwater containing MSM, and was purged with nitrogen gas to attain anoxic conditions before spiking with benzene to 30 mg/L. During the reaction period, the vessel was closed with an airtight cap to maintain anoxic conditions and the solution was mixed on a horizontal shaker (DS-500E, VWR International Co., PA, USA).

The activity of the degraders was tested monthly in a 250 mL amber bottle. One hundred fifty milliliters of synthetic groundwater containing MSM was mixed with 10 mL of enriched bacteria, purged with nitrogen gas to anoxic conditions before spiking with benzene to 10 mg/L. A control was also set up in another 250 mL amber bottle containing DI water and 10 mg/L of benzene. One hundred microliter samples were collected from both bottles at 1, 6, 12, 18, 24, 36, 48, 72, 96, and 120 hr for benzene analysis. The effect of mixing on benzene degradation was also tested using a parallel setup on the horizontal shaker.

3.2.6. Continuous Flow Experiments and Sand Columns Operation

A configuration of bench scale PRBBs and an experimental timeline are illustrated in Figure 3.1. Four sand columns (R1, R2, R3 and R4) were initially inoculated with 30 mL of the enriched benzene degraders (300 mg/L) from ER. A fifth column, which was not inoculated, was used as a control to measure abiotic losses due to volatilization and adsorption. Synthetic groundwater made of the MSM solution and benzene (10 mg/L) was pumped using a peristaltic pump from a gas bag through all the columns in an up-flow mode. The PRBBs were operated on a semi-continuous flow mode displacing 10 mL volume of reactor solution every day. Five pore volumes (5 PV = 150 mL) of synthetic

ground water were pumped through each of the columns, after which, benzene feeding was discontinued for a period of time and then reintroduced.

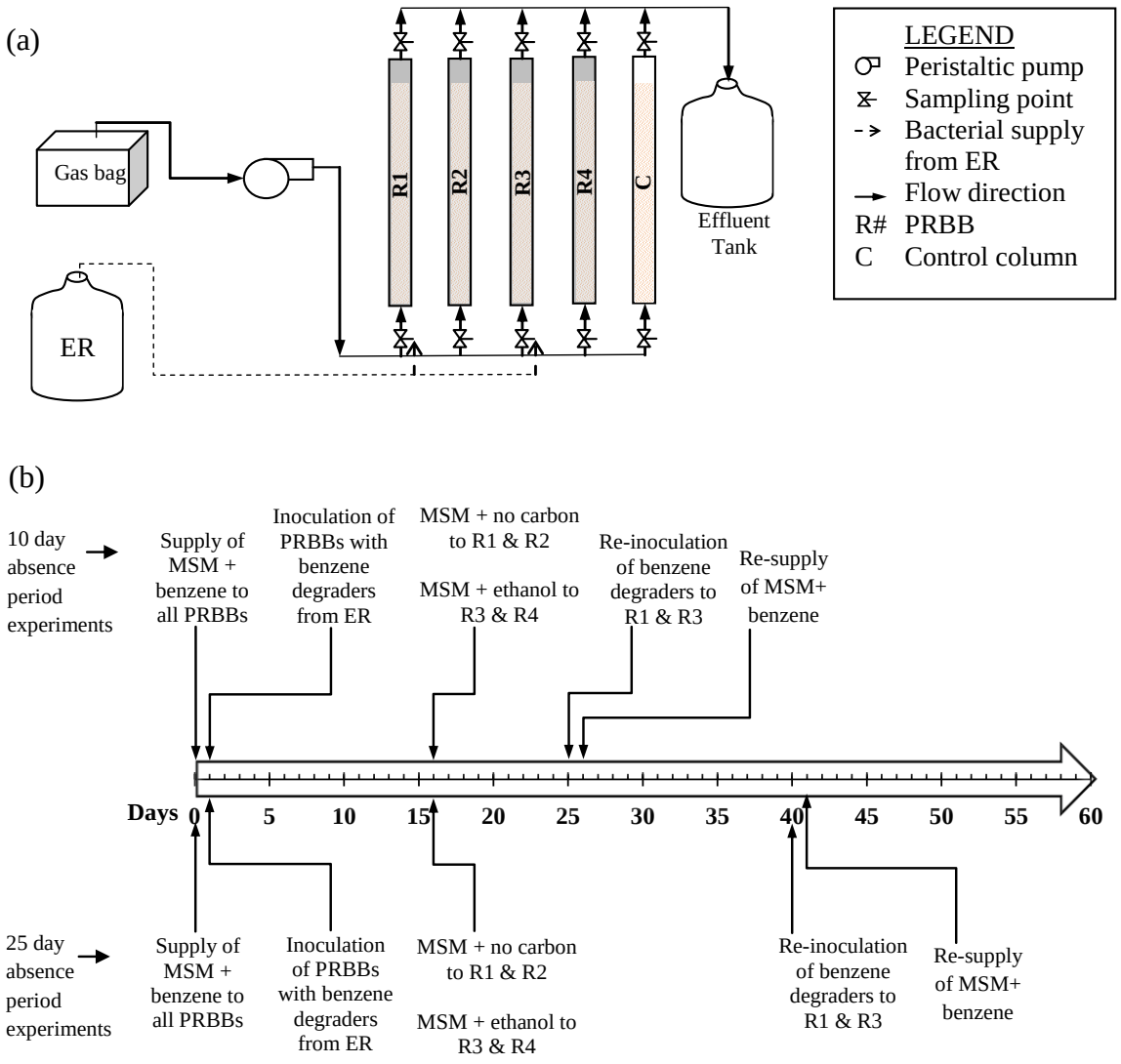


Figure 3.1. (a) Semi-continuous flow experimental setup for evaluating the performance of PRBB with and without ER and (b) Experimental timeline for semi-continuous flow experiments for 10 and 25-day absence periods.

Two different absence periods were tested, 10 days and 25 days. Two of the PRBBs (R3 and R4) received ethanol added MSM during the benzene absence periods, while the other two received MSM without any carbon source. Benzene degraders from ER were used to augment R1 and R3 just before the reappearance of benzene. Ten milliliters

of benzene degraders (300 mg/L) from the ER were used during the re-augmentation. After the benzene reappearance, four more pore volumes of synthetic ground water were pumped. Duplicate PRBB columns (five additional columns for R1-R4 and control) were also setup and operated in parallel for quality control.

Influent samples and effluent samples from both columns were collected daily. The volume of samples was 100 µL. The samples were immediately injected into 40 mL amber vials containing 40 mL of DI water and stored in the refrigerator at 4ºC before analysis. One microliter of fluorobenzene internal standard was added to all the samples, resulting in a 50 µg/L concentration. During the benzene absence period, 10 mL of effluent samples were collected daily and dissolved organic carbon (DOC) was measured as an indirect indicator of ethanol.

3.2.7. Bacterial Community Examination

The bacterial community dynamics in the PRBBs were examined using PCR amplification followed by SSCP. A PCR-SSCP procedure by Lin et al. (2007) was followed with a slight modification (time and number of cycles) in the PCR conditions. The entire procedure is described below. Total genomic DNA was extracted from samples collected from the PRBBs before the absence of benzene and just before the reappearance of benzene after a period of absence.

3.2.7.1. DNA extraction and amplification

The genomic DNA extraction procedure followed the instruction from the DNA extraction kit (Wizard Genomic DNA Purification Kit, Promega, USA). The V3 region (nucleotide positions 334-514 of *Esherichia coli*) of the extracted 16S rDNA was amplified with primers EUB1 (5'-CAG ACT CCT ACG GGA GGC AGC AG 3') and

UNV2 (5'-GTA TTA CCG CGG CTG CTG GCA C 3'). A 25 µL PCR reaction contained 1.5 mM of $MgCl_2$, 200 µM of dNTP, 5.0 µL of Taq Polymerase buffer 5x (Promega, CA, USA), 50 µM of each primer, 1.25U of Taq Polymerase (Promega, CA, USA), and 2 µL of DNA template. Dnase-free water was used for making up the volume of samples. The 0.2 mL PCR tubes were then placed in a thermocycler (Applied Biosystems 2720, Applied Biosystems Inc., Foster City, California, USA). The PCR conditions consisted of an initial denaturation at 94ºC for 5 min, 50 cycles at 94ºC for 30 sec, 55ºC for 30 sec, and 72ºC for 30 sec, and a final extension at 72ºC for 5 min. The presence of PCR products of expected size (approximately 200 base pairs) was confirmed by 3% agarose gel electrophoresis. The PCR products were stored at 4°C until next use.

3.2.7.2. SSCP gel electrophoresis and data analysis

The SSCP was carried out in a horizontal electrophoresis setup (Origins, Elchrom Scientific, Switzerland). The SSCP procedure followed the instructions from the manufacturer. Three microliters of PCR products were mixed with 7 µl of a denaturing solution (1 ml of formamide, 10 µl of 1 M NaOH, and 20 µl of 0.02% (w/v) bromphenol blue). The mixtures were heated at 95ºC for 5 min and immediately placed in ice until loading into the SSCP gel. The 10 µl denatured PCR products were loaded into a precast Elchrom's GMA™ gel (Elchrom Scientific, Switzerland). The gel was run at a constant voltage of 72 V for 10 hours. The gel temperature was maintained at 9ºC by circulating tris-acetate-ethylenediaminetetraacetic acid buffer. The gels were visualized by using a SYBR® Gold-stain method (Molecular probes, OR, USA). The relative positions of the normalized DNA bands in the SSCP gels were analyzed using the PHORETIX 1D software (TotalLab Ltd., UK). A hierarchical cluster analysis of the SSCP pattern was

performed by applying the Dice similarity index and unweighted pair-group method with arithmetic average algorithm for calculating the similarity of the dendrogram.

3.2.8. Analytical Methods

Benzene was analyzed using gas chromatography (GC) (Agilent 6890A PLUS with a capillary column, HP-5MS, 30 m long, and 0.25 mm inner diameter) and mass selective detector (Agilent 5973 Network) coupled with a purge and trap auto sampler system (Tekmar Dohrmann trap concentrator with Tekmar 2016 autosampler) using the EPA Method 624. The samples were loaded into the purge and trap concentrator and purged with helium gas at a flow rate of 35 mL/min for 11 minutes at ambient temperature. After sample purging, the trapped sample components were desorbed by heating the trap column at 225°C for 2 minutes. The purge and trap concentrator was in a bake mode between the analyses of samples for 6 minutes at 270ºC. The carrier gas for GC (He) had a flow rate of 1.5mL/min and the split gas (He) flow rate was 28 mL/min. The analyses was performed with an initial oven temperature of 40°C for 1 min, followed by a 5°C/min ramp to 45°C, then increased at 8°C/min to 125°C, and then increased at 25°C/min to a final temperature of 180°C where it was held for 1 min. The injector and detector temperatures were 250°C and 275°C, respectively.

The GC was calibrated with five benzene standards of varying concentrations over a linear response ranging from 5 µg/L to 50 µg/L (R^2 = 0.9978). The method detection limit for benzene was 4.4 µg/L. Fluorobenzene was used as an internal standard. A response factor method was used for the calibration and estimation of benzene in the samples (EPA Method 624).

DOC was analyzed according to Standard Methods (APHA et al., 1998) using an ultraviolet/persulfate oxidation total organic carbon (TOC) analyzer (Phoenix 8000, Tekmar Dohrmann, OH, USA). The analyzer was calibrated with 0, 1, 2.5, 5, and 10 mg TOC/L potassium hydrogen phthalate standard solutions. The analyzer has a detection limit of 0.002 mg/L.

3.3. Results and Discussion

3.3.1. Activity of Benzene Degraders in ER

Benzene degradation results from a batch study are shown in Figure 3.2. The results are based on average data from the duplicate experiments. The error bars are minimum and maximum values. Benzene-grown cells from the ER readily degraded benzene at a concentration of 8-10 mg/L in the batch with mixing (Figure 3.2 (a)). About 90% of benzene was removed in the first 72 hours. The degradation followed a first order kinetics ($R^2 = 0.97$) with a rate constant of 0.035 hr^{-1}. Results for the batch without mixing are shown in Figure 3.2 (b). The degradation was observed after a lag period of 48 hours after benzene spiking and followed a first order kinetics ($R^2 = 0.97$) with a rate constant of 0.026 hr^{-1}. The lag period could be due to an uneven distribution of microorganisms and nutrients, which also could be the reason for slower degradation compared to the batch with mixing. About 69% benzene removal was observed in the first 72 hours. No loss of benzene was observed in the controls for with and without mixing.

3.3.2. Tracer Study

In the tracer experiments (Figure 3.3), the benzene BTC shared a similar trend with the chloride BTC. The CXTFIT's equilibrium CDE model gave a good fit for both chloride and benzene BTC curves with determination coefficient (R^2) values of 0.999 and 0.997.

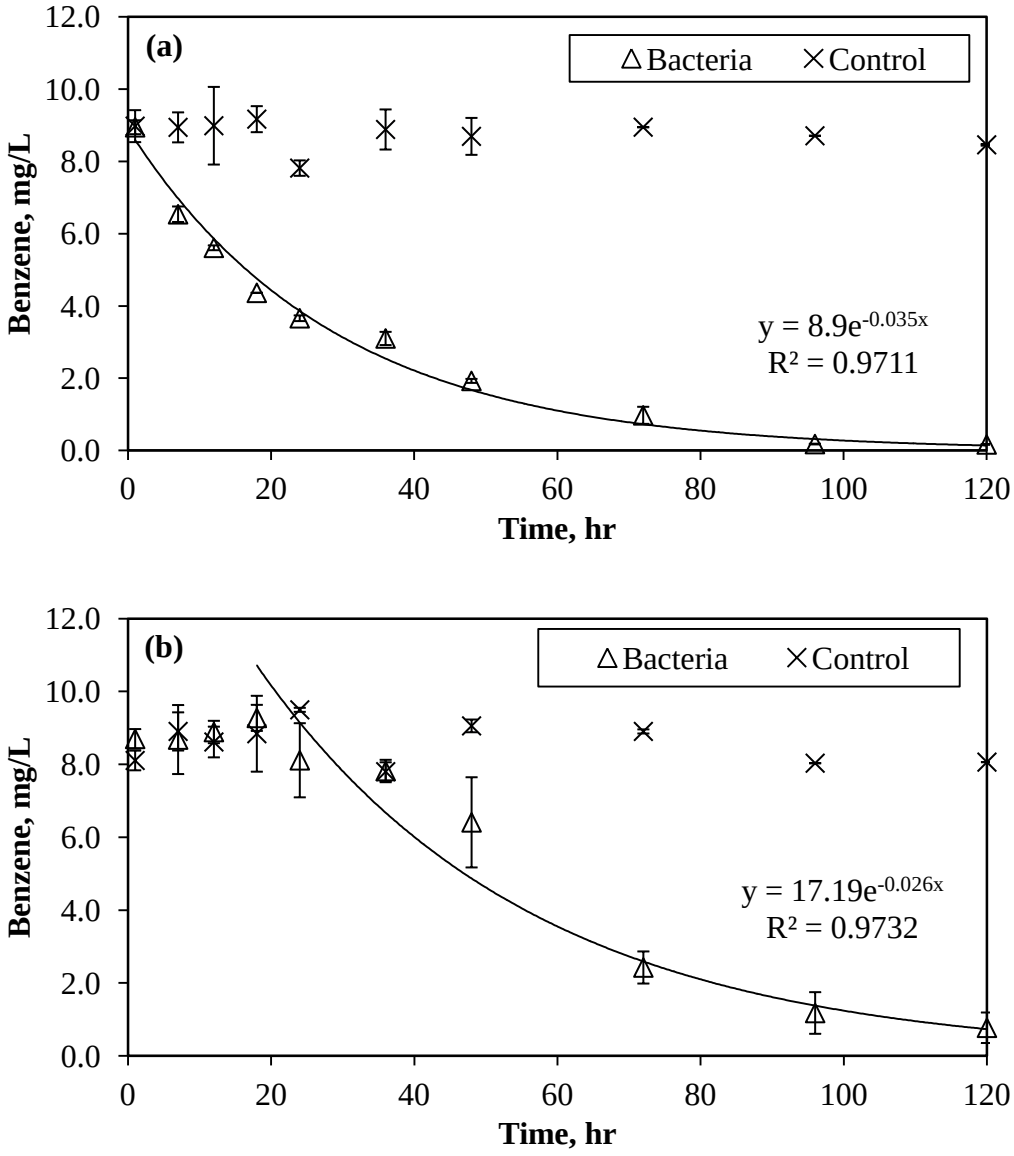


Figure 3.2. Activity test results for benzene degraders from ER in batch systems (a) with mixing and (b) without mixing

The estimated dispersion coefficients, retardation factors and velocities from the model are summarized in Table 3.2. Advection played a major role in the transport of chloride, while both advection and dispersion were major transport mechanisms for benzene. For the chloride tracer, C/C_0 approached 1 after 1.5 pore volumes while for benzene it reached 1 after 2.5 pore volumes. The predicted velocities of chloride and benzene were 5.55 and 10.3 cm/day respectively. The retardation factor values for both

chloride tracer and benzene derived from the model fit were small, which indicates that there was minimal retardation effect from walls of the glass columns (PRBBs) and/or the quartz sand.

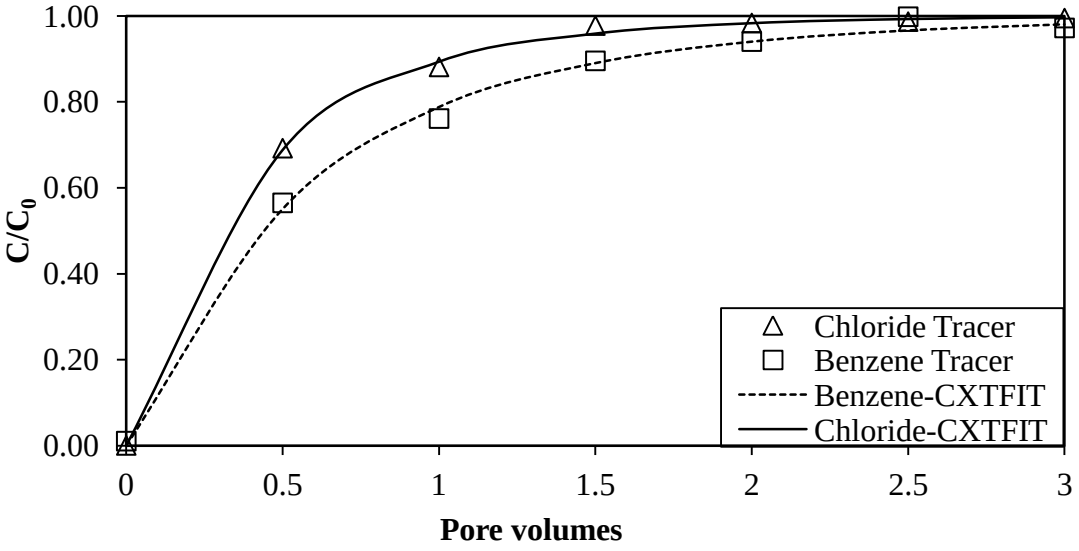


Figure 3.3. Tracer breakthrough curves.

Table 3.2. Summary of parameter estimation from CXTFIT.

Tracer compound	Velocity (V) (cm/day)	Dispersion coefficient (D) (cm^2/day)	Retardation factor (R)	Peclet Number (P)	Determination coefficient (R^2)
Chloride	5.55	126.60	0.10	1.31	0.999
Benzene	10.30	285.20	0.25	1.08	0.997

3.3.3. Column Experiments

The column experiment results are presented in Figures 3.4 and 3.5. For each absence period, the results from duplicate reactors were averaged. The values on y-axis represent the effluent concentrations of benzene normalized to the influent concentration. The x-axis values represent the number of pore volumes of water pumped through the PRBBs. The error bars represent minimum and maximum values.

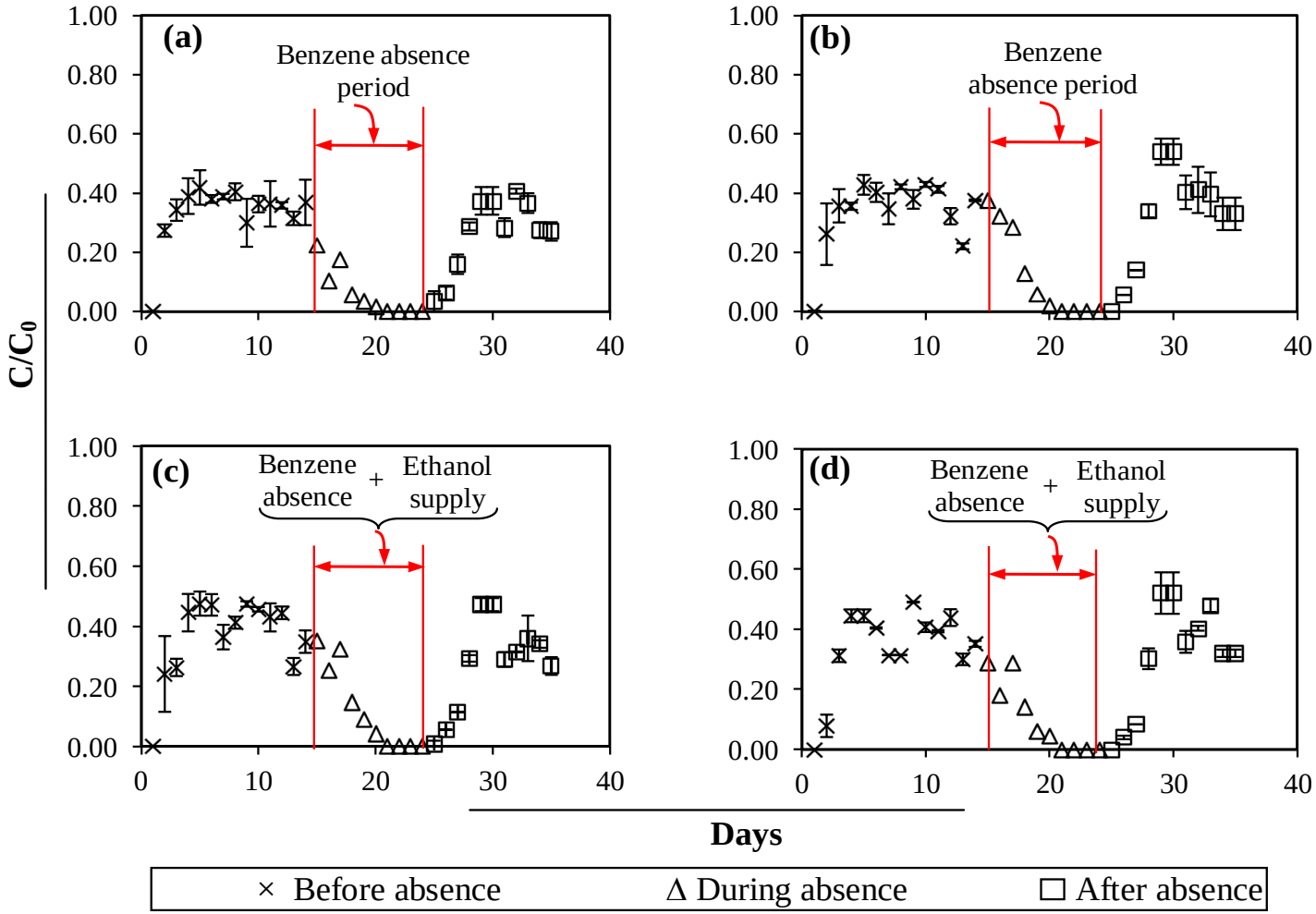


Figure 3.4. Performances of reactors for experiments with a 10-day benzene absence period. (a) R1, (b) R2, (c) R3, and (d) R4. Decrease in C/C_0 represents removal of benzene in the columns.

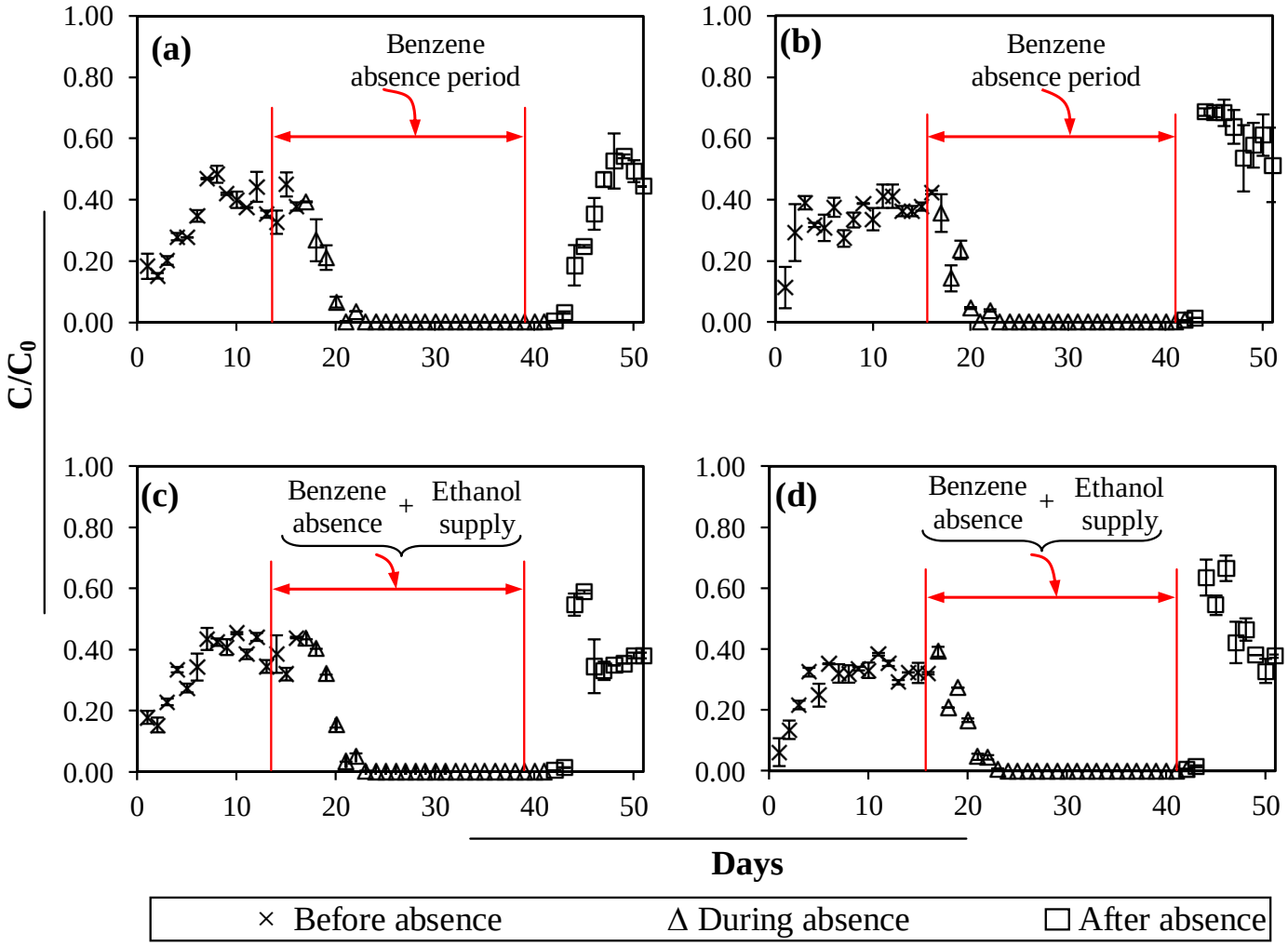


Figure 3.5. Performances of reactors for experiments with a 25-day benzene absence period. (a) R1, (b) R2, (c) R3, and (d) R4. Decrease in C/C_0 represents removal of benzene in the columns.

3.3.3.1. Ten day benzene absence period

Results from the experiment with a 10-day benzene absence period are shown in Figure 3.4. The PRBBs reached a steady removal performance in the first five days. The removal performances were 60%, 60%, 55% and 55% for R1, R2, R3 and R4 respectively, which were very close to the observed values of benzene degradation in the batch study without mixing (Figure 3.2 (b)). The small difference in the performances among PRBBs can be attributed to the variation in residence times, which could be a result of dissimilar void volumes caused by the arrangement of voids in the quartz sand in the PRBB columns. The PRBBs did not experience any initial lag because their environmental conditions were similar to that of the ER, where the activity of benzene degraders was maintained.

Ethanol supplied to R3 and R4 during the benzene absence period was removed above 95% (Figure 3.6). There was no lag period in ethanol consumption as effluent ethanol concentrations from the PRBBs were very low (less than 5% of the influent) from the first day of ethanol supply (or the benzene absence period). Benzene degraders were able to utilize ethanol as an alternative carbon source. As the amount of carbon supplied through ethanol and benzene was same, the ethanol supply should have either retained or enhanced the amount of biomass in these two PRBBs.

When benzene reappeared in the influent after the 10-day absence period, removal performances gradually reached to 60%, 45%, 55%, and 45% for R1, R2, R3 and R4 respectively in the first six days (two pore volumes). R1 and R3, the PRBBs that received benzene degraders from the ER just before benzene reappearance, did not experience any loss in their performances. R2 and R4 experienced 15% and 10% losses in their performances respectively. After two more pore volumes were pumped through all the

reactors, the removal performances were 70%, 65%, 70%, and 65% for R1, R2, R3 and R4 respectively. When compared to their performances before the benzene absence period, the performances of R1 and R2 have increased by 10% and 5% respectively while that of R3 and R4 increased by 15% and 10% respectively. The recovery period for both R2 and R4 was two days.

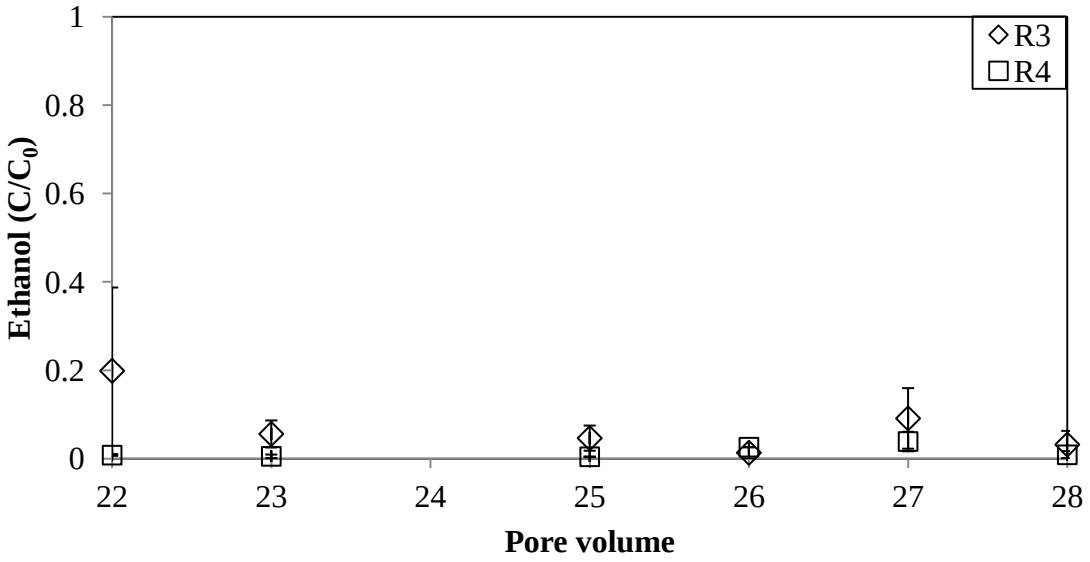


Figure 3.6. Ethanol removal in R3 and R4 during the benzene absence period.

3.3.3.2. Twenty five day benzene absence period

Results for experiments with a 25-day benzene absence period are shown in Figure 3.5. Similar to the experiments with the 10-day benzene absence period, the effluent concentrations in these experiments too increased gradually in the first five days for all the reactors before the absence period. All the reactors showed a steady performance of 60% removal. Ethanol was completely (more than 99%) consumed by the PRBBs R3 and R4 during the benzene absence period (Figure 3.7).

All PRBBs experienced losses in their removal performances when benzene reappeared after the 25-day absence period. The removal performance of R1 gradually

dropped to 45% in 8 days after the reappearance of benzene in the influent. For R2, R3, and R4, the removal performances quickly dropped to 30%, 45% and 35% respectively in 3 days after the reappearance of benzene in the influent. The amount of benzene degraders supplied from the ER to R1 and R3 just before benzene reappearance was the same for both of the absence periods. However, the supplied amount of acclimated benzene degraders was obviously not sufficient to maintain the PRBB removal performance after the 25-day absence period. Additionally, R3 experienced a quicker loss in performance as compared to R1. The performance loss for the 25-day absence period was higher than that for the 10-day absence period. The 25-day absence period caused 15%, 30%, 15%, and 20% losses, while the 10-day benzene absence period caused 0%, 15%, 0%, and 10% losses in the removal performances of R1, R2, R3 and R4 respectively.

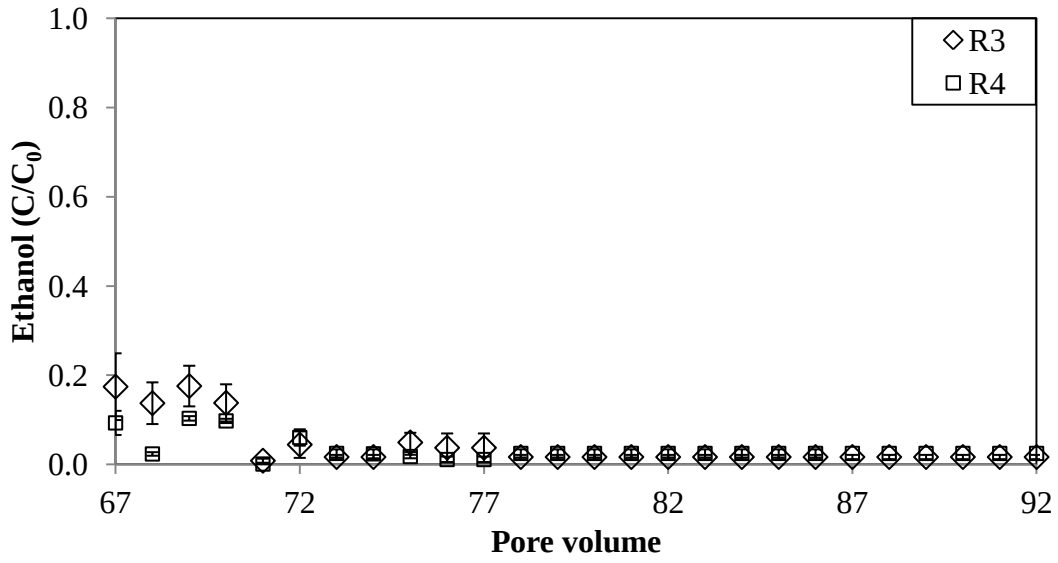


Figure 3.7. Ethanol removal in R3 and R4 during the benzene absence period.

The removal performances of all the PRBBs improved in the subsequent days to 60%, 50%, 70%, and 60% for R1, R2, R3, and R4 respectively. Except R2, all other PRBBs achieved the same or higher removal performances than those of before the

absence period. The recovery periods to reach the same performance as before the absence period were as follows: 4 days for R1, more than 12 days for R2, 3 days for R3, and 10 days for R4. The removal performance of R3 improved quicker than that of R1, while the removal performance of R4 improved quicker than that of R2.

A possible reason for the performance loss is the decrease in the number of benzene degraders due to the absence periods. In this study, R1 and R2 did not receive any carbon source during the benzene absence periods and could have experienced a loss of benzene degraders. Additionally, the higher losses in the performance due to the 25-day absence period compared to the 10-day absence period indicate that the PRBBs experienced a greater loss in the amount of benzene degraders. Many studies in the past have noted that bioreactors experience a reduction in the number of contaminant degraders during the starvation periods (Mathur et al., 2006; Chong et al., 2008). The starvation periods in these studies are similar to the benzene absence period for R1 and R2 in the present study.

Ethanol was provided to R3 and R4 during the benzene absence periods. Supply of ethanol at low concentrations (1.5 mg/L) was found to be beneficial for benzene degradation due to enhanced growth of the degraders (Lovanh et al., 2002). When ethanol was present in large quantities (> 5 mg/L), Lovanh et al. (2002) noticed inhibition of benzene degradation due to high consumption of electron acceptors and other necessary nutrients during ethanol degradation. This was not the case in the present study as sufficient amount of nutrients were constantly supplied through the influent. Also, the results for the 10-day absence period show that ethanol did not have any negative impact on the removal performances of R3 and R4 as they performed similar to R1 and R2 which did not receive any carbon source. Hence, loss of degraders does not completely explain

the performance loss for R4 due to the 10-day absence period and for both R3 and R4 due to the 25-day absence period.

In addition to the reduction in number of degraders, the absence of benzene could have caused a loss in biodegrading capability of the degraders remaining in the PRBB after an absence period. This particular loss can be observed when catabolic repression occurs due to suppression of enzymatic production required for benzene degradation. Enzymatic suppression could have taken place for the degraders in the PRBBs that did (R3 and R4) and did not (R1 and R2) receive an alternative carbon source (ethanol) during the benzene absence periods. The benzene degraders in R3 and R4 were able to utilize ethanol instantaneously because ethanol can be metabolized through constitutive enzymes. The benzene degraders in R1 and R2 were utilizing the lysis products from the dead bacterial cells, which also could be utilized through constitutive enzymes. Utilization of constitutive enzymes and absence of inducing compound could have suppressed the production of enzymes required for benzene degradation. When benzene reappeared in the influent, an immediate reproduction of these required enzymes did not occur. This enzyme suppression was more prominent for the 25-day absence period.

As mentioned earlier, the supply of ethanol increased the biomass of benzene degraders in R3 and R4 although it suppressed enzymatic production required for benzene degradation. When benzene reappeared in the influent, these degraders quickly started to respond to the benzene and provided superior removal performances for R3 and R4 as compared to R1 and R2 respectively.

3.3.4. Bacterial Community Examination

The SSCP profiles and the results of the cluster analysis of bacterial samples from the reactors during the experiments with 10-day and 25-day benzene absence periods are shown in Figure 3.8. The notations ‘B’ and ‘A’ in Figure 3.8 refer to samples collected just before an absence period and just before the reappearance of benzene, respectively. The notation ‘A2’ in Figure 3.8 (b) (25-day absence period experiments) refers to a sample collected when the reactors reached a steady performance after the reappearance of benzene after an absence period. The scale at the bottom of the figure indicates the percent similarity among the SSCP profiles. The value on the scale at each red dot indicates the percent similarity for the profiles connected at that point. For example, the SSCP profiles of R1-B, R2-B, and R3-B in Figure 3.8 (a) are 100% similar. A detailed description on cluster analysis is provided in Chapter 2, Section 2.7.1.

3.3.4.1. Ten day benzene absence period

The community structure in R1 and R2 after the benzene absence period was 9% different (or 91% similar) than that of the bacteria before the absence period. For R3 and R4 (these reactors received ethanol during benzene absence period), the community structure after the absence period was 29% different (or 71% similar) than those of the bacteria before the absence period. Additionally, there was 20% difference between the communities of R1/R2 and R3/R4. Thus, the community structure was affected by both the absence of benzene as well as the presence of ethanol when benzene was absent, while ethanol supply had caused greater changes in the community structure.

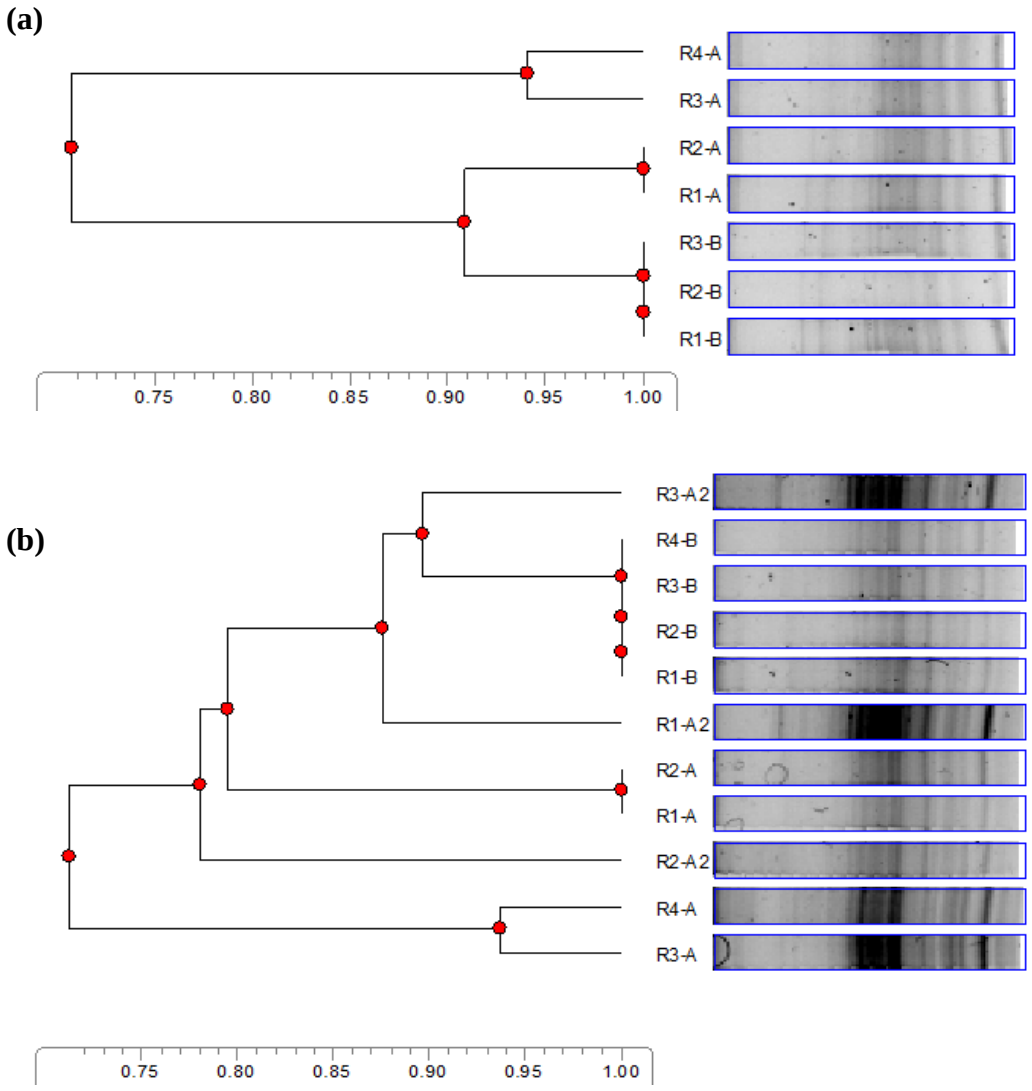


Figure 3.8. SSCP profiles and cluster analysis results of 16S rDNA samples from the PRBBs R1, R2, R3, and R4 before and after (a) 10-day benzene absence period and (b) 25-day benzene absence period. R4-A2 was not included in the profiles and cluster analysis due to unsuccessful DNA extraction from the reactor

3.3.4.2. Twenty five day benzene absence period

The community structure of bacteria in R1 and R2 after the benzene absence period was 17% different (or 83% similar) than that of the bacteria before the absence period. For R3 and R4 (reactors that received ethanol during the benzene absence period), the bacterial community structure after the absence period was about 29% different (or 71% similar) than before the absence period. The community structure in R1 at point A2 (when PRBB

reached a steady removal performance after the benzene absence period) was 88% similar compared to before the absence period. The community structure of R2 was 78% similar and that of R3 was 90% similar at point A2 compared to their respective community structures before the absence period.

The changes in the community structure for R1 and R2 due to the 25-day benzene absence period were almost two times more than the changes due to the 10-day absence period. The higher loss in the performance due to the 25-day absence period could be related to these greater changes in the community structure, which could be due to a complete loss of some of the benzene degrading species. The percent changes in the community structure due to the two absence periods were the same for R3 and also for R4, which were higher than the changes occurred in R1 and R2. Ethanol supply definitely caused changes in the community structure in these PRBBs; however, it had the same level of effect during the two absence periods. The shorter lag phases experienced by R3 and R4 before performance recovery (compared to R1 and R2, respectively) can be linked to these large changes in their bacterial community structure due to the presence of ethanol.

During the presence of ethanol, some of the bands with higher intensities were observed for R3 and R4 during the 25-day benzene absence period. These high intensity bands represent higher amounts of DNA being extracted from specific bacterial communities that were more competitive under the selective conditions used (Nakatsu et al., 2005). Spain et al. (2007) found that biostimulation with ethanol resulted in loss of diversity by enriching a specific group of bacteria. During the absence periods in the present study, some of the necessary degraders for benzene removal might have gradually decreased in number while the ones that grow on ethanol have increased.

Although there was an increase in microbial activity, the presence of ethanol during the benzene absence period might have resulted in the loss of enzymatic activity of microorganisms needed for metabolizing the target xenobiotic compound leading to a quick loss in the performances of R3 and R4 (Figure 3.5). However, R3 experienced a quicker performance recovery than R4 when benzene reappeared after the absence period, due to the augmentation from the ER just before the benzene reappearance. The increased biomass in R4 gradually recovered the necessary enzymatic activity and its performance quicker than R2, which did not receive any carbon source during the benzene absence period.

The absence periods studied in this research (10 and 25 days) are relatively small as compared to the real situations, which could be as long as several months. Such situations could lead to a complete loss of contaminant removal performance by PRBBs which may not be recoverable in a reasonable timeframe and without major expenditures and efforts to revive the contaminant degrading bacterial population. In the present study, the real situation was simplified to demonstrate the issue with the absence period and the need for an ER to maintain the performance of a PRBB. A cost-benefit analysis, which is beyond the scope of this study, is recommended as future work to further justify the value of an ER.

This study addresses the application of ER-PRBB to treat groundwater contaminated with benzene as a single contaminant appearing in batches. However, benzene in general, exists in groundwater along with toluene, ethylbenzene and xylenes (collectively known as BTEX). Similar to ethanol, some of these TEX compounds enhance the growth of benzene adapted microorganisms, while the others inhibit (Dou et al., 2008).

For the latter case, the PRBBs will experience a major loss in their removal performance. It is necessary to understand the synergistic and antagonistic interactions among the mixture of these compounds during bioremediation.

Moreover, in this study, the PRBBs that were augmented from the ER also experienced performance losses for short periods (2 to 3 days) after a longer benzene absence period. This could be due to inadequate augmentation from the ER leading to a less timely recovery of the performance of the PRBBs. Further studies need to be conducted to investigate the appropriate amounts of degraders needed from ER for different absence periods in order to effectively recover the removal performance of PRBBs. Additional molecular studies to identify the types and amounts of different microbial species present in the ER and PRBBs and to study the effects of absence periods on these microbial species are needed. Understanding of the changes at a microbial species level could provide better control on the ER operation and in turn better performance of the PRBBs. In addition, DOC data during the benzene presence period were not collected because it is known that intermediates in benzene degradation are more readily biodegradable than benzene itself. However, future work should be conducted to elucidate the biochemical pathways and to confirm benzene mineralization.

3.4. Summary

A new technique called ER-PRBB to treat an intermittently present contaminant in groundwater was developed. The removal performances of the PRBB system with ER were compared with a PRBB that did not have an ER. The ER-PRBB maintained the removal performance better than the PRBB that was not bioaugmented when benzene reappeared after two different absence periods tested in this study. Between the two

absence periods, the longer absence period caused greater performance losses for the PRBBs, while the PRBB with an ER recovered quicker than the PRBB without an ER. Molecular studies showed that the longer benzene absence period caused greater changes in microbial community structures. Additionally, ethanol availability during the benzene absence period was tested in this study and found that the availability of ethanol resulted in quicker performance recovery of PRBB after the longer absence period. This finding agrees with the practices of biostimulation, in which different types of carbon sources are used to stimulate the bacterial growth in a PRBB. Moreover, ethanol supply during the benzene absence period caused greater changes in the community structure when compared to no carbon source availability during the benzene absence period. This could be due to a decrease in bacterial diversity. In conclusion, the results from benzene removal performances and molecular studies showed that augmentation of enriched target contaminant degraders from the ER was necessary to achieve a more timely recovery of the performance for a PRBB.

CHAPTER 4. EFFECT OF CARBON SOURCE DURING ENRICHMENT ON BTEX DEGRADATION BY ANAEROBIC MIXED BACTERIAL CULTURES

4.1. Introduction

Supply of carbon, typically the target contaminant, during the enrichment in an ER is necessary to induce the necessary degradation abilities to the enriched microbial population. Benzene, which was the target contaminant in the previous chapter, was used as the sole carbon source to enrich the benzene degrading microbial culture. Compounds other than target contaminant were also used as a carbon source (or inducer compound) during the enrichment of contaminant degrading microbial cultures (Babcock and Stenstrom, 1993; Jitnuyanont et al., 2001). Typically, inducing compounds were less toxic, compounds that have structural similarity and/or are present in the degradation pathway of the target compound (Babcock and Stenstrom, 1993; Singleton et al., 2008). However, the degradation performance varies by the type of inducer compound used during enrichment. Bacterial cultures enriched on toluene removed benzene immediately, while *m*-xylene was removed after 300 days of lag period (Botton and Parsons, 2006). In the same study by Botton and Parsons, an early exposure to *m*-xylene induced the ability of the microbial communities to readily utilize benzene or toluene without an adaptation period. Babcock and Stenstrom (1993) also noticed different removal performances by the enrichment cultures grown on different inducing compounds.

Anaerobic bioremediation of BTEX has been reported in many studies (Blackburn, 1998; Boopathy et al., 2012; Da Silva and Alvarez, 2004; Margesin and Schinner, 2001b; Patterson et al., 1993; Weiner and Lovley, 1998). In the past, few studies have been conducted on the removal of BTEX mixture of compounds by enriched bacteria. Although

enriched bacteria have been used in bioaugmentation in some of the past studies, attention was not given to the enrichment procedure and/or the inducers during the enrichment. Handling and storage of toxic compounds such as BTEX would be cumbersome and may defeat the purpose of biodegradation if spills occur during the enrichment of degraders on the site. The storage of inducing hazardous compounds on site will raise the liability issues and requires proper care in designing the storage. Use of less number of hazardous chemicals will reduce the risk and liability associated with operations during the enrichment process. However, studies did not compare the effect of using individual BTEX compounds as inducers during the enrichment on the removal performance of the enriched culture for the removal of BTEX mixture. Additionally, the use of a common and/or less hazardous inducer(s) during the enrichment procedure was never investigated for a mixture of hazardous compounds. Moreover, past studies on microbial enrichment were conducted in a "black box" manner, while the underlying 'link' between the enriched bacterial community structures and their capabilities to degrade a specific compound or a group of compounds was not completely understood.

This chapter discusses a comprehensive investigation on the effects of different carbon sources (or inducers) for the enrichment of the microbial consortia on 1) the removal performances of individual BTEX compounds when they are present as a mixture, and 2) their bacterial community structures. Four different types of inducer compounds were considered for enrichment: (i) individual BTEX compounds, (ii) BTEX as a mixture, (iii) benzoate alone and (iv) BTEX mixture and benzoate. Benzoate was chosen as one of the carbon sources in this study because it is a common biodegradation intermediate for all BTEX compounds and was found to improve the BTEX degradation abilities of the mixed

bacterial cultures when used in biostimulation (Alvarez et al., 1998). Additionally, benzoate is a non-toxic compound which eliminates the deleterious effects of spills during handling and storage on the site during the enrichment of degraders.

Individual ERs were setup to acclimate mixed bacterial cultures obtained from a wastewater treatment plant to each of the BTEX compounds as a sole carbon source in denitrifying conditions (nitrate as an electron acceptor). Four additional ERs were setup where mixed bacterial cultures were fed with BTEX mixture and benzoate at varying ratios. Batch kinetic studies were conducted to study simultaneous removal of BTEX compounds by the enriched cultures. Additionally, removal of individual BTEX compounds by the degraders acclimated to individual BTEX compounds was also investigated. Microbiological studies were carried out in parallel using the ATP assay technique to study the growth kinetics. Total genomic DNA was extracted from bacterial samples collected from the batch reactors. PCR followed by SSCP were conducted to investigate the changes in bacterial community structures.

4.2. Materials and Methods

4.2.1. Enrichment of Cultures

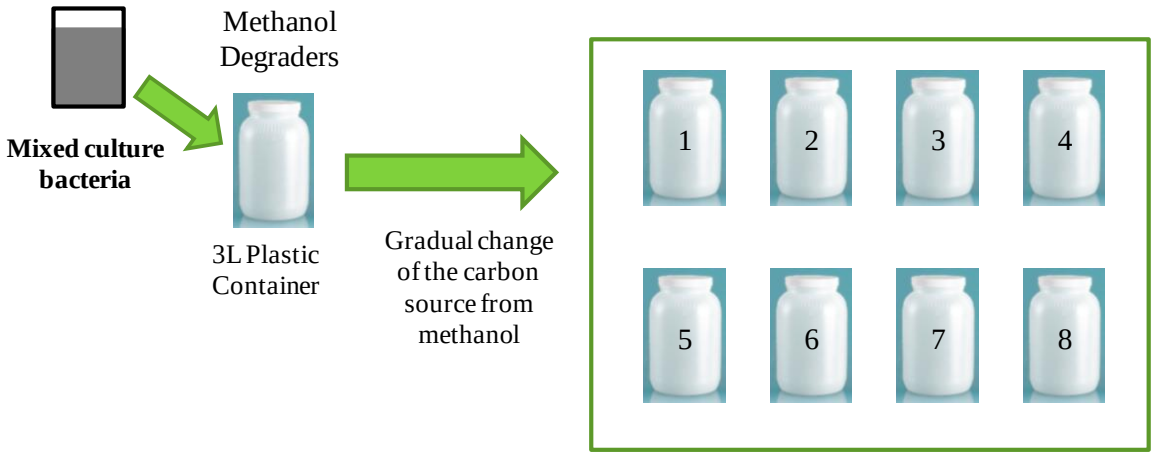
Different enrichment reactors were set up by acclimating a mixed bacterial culture from a wastewater treatment plant to eight different carbon sources (one reactor for each carbon source) under anoxic conditions: B, T, E, X, BTEX mixture, BTEX mixture and benzoate (two ratios), and benzoate alone. Benzene (99.5% purity), ethylbenzene (99% purity), and xylenes (99% purity) were purchased from Sigma-Aldrich Chemical Co., MO, USA. Toluene (99% purity) was purchased from VWR International Co., PA, USA. Acclimation procedures described in Chapter 3 for the enrichment of benzene enriched

degraders were followed in this study. MLSS from the Moorhead Wastewater Treatment Plant, Moorhead, MN, USA were used as the mixed bacterial culture source. Activated sludge has been successfully used as a source of cultures in some of the past bioremediation studies (Aburto-Medina et al., 2012; Tellez et al., 2002; Zhao et al., 2006). Anoxic conditions were created by purging with nitrogen gas. The culture was initially grown with methanol as a carbon source and fed with synthetic groundwater. Methanol is a simple and common substrate in wastewater treatment nutrient removal systems to enrich and maintain denitrifying cultures (Payne, 1973). Nitrate was supplied as the electron acceptor in the synthetic groundwater. Synthetic groundwater or MSM was prepared according to the composition described in Chapter 3.

The culture was later gradually adapted to B, T, E, X, BTEX mixture, BTEX mixture and benzoate (two ratios), and benzoate by increasing their concentrations in their respective reactors while reducing the methanol in the feed; the total mass of carbon (27.7 mg/L) supplied was kept constant during the gradual acclimation. The final concentrations in the synthetic groundwater that culture was exposed to are summarized in Table 4.1. A schematic of the enrichment procedure is presented in Figure 4.1.

Table 4.1. BTEX and benzoate concentrations in individual enrichment reactors.

Reactor #	**Reactor Name**	**Concentration (mg/L)**
1	Benzene only	30
2	Toluene only	30
3	Ethylbenzene only	30
4	Xylene only	30
5	BTEX mixture	8 mg/L of each BTEX compound
6	BTEX 50 + Benzoate 50	4 mg/L of each BTEX compound + 25 mg/L of benzoate
7	BTEX 25 + Benzoate 75	2 mg/L of each BTEX compound + 38 mg/L of benzoate
8	Benzoate only	50 mg/L of benzoate



Growth conditions
Mineral salts + vitamins + trace metals
Purge w/ N_2 gas to anoxic conditions

Carbon source	
1 – Benzene	5 – BTEX
2 – Toluene	6 – BTEX + Benzoate (1:1)
3 – Ethylbenzene	7– BTEX + Benzoate (1:3)
4 – Xylenes	8 – Benzoate

Figure 4.1. Enrichment procedure

4.2.2. BTEX Degradation Kinetics

A batch study on BTEX degradation kinetics by each of the eight enriched bacterial cultures was conducted in individual reactors. Each batch reactor received 10 mL of the enriched culture, 230 mL of MSM, and 2.5 mg/L of each BTEX compound. The solution in each reactor was purged with nitrogen gas to attain anoxic conditions before adding BTEX compounds. Samples were collected at different time intervals until no significant change in BTEX concentrations was observed. Then, the solids in each reactor were concentrated to 10 mL by centrifuging and the remaining solution was discarded. A new set of batch reactors were setup with these 10 mL bacterial cultures from each reactor, 230 mL of MSM and 2.5 mg/L of each BTEX compound. The solution in each reactor was

purged with nitrogen gas before adding BTEX compounds. Sample collection was repeated at different time intervals until no significant change in BTEX was observed.

Two different sets of samples were collected at each time interval: a one hundred microliter sample for analyzing BTEX compounds and a 2 mL sample for molecular analyses. Additionally, 100 µL samples from B, T, E, and X reactors were collected for bacterial growth analysis using the ATP assay. Two different types of controls were set up in 250 mL amber bottles containing DI water: one control received all BTEX with 2.5 mg/L of each and four more controls received individual BTEX compounds with 2.5 mg/L in each bottle. Duplicate reactors (including the controls) were setup for quality control purposes.

4.2.3. Modeling BTEX Degradation Kinetics

The Monod equation describing the biodegradation rate of a single compound when present as a sole carbon source can be expressed as:

$$\mu = -\frac{\mu_{max} C}{K_s + C} \tag{4.1}$$

where μ is the specific biomass growth rate (mg VSS/mg VSS-d), C is the liquid concentration (mg/L) of the growth substrate, μ_{max} is the maximum specific bacterial growth rate (mg VSS/mg VSS-d), and K_s is the half saturation constant (mg/L).

Modeling multiple substrate degradation requires the inclusion of inhibition interaction and/or simultaneous utilization. Some studies have included the effect of interactions by an additional term, K_i, inhibition constant (Bielefeldt and Stensel, 1999; Trigueros et al., 2010). According to Bielefeldt and Stensel (1999), the above equation for the degradation of benzene in a mixture of BTEX can be described as:

$$\mu_B = -\frac{\mu_{max,B} B}{B + K_{sB}(1 + \frac{T}{K_{sT}} + \frac{E}{K_{sE}} + \frac{X'}{K_{sX}})} \quad (4.2)$$

where, B, T, E, and X' are individual concentrations of BTEX compounds in a reactor with BTEX mixture. In the above equation, the effect of substrate interactions (e.g. inhibition, K_i) is described by K_s. If $K_{sB} = K_{sT} = K_{sE} = K_{sX}$ and B + T + E + X' = total BTEX concentration in the reactor, the above equation becomes:

$$\mu_B = -\frac{\mu_{max,B} B}{BTEX + K_{sB}} \quad (4.3)$$

Similar to benzene, equations for the removal of individual TEX compounds can also be developed. The overall growth rate of degraders in a reactor can be written as (Yoon et al., 1977):

$$\mu = \mu_B + \mu_T + \mu_E + \mu_X \quad (4.4)$$

Combining the equations (3.3) and (3.4) results in:

$$\mu = -\left(\frac{\mu_{max,B} B}{BTEX + K_{sB}} + \frac{\mu_{max,T} T}{BTEX + K_{sT}} + \frac{\mu_{max,E} E}{BTEX + K_{sE}} + \frac{\mu_{max,X} X'}{BTEX + K_{sX}} \right) \quad (4.5)$$

For relatively small initial biomass concentration, it is safe to assume that $\mu_{max,B} = \mu_{max,T} = \mu_{max,E} = \mu_{max,X} = \mu_{max}$. Equation (3.5) will become as:

$$\mu = -\frac{\mu_{max}(B + T + E + X')}{BTEX + K_{sB}} = -\frac{\mu_{max}(BTEX)}{BTEX + K_{sB}} \quad (4.6)$$

or,

$$\frac{dC}{dt} = -\frac{\mu_{max} X(BTEX)}{BTEX + K_{sB}} \quad (4.7)$$

where X is the biomass concentration in the reactor. The integrated form of equation (3.7) can be written as:

$$t = \frac{1}{\mu_{max} X}\left[K_{sB} \log\left(\frac{BTEX_0}{BTEX} \right) + (BTEX_0 - BTEX) \right] \quad (4.8)$$

where $BTEX_0$ is the initial BTEX concentration and BTEX is concentration at any given time (hr), *t*, during the batch degradation experiment. The best estimates of the rate coefficients (or the model parameters), μ_{max} and K_S, can be determined by fitting the integrated Monod equation to the experimental data for BTEX and *t*. Nonlinear regression analysis was used for generating values of the model parameters while minimizing the squared differences between predicted and experimentally observed values of *t*. Parameter estimation was carried out using the SOLVER in Microsoft Excel® (Microsoft Corp., Richmond, WA).

4.3. Growth Curve Using ATP (BacTiter-Glo™) Assay

4.3.1. Reagent Preparation and Optimization

The BacTiter-Glo buffer was mixed with the lyophilized BacTiter-Glo substrate and equilibrated at room temperature (22°C) to form the ATP reagent. The ATP assay was performed as described below.

4.3.2. The ATP Assay Procedure

The bioluminescence reaction was started by adding 100 µL of the BacTiter-Glo reagent to 100 µL of sample. The incubation time was 5 min at room temperature (22ºC). Bioluminescence was determined using a TN20/20 luminometer (Turner Designs, Sunnyvale, CA, USA). ATP per sample was expressed in terms of the bioluminescence signal (relative luminescence units, RLUs). The ATP concentration of different samples

and their corresponding RLUs were found to be linearly related for the BacTiter-Glo™ reagent (Wadhawan et al., 2010).

4.4. Molecular Analysis

The bacterial community dynamics in individual reactors were examined using PCR amplification followed by SSCP at three different stages during the batch degradation study: at the beginning of the study, before the second exposure of the BTEX compounds and at the end of the experiment. Two milliliters of samples from each reactor were collected for the molecular analyses.

The total genomic DNA was extracted from all the samples collected from each reactor. A detailed description of the extraction and PCR-SSCP procedures was provided in Chapter 3. The relative positions of the normalized DNA bands in the SSCP gels were analyzed using the Bionumerics 5.0. A hierarchical cluster analysis of the SSCP pattern was performed by applying the Dice similarity index and unweighted pair-group method with arithmetic average algorithm for calculating the similarity of the dendrogram.

4.5. Analytical Methods

BTEX were analyzed using GC/MSD coupled with a purge and trap auto sampler system using the EPA Method 524.2. The sample loading and GC conditions are described Chapter 3. The GC was calibrated with five BTEX standards of varying concentrations over a linear response ranging from 5 µg/L to 50 µg/L. The method detection limits were 0.4 µg/L for benzene, 0.11 µg/L for toluene, 0.06 µg/L for ethylbenzene, and between 0.05 and 0.13 µg/L for xylenes. Fluorobenzene was used as an internal standard. A response factor method was used for the calibration and estimation of BTEX in the samples (EPA Method 524.2).

4.6. Results and Discussion

4.6.1. Batch Degradation – BTEX Initial Exposure

Batch degradation results during the first exposure of BTEX for reactors with benzene enriched degraders, toluene enriched degraders, ethylbenzene enriched degraders, and xylene enriched degraders are presented in Figure 4.2. Figure 4.3 shows the degradation results during the first exposure of BTEX for reactors with BTEX enriched degraders, BTEX + benzoate enriched degraders, and benzoate enriched degraders. The data represents averages of duplicate reactors and the bars represent minimum and maximum values.

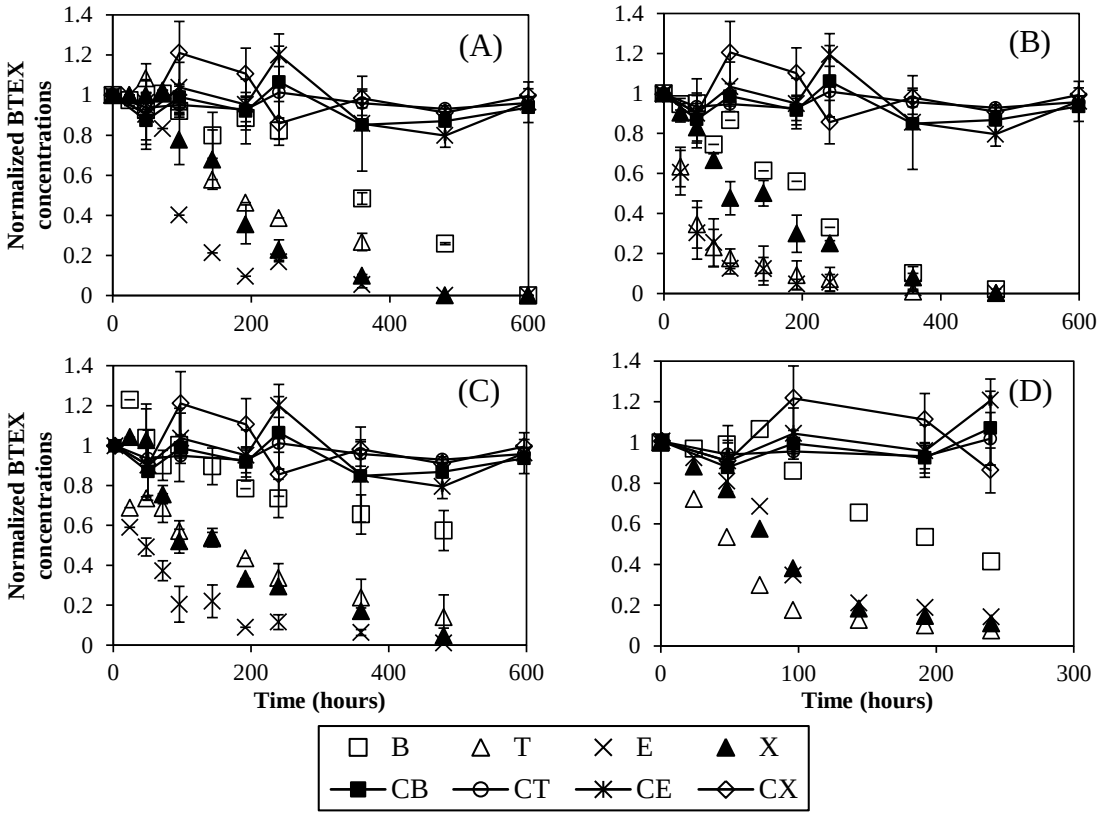


Figure 4.2. Removal performances of the enriched degraders during first time exposure. (A) Benzene enriched degraders (B) Toluene enriched degraders (C) Ethylbenzene enriched degraders (D) Xylene enriched degraders. CB, CT, CE, and CX represent BTEX concentrations in the control reactor, while B, T, E, and X represent BTEX concentrations in the reactor inoculated with enriched degraders.

Removal of all BTEX was observed in all the reactors. Minor losses of BTEX were noticed in the control reactor (Figures 4.2 and 4.3), which indicates that the loss of BTEX in the reactors seeded with bacteria was mainly by biological removal. However, the removal order and rates for individual BTEX compounds varied significantly within and among the reactors. During the first exposure, BTEX compounds were completely removed by the benzene enriched degraders (within 600 hours), the toluene enriched degraders (within 480 hours) and BTEX enriched degraders (within 360 hours). Except benzene, the remaining degraders removed TEX compounds almost completely in different time periods. Benzoate enriched degraders removed ethylbenzene and xylenes completely, and about 80% of benzene and toluene.

Benzene enriched degraders removed ethylbenzene, xylenes and toluene prior to the removal of benzene. Benzene removal was found to be hindered by the presence of toluene in many past studies (Da Silva and Alvarez, 2004; Zepeda et al., 2006). Similar to the results in this study, Da Silva and Alvarez (2004) also reported that microbial consortium enriched on benzene preferred toluene when both the compounds were present together. In a mixture of benzene, toluene and *m*-xylene, Zepeda et al. (2006) noticed that the removal of benzene started after a 6 hour lag period, while toluene and *m*-xylene were removed immediately. In contrast to the findings by Da Silva and Alvarez (2004), where benzene removal was noticed only in the reactors seeded with benzene enriched degraders, benzene removal was observed at varying levels in the reactors seeded with all types of enriched degraders in the present study.

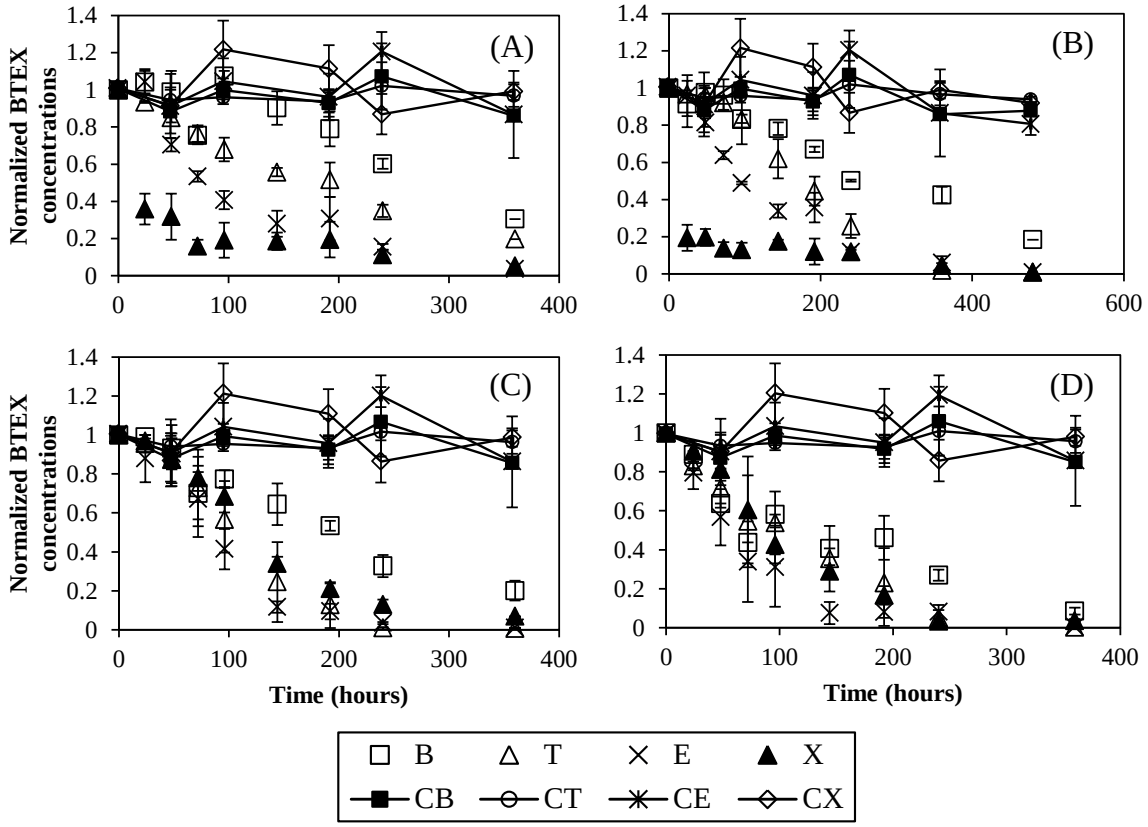


Figure 4.3. Removal performances of enriched degraders during the first exposure (A) Benzoate enriched degraders (B) BTEX + benzoate (1:1) enriched degraders (C) BTEX + benzoate (3:1) enriched degraders (D) BTEX enriched degraders. CB, CT, CE, and CX represent BTEX concentrations in the control reactor, while B, T, E, and X represent BTEX concentrations in the reactor inoculated with enriched degraders.

Although benzene was not preferred over toluene by the toluene enriched degraders, they still removed benzene faster (480 hours) than benzene enriched degraders (600 hours). Substrate inhibition could be more prominent in the case of benzene enriched degraders than toluene enriched degraders. Benzene removal has been recently identified as a syntrophic process (van der Zaan et al., 2012), which requires the existence of multiple species in the degradation process while only a limited number of benzene degrading strains has been identified. Hence, preferential utilization of TEX compounds by some of the benzene degrading strains could lower the removal rate. On the contrary, toluene

enriched degraders are known to contain a wide range of bacterial strains (Weelink et al., 2010), thus reducing the preferential utilization of TEX compounds.

Toluene and ethylbenzene were removed simultaneously by the toluene enriched degraders. Benzene removal was 40% by the ethylbenzene enriched degraders in 480 hours and 60% by xylene enriched degraders in 240 hours. Xylene enriched degraders preferred toluene over xylene. All the degraders enriched on BTEX and benzoate (at varying proportions) were able to remove benzene, although the degraders enriched on BTEX showed superior removal performance. BTEX enriched degraders did not show any distinct preference for any of the BTEX compounds, while benzoate enriched degraders preferred xylenes followed by ethylbenzene and toluene. Benzene was the least preferred by benzoate enriched degraders. The energy requirements for the degradation of BTEX compounds are in the following order: xylenes < ethylbenzene < toluene < benzene (Foght, 2008). Although bacterial strains that could degrade individual BTEX compounds were found to grow on benzoate, necessary enzymes and the intermediates were typically not found during their growth on benzoate (Rabus and Heider, 1998). Hence, the benzoate enriched degraders preferred the compounds with less energy requirements.

A significant initial lag was observed in the removal of almost all BTEX compounds by benzene enriched degraders and benzoate enriched degraders. To date only a few bacterial strains that can degrade benzene under denitrifying conditions have been identified (Weelink et al., 2010). Some of these strains such as *Dechloromonas aromatica* RCB, *Dechloromonas sp.* JJ, *Azoarcus sp.* DN11, can also utilize TEX compounds (Chakraborty et al., 2005; Coates et al., 2001). Competition for the same enzymes produced by these bacterial strains might have caused inappreciable amounts of initial

degradation for each BTEX compound, which appeared as lag times. Degraders enriched on benzoate, as described earlier, were not found to produce enzymes necessary for BTEX catabolism during the enrichment in the past studies (Rabus and Heider, 1998). Hence, the lag periods for these degraders were mainly due to the sequential utilization of BTEX compounds.

Except toluene enriched degraders and BTEX enriched degraders, significant lag periods were experienced in benzene removal by all other enriched degraders. The order of lag period for benzene removal was, benzoate enriched degraders > ethylbenzene enriched degraders > xylene enriched degraders > BTEX + Benzoate (1:3) enriched degraders > benzene enriched degraders. The lag period for benzene removal could be mostly due to the preferential inhibition (or diauxie), a sequential utilization of substrates, by the other TEX compounds. Nales et al. (1998) also observed that the presence of TEX inhibited the anaerobic benzene degradation in microcosms. This preferential degradation could be due to the less energy requirements for activation of TEX degradation than for activation of benzene degradation (Foght, 2008).

Benzene removal by benzene enriched degraders and xylene enriched degraders was observed after the removal of 70% to 80% of the other TEX compounds. However, benzene removal ceased after 80% of the toluene was removed in the reactors with ethylbenzene enriched degraders, which indicates that benzene was cometabolized with toluene by the ethylbenene enriched degraders. Studies on ethylbenzene degrading bacteria have identified very few pure cultures (EbN1, PbN1, and EB1) in anoxic conditions (Chakraborty and Coates, 2004; Kniemeyer and Heider, 2001) among which EbN1 was found to solely grow on toluene as well (Chakraborty and Coates, 2004; Champion et al.,

1999). However, Champion et al. (1999) found that EbN1 uses two distinct metabolic routes for the degradation of ethylbenzene and toluene. They proposed that ethylbenzene degradation includes formation of 1-phenylethanol, then to acetophenone, and subsequent carboxylation of acetophenone. In contrast, the proposed pathway for toluene degradation was through the generally recognized anaerobic activation of toluene through a fumarate-dependent formation of benzylsuccinate.

When present alone, benzene was removed by benzene enriched degraders in less than 72 hours (removal rate was 0.034 mg/L-hr or 0.833 mg/L-day). This high removal rate for benzene was normally observed in microcosms inoculated with enriched bacteria. Burland and Edwards (1999) reported that benzene removal rates of enriched microbial cultures (in denitrifying conditions) can be as high as 100 times to those of indigenous microorganisms. The removal rates reported by Burland and Edwards (1999) were 0.14 µmol/L-day (0.01 mg/L-day) for indigenous microorganisms and 13 µmol/L-day (0.936 mg/L-day) for enriched microorganisms. Dou et al. (2008) also reported benzene removal rates ranging between 0.45 and 1.2 mg/L-day by denitrifying bacteria enriched on BTEX mixture. However, benzene removal was greatly reduced when present as a mixture, which indicates the influence of inhibition. Moreover, the removal of benzene after the majority of TEX compound removal also indicates the preferential inhibition. Lag periods during the removal of benzene were also observed for BTEX + benzoate enriched degraders and benzoate enriched degraders. Preferential inhibition for benzene degradation by a pure culture (*Pseudomonas fluorescens*) was earlier noticed by Chang et al. (1993) due to the presence of toluene and *p*-xylene. Sequential utilization of toluene and *p*-xylene followed by benzene by a mixture of several pure cultures was observed by Oh and Choi (1997).

Although benzene enriched degraders received benzene as the sole carbon source for extensive periods (more than 2 years) during the enrichment process, preferential inhibition was still evident when benzene was present with TEX compounds.

4.6.2. Batch Degradation – BTEX Second Time Exposure

Batch degradation results during the second time exposure for degraders enriched on benzene, toluene, ethylbenzene and xylene are presented in Figure 4.4. The data represent averages of duplicate reactors and the error bars represent minimum and maximum values. The majority of the BTEX was removed by all of the enriched degraders much quicker during the second exposure (within 192 hours as compared to more than 600 hours during the first exposure). Contrary to the first exposure, ethylbenzene enriched degraders could not remove benzene as well as toluene after the majority of the ethylbenzene and xylenes were removed. Benzene during the initial and the second exposures was removed through cometabolism by ethylbenzene enriched degraders due to the presence of either toluene or ethylbenzene. Benzene removal ceased in the second exposure when these compounds were depleting.

The higher degradation rates of the degraders during the subsequent exposure to the target compounds could be due to the increased number of dormant bacterial cultures. During the enrichment of the degraders on individual BTEX compounds, the bacterial cultures, which could utilize compounds other than the inducer as growth substrates, could be present less in number. During the enrichment, these bacterial cultures were probably in a dormant stage until they were exposed to the BTEX mixture.

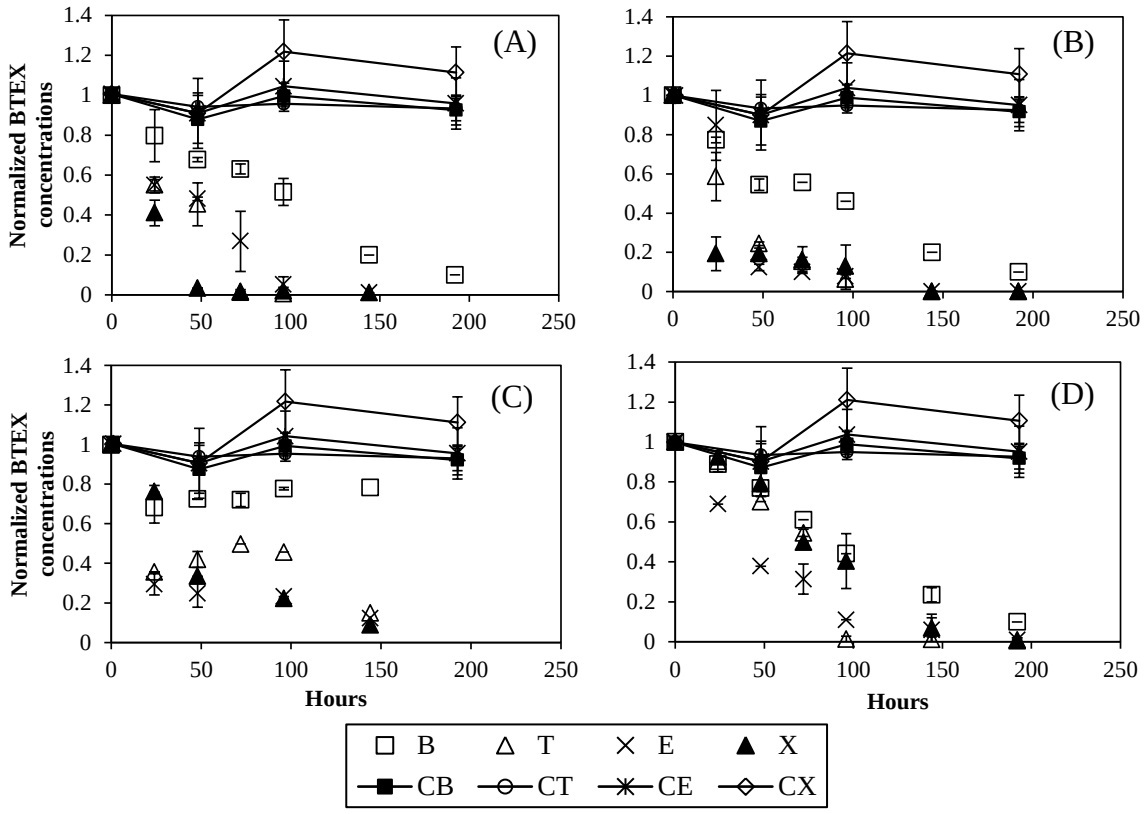


Figure 4.4. Removal performances of the enriched degraders during second time exposure (A) Benzene enriched degraders (B) Toluene enriched degraders (C) Ethylbenzene enriched degraders (D) Xylene enriched degraders. CB, CT, CE, and CX represent BTEX concentrations in the control reactor, while B, T, E, and X represent BTEX concentrations in the reactor inoculated with enriched degraders.

4.6.3. Modeling Degradation Kinetics

While estimating the model parameters (μ_{max} and K_S) for integrated Monod equation, several combinations of μ_{max} and K_s values were found that had very similar minimum sum of square errors. However, in this study, μ_{max}/K_s ratio was used as the fitting parameter. In a complex system such as the batch reactors in this study where mixed bacterial cultures were growing on mixture of substrates, individual consideration of μ_{max} and K_s can lead to misinterpretations. For example, some of the enriched degraders have similar K_s values; however, their growth rates differed considerably. In such cases, the ratio of μ_{max}/K_s can be used as a good parameter for comparison between different species

of microorganisms during the uptake under the same condition (Healey, 1980). The higher μ_{max}/K_s ratio indicates that the enriched degraders have higher affinity to degrade the BTEX compounds as compared to the degraders with lower μ_{max}/K_s ratio.

A summary of μ_{max}/K_s values for the first exposure is presented in Table 4.2. Toluene enriched degraders were found to have the greatest μ_{max}/K_s to degrade BTEX compounds (Table 4.2), while benzoate degraders had the least μ_{max}/K_s, although the values among different degraders are not significantly different from each other. Benzene enriched degraders, toluene enriched degraders, BTEX enriched degraders and BTEX + benzoate (3:1) enriched degraders have close μ_{max}/K_s, while the μ_{max}/K_s values for the remaining degraders are in close proximity. Benzoate degraders have a slightly better μ_{max}/K_s value than the ethylbenzene and xylene enriched degraders, which could be because of their superior removal performance for benzene.

Table 4.2. Integrated Monod kinetic parameters.

Reactor	**μ_{max}/K_s (L/mg-hr)**
1	0.00271
2	0.00296
3	0.00116
4	0.0018
5	0.00259
6	0.00235
7	0.00159
8	0.00126

Since the biomass concentration used in this study was small (4 µg/L), the growth rate (e.g. μ_B) of microbial cultures utilizing each BTEX compound (or B in equation 3.3)

would be almost identical. Relatively similar μ and μ_{max} values for each compound will lead to insignificant differences in K_S values. The reason for the use of small biomass in this study was to minimize the loss of BTEX compounds through biosorption, which allowed the use of the assumptions ($K_{sB} = K_{sT} = K_{sE} = K_{sX}$ and $\mu_{max,B} = \mu_{max,T} = \mu_{max,E} = \mu_{max,X} = \mu_{max}$) in this study. However, these assumptions have greatly simplified the real situation and could limit the use of the model to a wide range of applications where large amounts of biomass are needed. A further investigation is required to estimate inhibition and half saturation constants for each compound while present in a mixture.

In addition to the integrated Monod's equation, a least square regression analysis using Microsoft Excel (Microsoft Corp., Redmond, WA) was performed to determine the BTEX removal rates by each of the enriched degraders and the results are summarized in Table 4.3. Except benzene removal by the benzene enriched degraders, the first order removal rates were observed for all the BTEX compounds. A zero order removal for benzene by the benzene enriched degraders gave a good fit ($R^2 = 0.97$) after an initial lag period of 96 hours. Both zero-order and first-order removal has been observed for the biodegradation of all BTEX compounds (Suarez and Rifai, 1999). In this study, the R^2 value for benzene removal by benzene enriched degraders was very close for zero and first order removal, with zero-order being slightly higher (0.97 versus 0.89).

The first order removal rates determined for BTEX compounds in this study by all the enriched degraders are higher than the values reported in the studies with indigenous microorganisms under denitrifying conditions (Borden et al., 1997; Hunt et al., 1998; Hutchins et al., 1991). However, the removal rates observed in this study are comparable

Table 4.3. Kinetic rates and lag periods of degraders during the initial exposure to all BTEX together.

Compound ⇨ / Reactor		B			T			E			X			BTEX
#	Name	Model	Rate (hr^{-1})	Lag Period (hr)	Model	Rate (hr^{-1})	Lag Period (hr)	Model	Rate (hr^{-1})	Lag Period (hr)	Model	Rate (hr^{-1})	Lag Period (hr)	Rate (hr^{-1})
1	Benzene only	Linear	0.002*	96	Exp	0.012	96	Exp	0.012	48	Exp	0.013	96	0.005
2	Toluene only	Exp	0.006	N	Exp	0.014	N	Exp	0.013	N	Exp	0.01	N	0.009
3	Ethylbenzene only	Exp	0.002	96	Exp	0.004	N	Exp	0.009	N	Exp	0.007	48	0.004
4	Xylene only	Exp	0.006	72	Exp	0.012	N	Exp	0.009	N	Exp	0.01	N	0.007
5	BTEX mixture	Exp	0.006	N	Exp	0.014	N	Exp	0.014	N	Exp	0.01	N	0.009
6	BTEX 50 + Benzoate 50	Exp	0.004	24	Exp	0.018	72	Exp	0.013	N	Exp	0.008	N	0.007
7	BTEX 25 + Benzoate 75	Exp	0.004	96	Exp	0.011	72	Exp	0.009	N	Exp	0.008	N	0.006
8	Benzoate only	Exp	0.004	144	Exp	0.004	N	Exp	0.009	24	Exp	0.008	N	0.005

N – No lag period
*units are in mg/L-day

to those reported by Dou et al. (2008) and in the range of values from past studies conducted in denitrifying conditions as summarized in Suarez and Rifai (1999). The first order removal rates reported for microcosm studies conducted with enriched microorganisms are generally higher than those with the indigenous microorganisms. This could be due to an increased number of bacterial consortia capable of degrading the target contaminant during the enrichment process as compared to the indigenous microorganisms. Controlled environmental conditions during the enrichment process also aid in enhancing the biodegradation capability of the bacterial consortia.

Toluene enriched degraders and BTEX enriched degraders had the greatest removal rates for all the BTEX compounds. The removal rates for these two enriched degraders were almost the same for each of the BTEX compounds, which are in the order of: toluene > ethylbenzene > xylenes > benzene. Similar observations were made by Dou et al. (2008), where they used mixed microbial consortia enriched on BTEX compounds. The results also showed that toluene as the most easily degradable compound among all BTEX compounds, while benzene and *p*-xylene were found to be the least favorable or the slowly degradable compounds. On the other hand, Zheng et al. (2001) reported faster removal rates for toluene when it is present in a mixture with *o*-xylene, 1, 2, 4-trimethylbenzene, and naphthalene as compared to when present as a single compound. The reason for slow degradation of benzene was its molecular structure, which requires higher energies for the initial activation, while a faster degradation for the TEX compounds occurs due to the presence of methyl and ethyl groups on the benzene ring. Degradation of toluene and xylenes is initiated by an addition reaction of the methyl group to the double bond of fumarate to form benzylsuccinate or methylbenzylsuccinate, respectively, while the

degradation of ethylbenzene is initiated at the methylene carbon to form 1-phenylethanol (Widdel and Rabus, 2001). Benzene degradation in anaerobic conditions (denitrifying, iron reducing, and sulfate reducing) has been proposed to initiate via three plausible pathways: hydroxylation (producing phenol), methylation (producing toluene), or carboxylation (producing benzoate) (Foght, 2008). Although individual energy requirements for the activation of each BTEX compound is unknown, overall energy requirements for mineralization in denitrifying conditions are as follow: -2990 kJ/mol, -3554 kJ/mol, -4148 kJ/mol, and -4217 kJ/mol for benzene, toluene, ethylbenzene and xylene, respectively (Foght, 2008).

4.6.4. Microbial Growth Using ATP Assay

The ATP assay was used to quantify the growth of the enriched microbial degraders during the batch degradation experiments. Samples were taken over a period of time for the ATP analysis. Four microbial degraders used for growth estimates were benzene enriched degraders (BD), toluene enriched degraders (TD), ethylbenzene enriched degraders (ED), and xylene enriched degraders (XD). For testing the growth, a culture was grown separately on BTEX and on the carbon source (C) used during enrichment, such as benzene for BD. The ratios of the RLUs ($RLUs_{BTEX}/RLUs_{C}$) obtained were plotted as shown in Figure 4.5.

The $RLUs_{BTEX}/RLUs_{C}$ ratio was above one for all the time periods sampled. This indicates that the number of RLUs, which represents the number of bacteria, was higher when the degraders were grown on BTEX. A sudden and higher increase in the ratio for XD was observed within the first day. After 24 hours, the growth of XD was at least 4 times more than growth of other degraders, when grown on BTEX compared to its carbon

source. XD was able to grow about 12 times more on BTEX when compared to those that were grown on xylene alone. The utilization of different carbon sources in a mixture of contaminants and the corresponding growth vary among different bacterial strains. A future study is needed to identify bacterial strains in the enriched degraders and their growth and substrate consumption for BTEX compounds, when present individually and as a mixture.

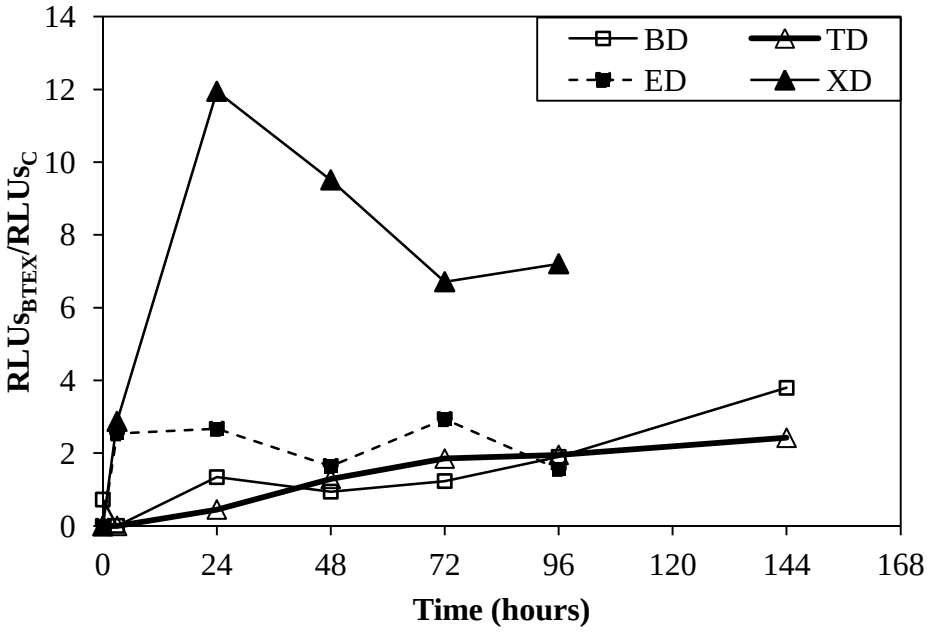


Figure 4.5. Relative ATP activity of enriched degraders in the presence of BTEX mixture.

A slight increase in the growth was observed for ED within the first few hours which then became stable. The growth of ED on BTEX was 2.9 times compared to ethylbenzene alone at 72 hrs. For BD and TD, gradual increases in growth were observed over 144 hrs. The growth of BD and TD on BTEX was at least two times higher than their growth on B and T, respectively, at several sampling time points. These results suggest that the degraders enriched on individual BTEX compounds were utilizing the rest of the BTEX compounds more efficiently as a growth substrate.

Growth rates reported in the past studies are highly specific to the type of bacterial species. Toluene removal in denitrifying conditions were 0.08 hr^{-1} for *Thauera aromatica* strain K172 (Leutwein and Heider, 1999) and 0.14 hr^{-1} for Strain T1 (Evans et al., 1992). The growth rates on individual BTEX compounds observed in the present study were: 0.022 hr^{-1}, 0.018 hr^{-1}, 0.01 hr^{-1}, 0.005 hr^{-1} for benzene, toluene, ethylbenzene, and xylene enriched degraders, respectively. These growth rates for each type of enriched degraders represent the cumulative of all the species present in the culture, which include both slow and fast growing strains. Hence, the observed values in this study are less than the reported values.

4.6.5. Microbiological Studies

Results from SSCP analysis are presented in Figure 4.6. The results from before the first exposure are presented in Figure 4.6 (a), after the first exposure in Figure 4.6 (b), and after the second exposure in Figure 4.6 (c). The notations started with BTEX represent samples from the reactors inoculated with the degraders from the ERs enriched using the BTEX + Benzoate mixture during the enrichment, while 0, 25, 50, and 100 before the hyphen indicate the percent of BTEX in the BTEX + Benzoate mixture. Individual letter notations B, T, E, and X represent the reactors inoculated with the degraders from the ERs enriched using individual BTEX compounds. The notations 0, 1, and 2 after the hyphen indicate before the first exposure, after the first exposure, and after the second exposure respectively.

Differences among bacterial communities are evident from Figure 4.6 (a) due to the carbon source during the enrichment. Although the modeling results show that degraders from benzene enriched ER, toluene enriched ER, BTEX enriched ER, and BTEX +

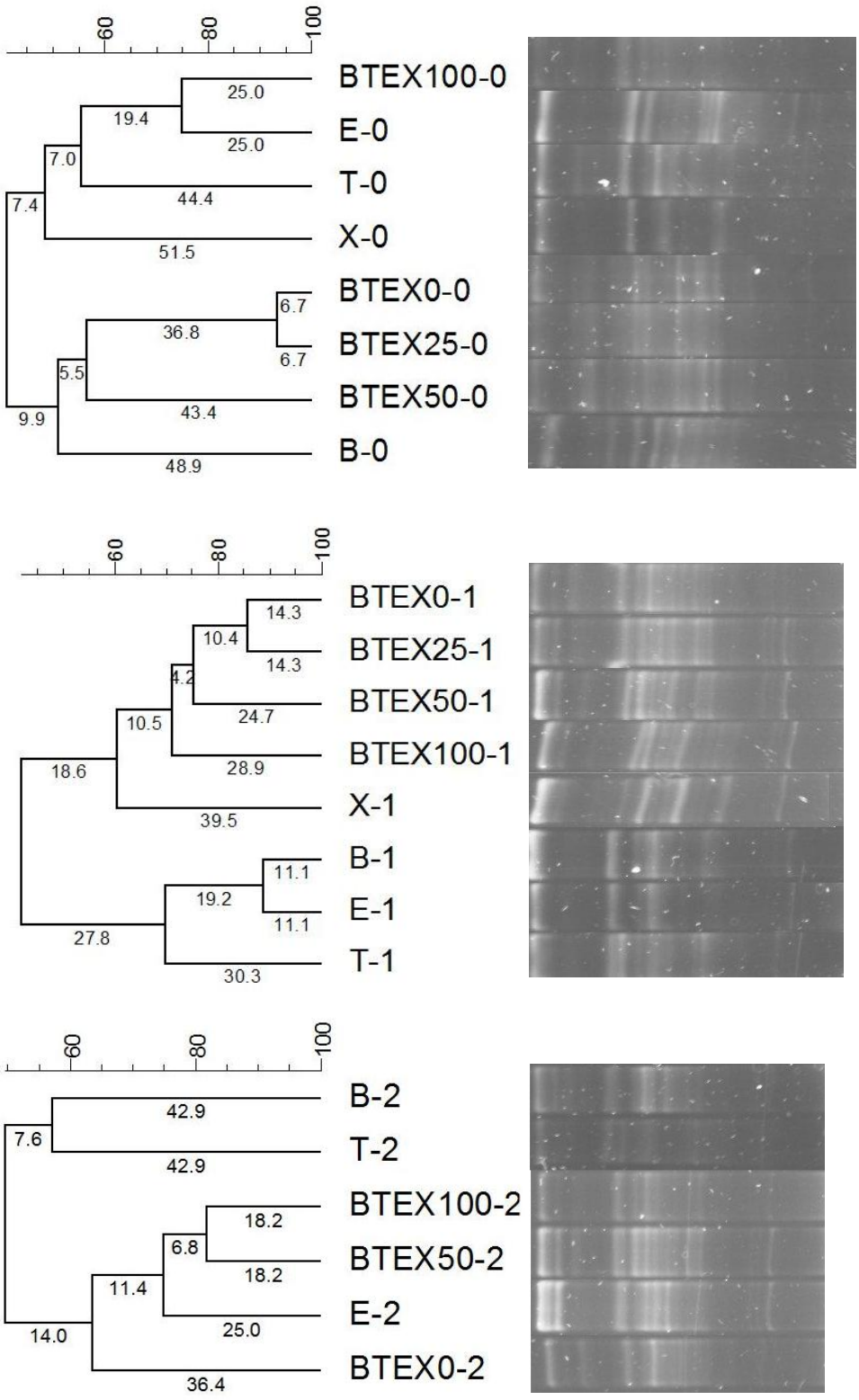


Figure 4.6. SSCP profiles of enriched degraders (A) before and (B) after the first and (C) after the second exposures to BTEX mixture.

benzoate (1:1) enriched ER have μ_{max}/K_s in the proximity, the community structures differed significantly. The benzene degraders were 37.5% similar to BTEX enriched degraders (BTEX100-0), and about 50% similar to toluene (T-0) and BTEX + benzoate (1:1) enriched degraders (BTEX50-0). On the other hand, BTEX + benzoate (1:3) enriched degraders (BTEX25-0) and benzoate enriched degraders (BTEX0-0) have communities that were 94% similar before their first exposure to BTEX mixture.

The differences in community structures decreased slightly after the subsequent exposures among various degraders. The community structures in benzene and toluene enriched degraders did not experience any change from before exposure and after the second exposure. These results in conjunction with μ_{max}/K_s values indicate that communities in these two different types of enriched degraders have the necessary enzymatic capabilities to degrade BTEX mixture, without the presence of all the BTEX compounds. The similarity between community structures in BTEX enriched degraders and BTEX + benzoate (1:1) enriched degraders has increased from 40% to 70% after the first exposure and then to 82% after the second exposure. Although the μ_{max}/K_s values for BTEX + benzoate (1:3) enriched degraders and benzoate enriched degraders were not in the close proximity to BTEX enriched degraders, the similarity between these degraders increased from 38% to 87% and 28% to 70% respectively after the first exposure. These results indicate that the use of benzoate as an inducer compound maintained the communities necessary to degrade BTEX mixture.

4.7. Summary

Enrichment of target contaminant degrading consortia is an important element in the bioremediation process because it helps in maintaining the performance of the process

when bioaugmentation is necessary for the site. For bioremediation of BTEX, a mixture of structurally similar contaminants, the effect of carbon source conditions during the enrichment of BTEX degrading consortia was investigated. The carbon sources tested were selected on the basis of minimization of the use of the toxic chemicals such as one BTEX compound versus BTEX mixture and benzoate (a non-toxic and a common intermediate compound for BTEX) versus BTEX compound(s). Results showed that individual BTEX compounds can be used as potential inducer compounds for enrichment of BTEX degrading consortia. Degraders enriched on one of the BTEX compounds were able to utilize remaining BTEX compounds also as growth substrates. Additionally, the results demonstrated the use of benzoate as a potential inducer; however with less degradation rates. When benzoate was used with BTEX mixture as the inducer, the degraders showed superior BTEX removal performances than those of degraders enriched on benzoate. Although degraders enriched on benzoate or benzoate-BTEX mixture may require relatively greater acclimation periods, the use of benzoate as inducer compound eliminates the potential for contamination due to accidental spills. The acclimation periods, however, would be relatively small for degraders enriched on benzoate or benzoate-BTEX mixture compared to typical biostimulation time for indigenous bacterial population.

CHAPTER 5. A NOVEL APPLICATION OF ER-PRBB FOR REMOVING A MIXTURE OF CONTAMINANTS WITH SUBSTRATE INTERACTIONS

5.1. Introduction

Successful PRBB application for a mixture of contaminants is often influenced by many different substrate interactions among individual contaminants in the mixture. These interactions can alter degradation rates of individual contaminants either synergistically or antagonistically (Arvin et al., 1989; Barbaro et al., 1992; Wang and Deshusses, 2007). Synergistic interactions promote the degradation rates of individual contaminants while the antagonistic interactions reduce the degradation rates through various inhibition processes. Antagonistic interactions such as preferential degradation or diauxie, which is sequential utilization of preferred substrates, can lead to lag phases before other substrates are consumed. A detailed description of substrate interactions is provided in Section 2.5.2 of Chapter 2. PRBBs are often augmented with mixed bacterial cultures that are adapted to target contaminants to address the substrate interactions. Mixed cultures are often found to be more effective than pure cultures in PRBBs because interspecies interactions may be necessary for the complete biodegradation of contaminant mixtures (Deeb and Alvarez-Cohen, 2000).

The transport of a contaminant or a mixture of contaminants in groundwater is unpredictable and can come in batches or discontinuous plumes as a result from trapped residual non aqueous phase liquids (Sahloul et al., 2002). Random disappearance of the target contaminants could cause a possible loss of some of the contaminant degraders and/or the biodegradation activity (Kasi et al., 2011), which are necessary for interspecies interactions for the biodegradation of contaminant mixture. This partial loss of degraders

can lead to increased antagonistic substrate interactions, such as diauxie, when the contaminant mixture reappears. In case of a mixture of structurally similar contaminants such as BTEX, increased diauxie effects after an absence period can be observed leading to unacceptable lag phases in degrading some of the contaminants in the mixture (Burbeck and Perry, 1993).

In addition to substrate interactions, contaminant mixture degradation can be suppressed by the presence of structurally dissimilar compounds as well. The presence of ethanol was found to repress the production of the inducible enzymes that are needed for starting BTEX degradation, leading to longer lag phases (Corseuil et al., 1998). BTEX are typically found along with ethanol in groundwater and ethanol is more soluble than BTEX and hence moves faster and reaches the PRBB sites sooner than BTEX.

A successful application of ER-PRBB to remediate groundwater contaminated with a single contaminant (benzene) when appears in batches is described in Chapter 3. ER is an offline reactor where bacterial culture is acclimated to target compounds and is used to augment a main treatment system, such as the PRBB. Appropriate growth conditions for culture enrichment, such as availability of nutrients and target compounds, and suitable environmental conditions (pH and temperature) are provided in the ERs to induce the desired degradation capability. Supplying the enriched bacterial culture from ER was found to maintain the performance of the PRBB when a single contaminant reappeared after a period of absence.

Supply of bacteria from an ER after an absence period can supplement the biobarrier with active bacterial culture to make up for the loss of degraders acclimated to the target contaminant. The activity of bacterial culture in the ER is maintained through the

supply of necessary growth materials as well as the target contaminant itself. Application of an actively enriched bacterial culture to address substrate interactions among mixtures of target contaminants, such as BTEX, has not been addressed. The active culture, although may not completely eliminate the antagonistic substrate interactions (inhibition), could minimize the effects of these interactions by maintaining the communities in sufficient numbers necessary to degrade each compound in the contaminant mixture. Hence, when used to augment to a PRBB, supply of this actively enriched culture can minimize the performance losses after a period of absence due to increased substrate interactions by providing the communities necessary to degrade all compounds in a contaminant mixture. For structurally similar compounds, the culture can also be enriched in a single ER by supplying all compounds in the same reactor or even supplying a single compound as inducer in the ER, which eliminates the cumbersomeness of enriching the necessary bacterial cultures on individual compounds in multiple ERs.

This chapter describes the application of ER-PRBB technique to address the performance loss of a PRBB due to substrate interactions among a mixture of contaminants in groundwater, when the mixture reappears after a period of absence. A mixture of BTEX was chosen as the model contaminants. Effect of ethanol during the BTEX absence period on the substrate interactions among BTEX was investigated. Ethanol is a common additive to gasoline in many countries and is commonly found with BTEX in contaminated groundwater. Toluene and BTEX as an inducer in ER were compared since bacterial cultures grown on toluene alone were found to effectively degrade BTEX compounds individually and as a mixture (Chapter 4). Changes in bacterial community structure in the

PRBBs due to BTEX absence period and the supply of ethanol during BTEX absence period were also investigated.

5.2. Methods

5.2.1. Cultivation of Toluene and BTEX Degraders

MLSS from the Moorhead Wastewater Treatment Plant, Moorhead, MN, USA were acclimated to toluene (toluene degraders or TD) and BTEX mixture (BTEX degraders or BTEXD) in two different ERs. Methanol degrading denitrifiers were gradually adapted to toluene and BTEX mixture by increasing their concentrations in their respective reactors while reducing the methanol in the feed; the total mass of carbon (27.7 mg/L) supplied was kept constant during the gradual acclimation. The degraders received the carbon source along with MSM, which was used to supplement necessary minerals for growth. The composition of MSM is described in Chapter 3. The final concentrations in the synthetic groundwater that culture was exposed to were 30 mg/L of toluene for TD and 8 mg/L of each BTEX compound for BTEXD.

5.2.2. ER Operation and Activity Test

The activities of the acclimated TD and BTEXD cultures were continuously maintained in their respective ERs. Synthetic groundwater containing MSM was constantly supplied with toluene for TD culture and BTEX for BTEXD culture. ERs were operated as SBRs in plastic vessels with a working volume of 3 liters. The operation of SBRs is described in Chapter 3. During the filling, the system received synthetic groundwater containing MSM, and was purged with nitrogen gas to attain anoxic conditions before spiking with toluene to 30 mg/L or BTEX with 8 mg/L of each BTEX compound. During the reaction period, the vessel was closed with an airtight cap to maintain anoxic

conditions and the solution was mixed on a horizontal shaker (DS-500E, VWR International Co., PA, USA).

The activity of the degraders was tested monthly in a 250 mL amber bottle. Two hundred and thirty milliliters of synthetic groundwater containing MSM was mixed with 10 mL of enriched bacteria, and purged with nitrogen gas to anoxic conditions. The concentrations of enriched bacteria used in the activity tests were 100 mg/L as volatile suspended solids (VSS) for toluene degraders and 85 mg/L as VSS for BTEX degraders. The reactors seeded with toluene degraders were spiked with toluene to 10 mg/L, while the reactors seeded with BTEX degraders were spiked with 2.5 mg/L of each BTEX compound. Control reactors consisting of MSM and 2.5 mg/L of each BTEX compound without bacterial culture were included. One hundred microliter samples were collected from both bottles at different time intervals for BTEX analysis.

5.2.3. Continuous Flow Experiments and Sand Columns Operation

Continuous-flow experiments conducted with sand columns representing PRBBs. Amount and type of sand in the sand columns were same as described in Chapter 3. Figure 5.1 shows the experimental setup of PRBBs and ER-PRBBs. Among the PRBBs, four columns (TD1, TD2, TD3, and TD4) were inoculated with 30 mL of TDs, and the remaining four columns (BTEXD1, BTEXD2, BTEXD3, and BTEXD4) were inoculated with 30 mL of BTEXDs from their respective ERs. The concentration of the degraders for both TDs and BTEXDs was 100 mg VSS/L. For all PRBBs, the sand was homogenously mixed with the degraders and loosely packed in the columns. A ninth column, which was not inoculated, was used as a control to measure abiotic losses due to volatilization and adsorption.

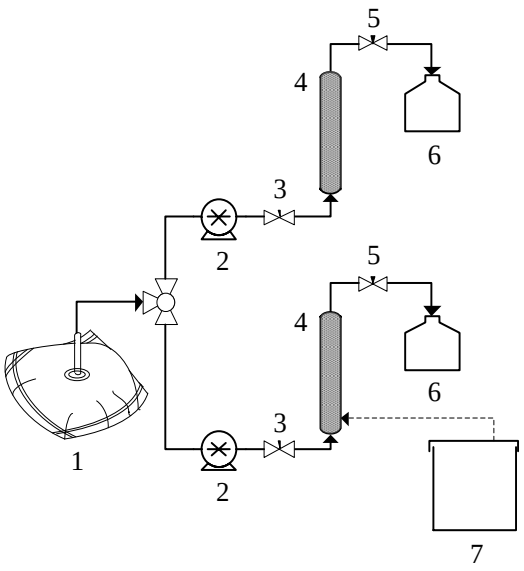


Figure 5.1. Experimental setup for PRBB and ER-PRBB. 1 – influent gas bag; 2 – peristaltic pump, 3 – influent sampling points, 4 – PRBB, 5 – effluent sampling points, 6 – effluent collection, 7 – ER.

Experiments were conducted in 3 phases. A summary of the phases is provided in Table 5.1. In phase 1 (Initial operation), all the columns initially received BTEX mixed with synthetic groundwater from a gas bag (Figure 5.1). Phase 2 (Absence period) was started after the columns showed steady removal performances in phase 1. Two parallel scenarios were implemented in phase 2: supply of no carbon source and supply of ethanol as an alternate carbon source. The first scenario was simulated by changing the influent source to a gas bag containing synthetic groundwater with no carbon source for four of the PRBBs, with two from each type of inoculation. The second scenario was simulated by changing the influent source to a gas bag containing ethanol mixed synthetic groundwater for the remaining columns. After 10 days, the influent source was changed back to the gas bag with BTEX (phase 3). Just before the start of phase 3 (BTEX reintroduction), PRBB with each type of inoculation from each scenario in the second phase received enriched degraders (one third of the initial inoculation) from their respective ERs. One hundred

microliters of influent and effluent samples were collected from each column. The samples were immediately injected into 40 mL amber vials containing 40 mL of DI water and stored in the refrigerator at 4ºC before analysis.

Table 5.1. Experimental design for continuous flow experiments. ER-PRBBs are highlighted in grey. Reactors that received ethanol during the BTEX absence period are boxed by dashed lines.

PRBB condition	**Initial inoculation**	**Phase 1 Initial operation (10 days)**	**Phase 2 Absence period (10 days)**	**Phase 3 BTEX reintroduction (18 days)**
TD-ER	Toluene degraders	BTEX + MSM in the influent	MSM alone in the influent	BTEX + MSM in the influent (ER supply)
TD	Toluene degraders	BTEX + MSM in the influent	MSM alone in the influent	BTEX + MSM in the influent
TD-ER-EtOH	Toluene degraders	BTEX + MSM in the influent	MSM + ethanol in the influent	BTEX + MSM in the influent (ER supply)
TD-EtOH	Toluene degraders	BTEX + MSM in the influent	MSM + ethanol in the influent	BTEX + MSM in the influent
BTEXD-ER	BTEX degraders	BTEX + MSM in the influent	MSM alone in the influent	BTEX + MSM in the influent (ER supply)
BTEXD	BTEX degraders	BTEX + MSM in the influent	MSM alone in the influent	BTEX + MSM in the influent
BTEXD-ER-EtOH	BTEX degraders	BTEX + MSM in the influent	MSM + ethanol in the influent	BTEX + MSM in the influent (ER supply)
BTEXD-EtOH	BTEX degraders	BTEX + MSM in the influent	MSM + ethanol in the influent	BTEX + MSM in the influent
C	Control	BTEX + MSM in the influent	MSM alone in the influent	BTEX + MSM in the influent

5.2.4. Bacterial Community Examination

The bacterial community dynamics in the PRBBs and ECPRBBs were examined using polymerase chain reaction (PCR) amplification followed by single strand conformation polymorphism (SSCP). A PCR-SSCP procedure described in Chapter 3 was followed. Total genomic DNA was extracted from samples collected from the PRBBs before the absence of BTEX and just before the reappearance of BTEX after a period of absence.

5.2.4.1. DNA extraction, amplification and gel electrophoresis

DNA from all the samples was extracted using a DNA extraction kit following the instructions from the kit and was amplified using a PCR reaction in a thermocycler. PCR reaction mix, configuration of primers, and temperature programming for the PCR reactions are described in Chapter 3. The PCR amplified DNA in each sample was denatured into single strands and was loaded in a precast Elchrom's GMA gel, which was run at a constant voltage and temperature in a horizontal electrophoresis setup. The denaturing conditions and electrophoresis setup are described in Chapter 3. The gels were visualized by using a SYBR Gold-stain method (Molecular probes, OR). The relative positions of the normalized DNA bands in the SSCP gels were analyzed using the Bionumerics 5.0. A hierarchical cluster analysis of the SSCP pattern was performed by applying the Dice similarity index and unweighted pair-group method with arithmetic average algorithm for calculating the similarity of the dendrogram.

5.2.5. Analytical Methods

BTEX were analyzed using gas chromatography GC/MSD. The GC flow and temperature conditions are described in Chapter 3. The GC was calibrated with BTEX standards as described in Chapter 4.

5.3. Results and Discussion

5.3.1. Activity of Toluene and BTEX Enriched Degraders

Activity test results for toluene degraders and BTEX degraders are presented in Figure 5.2. The data represent averages of duplicate experiments and the error bars represent minimum and maximum values. Toluene degraders completely removed 10 mg/L of toluene in 144 hours (Figure 5.2(A)) and 2.5 mg/L of toluene in a BTEX mixture in 240 hours (Figure 5.2(B)). BTEX degraders totally degraded toluene in a BTEX mixture in approximately 240 hours (Figure 5.2(C)). However, the rate for initial toluene degradation (first 80% of toluene) in the BTEX mixture by toluene degraders was faster than that by BTEX degraders. Toluene degradation by both types of degraders experienced inhibitions due to the presence of remaining BTEX compounds, which agreed with the results reported by previous studies (Alvarez and Vogel, 1991; Bielefeldt and Stensel, 1999; Kasi et al., 2012).

First order removal was observed for BTEX in the mixture by both toluene and BTEX degraders (Table 5.2). The order of removal rates of individual compounds in the BTEX mixture was toluene ≥ ethylbenzene > xylene > benzene by toluene degraders and BTEX degraders. First order removal rates by both types of degraders were similar for all compounds except ethylbenzene, for which BTEX degraders had slightly higher removal rate than toluene degraders.

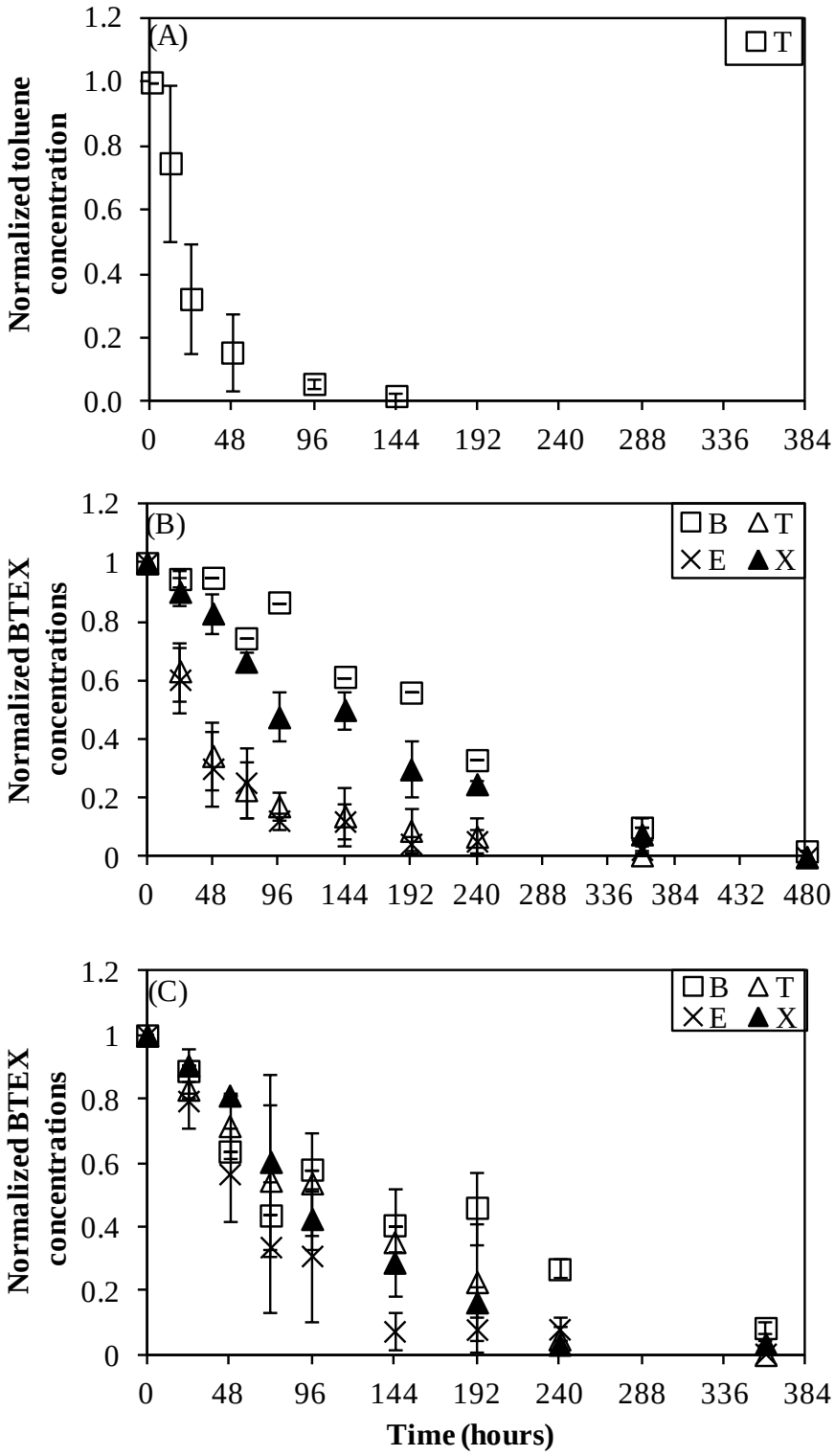


Figure 5.2. Activity test for (A) toluene degraders with toluene alone, (B) toluene degraders with BTEX mixture, and (C) BTEX degraders with BTEX mixture. B- benzene, T - toluene, E - ethylbenzene, and X - xylenes.

Table 5.2. Degradation rates of individual BTEX compounds in mixture during the activity tests

Compound	Toluene Degraders		BTEX Degraders	
	First order degradation		First order degradation	
	R^2	Rate (1/hr)	R^2	Rate (1/hr)
Benzene	0.88	0.006	0.9	0.006
Toluene	0.97 0.96[£]	0.014 0.029[£]	0.85	0.014
Ethylbenzene	0.9	0.013	0.95	0.014
Xylene	0.8	0.01	0.91	0.01

[£]Results from the activity test for toluene degraders with toluene alone

5.3.2. Continuous Flow Experiments

The continuous flow experiment results are presented in Figures 5.3 and 5.4. For each absence period, the results from duplicate reactors were averaged. The values on the y-axis represent the effluent concentrations (C) of benzene normalized by the influent concentration (C_0). The x-axis values represent operational time in days. The error bars represent minimum and maximum values.

Removal of all BTEX compounds was observed during the column experiment before the BTEX absence period. As shown in Figures 5.3 and 5.4, ethylbenzene and xylenes were not detected in the effluent from any of the PRBBs. The concentrations of benzene and toluene were mostly below 20% of the influent concentrations for all the PRBBs. BTEX losses in the control column were less than 20% due to the physical losses such as volatilization and/or adsorption. Hence, the majority of the benzene and toluene removal in the columns can be attributed to biological removal. In addition to biodegradation, ethylbenzene and xylene could have experienced higher biosorption. Previous studies reported that among BTEX compounds, ethylbenzene and xylenes

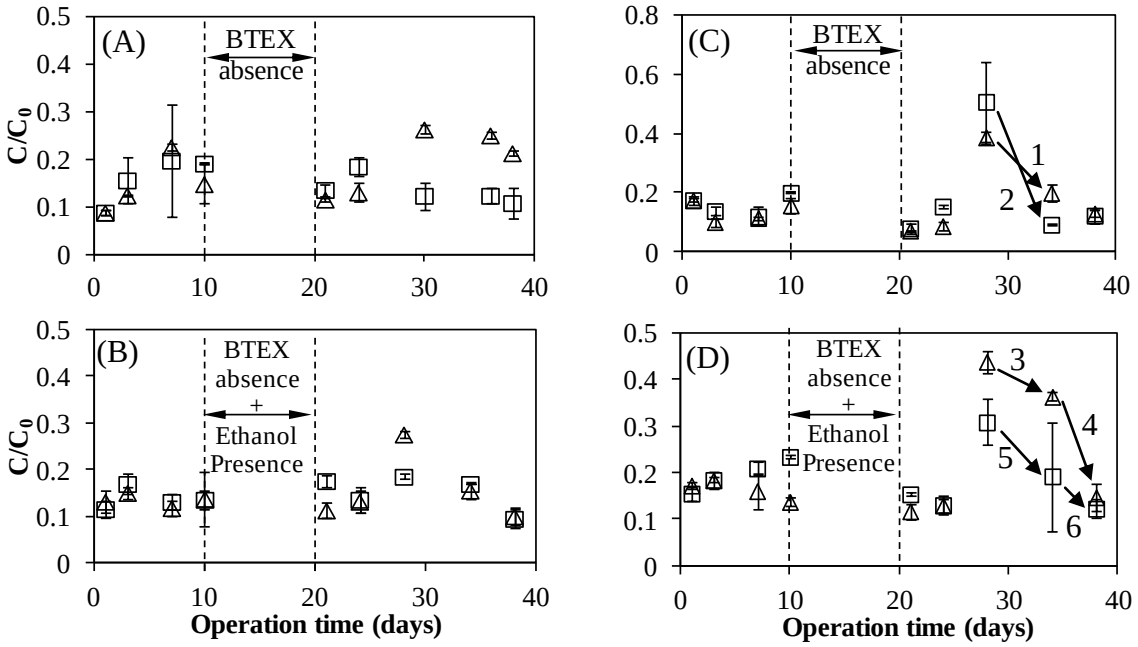


Figure 5.3. Removal performances of PRBBs inoculated with toluene degraders (A) TD-ER; (B) TD; (C) TD-ER-EtOH and (D) TD-EtOH. Triangle symbols represent benzene and square symbols represent toluene.

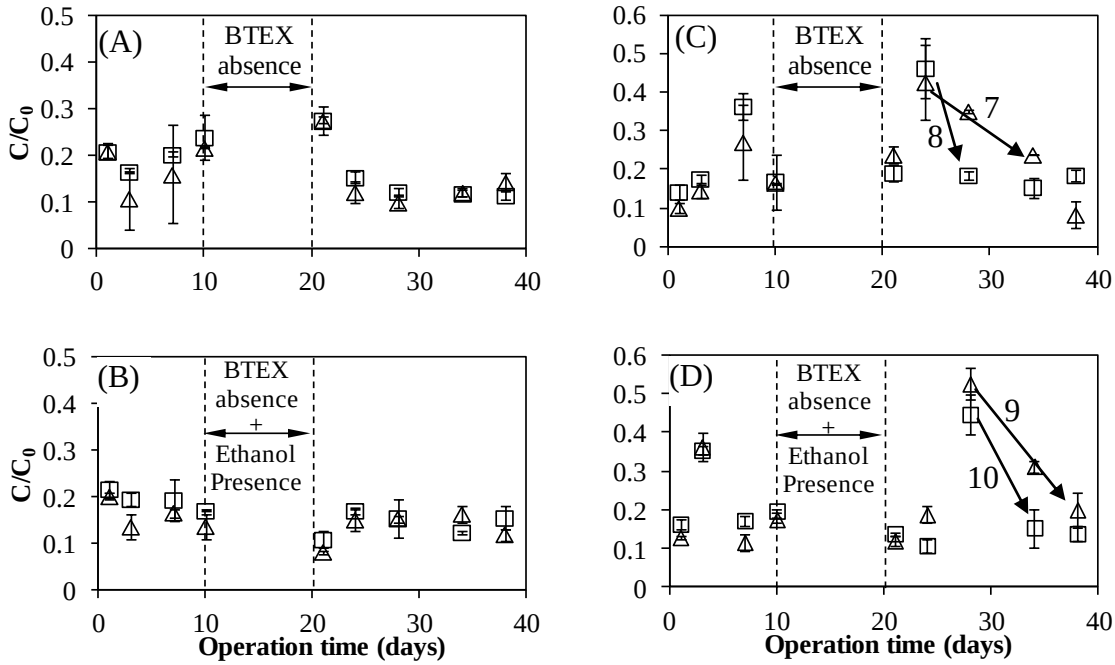


Figure 5.4. Removal performances of PRBBs inoculated with BTEX degraders (A) BTEXD-ER; (B) BTEXD; (C) BTEXD-ER-EtOH and (D) BTEXD-EtOH. Triangle symbols represent benzene and square symbols represent toluene.

experienced higher adsorption on to biological and/or non-biological adsorbents (Daifullah and Girgis, 2003; Costa et al., 2012). Higher values of octanol partition coefficient and molecular weight, and relatively very low solubility make them more favorable adsorbate than benzene and toluene.

Toluene was removed quicker than benzene in the activity tests by toluene degraders as well as BTEX degraders. However, the removal rates for benzene and toluene were almost identical in all the PRBBs inoculated with both toluene degraders and BTEX degraders before the absence period. Ethanol supplied during the BTEX absence period was completely removed by the PRBBs. Both toluene degraders and BTEX degraders were able to consume ethanol as an alternate carbon source.

5.3.2.1. Removal performances of PRBBs inoculated with toluene degraders

After the 10 day BTEX absence period, PRBBs initially inoculated with toluene degraders experienced varying levels of losses in removal performances for both benzene and toluene. Losses in removal performances were estimated from differences between average effluent concentrations before the absence period and the maximum effluent concentrations after the absence period. PRBBs that received augmentation from ER experienced relatively minimal loss of performance for benzene and toluene removal compared to the PRBBs without ER resupply. The orders of performance losses were TD-EtOH ≥ TD > TD-ER-EtOH > TD-ER for benzene removal and TD > TD-EtOH > TD-ER-EtOH ≥ TD-ER for toluene removal (Table 5.3). For TD-EtOH, the presence of ethanol during the absence period minimized the performance loss for toluene removal, while it caused more performance loss for benzene removal.

During the BTEX reappearance, all PRBBs recovered their removal performances, however at different recovery times and recovery rate. Recovery time was estimated as the number of days that a PRBB took to recover the performance equal to an average removal performance before the BTEX absence period. Recovery rates were calculated as the difference in effluent concentrations normalized by the higher concentration, divided by the duration of time associated with that concentration difference, and multiplied by 100% (Percent recovery per day). The PRBBs with ER supply (TD-ER and TD-ER-EtOH) recovered their removal performances for both benzene and toluene sooner than the PRBBs without ER supply (TD and TD-EtOH). TD-ER-EtOH had less recovery time for removal performance for benzene between the two PRBBs with ER supply (these PRBBs experienced slight losses in removal performances for toluene, hence the recovery was immediate and unremarkable), while TD had less recovery time for removal performance for both contaminants between the PRBBs without ER supply.

Table 5.3. Performance losses and recovery rates of PRBBs. Numbers in the parenthesis correspond to the numbers on arrows in Figures 5.3 and 5.4.

PRBB condition	**Performance loss (%)**		**Recovery rate(% per day)**	
	Benzene	**Toluene**	**Benzene**	**Toluene**
TD-ER	9.7	3.6		
TD	25.0	34.9	3.2 (1)	6.9 (2)
TD-ER-EtOH	14.1	4.7		
TD-EtOH	27.4	11.2	1.3 (3) 3.1 (4)	1.9 (5) 1.9 (6)
BTEXD-ER	6.1	5.9		
BTEXD	32.9	32.5	1.9 (7)	6.9 (8)
BTEXD-ER-EtOH	3.4	0.0		
BTEXD-EtOH	26.0	24.9	3.5 (9)	4.9 (10)

The recovery rates varied not only among PRBBs but also between the contaminants for each PRBB (Table 5.3). Between the two PRBBs without ER supply, TD had the fastest recovery rate for both benzene and toluene removal, which were 3.2% per day and 6.9% per day respectively (arrows '1' and '2' in Figure 5.3(C). However, it should be noted that TD had experienced the most performance losses as well. The recovery rates were influenced by the contaminant interactions, which were more evident in case of PRBB that received ethanol during the BTEX absence period but did not receive augmentation from ER (TD-EtOH). TD-EtOH recovered its benzene removal performance at a slower rate until toluene concentration in the effluent was substantially reduced. Benzene removal performance of TD-EtOH was recovered at 1.3% per day (arrow '3' in Figure 5.3(D)) until its toluene concentration in the effluent was reduced to the level same as before the BTEX absence period, then recovered at 3.1% per day (arrow '4' in Figure 5.3(D)). On the other hand, recovery rate for TD-EtOH did not change with benzene removal, which was 1.9% per day (arrows '5' and '6' in Figure 5.3(D)).

5.3.2.2. Removal performances of PRBBs inoculated with BTEX degraders

Among the four PRBBs inoculated with BTEX degraders (BTEXD-ER, BTEXD, BTEXD-ER-EtOH and BTEXD-EtOH), those that did not receive ER supply experienced major performance losses for both benzene and toluene removal due to the 10 day BTEX absence period. BTEXD-ER showed a small performance loss (approximately 6.0%) for both benzene and toluene removal in the first four days after BTEX reappearance; however, its performance quickly recovered (Figure 5.4(A)). BTEXD-ER-EtOH did not experience any performance loss for benzene and toluene removal. These two PRBBs

(BTEXD-ER and BTEXD-ER-EtOH) showed similar removal performances for benzene and toluene after the BTEX absence period.

The performance losses for BTEXD and BTEXD-EtOH were 32.9% and 25.5% for benzene removal and 22.5% and 24.9% for toluene removal when BTEX reappeared after the 10 day absence period. The maximum performance losses for benzene and toluene removal were close for BTEXD and BTEXD-EtOH. However, the removal performance recovery rates for these contaminants varied (Table 5.3 and Figure 5.4). The recovery rates for benzene removal were 1.9% per day (arrow '7' in Figure 5.4(C)) and 3.5% per day (arrow '8' in Figure 5.4(C)), while the recovery rates for toluene removal were 6.9% per day (arrow '9' in Figure 5.4(D)) and 4.9% per day (arrow '10' in Figure 5.4(D)) for BTEXD and BTEXD-EtOH, respectively. Contrary to the PRBBs inoculated with toluene degraders, minimal effects of toluene concentrations on the recovery rates for benzene removal were observed for PRBBs inoculated with BTEX degraders.

The removal performance recovery times differed between the PRBBs as well as the contaminants. As shown earlier, in addition to having a faster recovery rate for toluene removal, BTEXD also had a quicker recovery time for toluene removal. BTEXD recovered the toluene removal performance in 6 days, while it took 10 days for BTEXD-EtOH to recover its toluene removal performance. Although BTEXD-EtOH had a faster recovery rate for benzene removal, the recovery time was longer than BTEXD because the performance loss was higher for BTEXD-EtOH.

5.3.2.3. Toluene versus BTEX as inducer in the ER

Relative effluent concentrations from PRBBs inoculated with toluene degraders versus BTEX degraders were estimated and are presented in Figure 5.5. B_{TD}/B_{BTEXD} is the

ratio of effluent benzene concentrations from PRBBs inoculated by toluene degraders to those from PRBBs inoculated by BTEX degraders (Figure 5.5(A)). T_{TD}/T_{BTEXD} is the ratio of effluent toluene concentrations from PRBBs inoculated by toluene degraders to those from PRBBs inoculated by BTEX degraders (Figure 5.5(B)). Ratios less than 1 indicate that the PRBBs inoculated with toluene degraders have better removal performance than those inoculated with BTEX degraders and vice versa.

In general, PRBBs inoculated with toluene degraders showed equal or better performance in the removal of benzene and toluene before the BTEX absence period. TD-ER showed better benzene removal performance on day 21 (after the absence period) than BTEXD-ER, while BTEXD-ER showed better benzene removal performance than TD-ER after day 24 (dotted line with square symbols on days 21 and 24 in Figure 5.5(A)). After day 24, BTEXD-ER showed higher benzene removal performance than before the absence period, while TD-ER had experienced a small loss in benzene removal performance. On the contrary, TD-ER-EtOH showed better benzene removal performance than BTEXD-ER-EtOH immediately after the absence period and equal benzene removal performance after day 24 (solid line with triangle symbols on days 21 and 24 in Figure 5.5(A)). Among the PRBBs that did not receive ER augmentation, TD-EtOH experienced a slightly higher performance loss than BTEXD-EtOH for benzene removal on day 28, which resulted in a slightly higher B_{TD}/B_{BTEXD} value of 1.8 (thick dotted line with cross symbols in Figure 5.5(A)).

For toluene removal after the absence period, PRBBs initially inoculated with toluene degraders showed equal or better removal performances than those initially

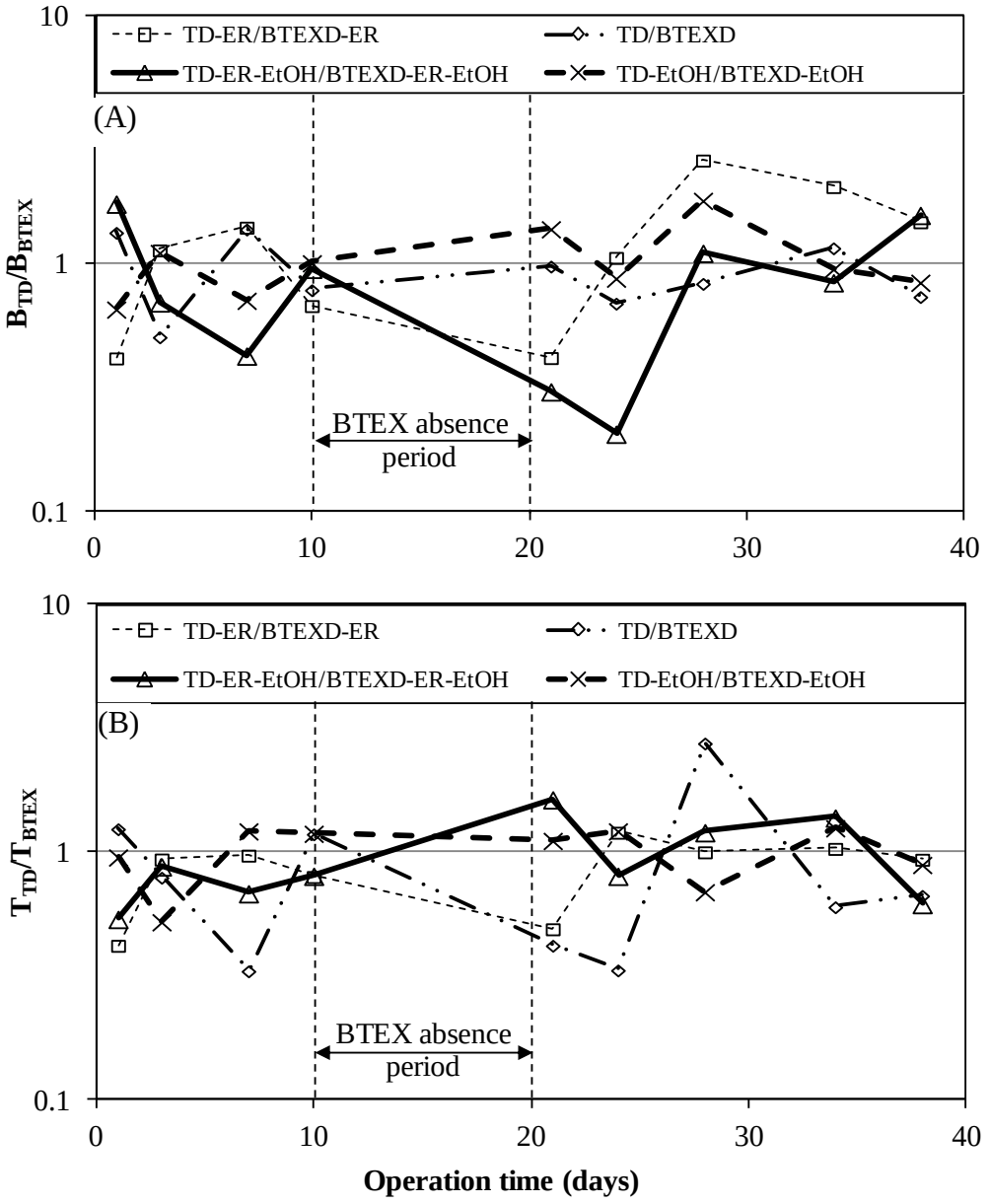


Figure 5.5. Relative removal of benzene (A) and toluene (B) by the toluene degraders and BTEX degraders.

inoculated with BTEX degraders, except for TD and BTEXD (double dotted dash line with diamond symbols in Figure 5.5(B). TD showed better toluene removal performance on days 21 and 24 ($T_{TD}/T_{BTEXD} < 0.4$), however, BTEXD showed a better toluene removal performance than TD on day 28 ($T_{TD}/T_{BTEXD} = 2.75$). The reason for this was that BTEXD

experienced the maximum performance loss for toluene removal on day 24 (normalized effluent toluene concentration = 0.46) and recovered its removal performance by day 28 (normalized effluent toluene concentration = 0.2). On the other hand, TD experienced its maximum performance loss for toluene removal on day 28 (normalized effluent toluene concentration = 0.51), which resulted in the T_{TD}/T_{BTEXD} being less than 1 before day 24 and greater than 1 after day 28.

5.3.2.4. Bacterial community examination

The SSCP profiles and the results of the cluster analysis of bacterial samples from the PRBBs during the experiments with 10-day BTEX absence period are shown in Figure 5.6. The notations "B" and "A" refer to samples collected just before the BTEX absence period and just after the reappearance of BTEX and augmentation, respectively, while "ER*" indicates sample collected from ER during the normal operation. Since the conditions for all PRBBs were the same except the inoculation source before the absence period, only one sample from each type of PRBB (inoculated with toluene degraders or BTEX degraders) was collected. The scale at the top of the figure indicates the percent differences among the SSCP profiles. A detailed description on cluster analysis is provided in Chapter 2, Section 2.7.1.

Just before the absence period, bacterial community in the PRBBs inoculated with toluene degraders were 29.4% different from (70.6% similar to) their source (ER) (TD-ER versus TD-B in Figure 5.6(A)). The PRBBs received BTEX contaminated synthetic groundwater until stable removal performances were observed. During this period, the bacterial community in the PRBBs could have acclimated to the BTEX compounds and the communities that could grow on all four BTEX compounds would have outcompeted the

communities that could grow on toluene alone, which resulted in the differences in community structures.

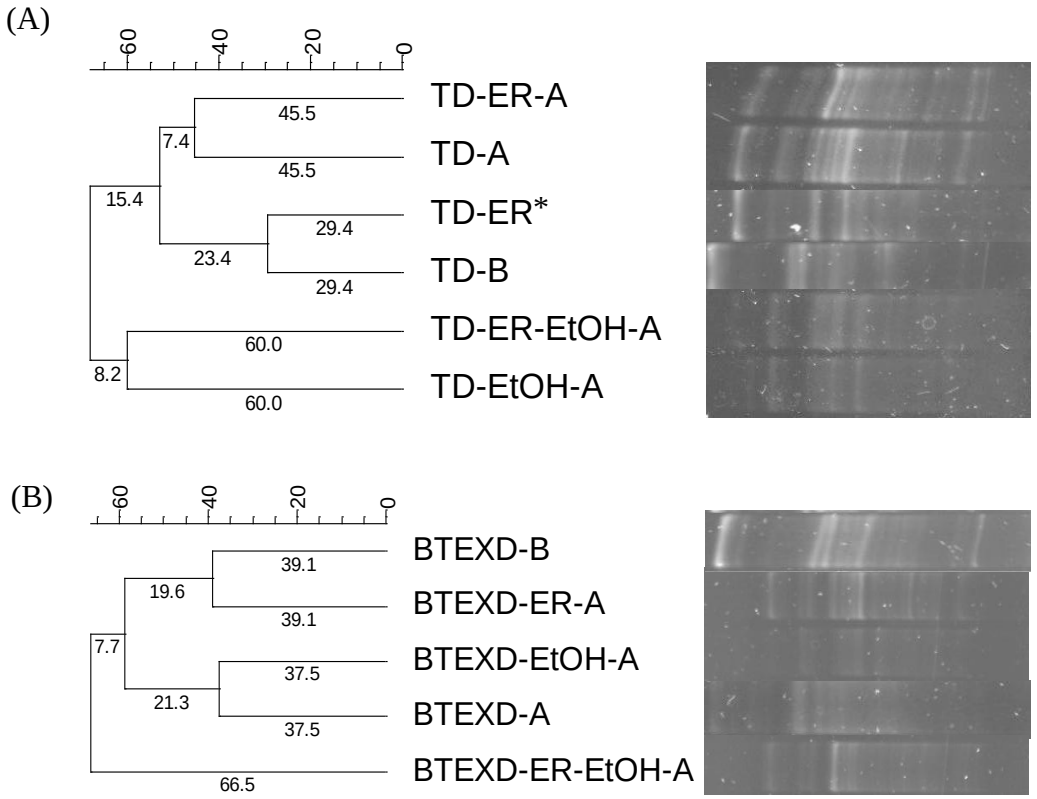


Figure 5.6. SSCP profiles and cluster analysis results of 16S rDNA samples from the PRBBs inoculated with toluene degraders (A) and BTEX degraders (B) before and after a 10-day BTEX absence period.

The communities in all PRBBs inoculated with toluene degraders differed greatly after the BTEX absence period from those before the absence period (TD-B versus TD-A, TD-ER-A, TD-EtOH-A, and TD-ER-EtOH-A in Figure 5.6(A)). The differences were in the order of TD-ER-EtOH > TD-EtOH > TD-ER > TD. TD-ER-EtOH had experienced the most community change because of three possible reasons: BTEX absence, ethanol presence, and augmentation of enriched degraders from toluene degraders. The community change due to augmentation was because the culture maintained in ER was 29.5% different from the culture adapted to BTEX compounds in PRBB.

PRBBs inoculated with BTEX degraders also experienced significant community changes due to the 10-day BTEX absence period (Figure 5.6(B)). The changes were in the order of BTEXD-EtOH > BTEXD > BTEXD-ER-EtOH > BTEXD-ER. In contrary to PRBB inoculated with toluene degraders, BTEXD had experienced greater changes in bacterial communities than the PRBBs that were augmented from ER. Also, the PRBBs that received ethanol during the BTEX absence period experienced greater changes than those did not receive ethanol. However, the presence of ethanol during the absence period did not help in recovering the toluene removal performance as it did for the PRBBs inoculated with toluene degraders, but it caused a further loss of benzene removal performance. BTEXD-EtOH experienced a 10% more loss in benzene removal performance than BTEXD after the BTEX absence period, while the toluene removal performances for both the PRBBs were almost equal. As compared to the TD-EtOH, the BTEXD-EtOH experienced a 15% more loss in toluene removal performance after the BTEX absence period.

Although TD experienced the least community change, it experienced the most performance loss, especially for toluene removal. Compared to TD, TD-EtOH experienced 20% less performance loss for toluene removal and 6% more performance loss for benzene removal after the BTEX absence period. Hence, the presence of ethanol during the BTEX absence period had a positive impact on toluene removal and an adverse effect on benzene removal.

Ethanol presence could have either maintained or increased the biomass in the PRBBs (Lovanh et al., 2002). However, the retained bacterial culture preferred toluene over benzene. The preference could have been due to either suppression of enzymatic

activity or the loss of necessary bacterial cultures due to the presence of ethanol during the BTEX absence period (Chakraborty and Coates, 2004). This indicates a possible diauxie effect by toluene utilizing the enzymes necessary for benzene degradation, since toluene is more easily degradable than benzene. If the number of degraders was reduced due to starvation during the absence period, there is a possibility for reduction in the concentration of enzymatic release. This reduced the availability of these enzymes for benzene degradation until the bacterial number returned to a critical concentration necessary to produce enough enzymes for the degradation of both benzene and toluene or until toluene was mostly degraded.

5.3.2.5. Toluene as inducer in the ER

PRBBs initially inoculated with toluene degraders showed better recovery (recovery time as well as recovery rate) of removal performance for toluene when ethanol was present during the BTEX absence period. Although the communities in PRBBs differed significantly between the ER and PRBB for the toluene degraders, the PRBBs did not require any acclimation period or lag time in the performance recovery for the removal of benzene and toluene. Use of toluene as inducer in the ER was successfully tested indicating that degraders grown on toluene alone can recover the removal performances after BTEX absence period equally or better than degraders grown on BTEX mixture. This demonstrates that the use of a single compound to maintain the target contaminant mixture degrading bacteria is possible.

The ability to use toluene as a sole inducer reduces the need for using multiple toxic chemicals. It should be noted that toluene had less toxicity than benzene. It may be possible to use a non-toxic compound such as benzoate as an inducer in the ER to maintain

the target degrading bacterial culture for augmentation in PRBBs. In a previous study described in Chapter 4 on the effects of carbon sources during the enrichment on the BTEX degradation ability of the enriched degraders, benzoate as an inducer was able to maintain a culture that could degrade BTEX (Kasi et al., 2012). The study also showed that benzoate plus BTEX mixture as the inducer maintained degraders with superior BTEX removal performances than the degraders enriched on benzoate alone. A future study is recommended to identify appropriate inducer conditions in an ER to augment the PRBBs when BTEX reappear after an absence period.

5.4. Summary

Performance loss of a PRBB due to inhibition interaction among BTEX compounds when the BTEX mixture reappeared after an absence period was addressed using a novel groundwater remediation technique, ER-PRBB. Two different inducer conditions in the ER were evaluated: toluene alone and BTEX mixture. PRBBs augmented with actively enriched bacteria from an ER experienced minimal or no performance loss when BTEX reappeared, regardless of the type of initial inoculation (toluene enriched or BTEX enriched bacteria); while PRBBs without ER augmentation experienced greater performance losses. The presence of ethanol as a carbon source during the BTEX absence period improved the removal performance of ER-PRBBs for both benzene and toluene; however, for PRBBs without ER, it improved the treatment performance for toluene but reduced benzene removal. Either loss of benzene degrading community or suppression of benzene degradation activity could have caused preferential degradation (one type of inhibition) of toluene over benzene. Additionally, ER-PRBBs with toluene enriched degraders showed equal or better removal performances for benzene and toluene than ER-

PRBBs with BTEX enriched degraders after the absence period, especially when ethanol was present during the absence period. ER augmentation was found to minimize the inhibition effects among BTEX compounds in a PRBB after an absence period and toluene as a single inducer in ER could be a better option than BTEX due to a possibility of minimizing the use of toxic chemicals as inducers in the ER. Future studies are recommended to investigate the use of a non-toxic compound as inducer and identify its relationship to the types of enzymes triggered in the PRBB when the enriched bacterial culture is exposed to target contaminant mixture, which will greatly help in optimizing the inducer conditions in the ER and minimizing the substrate interactions in the PRBB.

CHAPTER 6. CONCLUSIONS AND FUTURE WORK RECOMMENDATIONS

6.1. Conclusions

PRBB is considered as an environmentally friendly technique for treating contaminated groundwater. This technique highly depends on proper environmental conditions and a continuous supply of materials (including target contaminant) necessary to maintain the degradation activity of microorganisms. Discontinuous presence of target contaminant can disrupt the activity of microorganisms, which can lead to the failure of a PRBB. A novel bioaugmentation technique, ER-PRBB system, was developed to treat groundwater with contaminants that appear in batches. ER is an offline reactor used to enrich contaminant degraders by supplying necessary growth materials and the enriched degraders are used to augment PRBB to increase its performance after a period of contaminant absence. The technique was originally developed to augment biological treatment systems in wastewater treatment to treat hazardous contaminants that appear intermittently. However, the application of ER for groundwater treatment has never been tested. Moreover, the applicability of ER for mixture of contaminants that exhibit antagonistic substrate interactions has never been investigated.

Bench scale experiments on PRBBs with and without bacterial supply from the ER were conducted to evaluate PRBB removal performances for benzene, which was used as a model contaminant. Benzene absence periods of 10 and 25 days were tested in the presence and absence of ethanol. PRBBs without the bioaugmentation from the ER experienced greater performance losses when benzene reappeared, which were greater for longer absence period. However, the presence of ethanol accelerated the benzene removal

performance recovery of PRBBs. ER augmentation greatly reduced the performance loss and helped in timely recovery of the PRBBs.

Carbon source plays an important role during the enrichment of target contaminant degraders in an ER to maintain their degradation activity. Target contaminant(s) itself is often used as carbon source during the enrichment. Offline enrichment can become an unattractive technique for bioaugmentation, if accidental spills occur during the enrichment process. The use of less toxic and/or non-hazardous compounds as inducers was investigated. The effect of type of inducer compound during the enrichment of degraders on their removal performances of BTEX compounds when present as a mixture was studied. Batch BTEX removal kinetic experiments were performed using cultures enriched with individual BTEX compounds or BTEX as a mixture or benzoate alone or benzoate-BTEX mixture. An integrated Monod-type non-linear model was developed and a ratio between maximum growth rate (μ_{max}) and half saturation constant (K_s) was used to fit the non-linear model. A higher μ_{max}/K_s indicates a higher affinity to degrade BTEX compounds.

Complete removal of BTEX mixture was observed by all the enriched cultures; however, the removal rates for individual compounds varied. Degradation rate and the type of removal kinetics were found to be dependent on the type of carbon source during the enrichment. Cultures enriched on toluene and those enriched on BTEX mixture were found to have the greatest μ_{max}/K_s and cultures enriched on benzoate had the least μ_{max}/K_s. Removal performances of the cultures enriched on all different carbon sources, including the ones enriched on benzoate or benzoate-BTEX mixture were also improved during a second exposure to BTEX. A molecular analysis showed that after each exposure to the

BTEX mixture, the cultures enriched on benzoate and those enriched on benzoate-BTEX mixture had increased similarities to the culture enriched on BTEX mixture, which indicated that the use of benzoate as an inducer compound maintained the communities necessary to degrade BTEX mixture.

Finally, ER-PRBB was tested to address the performance loss of a PRBB due to substrate interactions among a BTEX mixture in groundwater, when the mixture reappeared after 10 days of absence. Effect of ethanol, a common additive to gasoline, during the BTEX absence period on the substrate interactions among BTEX was investigated. Based on the investigation on effects of carbon source during enrichment, toluene and BTEX were selected as inducers in ER, which had the greatest BTEX degradation rates. Also, these inducers were compared to investigate toluene as a potential single inducer in ER.

Augmentation of degraders from ERs greatly reduced the influence of substrate interactions and recovered the removal performances of PRBBs in relatively short time periods. PRBBs that did not receive ER supply experienced the most performance losses. The presence of ethanol as compared to no carbon source during the BTEX absence period improved the removal performance of ER-PRBBs for both benzene and toluene; however, for PRBBs without ER, it improved the removal performance for toluene but reduced for benzene. ER-PRBBs inoculated with toluene enriched degraders showed equal or better removal performances for benzene and toluene than ER-PRBBs with BTEX enriched degraders after the absence period, especially when ethanol was present during the absence period. Moreover, it is easier to handle one hazardous compound than multiple compounds during enrichment. Hence, toluene as a single inducer in an ER was found to be a better

option than BTEX mixture due to a possibility of minimizing the use of toxic chemicals as inducers in the ER.

6.2. Future Work Recommendations

The research described in this dissertation was an initial step in developing and implementing the ER-PRBB concept for treating contaminated groundwater. The results demonstrated a successful application of this concept for BTEX removal. However, several areas of this research require further investigation to optimize the operational conditions, expand its applicability in groundwater remediation, and develop strategies for field applications. Some of the areas are summarized below.

- Molecular studies to identify the types and amounts of different microbial species present in the ER and PRBBs and the effects of absence periods on these microbial species are needed. Furthermore, DNA sequencing will help in identifying key players among the enriched degraders involved in BTEX removal. Understanding the changes at a microbial species level could provide better control on the ER operation and, in turn, better performance of the PRBBs.
- Toluene as an inducer for degrader enrichment showed promising results in maintaining the performance of the PRBBs when BTEX reappeared. However, benzoate is less toxic than toluene and should be evaluated as a potential inducer. Additionally, proteomic and transcriptomic studies should be performed on the enriched degraders. This will help in identifying the role of inducer in triggering different protein and/or gene expressions responsible for degradation of a contaminant, which would be greatly beneficial in case of a mixture of contaminants.

- The ER-PRBB application should also be evaluated for structurally dissimilar organic compounds. Methyl *tert*-butyl ether (MTBE) is also a common additive to gasoline which serves as an oxygenate to increase the octane rating and improve combustion efficiency. MTBE is commonly found in groundwater along with BTEX. As BTEX compounds are structurally different from MTBE, evaluation of types of inducers and enrichment conditions different from those used in Chapters 4 and 5 could be necessary. Additionally, substrate interactions among MTBE and BTEX (Deeb et al., 2001) should also be considered during the investigation of ER conditions.
- Some inorganic and/or organic contaminants can be toxic to target contaminant degraders in the PRBBs. Arsenic, cadmium, uranium and poly aromatic hydrocarbons are some of the examples. The random appearance of these compounds should be evaluated for the performance loss in the PRBBs.
- Cell entrapment is a method of fixating cells in a matrix for many different biological applications, such as wastewater treatment and bioremediation. One of the draw backs for PRBB which was not evaluated here is the possible loss of number of degraders with the flow of groundwater. Using cell entrapment in combination with ER-PRBB will have a three-fold advantage: minimization of loss in the number of degraders, protection of degraders from environmental stresses (such as pH and temperature), and potential for reactivation of the degraders by bringing the degraders from PRBB to ER. Shock loading of contaminants, during which the concentration of contaminants can vary between extreme values can also be considered as one of the environmental stresses. Shock loading has been reported to upset biological treatment systems due to

the toxic effects of contaminants at high concentrations. Combination of ER and cell entrapment can be a good solution to address shock loading issues.

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