THE KINETICS, BIOCHEMICAL PATTERNS, AND MICROBIAL ECOLOGY IN MULTIREDOX (ANOXIC, MICROAEROBIC, AEROBIC) ACTIVATED SLUDGE SYSTEMS TREATING BTX CONTAINING WASTEWATER

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(ABSTRACT)

BTX biodegradation rates, biochemical expression patterns and microbial ecology were studied under anoxic (denitrifying), anoxic/microaerobic/aerobic, and anoxic/microaerobic conditions in activated sludge sequencing batch reactors. The studies showed that toluene and *m*-xylene were denitrified via benzoyl-CoA reductase. Although benzene, *o*-, and *p*-xylene were recalcitrant under denitrifying conditions, they were biodegraded under microaerobic (< 0.2 mg/L dissolved oxygen) and nitrate or nitrite (NO_x)-supplemented microaerobic conditions. The patterns of the specific enzymes associated with BTX biodegradation under microaerobic conditions indicated that the three compounds were metabolized by oxygen-dependent pathways. The expression levels of catechol 1, 2-dioxygenase and catechol 2, 3-dioxygenase under microaerobic conditions were induced to levels as high as under aerobic conditions (> 4 mg/L dissolved oxygen). Benzene, *o*-, and *p*-xylene biodegradation rates were twice as fast under NO_x-supplemented compared to NO_x-free microaerobic conditions, and the specific biodegradation rates under aerobic and NO_x-supplemented microaerobic conditions were comparable.

16S rRNA probes targeting representative toluene-degraders were used to investigate the microbial communities in the three sequencing batch reactors by using a dot blot hybridization technique. The hybridization results suggest that multiple redox environments fostered a more diverse microbial community and the activities of the target organisms in the reactors with multiple redox environments were higher than in the single redox reactor. Additionally, facultative toluene-degraders appeared to play a less significant role than the strict anoxic and aerobic toluene-degraders in all three SBRs.

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TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 1. LITERATURE REVIEW

CHAPTER 2. KINETIC, BIOCHEMICAL AND REDOX RESPONSES ASSOCIATED WITH ANOXIC, MICROAEROBIC AND AEROBIC METABOLISM

CHAPTER 4. THE DISTRIBUTION OF BTX DEGRADERS IN BTX-FED BIOLOGICAL REACTORS UNDER DIFFERENT REDOX CONDITIONS

LIST OF FIGURES

TABLE OF ABBREVIATION

AER: aerobic

ANX: anoxic

BTX: benzene, toluene, *o*-xylene, *m*-xylene, *p*-xylene

BOD: biological oxygen demand

COD: chemical oxygen demand

CSPD: Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.13,7]decan}-4-yl) phenyl phosphate

C12O: catechol 1, 2-dioxygenase

C23O: catechol 2, 3-dioxygenase

DEPC: diethyl pyrocarbonate

DNA: deoxyribonucleic acid

MA: microaerobic

MLSS: mixed liquor suspended solids

MLVSS: mixed liquor volatile suspended solids

NESHAP: National Emission Standards for Hazardous Air Pollution

ORP: oxidation-reduction potential

PCR: polymerase chain reaction

RNA: ribonucleic acid

SBR: sequencing batch reactor

SOUR: specific oxygen uptake rate

VOC: volatile organic compounds

INTRODUCTION

Currently, conventional aerobic biological wastewater treatment processes are commonly used to treat wastewater streams containing benzene, toluene, *o*-, *m*-, and *p*-xylene (BTX) ; however, these processes are often out of compliance with the current National Emissions Standards for Hazardous Air Pollutants (NESHAP) regulations due to stripping of the volatile compounds from the aqueous phase into the atmosphere. As a consequence, cost-effective and environmentally friendly approaches for removing BTX contaminants from wastewaters are desired. Additionally, the ultimate goal of an engineered system is to retain a robust microbial community with a high level of biodegradation activities. Unfortunately, knowledge on the effect of reactor configurations and environmental conditions, including redox states, on the structure and function of microbial communities in engineered systems is very limited.

Previous studies suggested that toluene (2, 3) and possibly benzene (1) biodegradation rates were enhanced under nitrate-supplemented microaerobic conditions. Additionally, in a study (4) on aerobic BTX biodegradation using aquifer microcosms, it was shown that cycling redox conditions between aerobic and anoxic conditions fostered favorable changes in the composition of the microbial consortium. As a result, aerobic biodegradation rates of benzene, toluene, and *o*-xylene were increased after the aquifer consortium was exposed to the alternating redox environments.

It was hypothesized in this research that multiredox conditions will enable enhanced biodegradation of BTX compounds due to (a) the retention of a more diverse BTX-degrading microbial consortium, (b) the induction of different specific enzymes associated with BTX biodegradation by alternating multiredox environments, and (c) an enhanced biodegradation rate with the presence of microaerobic conditions. To test this hypothesis, the objectives of this research were to: characterize BTX biodegradation patterns and the specific enzyme activity levels associated with BTX biodegradation under different redox conditions, and to investigate the distribution patterns of representative toluene-degraders under different redox conditions. Specific tasks were addressed and included:

1. To seek a reliable tool to monitor microaerobic conditions since the dissolved oxygen (DO) level may not be high enough to be detected by a DO probe.

2. To investigate whether BTX compounds are amenable to biodegradation under microaerobic conditions. It may be possible to modify wastewater treatment strategies by alternating anoxic and microaerobic conditions to treat BTX or other VOC containing wastewaters.

3. To study the kinetics of BTX biodegradation under microaerobic conditions and to determine the effect of nitrate or nitrite on BTX biodegradation rates.

4. To study the expression of specific enzymes associated with BTX biodegradation under anoxic, microaerobic, and aerobic conditions.

5. To study the microbial activities of the representative toluene-degraders under different reactor configurations by using dot blot hybridization techniques.

6. To investigate whether an alternating multiredox biological reactor encourages a more diverse microbial community.

Chapter 2 addresses items 1 through 4, Chapter 3 addresses item 2, and Chapter 4 addresses items 5 and 6. Chapter 5 addresses the engineering significance of this work.

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CHAPTER 1. LITERATURE REVIEW

Introduction

Benzene, toluene, *o*-, *m*-, and *p*-xylene (BTX) are volatile xenobiotic compounds that are prevalent in industrial wastewaters (21, 24, 48) and gasoline or petroleum-contaminated groundwaters (50). Cost-effective and environmentally friendly approaches for removing BTX contaminants from wastewater and groundwater streams are desired due to the hazards associated with BTX compounds (12, 40, 43, 49) and current National Emissions Standards for Hazardous Air Pollutants (NESHAP). More recently, studies suggested that microaerobic conditions (low oxygen levels) enhanced the biodegradation abilities of toluene (26, 28), and possibly benzene (2). Although these studies were conducted either with pure (26, 28) or subsurface (2) cultures, the concept of microaerobic metabolism is encouraging and may provide an alternative for enhancing the effectiveness of treating wastewaters containing BTX or other volatile organic compounds (VOC).

The microbial community in a biological wastewater treatment system largely defines the system's treatment efficiency; however, consideration of the effect of reactor configuration and environmental conditions, including redox conditions, on the microbial ecology in an engineered system is often overlooked by engineers. By using aquifer microcosms to study aerobic BTX biodegradation, a study suggested that cycling electron acceptor conditions between aerobic and anoxic (denitrifying) conditions fostered favorable changes in the composition of the microbial consortium and showed improved aerobic benzene, toluene, *o*-xylene biodegradation abilities (51). The total heterotrophic plate counting method was used to investigate the microbial composition of this consortium, and the results led to speculation that a more diverse microbial ecosystem may develop when the microbial community is exposed to different redox conditions. However, knowledge about the microbial ecology in activated sludge systems with multiple redox conditions is limited.

Current processes in treating BTX containing wastewater

Conventional aeration processes are commonly used in treating BTX or other VOC containing industrial wastewater (35); however, the volatility of these compounds often results in a significant amount of removal by stripping from various processes in engineered wastewater treatment systems (15, 24, 35, 59). This approach to treating BTX-containing wastewater simply transfers the problem from one medium to another rather than converting the contaminants into innocuous products, as would be achieved with biodegradation. Additionally, aeration is among the most costly operational expense associated with wastewater treatment systems (5, 7). On the other hand, the high volatility of BTX compounds have enticed industries to employ physical processes such as gas stripping in which the volatile compounds are transferred from the wastewater to the atmosphere (9, 15, 59). Current National Emissions Standards for Hazardous Air Pollutants (NESHAP) regulate volatile organic compounds emanating from various sources; therefore, these processes alone are no longer sufficient to meet the stringent standards in many cases.

BTX biodegradation under aerobic conditions

All five BTX compounds have been shown to be biodegradable as a sole carbon and energy source under aerobic condition (1, 10, 13, 14, 22, 29, 31), but no single strain has demonstrated the ability to metabolize each of them as a sole carbon source (10, 13, 14, 29, 34, 42, 45, 55, 56, 58). Table 1 summarizes the substrate utilization abilities of different isolates and strains that aerobically degrade BTX compounds. Biodegradation of *o*-, *m*-, and *p*-xylene in conjunction with toluene was observed by Olsen et al. (34) with *Pseudomonas pickettii*. Similar phenomenon was also observed with *Pseudomonas* sp. strain JS150, although in this case, *m*- and *p*-xylene were biodegraded (22).

Figure 1 shows the five major aerobic biodegradation pathways for toluene that have been identified to date. The best characterized pathway is the TOL plasmid pathway of *Pseudomonas putida* PaW1. In the TOL plasmid pathway, toluene is converted to benzyl alcohol, benzaldehyde, benzoate, and finally to catechol. Catechol then undergoes *meta* cleavage (58). In the degradation of toluene by *Pseudomonas putida* F1, toluene is transformed into *cis*-toluene dihydrodiol, and then 3-methylcatechol, which undergoes

Table 1. Substrate utilization abilities of bacterial strains under aerobic conditions.

Strain	Growth response						Reference
	Benzene	Toluene	Ethylbenzene	*o*-Xylene	*m*-Xylene	*p*-Xylene	
Laboratory strain							
Pseudomonas pickettii PKO1	+	+	+	Induction with toluene	Induction with toluene	Induction with toluene	(34)
*P*seudomonas sp. Strain JS150	+	+	+		Induction with toluene	Induction with toluene	(22, 25)
Bioreactor isolate							
Pseudomonas fluorescens CA4	+		+				(10)
Pseudomonas cepacia G4	-	+	+	+	-	±	(13, 42)
Soil isolates							
Pseudomonas cepacia M1C4	+	+	+		-	-	(13)
Pseudomonas cepacia O2C1	-	+	-		+	+	(13)
Pseudomonas fluorescens R2AT2	+	+	+		-	-	(13)
Pseudomonas fluorescens A4C2	-	+	-		+	+	(13)
Pseudomonas fluorescens CFS215	+	+	+			+	(31, 34)
Pseudomonas mendocina KR1	-	+	+	-	-	-	(13, 55)
Pseudomonas putida F1	+	+	+	-	-	-	(13, 45)
Pseudomonas putida PaW1	-	+	-	-	+	+	(13, 56,
Pseudomonas putida A3AT1	+	+	+		-	-	(13)
Pseudomonas putida M1D2	-	+	-		+	+	(13)
Pseudomonas sp. W31		+					(26)
Rhodococcus sp. Sm-1	+	+	In the presence with propane	In the presence with propane	In the presence with propane	In the presence with propane	(29)
Rhodococcus sp. Wrink	+	+	In the presence with propane	In the presence with propane	In the presence with propane	In the presence with propane	(29)

+: growth as sole carbon source; -: no growth on carbon source; ±: weak growth as sole carbon source; blank: not tested.
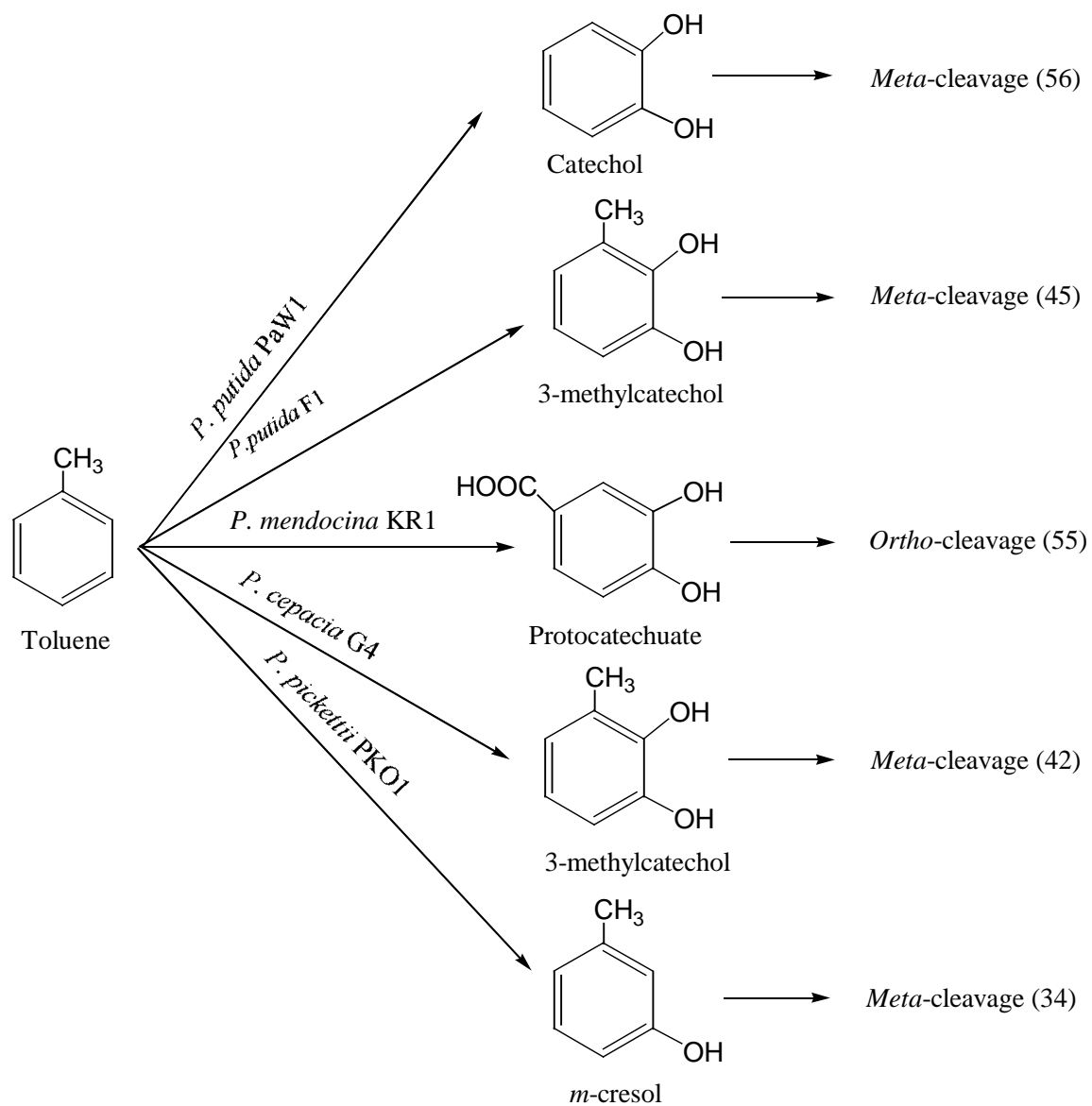
Figure 1. Aerobic biodegradation pathways for toluene (numbers in parentheses are relevant citations)

meta cleavage (45). A third pathway for toluene degradation is found in *Pseudomonas mendocina* KR1, in which toluene is oxidized into protocatechuate. Protocatechuate is further metabolized through *ortho* cleavage (55). *Pseudomonas cepacia* G4 converts toluene into *o*-cresol and then to 3-methylcatechol which undergoes *meta* cleavage (42). The fifth pathway for the degradation of toluene is carried out by *Pseudomonas pickettii* PKO1. Toluene is converted into *m*-cresol, which undergoes *meta* cleavage (34). The major differences between these pathways relate to the location where ring oxidation occurs relative to the methyl group, and the point of aromatic ring cleavage.

BTX biodegradation under anoxic conditions

Extensive research has been conducted on BTX biodegradation using electron acceptors other than oxygen due to the fact that it can be difficult and expensive to provide sufficient oxygen into contaminated subsurface environments. It has been repeatedly shown that biodegradation of toluene under denitrifying conditions is a common phenomenon among a broad range of bacteria (4, 17, 19, 36, 47). The xylene isomers have all been shown to undergo denitrification (2-4, 19, 36, 39, 47), although in some instances, *o*- and *m*-xylene were consumed at a slower rate than toluene (4). Additionally, transformation of *o*-xylene was observed to occur either in conjunction with toluene (17) or *m*- and *p*-xylene (47) biodegradation. The potential for benzene biodegradation under nitrate-reducing conditions was recently reported by Burland and Edwards (8), but most studies have shown that benzene is recalcitrant to biodegradation under denitrifying conditions (2, 3, 27).

Table 2 lists pure cultures that have the ability to biodegrade BTX under denitrifying conditions. *Thauera aromatica*, and sediment isolates EbN1, PbN1, ToN1, and mXyN1 are isolates which have been identified as strict anoxic BTX-degraders (4, 17, 36). Despite being isolated from denitrifying environments, some strains showed positive aerobic BTX biodegradation activities (19).

BTX biodegradation under microaerobic conditions

In recent years, interest has been directed toward studying BTX biodegradation under low dissolved oxygen, or microaerobic, conditions. Unfortunately, the methods

Table 2.   Substrate utilization abilities of bacterial isolates under denitrifying conditions.

Isolates	Growth response						reference
	Benzene	Toluene	Ethylbenzene	*o*-xylene	*m*-xylene	*p*-xylene	
Bioreactor isolate							
Thauera aromatica K172		+	-	+	+	+	(4)
Sediment isolate							
EB1	-	-	+	-	-	-	(3)
Thauera aromatica T1	-	+	-	In the presence of toluene	-	-	(17)
EbN1	-	+	+	-	-	-	(36, 37)
PbN1	-	-	+	-	-	-	
ToN1	-	+	-	-	-	-	
Azoarcus sp. mXyN1	-	+	-	-	+	-	
Sewage sludge isolate							
Pseudomonas maltophilia SU1	-	+***		In the presence of *m*-, *p*-xylene	+***	+***	(47)
Soil and aquifer isolate							
Azoarcus tolulyticus Tol-4	-	+ **	- **	-	-	-	(19)
Azoarcus tolulyticus Td-1	-	+ **	- **	-	-	-	
Azoarcus tolulyticus Td-2	-	+ *	-	-	-	-	
Azoarcus tolulyticus Td-3	- *	+ *	-	-	-	-	
Azoarcus tolulyticus Td-15	-	+ **	-**	-	+	-	
Azoarcus tolulyticus Td-17	- **	+ **	- **	-	-	-	
Azoarcus tolulyticus Td-19	-	+ *	-	-	-	-	
Azoarcus tolulyticus Td-21	- **	+ **	- **	-	-	-	

+: growth as sole carbon source; -: no growth on carbon source;***:enhanced activity in the presence of 2% oxygen;**: showed positive activity under aerobic conditions; *: activity often delayed under aerobic conditions; blank: not tested

used to control microaerobic conditions in laboratory experiments and to monitor low concentrations of residual dissolved oxygen are not standardized, and the extent of oxygen limitation varies considerably from study to study. For example, initial dissolved oxygen (DO) concentrations of 2 and a poised constant level of less than 1 mg/l were both considered to be microaerobic in separate studies (26, 28) while in another study, microaerobic conditions were created by adding sufficient levels of biodegradable material to an aerobic (DO = 8.9 mg/L) solution so that the oxygen demand exceeded the oxygen available and DO was depleted over time (2). In a fourth study, cultures were incubated in sealed serum bottles under 98% N_2:2% O_2 headspace (47). Despite these differences in oxygen levels, it has been shown that toluene biodegradation rates were enhanced in selected bacterial strains under microaerobic (< 1 mg/L DO) conditions when nitrate was present (28). Additionally, Kukor and Olsen (26) demonstrated the simultaneous utilization of nitrate and O_2 in pure cultures during toluene biodegradation under microaerobic (2 mg/L DO and less) conditions. Similarly, Alvarez and Vogel showed (2) enhanced benzene biodegradation in a mixed aquifer culture grown under batch conditions in the presence of aerobic, then microaerobic conditions with nitrate. However, they did not demonstrate whether benzene biodegradation could be initiated under microaerobic conditions. It has been suggested that a minimum dissolved oxygen threshold exists below which biodegradation of aromatic hydrocarbons is not supported (57); however, such a threshold has not been experimentally determined.

Enzymes associated with BTX biodegradation

One method to determine the biochemical pathway used for BTX biodegradation is to measure enzyme activities. The BTX biodegradation enzymes under aerobic conditions are well characterized. A crucial step in BTX biodegradation under aerobic conditions is aromatic ring cleavage. Catechol 2, 3-dioxygenase (C23O), an enzyme encoded for on the TOL plasmid, is the enzyme responsible for *meta* cleavage, while the chromosomally associated enzyme, catechol 1, 2-dioxygenase (C12O), cleaves the aromatic ring at the *ortho*-position (34).

Current information on oxygen-independent biodegradation of aromatic compounds shows that these compounds are metabolized via a common intermediate called benzoyl-CoA (23). In this case, the aromatic ring is reduced prior to ring cleavage rather than oxidized as in oxygen-dependent BTX biodegradation pathways. Benzoyl-CoA reductase is the inducible

enzyme responsible for this reduction step and was first detected in cell extracts of *Thauera aromatica* (4, 6).

Under nitrate or nitrite (NO_x) supplemented microaerobic conditions, it is equally important to monitor both specific aerobic and anoxic BTX biodegradation enzymes since both oxygen and NO_x are available as electron acceptors for BTX biodegradation. Kukor and Olsen (26) found measurable C23O activities in pure cultures grown under oxygen-limited conditions. On the other hand, the exact effect of oxygen on the strictly anaerobic enzyme activity under NO_x-supplemented microaerobic conditions is unknown.

Microbial ecology in activated sludge systems

The ultimate goal of studying microbial ecology in biological wastewater treatment systems is to understand how reactor configurations can influence the structure and function of bacteria groups, which ultimately influence the performance of treatment systems. Techniques available to study distributions of specific bacterial groups in mixed cultures fall into two categories: culture-dependent and culture-independent methods. It is well known that in oligotrophic engineered wastewater treatment systems, microbial communities are predominated by slow growing or dormant bacteria (52); therefore, conventional cultivation-dependent methods such as viable plate count or the most-probable-number techniques, when used in identifying microbial communities in those systems, gave highly biased and inaccurate results (52, 53). More recently, the employment of culture-independent molecular biology techniques targeting 16S rRNA is able to overcome this drawback and allow researchers to explore an oligotrophic microbial ecology in more detail. Labeled 16S rRNA probes have been used in studying diverse aspects of environmental interests including: the activities (20) or the distribution (41, 54) of ammonia-oxidizing bacteria in biofilms; the distribution of sulfate-reducing bacteria (SRB) in biofims (38) or in activated sludge (30); identification of a foaming filamentous bacterium in activated sludge (16); the population analysis of denitrifiers in biofilms (33); and the methanogens in anaerobic reactors (44). In these studies, hybridization is carried out either *in situ* or with bulk RNA extracts. It has been shown that *in situ* hybridization revealed the best cell number recovery (52) while dot blot hybridization was a valid tool when the metabolic activity of a certain microbial group is of special interests, as ribosomal contents in cells can be related to metabolic activities (46, 52). Organism activity and distribution can be

estimated from the fractional contribution of species-specific or genus-specific rRNA relative to domain-specific rRNA.

Recently, new methods such as denatured gradient gel electrophoresis (DGGE) or temporal temperature gel electrophoresis (TTGE) have been developed to study heterogeneous microbial communities from complex samples, including soil (18), biofilm (32), and activated sludge (11). These methods amplify 16S rRNA genes using universal probes, then separate the PCR products based on electrophoretic mobilities in polyacrylamide gels containing a linearly increasing gradient of denaturants or temperature. In studying biological treatment processes, these methods show promise for identifying shifts in microbial populations in response to environmental changes.

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CHAPTER 2. KINETIC, BIOCHEMICAL AND REDOX RESPONSES ASSOCIATED WITH ANOXIC, MICROAEROBIC AND AEROBIC BTX METABOLISM

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ABSTRACT: Activated sludge sequencing batch reactors were used to study BTX metabolism under anoxic (denitrifying), anoxic/microaerobic/aerobic, and anoxic/microaerobic conditions. Toluene and *m*-xylene were denitrified via benzoyl-CoA reductase. Although benzene, *p*- and *o*-xylene were recalcitrant under anoxic treatment, all three were biodegraded under microaerobic (< 0.2 mg/L dissolved oxygen) and nitrate or nitrite (NO_X)-supplemented microaerobic conditions. Benzoyl-CoA reductase was repressed under all microaerobic conditions while catechol 1,2-dioxygenase (C12O) and catechol 2,3-dioxygenase (C23O) were induced, indicating that the aromatic hydrocarbons were metabolized by aerobic pathways, even in the presence of NO_X and in the absence of measurable dissolved oxygen levels. The degree of C12O and C23O expression under microaerobic conditions was comparable to levels found under aerobic (dissolved oxygen > 4 mg/L) conditions. Benzene, *p*- and *o*-xylene biodegradation were twice as fast under NO_X-supplemented compared to NO_X-free microaerobic conditions, and specific biodegradation rates under aerobic and NO_X-supplemented microaerobic conditions were comparable. Oxidation reduction potential successfully differentiated between the various electron acceptor conditions and proved to be a sensitive indicator. An electron balance suggested that NO_X enhanced the rate of benzene, *p*- and *o*-xylene biodegradation under microaerobic conditions because endogenous respiration could be supported in part by denitrification, thus making oxygen more available for oxygenase enzymes in the aerobic BTX biodegradation pathways.

Keywords: Benzene, Toluene, Xylene, Enzymes, Energy Balance, Redox

INTRODUCTION

Benzene, toluene, *o*-, *m*-, and *p*-xylene (BTX) are volatile xenobiotic compounds that are prevalent in industrial wastewaters (22, 27, 50) and gasoline or petroleum-contaminated groundwaters (52). In addition to being common pollutants, there are health implications associated with human (14, 48) and ecological (47) exposure to these compounds, especially benzene (51). Current National Emissions Standards for Hazardous Air Pollutants (NESHAP) regulate volatile organic compounds emanating from various sources, including wastewater streams (29, 30). Consequently, cost-effective and environmentally friendly approaches for removing BTX contaminants from wastewaters and groundwaters are desirable.

Whereas a substantial amount of research has been directed toward elucidating biodegradation mechanisms of BTX compounds in subsurface environments (2, 6, 26, 28, 46), limited research has occurred in suspended culture biological wastewater treatment systems. Although all five BTX compounds have been shown to be biodegradable as a sole carbon and energy source under aerobic conditions (1, 13, 15, 16, 23, 35, 37), the volatility of these compounds often results in a significant amount of removal by stripping from various processes in engineered wastewater treatment systems (17, 27, 42, 54). To avoid uncontrolled removal and operator exposure, benzene is often stripped from wastewaters upstream of treatment systems using abiotic means (43). This approach to treating BTX-containing wastewater can be costly, and simply transfers the problem from one medium to another rather than converting the contaminants to innocuous products, as would be achieved with biodegradation.

Extensive research has been conducted on BTX biodegradation using electron acceptors other than oxygen due to the fact that it can be difficult and expensive to provide sufficient oxygen into contaminated subsurface environments. Similarly, aeration is among the most costly operational expenses associated with wastewater treatment systems (8, 10). As a result, denitrification may serve as a viable alternative or supplement to conventional activated sludge and biofilm treatment systems for biodegrading those volatile constituents which are amenable to denitrification. It has been repeatedly shown that denitrification of toluene is a common phenomenon among a broad range of bacteria (7, 18, 20, 45, 49). The xylene isomers have all been shown to undergo denitrification (2, 6, 7, 20, 45, 46, 49), although in some instances, *o*- and *m*-xylene were consumed at a slower rate than toluene (7). Additionally, transformation of *o*-

xylene was observed to occur either in conjunction with toluene (18) or *m-* and *p*-xylene (49) biodegradation. The potential for benzene biodegradation under nitrate-reducing conditions was recently reported by Burland and Edwards (11), but most studies have shown that benzene is recalcitrant to biodegradation under denitrifying conditions (2, 6, 33). Collectively, these results suggest that denitrification may be used in wastewater treatment systems to biodegrade some of the BTX compounds. Use of a denitrifying reactor system is beneficial because it involves less agitation and less opportunity for volatilization of the contaminants (41), as well as cost savings by eliminating aeration. However, it is unclear if all constituents will undergo timely biodegradation under these conditions, and a strategy is needed to encourage cost effective biodegradation of the remaining BTX constituents.

In recent years, interest has been directed toward studying BTX biodegradation under low dissolved oxygen, or microaerobic, conditions. Unfortunately, the methods used to control microaerobic conditions in laboratory experiments and to monitor low concentrations of residual dissolved oxygen are not standardized, and the extent of oxygen limitation varies considerably from study to study. For example, initial dissolved oxygen (DO) concentrations of 2 mg/l and a poised constant level of less than 1 mg/l were both considered to be microaerobic in separate studies (32, 34) while in another study, microaerobic conditions were created by adding sufficient levels of biodegradable material to an aerobic (DO = 8.9 mg/L) solution so that the oxygen demand exceeded the oxygen available and DO was depleted over time (2). In a fourth study, cultures were incubated in sealed serum bottles under 98% N_2:2% O_2 headspace (49). Despite these differences in oxygen levels, it has been shown that toluene biodegradation rates were enhanced in selected bacterial strains under microaerobic (< 1 mg/L DO) conditions when nitrate was present (34). Additionally, Kukor and Olsen (32) demonstrated the simultaneous utilization of nitrate and O_2 in pure cultures during toluene biodegradation under microaerobic (2 mg/L DO and less) conditions. Similarly, Alvarez and Vogel (2) showed enhanced benzene biodegradation in a mixed aquifer culture grown under batch conditions in the presence of aerobic, then microaerobic conditions with nitrate. However, they did not demonstrate whether benzene biodegradation could be initiated under microaerobic conditions. It has been suggested that a minimum dissolved oxygen threshold exists below which biodegradation of aromatic

hydrocarbons is not supported (53); however, such a threshold has not been experimentally determined.

One method for determining the biochemical pathway used for BTX biodegradation is to measure enzyme activities. A crucial step in BTX biodegradation under aerobic conditions is aromatic ring cleavage, using either a *meta* cleavage process via catechol 2,3-dioxygenase (C23O), or an *ortho* cleavage process via catechol 1,2-dioxygenase (C12O) (40). Current information on oxygen-independent biodegradation of aromatic compounds shows that these compounds are metabolized via a common intermediate called benzoyl-CoA (24). In this case, the aromatic ring is reduced rather than oxidized prior to ring cleavage. Benzoyl-CoA reductase is the inducible enzyme responsible for this reduction step and was first detected in cell extracts of *Thauera aromatica*, a strict anoxic toluene, *o-, m-,* and *p*-xylene degrader (7, 9).

Existing experimental evidence strongly suggests that combining denitrifying (anoxic) and microaerobic environments in engineered biological treatment systems will result in complete and cost effective biodegradation of BTX, and possibly other volatile compounds. However, carefully controlled experiments have not been conducted to study the metabolism of microorganisms grown under sequential treatment strategies that include microaerobic conditions. In addition, application of microaerobic and denitrifying treatment zones into biological treatment reactors will necessitate the use of monitoring technologies for process control purposes that function under these environments. We propose oxidation-reduction potential (ORP) as a valid indicator. Finally, kinetic values representing BTX biodegradation under microaerobic and aerobic treatment conditions are not available for application to engineered biological treatment systems. Therefore, the objective of this study was to address these needs using a laboratory activated sludge system. In order to conduct experiments under anoxic and microaerobic conditions that minimized BTX losses due to volatilization, a novel experimental reactor system was designed for use during this study and is described herein. The experimental setup allowed an electron balance to be calculated and the ultimate fate of the O_2 introduced into the system to be inferred.

MATERIALS AND METHODS

Reactor set up and operation.

Glass fermentation systems (LH Fermentation, Hayward, CA) were operated as activated sludge sequencing batch reactors (SBRs) for all bioreactor experiments. The bioreactors were sealed with a gasketed stainless steel lid and operated using a working liquid volume of 2 liters and a reactor headspace volume of 0.5 liter. Additional headspace volume was provided through use of a Tedlar bag, as described below. The cultures were maintained at 25°C with a temperature controller (Valley Instrument Company, Exton, PA) and were mixed continuously at 200 rpm during fill and reaction cycles. pH was maintained near neutral with a pH controller (Valley Instrument Company, Exton, PA) coupled with 1.0 N NaOH and 0.5 N phosphoric acid. The initial activated sludge inoculum came from a blend of industrial (chemical manufacturing industry) and domestic (Blacksburg, Virginia) mixed liquors. The SBRs were operated using a 24 hour cycle including fill (0.5 hour), react (21.5 hours), settle (1.5 hours) and draw (0.5 hour) phases. Phases were controlled electronically by programmable timers (ChronTrol Corp., San Diego, CA). Three reactor configurations were studied and included the following reaction times: 21.5 hours denitrifying anoxic (ANX), 9/2/10.5 hours ANX/microaerobic (MA)/aerobic (AER), and 9/12.5 hours ANX/MA. Each experimental condition was operated for at least 30 days before intensive sampling was initiated.

Solids retention time (SRT) and hydraulic retention time (HRT) were maintained at 15 days and 4 days, respectively. Peristaltic pumps (Cole Parmer Instrument Co., Chicago, IL) were used to add feeding solutions and to decant settled supernatant. Reactor offgas was collected via Teflon tubing into a Tedlar air sampling bag (SKC Inc., Eighty Four, PA) during reaction phases and vented to a fume hood during fill phases. The Tedlar bag was filled at least half-way with $\geq$99.998% N_2 gas everyday before the start of the reaction phase to serve as a N_2 reservoir in order to maintain a positive gas pressure in the reactors, especially during intensive sampling periods. The N_2 reservoir also helped to ensure an oxygen free headspace during the anoxic phases of all SBR experiments. All reactors were purged with N_2 gas via spargers during the feeding phase (before BTX was added) in order to remove any entrained oxygen. To avoid accumulation of unbiodegraded BTX and possible problems with floating sludge during settling caused by gas entrainment in the sludge flocs, the reactors were also purged with N_2 at the end of

each reaction phase. Therefore, the effective residence time of unbiodegraded BTX compounds was less than one day.

All reactors were fed a synthetic biogenic organic substrate that included proteins, sugars, and organic acids (5), and was amended with 5 mg/l of each BTX compound to encourage the growth and maintenance of a diverse microbial community while maintaining the BTX degraders in the mixed liquid suspended solids. The BTX was added manually into the reactor daily during the feed cycle (after sparging) by submerging a pipette tip below the surface of the mixed liquor. The initial theoretical oxygen demand (ThOD) of the biogenic and BTX constituents added to the reactors per cycle was approximately 670 mg/l. A mineral salts solution containing nitrate for use as an electron acceptor was also fed to the reactors and the stock solution contained the following: 0.144 mM $CaCl_2$, 0.044 mM $FeCl_3.6H_2O$, 0.005 mM $CoCl_2.6H_2O$, 0.009 mM $ZnCl_2$, 0.002 mM $CuCl_2.2H_2O$, 0.002 mM H_3BO_3, 0.487 mM $MgSO_4.7H_2O$, 0.021 mM $MnSO_4.H_2O$, 0.002 mM $Na_2MoO_4.2H_2O$, 4 mM KH_2PO_4, and 48 mM (672 mg/l) NO_3^--N. This solution was diluted 1:4 when added into the reactors. The mineral salts were acidified with nitric acid to avoid precipitation of essential nutrients; half of the NO_3-N in the mineral salts solution was added as nitric acid while the rest was added as KNO_3.

Reactor Aeration

The volatile nature of BTX compounds prevented the use of diffused air to provide oxygen for the microaerobic and aerobic conditions. Oxygenation via bubbleless membranes are a likely alternative for generating microaerobic conditions under full-scale application, but were difficult to control within the 2 liter laboratory scale system used here. Therefore, an alternative approach was used during this study in order to provide bubbleless and controllable oxygenation in the laboratory reactors. In the ANX/MA/AER SBR, 4% and 2% (w/w) solutions of hydrogen peroxide (H_2O_2) (Sigma chemicals, St. Louis, MO) coupled with a solution containing 1.47×10^4 units/mL of catalase (Sigma chemicals, St. Louis, MO) were added at a flow rate of 0.03 ml/min each to provide oxygen under aerobic and microaerobic conditions, respectively. The slow flow rates were used to avoid significantly changing the reactor volume during each reactor cycle. The active catalase concentration added with the H_2O_2 was at least 7 times greater than the stoichiometric amount required in order to ensure rapid and complete reaction. During the

microaerobic phase of the ANX/MA SBR, 0.03 ml/min of catalase (1.47×10^4 units of catalase/mL) and H_2O_2 (2% for 8.5 hours, 1% for 4 hours) stock solutions were added. A polarographic dissolved oxygen (DO) probe (Mettler-Toledo Process Analytical, Inc., Wilmington, MA) with a detection limit of 0.2 mg/L was used in an attempt to measure the DO concentration under microaerobic conditions. The DO probe failed to detect DO; therefore, the microaerobic phase used during this study was sustained at< 0.2 mg/L DO.

An experiment was conducted to determine if the H_2O_2 + catalase approach for providing oxygen to the activated sludge imposed toxicity to the microorganisms. Toxicity was measured by conducting specific oxygen uptake rates (SOURs) using mixed liquor collected fresh from a local domestic wastewater treatment plant that was not previously exposed to comparable doses of H_2O_2. The SOUR assays were conducted in 300 ml BOD bottles by adding 40 ml of mixed liquor plus 260 ml of mineral salts to each of two bottles. One bottle was oxygenated using conventional aeration with compressed air and a diffuser stone to generate an initial dissolved oxygen (DO) concentration around 8 mg/l. The other bottle was deoxygenated by purging for 20 minutes with N_2, then oxygenated using 127 µl each of 4 % H_2O_2 and 1.47×10^4 units/ml catalase, and generated an initial DO concentration of approximately 8 mg/l. Biogenic organic substrate stock was added into each BOD bottle to reach an initial concentration of 300 mg/l as chemical oxygen demand (COD). Dissolved oxygen (DO) concentration was recorded every minute for 20 minutes using a YSI DO probe (YSI Inc., Yellow Springs, Ohio). This comparative assay method was repeated 6 times on different days in order to obtain 6 independent measurements for statistical analysis.

Monitoring and Analytical Methods

Each reactor configuration was monitored for effluent BTX and anions daily, effluent COD every other day, and both mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) once a week. A minimum of two profiles were completed across SBR reaction cycles for each experimental condition. A ten day (minimum) recovery period was allowed between each profile. A minimum of twelve samples were collected along each profile and were analyzed for BTX concentrations, NO_3^-, NO_2^-, and SO_4^{2-} concentrations, oxidation-reduction potential, and specific enzyme activities. Abiotic tests were conducted with the ANX

and ANX/MA/AER reactor configurations to determine the potential for loss of BTX via nonbiological processes.

BTX compounds were analyzed with a Hewlett Packard 5890 gas chromatograph (GC) equipped with a 30 m poly(alkylene glycerol) 0.25 mm i.d. capillary column , with a film thickness of 0.25 µm (Supelco Inc., Bellefonte, PA), and a 5 m guard column. A split injection was used at a ratio of 1:30. The injection port temperature was 250°C, and the FID temperature was 260°C. Helium was used as a carrier gas at a column head pressure of 15 psi and a column flow rate of 1.46 ml/min. The initial column temperature was 70°C for 10 minutes, then the temperature was ramped at 10°C/min to a final temperature of 120°C. BTX analytical external standards were made using hexanes as solvent. The BTX concentration in mixed liquid suspended solids samples collected from the reactors was determined by directly extracting with hexanes for 2 hours on a rotary mixer at an extraction ratio of 7 parts sample to 1 part hexanes in a 16 ml vial containing a teflon-lined cap. After extraction, the liquid and solvent mixture was separated by centrifugation (1900 ×g) and the extract was transferred into a 2 ml GC autosampler vial and stored at 4 °C until analyzed.

Anions, including nitrate-N, nitrite-N and sulfate, were analyzed by a Dionex 2010I ion chromatograph (IC) with an IONPAC AS4A-SC column and an electrochemical conductivity detector (Dionex Corp., Sunnyvale, CA). The eluent for the IC was 1.7 mM sodium bicarbonate-1.8 mM sodium carbonate. External anion standards were used. Mixed liquid suspended solids samples were collected from the reactors and centrifuged at 12,100 ×g for 10 minutes and the centrate was filtered through 0.2 µm Supor$^®$ filters (Gelman Sciences, Ann Arbor, MI).

Oxidation reduction potential (ORP) was measured during the anoxic, microaerobic, and aerobic phases of each reactor configuration using an ORP probe (Cole Parmer Instrument Co., Chicago, IL) and a pH/mV meter (Fisher Scientific, Pittsburgh, PA). The probes were standardized with Light's solution as specified in Standard Methods (3) and are reported on a Ag/AgCl reference basis. COD, MLSS, and MLVSS were analyzed according to Standard Methods (3). Excel Version 6 was used for conducting all statistical analyses.

Samples for enzyme activity and protein assays were collected from the SBRs in an anaerobic manner using N_2 purged sample vials, and were processed in an anaerobic glove box (Plas Labs, Lansing, MI) with a positive pressure N_2 headspace. Sample vials were capped and

wrapped with teflon tape inside the anaerobic glove box to keep an anaerobic headspace while centrifuging. Ten ml of mixed liquor was centrifuged at 12,100×g at 4 °C and was resuspended with 10 ml pH 7.5, 0.1 M phosphate buffer. The mixture was centrifuged again and the final pellet was resuspended in 1 ml phosphate buffer. The suspension was sonicated on ice with a Sonifier® cell disruptor (Branson Ultrasonics Corporation, Danbury, CT). The disrupted sample was centrifuged again (12,100×g at 4 °C) and the supernatant cell free extract (CFE) was recovered. The CFE was immediately assayed for C12O, C23O and/or benzoyl-CoA reductase and residual CFE was stored at –20°C for protein determination at a later time.

The methods of Nakazawa and Nakazawa (38), and Nozaki (39), were used to assay C12O and C23O, respectively. One unit of C12O activity is defined as the amount of catechol which forms one µmole of *cis, cis*-muconic acid per minute at 24°C (38). One unit of C23O activity equals the amount of catechol which forms one µmole of 2-hydroxymuconic semialdehyde per minute at 24°C (39). Benzoyl-CoA reductase activity was determined using the spectrophotometric method described by Boll and Fuchs (9). All the solutions used in this assay were purged with high capacity gas purified N_2 (Supelco, Inc., Bellefonte, PA). One unit of benzoyl-CoA reductase activity is defined as the amount of enzyme catalyzing the oxidation of 2 µmol methyl viologen per minute which is assumed to be equivalent to the reduction of 1 µmol benzoyl-CoA per minute (9). The molar extinction coefficient for the oxidation of reduced methyl viologen was determined to be 3400 $M^{-1}cm^{-1}$ at a wavelength of 600 nm. Cell free extracts of *T. aromatic* strain T1 grown on ATCC medium 2050 and *Escherichia coli* JM109 grown on ATCC medium 1065 were maintained and processed under anoxic conditions, and were used as positive and negative controls, respectively, while assaying for benzoyl-CoA reductase in activated sludge samples. Total protein was measured in the CFE using the bicinchoninic acid protein assay kit (Sigma Procedure No. TPRO-562, Sigma Chemicals, St. Louis, MO). Protein standards were prepared by using bovine serum albumin (Sigma Chemicals, St. Louis, MO) dissolved in pH 7.5, 0.1 M phosphate buffer. All enzyme activities were normalized back to total protein concentrations to obtain specific enzyme activities.

RESULTS AND DISCUSSION

Toxicity Assays

Six independent SOUR tests were conducted on activated sludge from a local domestic wastewater treatment plant that were oxygenated by either conventional diffused aeration or H_2O_2 coupled with catalase. A pooled student t test (2 tails, assume equal variance) indicated that at a test level of 5%, the means of the two groups are not significantly different. Therefore, it can be concluded that use of H_2O_2 coupled with excess catalase in the manner described in this paper failed to impose toxicity as measured by SOUR. We believe this is due to the rapid rate of H_2O_2 conversion into O_2 and H_2O in the presence of excess catalase and conclude that the laboratory-scale method used here for oxygenating the biomass enabled the establishment of highly controllable microaerobic (<0.2 mg/L DO) conditions without imposing toxicity.

Reactor profiles

Anoxic Reactor Configuration

Figure 1 shows a typical profile throughout a reaction cycle for the ANX SBR for BTX, nitrate and nitrite (NO_X) and sulfate concentrations, ORP and enzyme activities. ORP remained between -200 mV and -100 mV as shown in Figure 1a. Figure 1b shows that nitrite-N was present at time zero because the oxidation of biogenic organic matter occurred rapidly as the feed solutions were pumped into the reactor. Accumulation of nitrite-N during nitrate dissimilation occurred, and is common in denitrifying systems receiving high levels of nitrate (21, 36, 44). No significant consumption of sulfate was observed, suggesting that a sulfate-reducing condition was not developed in the reactor. ORP decreased slightly along the reaction phase as nitrate-N and nitrite-N were consumed, indicating that a more reduced environment was developed along the cycle. The profile of BTX concentrations shown in Figure 1c demonstrated that the complete dissolution of BTX took about 30 minutes after being fed into the reactor. An abiotic test showed that BTX losses due to non-biological mechanisms were minimal under the ANX reactor conditions (data not shown); therefore, the losses observed during the biotic experiments are believed to be due to biodegradation. Toluene and *m*-xylene were completely biodegraded under rigorously-controlled denitrifying conditions within 6 and 10 hours, respectively, based on their disappearance from the gas chromatograms and the fact that no intermediate peaks were detected.
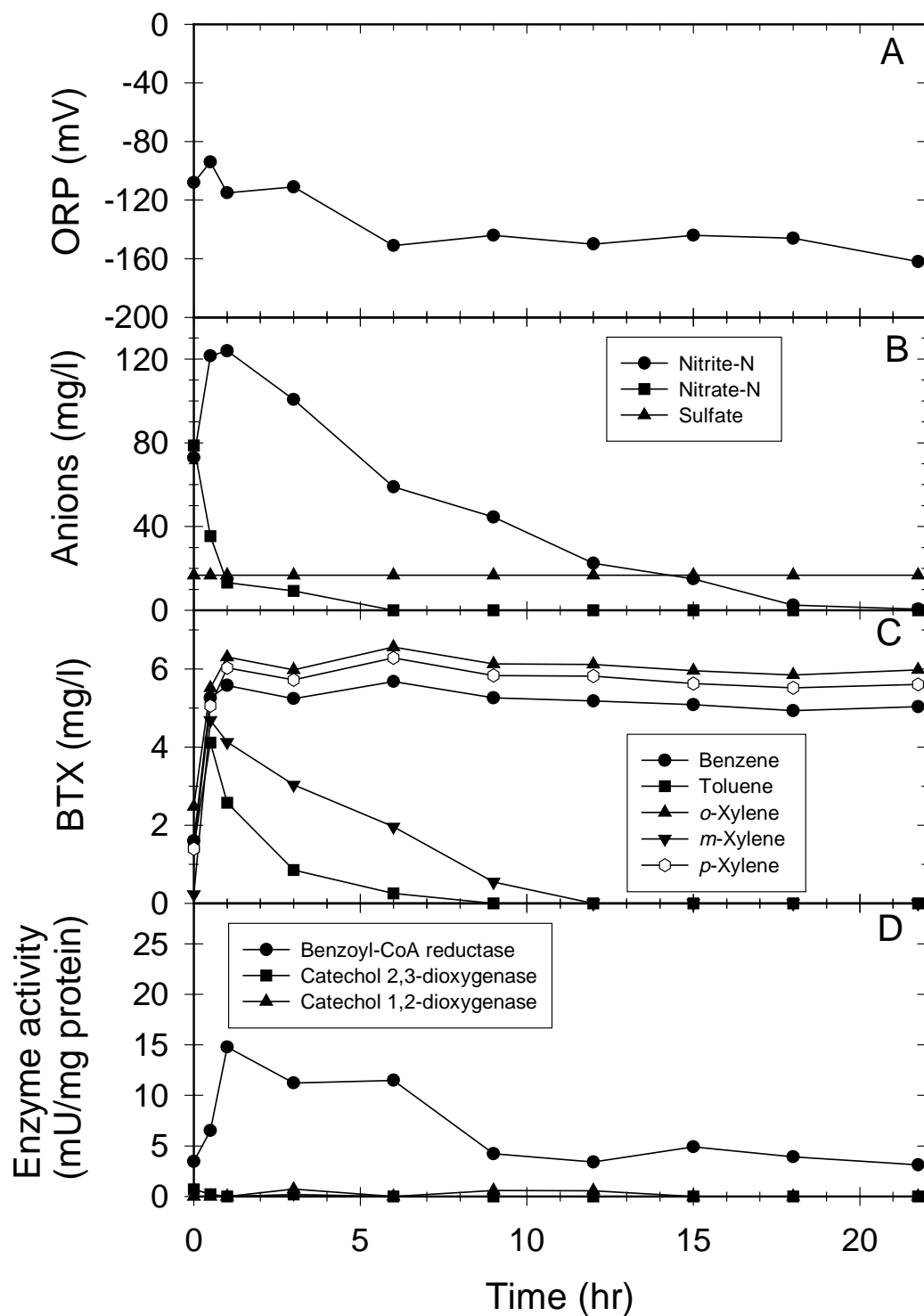
Figure 1.   Profile during an anoxic SBR experiment showing (a) redox potential, (b) electron acceptor concentrations, (c) BTX concentrations and (d) aerobic and anaerobic specific enzyme activities.

On the other hand, benzene, *o*-, and *p*-xylene were recalcitrant to denitrification. This same BTX biodegradation pattern was observed under denitrifying conditions with the ANX culture for over 3 years in our laboratory (19). Figure 1d summarizes the specific enzyme activities over the reaction cycle. C12O and C23O were not induced to significant levels in the ANX SBR; however, benzoyl-CoA reductase was induced, and changes in its specific activity level corresponded with the biodegradation of toluene and *m*-xylene. This confirms that the anaerobic pathway was involved with toluene and *m*-xylene biodegradation during the ANX phase.

These biodegradation patterns are consistent with most past reports on toluene and benzene fate under denitrifying conditions (2, 6, 33), but not with a recent report showing benzene denitrification by an enrichment culture from a subsurface environment (11). *m*-Xylene denitrification was also observed by other researchers (2, 6, 46). Although the remaining xylene isomers were shown to be recalcitrant to denitrification here, others have shown *p*- (2, 6, 46) and *o*-xylene (2, 46) biodegradation under denitrifying conditions, although *o*-xylene metabolism is most often linked to co-metabolism with toluene (2, 4, 6, 18, 19) or the other xylene isomers (49).

Anoxic/Microaerobic/Aerobic Reaction Profiles

A typical profile for the ANX/MA/AER SBR is shown in Figure 2. During the anoxic phase, the reactor exhibited similar patterns to those observed in the ANX SBR. Dual electron acceptors (nitrite-N and oxygen) existed during the 2 hour microaerobic phase. The ORP values increased rapidly from -170 mV at the end of the anoxic phase to -30 mV at the end of the microaerobic phase, as indicated in Figure 2a. When the reactor was shifted to the microaerobic phase, biodegradation of benzene, and *p*-xylene was initiated (Figure 2c), and C12O and C23O were induced to significant levels (Figure 2d). Degradation of *o*-xylene was not obvious during the brief microaerobic phase. During the aerobic phase, the ORP measurements further increased to around +75 mV and the DO was routinely ≥ 4 mg/L (data not shown). Benzene, *o*- and *p*-xylene were completely biodegraded by the end of the aerobic phase, and both C12O and C23O were continuously present at high to moderate levels, although specific enzyme activity levels decreased as the hydrocarbon concentrations decreased. The specific activity of benzoyl-CoA reductase decreased once the reactor entered the microaerobic phase and remained low during
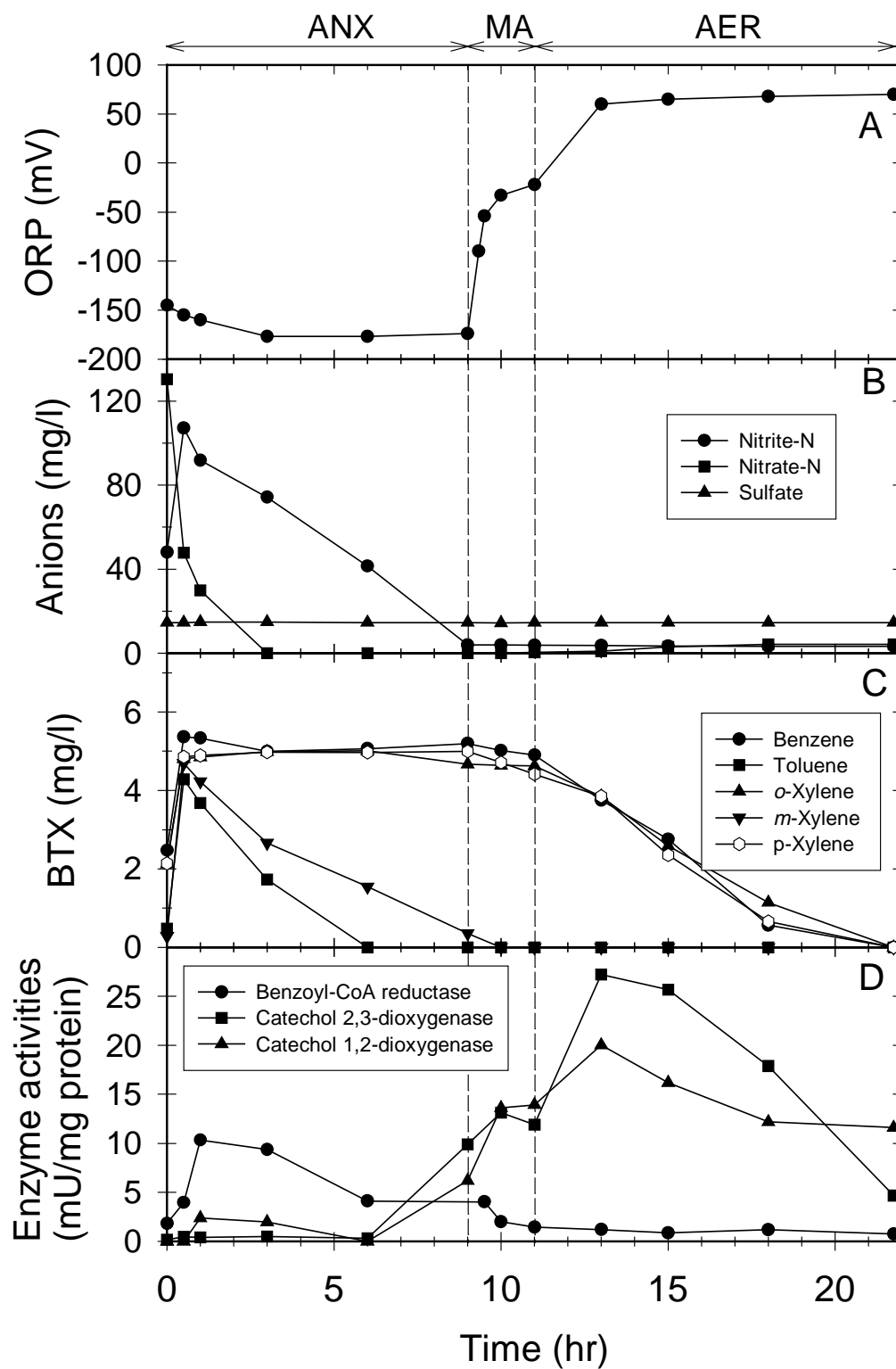
Figure 2.   Profile during an anoxic/microaerobic/aerobic SBR experiment showing (a) redox potential, (b) electron acceptor concentrations, (c) BTX concentrations and (d) aerobic and anaerobic specific enzyme activities.

the aerobic phase. An abiotic test was conducted using this reactor configuration and all five BTX compounds were retained in solution at their target concentrations for the duration of a complete reaction cycle (data not shown).

The initiation of benzene and *p*-xylene biodegradation during the microaerobic (DO < 0.2 mg/L) phase of the ANX/MA/AER SBR, and the increase in C12O and C23O activities at the expense of benzoyl-CoA reductase activity under microaerobic conditions suggest that the culture had the potential to biodegrade these monoaromatic hydrocarbons using aerobic pathways under low oxygen tension conditions. In addition, the data showed that significant C12O and C23O

expression levels can be realized under microaerobic conditions where DO levels are below detection, and suggest that a longer microaerobic reaction phase may be used to enable complete benzene, *p*- and *o*-xylene biodegradation. Consequently, a third reactor configuration (ANX/MA) was designed to test the biodegradation potential of benzene, *o*- and *p*-xylene under longer microaerobic conditions.

Anoxic/Microaerobic Profiles

Figure 3 summarizes a typical profile in the ANX/MA SBR. Again, the trends exhibited under the anoxic phase were similar to those in the ANX SBR (Figure 1). The ORP measurements under both anoxic and microaerobic phases agreed with the values observed in the same environments in the ANX/MA/AER SBR (Figure 2a). Due to nitrite bleed through from the anoxic zone, both oxygen and nitrite-N were present and available as electron acceptors throughout the microaerobic phase, and nitrite-N was shown to be consumed throughout (Figure 3b). Additionally, benzene, *o*- and *p*-xylene were completely oxidized by the end of the microaerobic phase (Figure 3c). The data show that C12O and C23O specific activities were induced during the microaerobic phase to levels comparable to those observed under aerobic conditions during the ANX/MA/AER SBR, while benzoyl-CoA reductase activity was repressed (Figure 3d), showing that benzene, *p*- and *o*-xylene were biodegraded at least partway by aerobic pathways under NO_2-supplemented microaerobic conditions. C12O and C23O specific activity levels remained elevated even after the aromatic hydrocarbons were depleted, but were routinely gone by the beginning of the next reactor cycle and were presumably degraded during the 2 hour
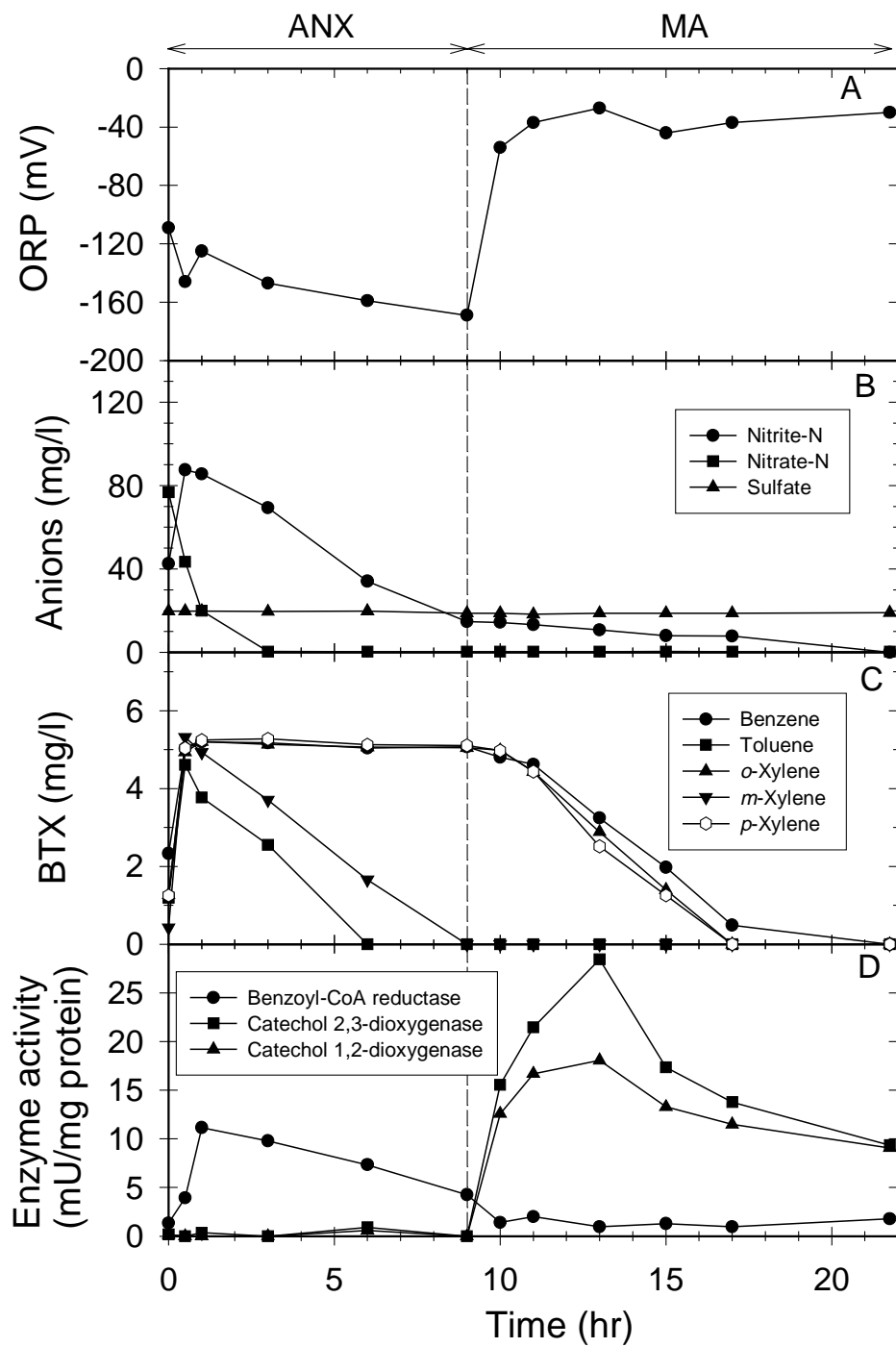
Figure 3.   Profile during an anoxic/microaerobic SBR experiment showing (a) redox potential, (b) electron acceptor concentrations, (c) BTX concentrations and (d) aerobic and anaerobic specific enzyme activities.

settling and decant phases. Although benzene, *o-* and *p*-xylene were biodegraded during the MA phase, the effect of nitrate-N or nitrite-N on this biodegradation was unclear.

NO$_X$-N-Supplemented Microaerobic Biodegradation

Two additional ANX/MA profiles were completed so that nitrite would be depleted part way through the microaerobic zone in order to discern the effect of nitrate or nitrite on microaerobic benzene, *o-* and *p*-xylene biodegradation. For one profile (Figure 4), nitrite-N was depleted by the 12th hour after the reaction cycle started and was operated under microaerobic conditions without NO_2^- or NO_3^- for 3.5 hours before an aliquot of deoxygenated nitrate solution was spiked into the reactor to a final concentration of 25 mg/l as NO_3^--N at 15.5 hours (Figure 4b). Consequently, the microaerobic phase in this reaction cycle is divided into three distinct stages, including hours 9-12 (microaerobic O_2 plus NO$_2$-N), hours 12-15.5 (microaerobic O_2 only), and hours 15.5-19 (microaerobic O_2 and NO$_3$-N). Figure 4a shows that ORP was slightly higher in the presence of nitrite-N or nitrate-N under the microaerobic phase than when nitrite-N and nitrate-N were depleted. Figure 4c shows that benzene, *o-* and *p*-xylenes were biodegraded throughout the entire microaerobic phase, whether in the presence or absence of the alternative electron acceptors. In particular, the data in Figure 4 show that small amounts of oxygen entrainment can be enough to enable significant benzene biodegradation, even though dissolved oxygen measurements may imply that an environment is anaerobic.

Our data suggest that NO$_X$ enhanced the rate of benzene, *p-* and *o*-xylene biodegradation under microaerobic conditions. A summary of microaerobic (with and without NO$_X$) and aerobic biodegradation kinetics normalized to MLVSS are presented in Table 1. The data show that in the presence of NO$_X$ the biodegradation rates of benzene, *o-*, and *p*-xylenes were twice the rates observed in the absence of NO$_X$ with no significant distinction between NO$_3$ and NO$_2$. The elevated biodegradation rates observed in this study concur with the results presented by other researchers working with pure cultures when toluene was used as a substrate (32, 34) or with benzene biodegradation with subsurface cultures where enzymes were originally induced under O_2 saturated aerobic conditions (2). An important distinction in this study is that the data show rapid initiation of benzene, *p-* and *o*-xylene biodegradation and C12O/C23O expression under nitrite-supplemented microaerobic (DO<0.2 mg/L) conditions without first being exposed to aerobic (DO > 4 mg/L) conditions. Additionally, it is important to note
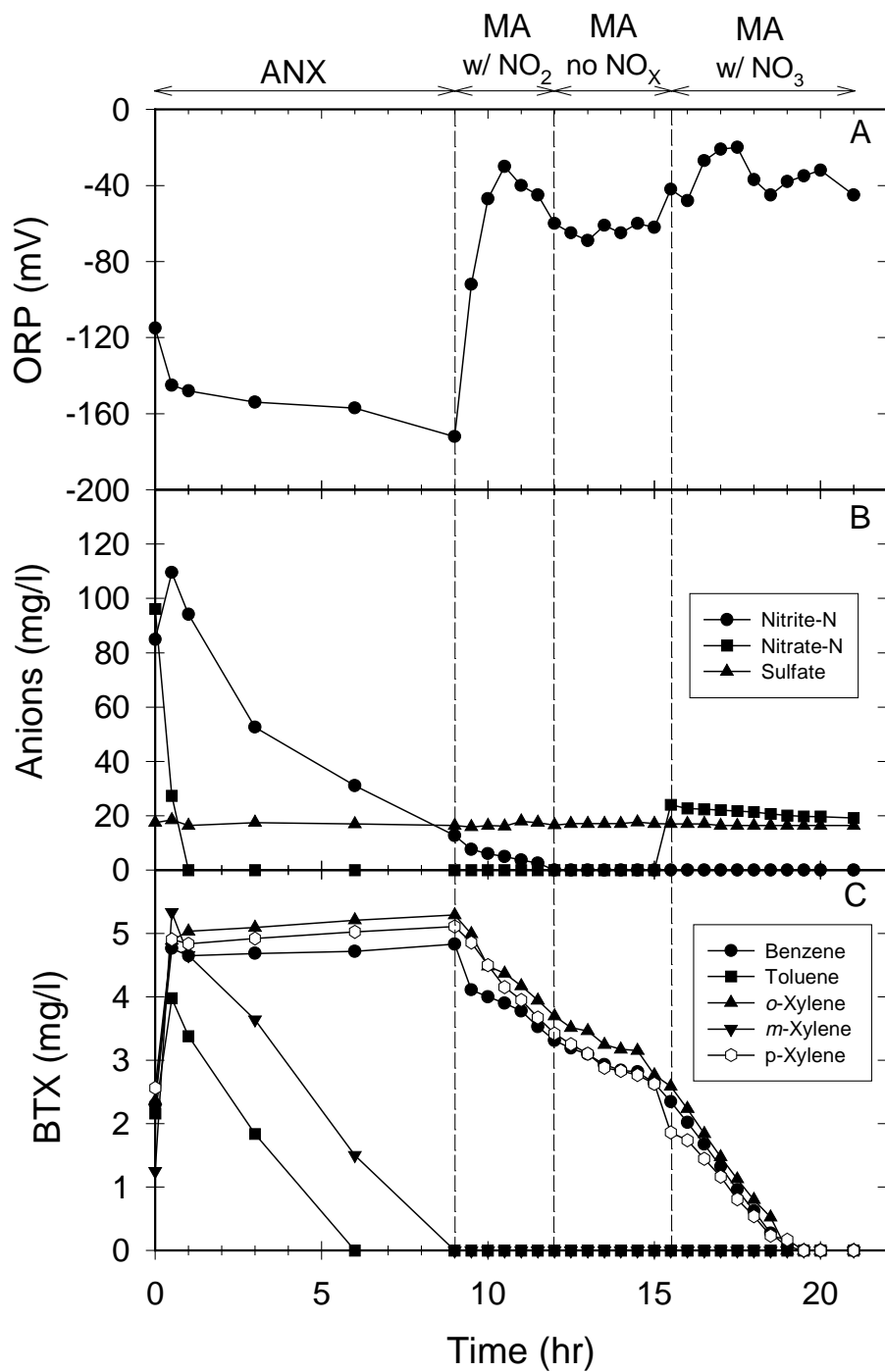
Figure 4.   The effect of nitrate-N and nitrite-N on biodegradation of benzene, p-xylene and o-xylene in an anoxic/microaerobic SBR: (a) redox potential, (b) electron acceptor concentrations, and (c) BTX concentrations.

that the biodegradation rates under microaerobic conditions in the presence of NO_X are comparable to aerobic biodegradation rates. This suggests that nitrate-supplemented microaerobic conditions may be competitive with aerobic treatment strategies for wastewaters or groundwaters containing BTX or other volatile compounds amenable to microaerobic metabolism. Although the experimental system studied here was operated in a way that allowed NO_X to bleed through into the microaerobic or aerobic phases, the more typical condition would be NO_X limitation at some point along the reactor cycle in an engineered system (assuming that nitrification is being used to sustain denitrification). The kinetic data show that BTX biodegradation would be slower under microaerobic conditions without supplemental NO_X, which would translate into larger and more expensive basins. This would be compensated in part by a smaller air handling system and lower operating costs. Further work is needed to optimize the costs associated with supplementing with NO_X versus constructing and operating a larger treatment system.

Table 1.   Biodegradation rates for benzene, *o*-, and *p*-xylene under aerobic and microaerobic conditions.

| Compound | Biodegradation rate[a] (mg/g MLVSS-hr) | | | |
	Aerobic	Microaerobic w/ NO_2^--N	Microaerobic w/o NO_x-N	Microaerobic w/ NO_3^--N
Benzene	0.229±.045	0.211±.013	0.112±.010	0.291±.039
o-Xylene	0.251±.049	0.238±.006	0.128±.0071	0.303±.056
p-Xylene	0.258±.031	0.250±.030	0.145±.037	0.247±.008

[a] Biodegradation rates were calculated using zero-order kinetics. Each biodegradation rate was generated by the average of two separate experiments. The regression coefficients for all regressions were between 0.85 and 0.99.

The aromatic hydrocarbon biodegradation that was observed under microaerobic conditions with and without NO_X occurred via oxygen-dependent ring-cleavage, as demonstrated by the coordinated induction of C12O and C23O and repression of benzoyl-CoA reductase. Additional experiments were conducted on testing whether benzoyl-CoA reductase is inducible under microaerobic conditions in the presence of nitrite-N and toluene. In this experiment,

toluene was spiked into the reactor to reach a final concentration of 5 mg/l when the ANX/MA SBR shifted to the microaerobic phase. Although toluene biodegraded rapidly, benzoyl-CoA reductase was not induced (data not shown).

Energy Balance

Due to the manner in which O_2 was introduced into the SBRs, it was possible to conduct an electron balance for two separate ANX/MA profiles experiencing microaerobic conditions with and without NO_X. Oxygen demand units are used for the electron balance and is an appropriate unit for this purpose based on the notion that up to 4 electrons can be accepted by O_2 per mole. We converted all constituents of the electron balance to oxygen demand units using the conversions shown in Table 2. The constituents included in the electron balance include electron acceptors provided (O_2 calculated from stoichiometry based on the amount of H_2O_2 added, or NO_2^-- N and NO_3^--N measured by chromatography), which should balance the electron acceptors demanded (through substrate consumption and endogenous respiration). It is known from previous studies conducted in our lab that the biogenic substrates were routinely depleted within 1 hour in the SBRs (19). In addition, the following assumptions were made when calculating the electron balances: (1) H_2O_2 was completely and rapidly converted to oxygen via excess catalase during the microaerobic phases, (2) the endogenous respiration rate measured by NO_X uptake late in the ANX phase reflects the endogenous respiration rate throughout the ANX/MA phase, and (3) both NO_3^- and NO_2^- are reduced all the way to N_2 during denitrification. Finally, experimentally-determined true growth yields of 0.34 mg biomass COD/mg BTX COD, and 0.41 mg biomass COD/mg biogenic substrate COD were used for the calculations (19).

The energy balance for two independent ANX/MA profiles are shown in Figure 5, and reflect well balanced conditions. For the anoxic phase of each profile, the electron acceptors provided (O_2 demand) balanced within 92 percent of the electron acceptors consumed (O_2 demand satisfied) for both cases. Figure 5 also shows that the oxygen supplied to the reactor under microaerobic conditions was consistently in excess of that needed for complete substrate consumption alone, but less than the total demanded for substrate consumption and endogenous respiration. The presence of nitrate-N or nitrite-N supplemented the electron acceptor pool under the microaerobic phases to support this demand. On the other hand, when O_2 was the sole

electron acceptor under the microaerobic phase, it had to satisfy the demands for both substrate consumption and endogenous respiration, leaving less O_2 available to support oxygenase reactions. Half saturation constants for O_2 respiration (k_{O2}) in flocculant activated sludge cultures are typically estimated to be between 0.07 and 0.2 mg/L O_2 (25, 53) and are only slightly larger than k_{O2} values (0.03 to 0.06 mg/L O_2) determined for C23O enzymes in hypoxic pure cultures that demonstated an ability to degrade toluene at enhanced rates under nitrate-supplemented microaerobic (< 2 mg/L) conditions (32). Consequently, it is possible that under microaerobic conditions, oxygenase enzymes in hypoxic strains may demonstrate a slight preference for O_2 over cytochromes (e.g., aa_3 and o), which use O_2 during respiration. We propose that the dual presence of NO_X and O_2 in the experiments reported here resulted in use of NO_X to support some fraction of the endogenous respiration demand, thereby allowing the redirection of more O_2 molecules for use in strict O_2-dependent oxygenase reactions in the aerobic aromatic biodegradation pathway. This helps to explain why the biodegradation rates of benzene, *o*-, and *p*-xylene are faster in the presence of NO_X than in the absence of NO_X under microaerobic conditions. It is also possible that some fraction of the NO_X present supported the respiration of BTX intermediates generated downstream of the oxygenase reactions, as suggested by Wilson and Bouwer (1997).

Table 2.　　　Theoretical oxygen demand conversions for energy balance calculations[a].

Constituent	Theoretical Oxygen Demand (ThOD) Conversion Factor	No. of electrons accepted or donated
Benzene	3.07 mg ThOD/mg benzene	5 donated per carbon
Toluene	3.13 mg ThOD/mg toluene	5.14 donated per carbon
m-, *p*- and *o*-xylene	3.17 mg ThOD/mg xylene	5.25 donated per carbon
NO_2^- - N	1.71 mg ThOD/mg NO_2^- - N	3 accepted per nitrogen
NO_3^- - N	2.86 mg ThOD/mg NO_3^- - N	5 accepted per nitrogen

[a] Energy conversions were calculated as follows:

$$\frac{\text{ThOD}}{\text{g constituent}} = \frac{\text{moles C or N}}{\text{moles constituent}} \times \frac{\text{mole constituent}}{\text{g (molecular wt)}} \times \frac{\text{\# electrons transferred}}{\text{mole C or N}} \times \frac{8\,\text{g O}_2}{\text{electron}}$$
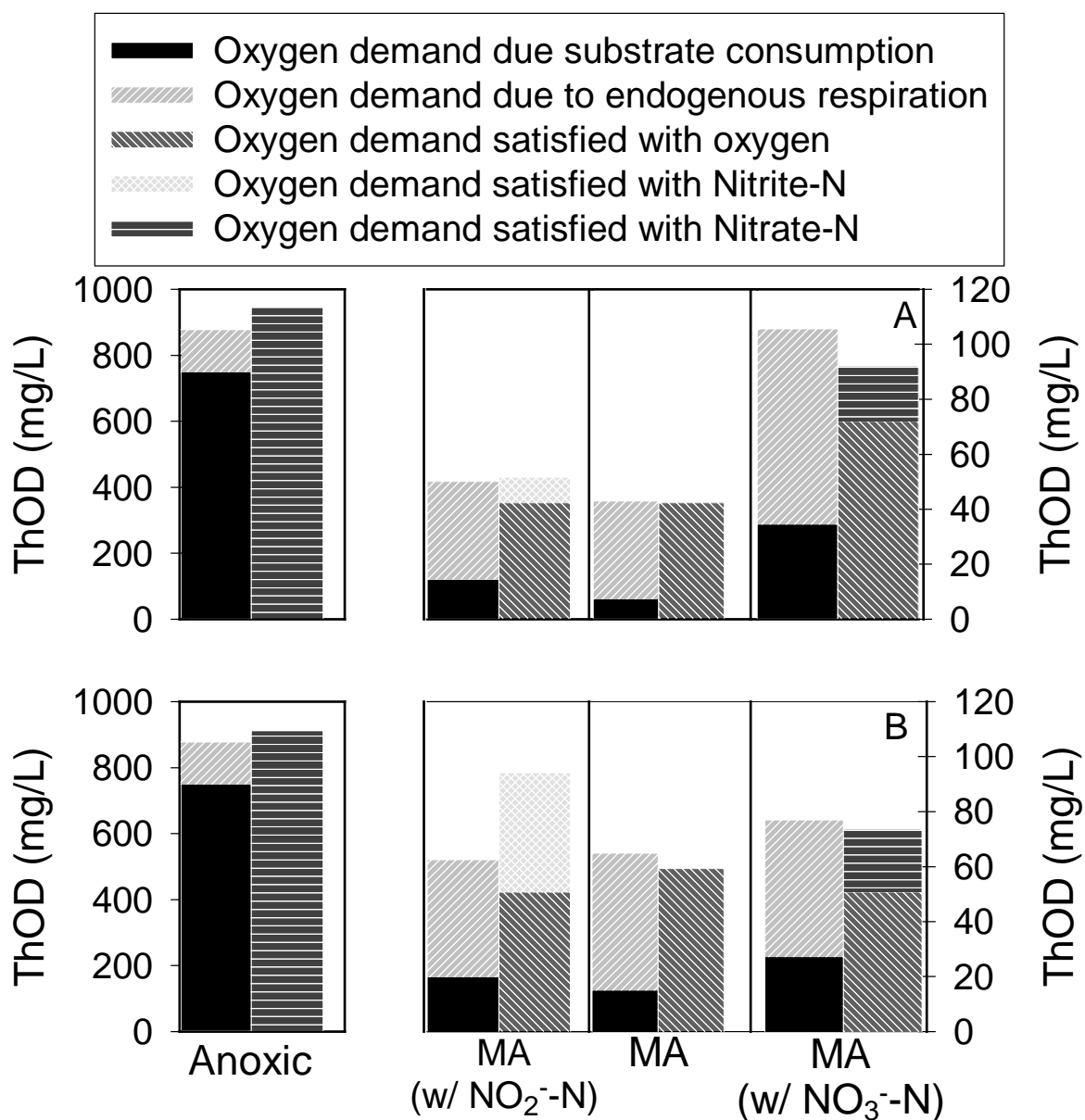
Figure 5. An electron balance in terms of oxygen demand units for two anoxic/microaerobic profiles undergoing microaerobic biodegradation with and without nitrate or nitrite.

For the two profiles shown, there were differences in the energy balance during the microaerobic phases in the presence of nitrite-N which were reflected in differences in observed ORP measurements (Figure 6). In Profile B (Figure 5b), the amount of nitrite-N consumed and oxygen provided (oxygen demand satisfied) were in excess of the oxygen demanded; therefore, the surplus oxygen remained in the reactor during the next stage or degassed into the headspace. On the other hand, the electron acceptors provided just satisfied the demands during profile A at the same point in the reaction stage (Figure 5a). This difference in residual oxygen was reflected in the ORP values measured (Figure 6). During profile A, demand was well balanced by the electron acceptors provided and complete consumption of O_2 was likely to have occurred, resulting in a greater ORP reduction during the microaerobic only stage. In contrast, the ORP remained 30 mV higher during profile B when excess O_2 was present.

Oxidation-Reduction Potential

The data show that oxidation reduction potential proved to be a valid and sensitive monitoring device which could be used to discriminate between different electron acceptor conditions in the system studied. The values observed for anoxic and aerobic conditions during this study are consistent with those reported by others (31); however, previous reports describing measured ORP levels under microaerobic conditions were not found. The sensitivity of ORP to different degrees of microaerobicity was also demonstrated through use of an electron balance. It has been reported that ORP is not a good indicator of electron acceptor condition for use in more reduced subsurface environments where multiple electron acceptors and H_2–generating fermentative microorganisms generate lower redox couples (12). In the suspended growth denitrifying/microaerobic activated sludge cultures studied here, the redox environments were not low enough to support sulfate reducing or significant H_2-generating fermentation or methanogenic reactions, which Chapelle and co-workers studied. Our data showed that an ORP probe was very sensitive and responsive to changing electron acceptor conditions, and was able to differentiate between microaerobic conditions in the presence or absence of NO_X, and in the presence of different amounts of available O_2 under microaerobic and denitrifying conditions. Therefore, we conclude that ORP can be very useful as a process control device for engineered suspended growth treatment systems that incorporate denitrifying and/or microaerobic treatment phases.
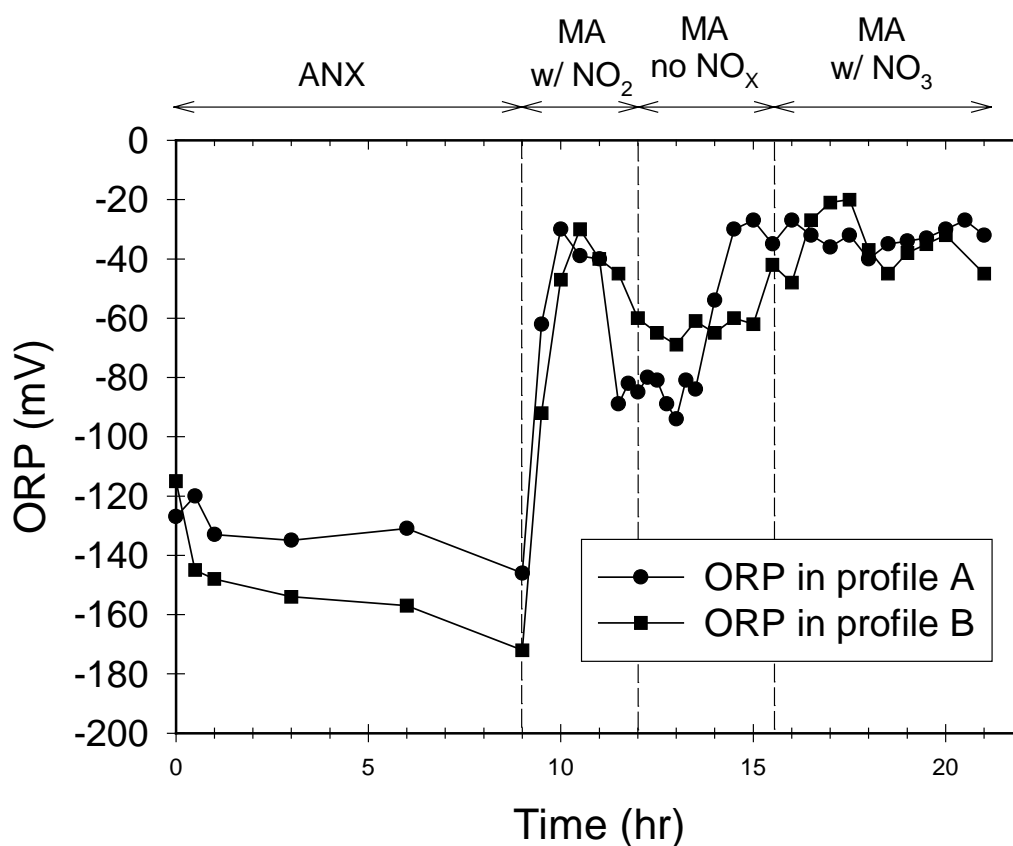
Figure 6.   ORP profiles for the anoxic/microaerobic data shown in Figure 5.

SUMMARY AND CONCLUSIONS

1. Toluene and *m*-xylene were consistently biodegradable under denitrifying conditions via a ring-reducing anaerobic pathway while benzene, *p*- and *o*-xylene were recalcitrant to denitrification. However, the latter aromatic hydrocarbons were biodegraded by aerobic oxygenase pathways under aerobic (DO > 4 mg/L) and microaerobic (DO < 0.2 mg/L) conditions.

2. Biodegradation of benzene, *p*- and *o*-xylene was twice as fast under nitrate or nitrite-supplemented microaerobic conditions compared to NO_X-deficient microaerobic conditions.

3. Biodegradation rates for benzene, *p*- and *o*-xylene under NO_X-supplemented microaerobic conditions and aerobic conditions were comparable.

4. Microaerobic metabolism of benzene, *p*- and *o*-xylene was facilitated by catechol 1,2-dioxygenase and catechol 2,3-dioxygenase enzymes, which were capable of being

induced under NO_X-supplemented microaerobic conditions to levels comparable to those observed under aerobic conditions.

5. Oxidation-reduction potential proved to be a very sensitive indicator of the electron acceptor condition present in the sequencing batch reactor system used and shows promise for use in full-scale systems that incorporate denitrifying and/or microaerobic treatment strategies.

6. A novel reactor design incorporating a Tedlar bag to create supplemental and flexible headspace was demonstrated for use in laboratory experimentation where intensive sampling may otherwise cause uncontrolled oxygen entrainment, and may be used to study strictly anoxic or microaerobic metabolism under controlled conditions. Use of low amounts of H_2O_2 coupled with excess catalase yielded a controlled, bubbleless and nontoxic reaction which generated dissolved oxygen directly.

It is anticipated that microaerobic metabolism has a role to play in the treatment of industrial or hazardous wastewaters, or contaminated groundwaters containing volatile or semivolatile compounds. In particular, this study shows the utility of using NO_X supplemented microaerobic conditions to treat wastewaters containing aromatic hydrocarbons. Bubbleless membrane technologies are continually under development, and this study demonstrates a possible application for that technology, which may be used to treat VOC-contaminated wastewaters that are regulated by stringent NESHAP guidelines.

ACKNOWLEDGEMENTS

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CHAPTER 3. BIODEGRADATION OF BTX IN ANOXIC AND MICROAEROBIC SEQUENCING BATCH REACTORS

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ABSTRACT

This research proposed a new treatment process for BTX containing wastewater by using an alternating anoxic/microaerobic sequencing batch reactor to overcome the stripping of BTX compounds into the atmosphere, as often occurs in conventional aerobic treatment processes. An ORP probe was successfully used to monitor different electron acceptor conditions in sequencing batch reactors whereas a DO probe failed to detect the low DO concentration under microaerobic conditions. Toluene and *m*-xylene were amenable to anoxic (denitrifying) metabolism while benzene, *o*-, and *p*-xylene were biodegradable under microaerobic conditions. Compared to conventional aerobic treatment processes, this approach can eliminate vigorous aeration and significantly reduce stripping of BTX (and other volatile contaminants amenable to anoxic/microaerobic biodegradation) caused by aeration.

KEYWORDS: anoxic; BTX; microaerobic; ORP; SBR.

INTRODUCTION

One of the major concerns associated with using sequencing batch reactors for wastewater treatment is the uneven oxygen demands encountered throughout a reaction cycle, with much higher oxygen demands at the beginning. Such an oxygen demand profile leads to either a complicated operation strategy or high peak energy consumption. This particular problem associated with sequencing batch reactors can be overcome by incorporating an anoxic environment into the reaction cycle. This paper presents a study on the biological treatment of a wastewater stream containing benzene, toluene, *o*-, *m*-, and *p*-xylene (BTX) by using sequencing batch reactors with alternating anoxic and microaerobic conditions.

BTX are a group of toxic and volatile aromatic compounds prevalent in many industrial wastewaters and gasoline or petroleum contaminated subsurfaces. Traditionally, BTX containing wastewater is treated by conventional aerobic wastewater treatment processes; however, the removal mechanisms are controversial since these compounds tend to be readily stripped from the aqueous phase to the atmosphere due to their volatile nature (7, 8). Therefore, alternative treatment strategies are needed.

Studies with pure cultures grown under anoxic conditions demonstrated the biodegradation abilities of toluene (5, 9, 11, 17, 19), as well as *o*- (5), *m*- (5, 11, 17, 19), and *p*-xylene (5, 19). Studies on mixed cultures grown under anoxic conditions exhibited similar patterns. Batch incubation tests (1, 4) or *in situ* tests (18) with subsurface cultures showed the anoxic biodegradation abilities of toluene, *m*- and *p*-xylene. In a study on activated sludge (10), the biodegradation of toluene and *m*-xylene was observed. The transformation of *o*-xylene in the presence of toluene biodegradation might be a common feature under anoxic conditions (1, 4, 10). The potential for benzene biodegradation under anoxic conditions is debatable. Most studies have shown that benzene is recalcitrant to anoxic biodegradation (1, 4, 10, 15), although a recent study suggested otherwise (6).

In recent years, there have been several reports of BTX metabolism under oxygen-limited, or microaerobic conditions, in the presence of alternative electron acceptors such as nitrate-N (NO_3^--N) or nitrite-N (NO_2^--N) (1, 14, 16, 19). Unfortunately, the definition of microaerobic is vague and the extent of oxygen limitation varies from study to study due to differences in the methods used to both control microaerobic conditions and to monitor residual dissolved oxygen concentration. Nevertheless, those studies showed enhanced BTX

biodegradation abilities under microaerobic conditions in the presence of alternative electron acceptors. Most importantly, Alvarez and Vogel (1) showed that benzene biodegradation was significantly improved in the presence of nitrate under oxygen-limiting conditions.

The purpose of this research is to investigate an alternative approach for treating BTX containing wastewater in order to comply with U.S. Clean Air Act regulations. One of the significant features of this work is to take full advantage of BTX biodegradation potentials under anoxic conditions and microaerobic conditions so that BTX stripping into the atmosphere is minimized and a low yet uniform oxygen demand profile throughout a reaction cycle can be achieved. Finally, we investigate oxidation-reduction potential (ORP) as a monitoring device for process control of microaerobic conditions.

METHODS

Reactor setup

Two activated sludge sequencing batch reactors (SBRs), each with a working volume of 2 liters and a headspace of 0.5 liter were used. The glass reactors (LH Fermentation, Hayward, CA) were maintained at 25 ℃ and continuously mixed at 200 rpm during react and feed phases. The pH in the reactors was controlled at neutral with pH controllers (Valley Instrument Company, Exton, PA). Both of the SBRs were operated with a 24-hour cycle including fill (0.5 hour), react (21.5 hours), settle (1.5 hours) and draw (0.5 hour). One SBR (ANX) was operated so that the 21.5 hr react phase remained anoxic (denitrifying) while the second SBR (ANX/MA) was operated with 9 hr of an anoxic and 12.5 hr of a microaerobic condition. Biomass for the ANX SBR was acquired from an industrial activated sludge wastewater treatment facility, whereas the ANX/MA SBR received biomass from the same industrial activated sludge wastewater facility (3/4 volume) as well as a local domestic wastewater treatment facility (1/4 volume). Sludge retention time (SRT) and hydraulic retention time (HRT) were maintained at 15 days and 4 days, respectively, in each of the reactors.

The reactor offgas was collected into a 1.5L tedlar air sampling bag (SKC Inc. Eighty Four, PA) during the reaction phase and vented to a fume hood during the fill phases. The tedlar bags were filled at least half way with 99.998% N_2 gas everyday before the start of the reaction phase and served as a N_2 gas reservoir in order to maintain positive gas pressure in the reactors during extensive sampling periods. The N_2 reservoirs also helped to ensure an oxygen-free

headspace for the ANX SBR, and for the anoxic phase of the ANX/MA SBR. The reactors were purged with N_2 gas via spargers during the feeding phase in order to maintain anoxic conditions. To avoid accumulation of BTX and possible problems of floating sludge during settling caused by gas entrained in the sludge flocs, the reactors were also purged with N_2 at the end of each reaction phase. Therefore, the effective residence time of any nonbiodegraded BTX compounds was less than one day.

To encourage the growth of a diverse microbial community while maintaining BTX degrading bacteria in the mixed liquid suspended solids (MLSS), the SBRs were fed with 600 mg/l COD of a biogenic organic substrate including proteins, sugars, and organic acids (3), and amended with 5 mg/l of each BTX compound at the beginning of each cycle. Mineral salts medium was prepared to provide essential nutrients for biomass growth and nitrate as an electron acceptor under anoxic conditions. The concentration of components in the stock mineral salts solution was as following: 0.144 mM $CaCl_2$, 0.044 mM $FeCl_3.6H_2O$, 0.005 mM $CoCl_2.6H_2O$, 0.009 mM $ZnCl_2$, 0.002 mM $CuCl_2.2H_2O$, 0.002 mM H_3BO_3, 0.487 mM $MgSO_4.7H_2O$, 0.021 mM $MnSO_4.H_2O$, 0.002 mM $Na_2MoO_4.2H_2O$, 4 mM KH_2PO_4, and 48 mM NO_3^--N. The concentration of NO_3^--N was slightly reduced for the ANX/MA SBR to avoid the accumulation of NO_2^--N or NO_3^--N. Half of the NO_3^--N provided was from concentrated nitric acid while the rest was from KNO_3. The mineral salts solution was diluted by 1:4 in the SBRs.

The volatile nature of BTX compounds prevented use of diffused air to provide oxygen for the microaerobic conditions. In order to provide oxygen to the laboratory ANX/MA SBR without agitation, we used hydrogen peroxide coupled with excess catalase (7 times the amount needed according to the stoichiometric reaction) to generate dissolved oxygen directly. Therefore, the microaerobic zone was oxygenated by adding 2% (w/w) hydrogen peroxide for 8.5 hours following by 1% hydrogen peroxide for 4 hours, coupled with 1.47×10^4 units/mL catalase (Sigma chemicals, St. Louis, MO) whenever hydrogen peroxide was added. All solutions were added at a flow rate of 0.03 ml/min. The low flow rate was used to avoid significantly changing of the reactor volume. Abiotic tests conducted in the reactor under anoxic and hydrogen peroxide-catalase generated microaerobic conditions showed that the BTX constituents remained in solution for the duration of the reaction phase.

Analytical methods

BTX compounds were analyzed with a Hewlett Packard 5890 gas chromatograph (GC) equipped with a 30 m poly(alkylene glycerol), 0.25 mm i.d. capillary column, with a film thickness of 0.25 μm (Supelco Inc., Bellefonte, PA), and a 5 m guard column. A split injection was used at a ratio of 1:30. The injection port temperature was 250 ℃, and the FID temperature was 260 ℃. Helium was used as a carrier gas at a column head pressure of 15 psi and a column flow rate of 1.46 ml/min. The initial column temperature was 70 ℃ for 10 minutes, then the temperature was ramped at 10 ℃/min to a final temperature of 120 ℃. BTX analytical external standards were made using hexanes as solvent. The BTX concentration in mixed liquid suspended solids samples collected from the reactors was determined by directly extracting with hexanes for 2 hours on a rotary mixer at an extraction ratio of 7 parts sample to 1 part hexanes in a 16 ml vial equipped with a teflon-lined cap. After extraction, the liquid and solvent mixture was separated by centrifugation (1900 ×g) and the extract was transferred to a 2 ml GC autosampler vial and stored at 4 ℃ until analyzed.

Anions including nitrate-N, nitrite-N, and sulfate were analyzed by a Dionex 2010I ion chromatograph (IC) with an IONPAC AS4A-SC column and an electrochemical conductivity detector (Dionex Corp., Sunnyvale, CA). The eluent for the IC was 1.7 mM sodium bicarbonate-1.8 mM sodium carbonate. External anion standards were used. Mixed liquid suspended solids samples were collected from the reactors and centrifuged at 12,100 ×g for 10 minutes and the supernatant was filtered through 0.2 μm Supor® filters (Gelman Sciences, Ann Arbor, MI). prior to analysis.

ORP probes (Cole Parmer Instrument Co., Chicago, IL) and pH/mV meters (Fisher Scientific, Pittsburgh, PA) were used to monitor the ORP in both SBRs. The probes were standardized with Light's solution according to Standard Methods (2). The ORP data reported below are referenced to the silver/silver chloride electrode.

RESULTS AND DISCUSSIONS

Toxicity assessment

To evaluate the possible toxicity caused by using hydrogen peroxide coupled with catalase to provide oxygen in the microaerobic phase of the ANX/MA SBR, specific oxygen uptake rates (SOUR) were measured using biomass from a local domestic wastewater treatment

plant that was not acclimated to hydrogen peroxide (Blacksburg, VA). Two oxygen supply methods, conventional diffused aeration and hydrogen peroxide coupled with catalase, were employed in the tests. SOUR data were collected from 6 independent tests for each oxygenation method (data not shown). At a test level of 5%, a pooled student t test (2 tails, assume equal variance) indicated that the mean of SOUR from each oxygenation method was not significantly different. Therefore, it is concluded that hydrogen peroxide did not impose toxicity on biomass in the ANX/MA SBR.

BTX biodegradation in the ANX SBR

The ANX SBR was maintained for approximately 6 months after stable effluent quality was observed. Figure 1 shows the effluent BTX collected during this stable operating period. It can be concluded from Figure 1 that toluene and *m*-xylene were biodegradable under denitrifying conditions except on the days immediately after intensive samplings, which consumed 1/3 of the biomass volume. Benzene, *o*-, and *p*-xylene were recalcitrant to anoxic biodegradation throughout the study.

A typical performance profile is shown in Figure 2. ORP was maintained between -200 mV and -100 mV under anoxic conditions as shown in Figure 2a. This range concurs with the typical values observed under anoxic conditions (13). Nitrite-N and nitrate-N were used as electron acceptors, as shown in Figure 2b. The presence of nitrite-N at time zero is due to the oxidation of biogenic substrates as the substrates and mineral salts were pumped into the reactor. The accumulation of nitrite occurred due to the influent $COD:NO_3^-$-N ratio and is typical in denitrifying systems (12). Figure 2c shows that it took approximately 6 and 9 hours to completely biodegrade toluene and *m*-xylene in the ANX SBR, respectively. The concentration of benzene, *o*-xylene and *p*-xylene remained relatively constant throughout the reaction cycle, confirming that these compounds were recalcitrant to anoxic biodegradation.

BTX biodegradation in the ANX/MA SBR

All five BTX compounds were biodegraded in the ANX/MA SBR since the effluent BTX concentrations were not detectable (data not shown). A typical profile is shown in Figure 3. Under anoxic conditions, the ANX/MA SBR showed the same pattern as the ANX SBR. When the reactor environment shifted to microaerobic conditions, benzene, *o*-, and *p*-xylene, the
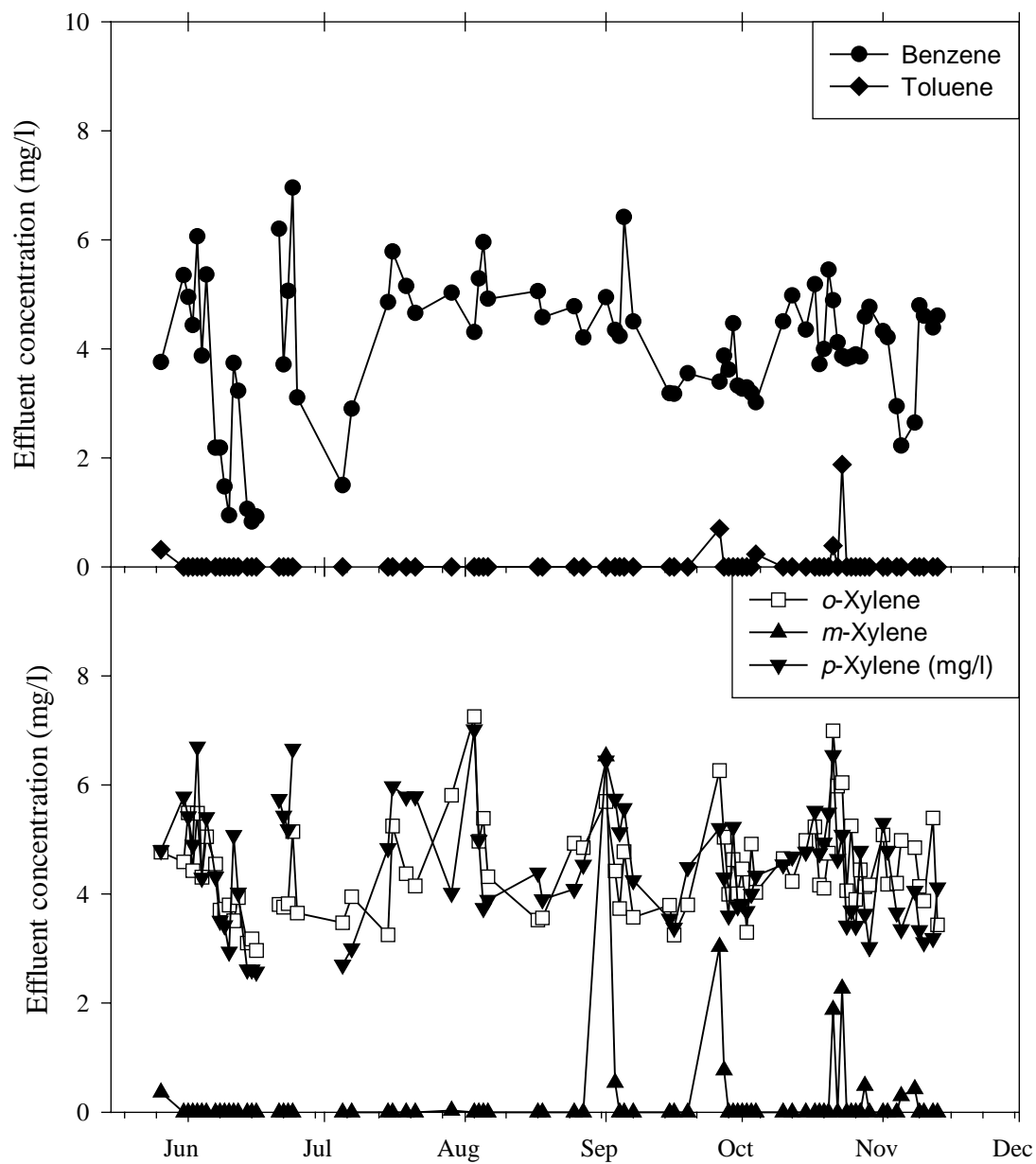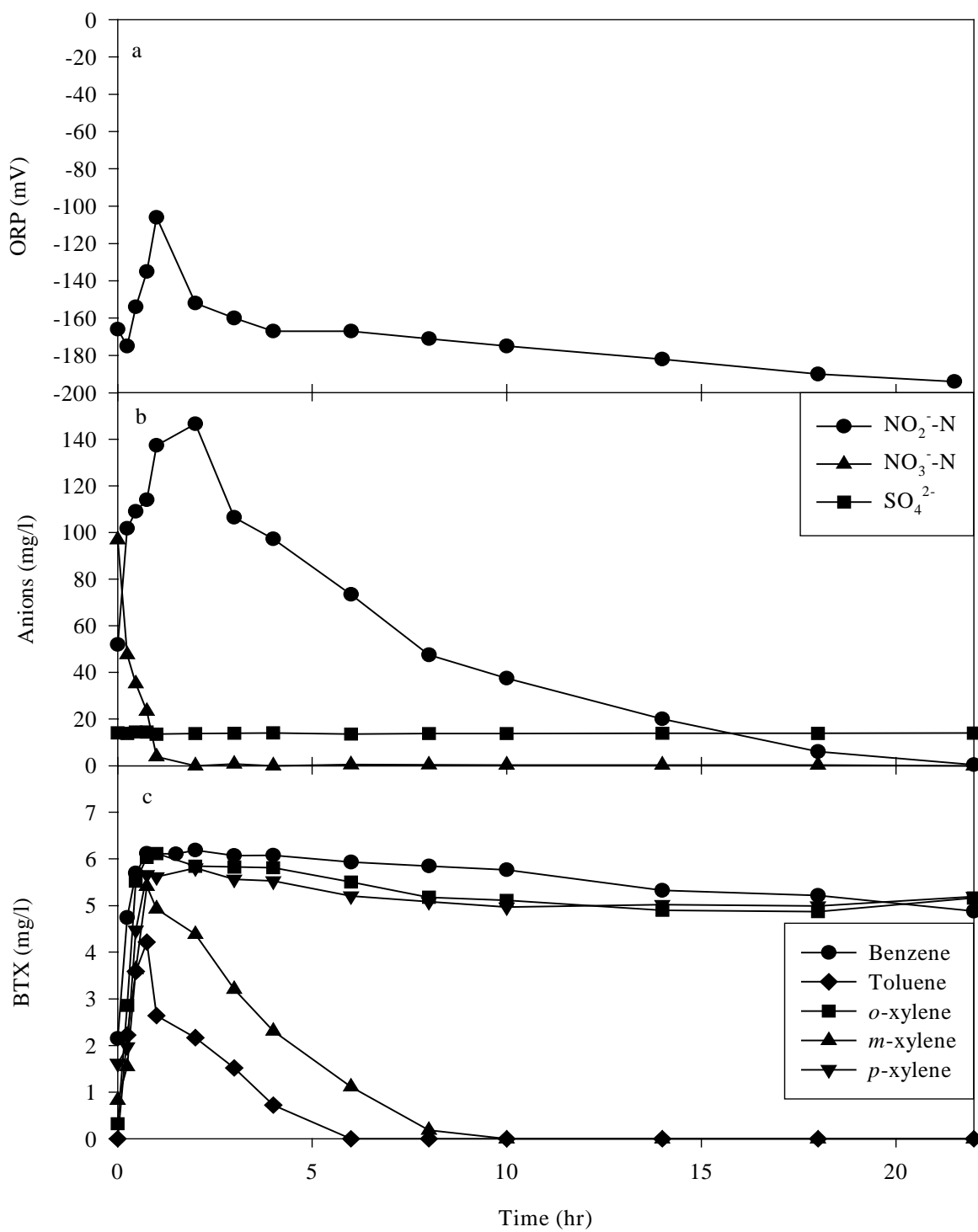
Figure 1. BTX biodegradation in anoxic SBR

Figure 2. Profile of the anoxic SBR (ANX SBR)

compounds that were recalcitrant to anoxic metabolism, were biodegraded under microaerobic conditions as shown in Figure 3c. The anions data in Figure 3b showed that nitrite-N was used as an alternative electron acceptor in addition to oxygen under microaerobic conditions. Initially, we attempted to use a dissolved oxygen (DO) probe with a detection limit of 0.2 mg/l to monitor DO in the microaerobic zone, but DO was routinely below the level of detection. However, Figure 3a shows that the two different redox environments can be distinguished by using an ORP probe. Under microaerobic conditions, the ORP readouts were between -50 mV to -20 mV.

CONCLUSIONS

This study showed that only toluene and *m*-xylene were amenable to anoxic biodegradation while benzene, *o*-, and *p*-xylene were biodegradable under microaerobic conditions which were generated without agitation. It is noteworthy to emphasize that the activated sludge used in this research was exposed to anoxic conditions for over three years (this study plus a previous study), and anoxic benzene biodegradation was never observed during that time. We successfully used an ORP probe to distinguish between microaerobic conditions and anoxic (denitrifying) conditions. Therefore, ORP probes may be used for process control purposes within SBR systems employing microaerobic treatment strategies.

The incorporation of anoxic conditions in sequencing batch reactors for treating BTX containing wastewater has several advantages. Firstly, nitrate-N serves as an electron acceptor for the biodegradation of biogenic substrates; therefore, high oxygen supplies at the beginning of reaction cycles that are commonly seen in SBRs can be eliminated. If nitrate is not generated internally through nitrification, a cost analysis of adding nitrate versus oxygen would have to be conducted to determine the feasibility of this approach. Secondly, since toluene and *m*-xylene are biodegradable under anoxic conditions, the stripping of BTX caused by aeration was reduced. In addition, this study showed that benzene, *o*- and *p*-xylene were amenable to microaerobic metabolism. The stripping of BTX under microaerobic conditions can be minimized by employing bubbleless membrane aeration systems which will minimize vigorous aeration for full scale applications. Therefore, the alternating anoxic/microaerobic SBR may provide an alternative process other than conventional aerobic process for treating wastewaters containing BTX (and possibly other volatile) compounds. This may enable compliance with current U.S. National Emission Standards for Hazardous Air Pollutants (NESHAP) regulations (20).
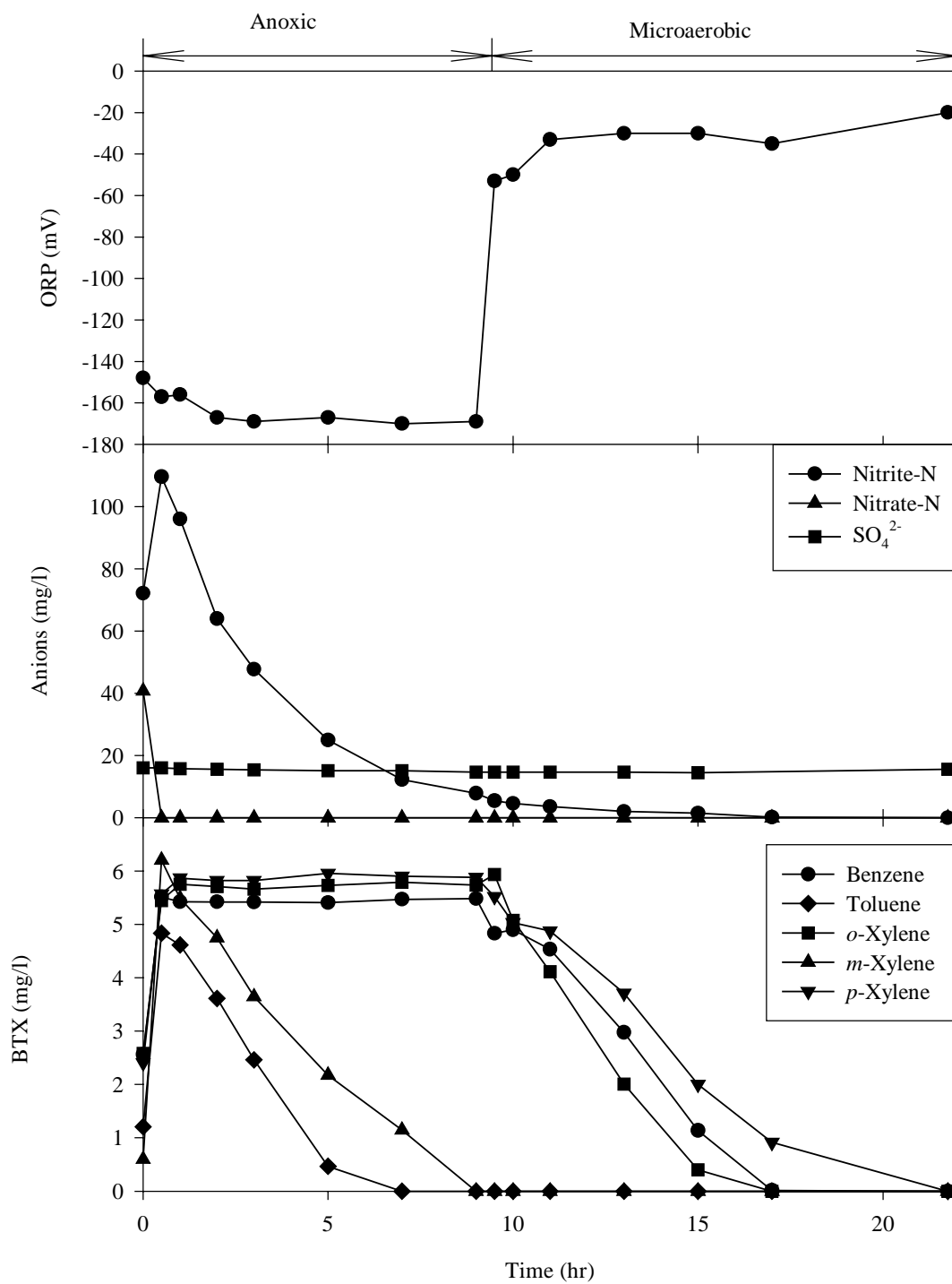
Figure 3. BTX biodegradation in ANX/MA SBR

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CHAPTER 4. THE DISTRIBUTION OF BTX DEGRADERS IN BTX-FED BIOLOGICAL REACTORS UNDER DIFFERENT REDOX CONDITIONS

ABSTRACT

The distribution of representative anoxic, facultative, and aerobic toluene-degraders was studied in anoxic (denitrifying), anoxic/microaerobic/aerobic, and anoxic/microaerobic sequencing batch reactors (SBRs) receiving benzene, toluene, o-, m-, and p-xylene and biogenic substrates. 16S rRNA probes for the investigated toluene-degraders were designed and optimized. The RNA extractions from reference cultures were quantified by a mass ladder approach and used to make standard blots for each respective probe. The results of the dot blot hybridization with reactor samples suggested that reactors with multiple redox environments fostered a more diverse microbial community and the activities of the target organisms were higher than in the single redox reactor. The normalized activities of the target organisms were between 0.2-1.2%. The activities of the facultative toluene-degraders were lower than the activities of the organisms that biodegrade toluene only under anoxic and aerobic conditions in all three SBRs. Our results also suggest that in a biological reactor receiving mixed substrates, the influence of the other substrates on the microbial activities of the organisms degrading a specific compound cannot be ignored.

Keywords: 16S rRNA, dot blot hybridization, microbial activity, toluene-degraders, probe, standard blots

INTRODUCTION

For years, we have studied benzene, toluene, *o*-, *m*-, and *p*-xylene (BTX) biodegradation patterns and enzyme activities in BTX-fed activated sludge sequencing batch reactors under different redox conditions (24) . One of our hypotheses was that exposing a culture to multiple redox conditions would develop a more diverse microbial ecology in a single-sludge activated sludge system. The microbial community in a biological wastewater treatment system defines the system's treatment efficiency; however, knowledge on the effect of reactor configurations on microbial ecology is very limited. This dilemma is slowly being addressed by applying molecular biological tools to investigate the environmental microbial communities.

Currently, techniques which are available to study distributions of specific bacterial groups in mixed cultures fall into two categories: culture-dependent and culture-independent methods. It is well known that in oligotrophic engineered wastewater treatment systems, the microbial communities are dominated by slow growing or dormant bacteria (42); therefore, conventional cultivation dependent methods such as viable plate count or the most-probable-number techniques gave highly biased and inaccurate results when used in identifying microbial communities in those systems (42, 43). More recently, the employment of culture-independent molecular biology techniques targeting 16S rRNA has been able to overcome this drawback and allow researchers to explore oligotrophic microbial ecosystems in more detail. Labeled 16S rRNA probes have been used in studying diverse aspects of environmental interests including: the activities (17) or the distribution (34, 44) of ammonia-oxidizing bacteria in biofilms; the distribution of sulfate-reducing bacteria (SRB) in biofims (28) or in activated sludge (25); identification of a foaming filamentous bacterium in activated sludge (9); the population analysis of denitrifiers in biofilms (26); and the methanogens in anaerobic reactors (38). In these studies, hybridization is carried out either *in situ* or with bulk RNA extracts. It was shown that *in situ* hybridization revealed the best cell number recovery (42) while dot blot hybridization was a valid tool when the metabolic activity of a certain microbial group was of special interest, as ribosome content in cells can be related to metabolic activity levels (39, 42).

A diverse range of electron acceptor conditions may be indicative of the typical environment encountered by microorganisms in nature, which tend to accumulate at the anaerobic/aerobic interface. In a study using aquifer microcosms for aerobic BTX biodegradation, Vermace et al. (41) suggested that cycling between aerobic and anoxic

(denitrifying) conditions resulted in increased number of aerobic toluene-degraders in the microbial consortium as determined by a total heterotrophic plate counting method.

The objective of this work was to investigate the distribution and activities of model BTX-biodegrading bacteria which were metabolically diverse in sequencing batch reactors exposed to a range of redox conditions. Dot blot hybridization was used to characterize the population with probes targeting the 16S rRNA of these bacterial groups. When selecting model bacteria representing strict anoxic, strict aerobic, and facultative BTX-degraders, we fully utilized the current knowledge on BTX-degraders under different redox conditions. In the mean time, efforts were made to isolate BTX-degraders from the laboratory sequencing batch reactors so that they could serve as model organisms known to be present in the original activated sludge.

MATERIALS AND METHODS

Isolation of toluene degraders.

A 500 ml anoxic enrichment sequencing batch reactor (SBR) was set up and inoculated with a toluene-degrading activated sludge from another anoxic SBR (13). The 500 ml enrichment reactor was initially maintained in the same manner as the primary reactor (13), which was fed 5 mg/l of each BTX compound, 600 mg/l as COD of a complex biogenic substrate and nitrate as electron acceptor over two months. The fraction of toluene in the feed to the enrichment reactor was gradually increased from 5 mg/l to 25 mg/l and the concentration of the complex biogenic substrate was reduced until it was completely excluded. The anoxic culture in the enrichment reactor continually received 25 mg/l of toluene as the sole energy and carbon source and sufficient nitrate as electron acceptor for a month before it was transferred to serum bottles for further enrichment. In an attempt to isolate anoxic toluene-degraders, the diluted (dilution ratio is 1:5) mineral salts solution (24) was amended with 0.267 mM NH_4Cl, autoclaved, and equilibrated in an anaerobic bag (Coy Laboratory Products, Inc., Grass Lake, MI) with a headspace of 95% N_2 and 5% H_2 for 24 hours. An aliquot of filter sterilized toluene was then added into the mineral salts solution to reach a final concentration of 5 mg/l before being transferred into 160 ml serum bottles. Serum bottles were capped with teflon-lined mininert valves (Supelco, Inc., Bellefonte, PA). The culture in the serum bottles was incubated under static conditions at 26ºC in the anaerobic bag and transferred into fresh mineral salts solution every week for a period of two months. The enrichment culture was then streaked on FN agar

medium (5) in the anaerobic bag to obtain individual isolates and re-streaked three times from each single colony. Isolates were inoculated into liquid FN medium to obtain a desired culture density under either anoxic or aerobic conditions, depending on test conditions of the next step. These cultures were then tested for their abilities to utilize toluene and the other BTX compounds as sole carbon and energy source in serum bottles under either anoxic or aerobic conditions. Under anoxic conditions, 17 mg/l of nitrate-N was provided in each serum bottle to serve as an electron acceptor. When the BTX biodegradation abilities of isolates were tested under aerobic conditions, nitrate-N was excluded from the mineral salts medium and the sterile mineral salts solution was equilibrated with air over 2 days before being transferred into serum bottles.

The basic morphological characteristics of isolates were examined by phase-contrast microscopy. Gram staining was performed using a Difco Gram staining kit (Difco Laboratories, Detroit, MI). One aerobic toluene-degrading isolate (hereafter designated GM1) was characterized further using phylogenetic methods (described below).

Selecting representative targeting toluene-degraders.

Three bacterial groups that represent anoxic, facultative, and aerobic toluene-degraders were selected based on their metabolic abilities. Table 1 summarizes the model toluene-degrading bacteria selected and their substrate utilization abilities. *Thauera aromatica* (strains T1 and K172) and *Azoarcus* sp. mXyN1 represent strict anoxic toluene degraders which also display some ability to metabolize the xylene isomers under anoxic conditions. All three can grow on biogenic substrates under anoxic or aerobic conditions. *Azoarcus tolulyticus* (strains Tol-4, Td-1, Td-2, Td-3, Td-15, Td-17, Td-19, and Td-21) are facultative toluene degraders, and some strains degrade benzene aerobically or *m*-xylene anoxically. *Pseudomonas putida* (strains F1 and PaW1) and isolate GM1 represent strict aerobic toluene-degraders with varying degrees of ability to biodegrade other BTX compounds. In addition, the *P. putida* strains are strictly aerobic metabolizers in general whereas isolate GM1 metabolizes biogenic substrates in a facultative manner.

Table 1. Investigated BTX-degraders and their substrate utilization abilities

Strain	Benzene		Toluene		o-Xylene		m-Xylene		p-Xyelne		Biogenic substrates		Reference
	Aer	Anx	Aer	Anx	Aer	Anx	Aer	Anx	Aer	Anx	Aer	Anx	
Thauera aromatica T1	-	-	-	+	-	-	-	-	-	-	+	+	(10, 11)
Thauera aromatica K172	-	-	-	+	-	±	-	±	-	±	+	+	(4, 7)
Azoarcus sp. mXyN1	nt	-	-	+	nt	-	-	+	nt	-	+	+	(27)
Azoarcus tolulyticus Tol-4	-	-	+	+	-	-	-	-	-	-	+	+	(14)
A. tolulyticus Td-1	-	-	+	+	-	-	-	-	-	-	+	+	(14)
A. tolulyticus Td-2	-	-	+*	+	-	-	-	-	-	-	+	+	(14)
A. tolulyticus Td-3	+*	-	+*	+	-	-	-	-	-	-	+	+	(14)
A. tolulyticus Td-15	-	-	+	+	-	-	-	+	-	-	+	+	(14)
A. tolulyticus Td-17	+	-	+	+	-	-	-	-	-	-	+	+	(14)
A. tolulyticus Td-19	-	-	+*	+	-	-	-	-	-	-	+	+	(14)
A. tolulyticus Td-21	+	-	+	+	-	-	-	-	-	-	+	+	(14)
Pseudomonas putida F1	+	-	+	-	+	-	+	-	+	-	+	-	(16)
Pseudomonas putida PaW1	+	-	+	-	+	-	-	-	+	-	+	-	(45)
Isolate GM1	-	nt	+	-	-	nt	+	nt	+	nt	+	+	This study

Aer: aerobic; Anx: Anoxic; +: growth as sole carbon source; -: no growth on carbon source; ±: very slow growth; +*: degradation activity often delayed; nt: not tested.

Culture medium and growth conditions.

All strains, except for GM1, were obtained from the American Type Culture Collection (ATCC) (Vienna, VA). *T. aromatica* T1 (ATCC 700625), *A. tolulyticus* Tol4 (ATCC 51758), *P. putida* strain PaW1 (ATCC 33015) and F1 (ATCC 700007) were grown at 30ºC on ATCC medium 2050, 1981, and 3, respectively. *A. indigens* VB32^T (ATCC 51398) and *Escherichia coli* JM109 (ATCC 53323) were grown at 37ºC on ATCC medium 3 and 1065, respectively. Isolate GM1 was grown on FN medium at 30ºC. A Growth curve of each organism except *A. indigens* VB32^T was determined and cells were collected at late-log phase for RNA extraction. The clumpy growth of *A. indigens* VB32^T on ATCC medium 3 made it very difficult to determine a growth curve; therefore, *A. indigens* VB32^T was collected 15 hours after inoculation.

The initial activated sludge inoculum in the anoxic SBR came from a blend of industrial (chemical manufacturing industry) and domestic (Blacksburg, VA) mixed liquors. An anoxic/microaerobic/aerobic SBR was initiated about two years after the anoxic SBR. Three fourths of the inoculum in this reactor came from the anoxic reactor and the rest from a fresh sample of the domestic mixed liquor. The anoxic/microaerobic/aerobic SBR was reconfigured to an anoxic/microaerobic SBR after being operated for one year. Additional information on operation and performance of the SBR systems are given elsewhere (24).

Activated sludge sample collection and preservation.

At each designated sampling point, 0.7 ml of activated sludge from sequencing batch reactors was added into 2 ml screw-cap vials (VWR Scientific Products, West Chester, PA 19380) containing 1.5 g sterilized glass beads (0.1 mm diameter, Biospec Products, Bartlesville, OK). Vials were immediately frozen at -20ºC, then were transported to a -50ºC freezer within 24 hours, and were kept in the freezer until analyzed. Pure culture samples samples were prepared in the same manner.

Nucleic acid extraction.

Vials containing samples and glass beads received 50 µl of 10% SDS and the rest of the volume was filled with warm (60ºC) pH=4.3 phenol (Fisher Scientific, Pittsburgh, PA)(33) in order to preferentially extract RNA. For preferential extraction of DNA, room temperature pH 8.0 phenol was used instead. Cells were beaten by mechanical disruption on a mini-beadbeater

(Biospec Products, Bartlesville, OK) at a speed of 4600 rpm for 90 seconds twice with a 30 second interval. The mechanical disruption insured uniform extraction of nucleic acids from a variety of microorganisms including gram-positive and gram-negative bacteria. The aqueous phase was collected and further extracted at least twice with phenol:chloroform:isoamyl alcohol (100:24:1, prewarmed to 60ºC) until the aqueous-organic interface was clear. The aqueous phase was then extracted with chloroform:isoamyl alcohol (24:1) once to remove residue phenol. The extracted nucleic acids were precipitated at -50ºC overnight in 1/10 volume of 4 M LiCl and 2 volumes of pre-chilled ethanol.

After being vacuum dried, ethanol precipitated RNA was resuspended in RNA storage buffer (10 mM Tris-HCl, pH=8.0, 0.1 M NaCl, 1 mM $MgCl_2$) whereas DNA was resuspended in sterile deionized and distilled water.

It is well known that RNA is subject to degradation by RNases (21). In addition, some regions in the rRNA are more vulnerable to RNase attack than others, which may have a detrimental effect on the quantitative hybridization procedure (29). As a consequence, it is important to work with intact 16S rRNA when performing quantitative hybridization (see below). In this research, all solutions used for RNA extraction and storage were treated with diethyl pyrocarbonate (DEPC) according to Sambrook et al. (32) in order to inhibit RNase activity. Additionally, glassware was baked at 475ºC for 6 hours and disposable RNase-free plastic tubes were used. All the RNA extractions from the reactor samples were checked by 1.5% agarose gel electrophoresis to confirm the integrity of 16S rRNA before dot blot hybridization was performed.

16S rRNA amplification, sequencing, and phylogenetic analysis.

Isolate GM1 was phylogenetically characterized by amplifying and sequencing the 16S rRNA gene. A polymerase chain reaction mix (100 µl) was prepared and contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 800 µM of dNTP mixture (Roche, Molecular Biochemicals, Indianapolis, IN), 1 µM each of forward primer S-D-Bact-0011-b-S-20 and reverse primer S-D-Bact-1492-b-A-21 (see Table 2), 5 U of Taq polymerase (Fisher Scientific, Pittsburgh, PA), and 90 ng of template DNA (extracted following the method described below) . The primers used here are slightly different from the sequences published previously (19) in that the first three nucleotides in the primer S-D-Bact-0011-b-S-20 are extra

and the 5th nucleotide in primer S-D-Bact-1492-b-A-21 is T instead of Y (C or T) in the previously published sequence. A DNA thermal cycler (Perkin-Elmer, Norwalk, CT) was used and programmed as follows: (A) an initial denaturing temperature of 95ºC for 5 min; (B) a run of 30 cycles, with each cycle consisting of 1 min at 95ºC (denaturing), 1 min at 60ºC (annealing), and 4 min at 72ºC (elongation), and (C) 5 min at 72ºC to allow for final elongation. The reaction mixture was held at 4ºC until it was collected. 10 µl of the PCR product was viewed by electrophoresis in a 1.0% agarose gel stained with ethidium bromide. The rest of the PCR product was purified with the Qiagen PCR purification kit (Qiagen Inc., Valencia, CA). The purified PCR products was sequenced by the DNA Facility at the University of Iowa with two additional internal primers, S-*-Univ-0907-a-A-22 (3) and S-D-Bact-0704-a-S-20 (37).

The SeqMan program in Lasergene99 was used to assemble the 16S rRNA gene sequence of isolate GM1 based on the location of the four primers, and the MegAlign program was used to generate a phylogenetic tree. All other 16S rRNA gene sequences considered were retrieved from either Genebank or European Molecular Biology Laboratory (EMBL).

Oligonucleotide probe design, labeling, and T_d determination.

The retrieved 16S rRNA gene sequences of the target and closely related non-target organisms were aligned using MegAlign in Lasergene99. The sequences that are consensus among target organisms, but have mismatches to non-target organisms were

Table 2. List of primers

Primer[a]	Sequence 5'-3'	Position[b]	Specificity	Reference
S-D-Bact-0008-b-S-20	AGAGTTTGATCCTGGCTCAG	8-27	Domain *Bacteria*	(19)
S-D-Bact-1492-b-A-21	ACGGTTACCTTGTTACGACTT	1472-1792	Domain *Bacteria*	(19)
S-*-Univ-0907-a-A-22	CCCCGTCAATTCCTTTGAGTTT	886-907	Universal	(3)
S-D-Bact-0704-a-S-20	GTAGCGGTGAAATGCGTAGA	704-723	Domain *Bacteria*	(37)

a: The nomenclature is standardized according to the methods suggested by Alm et al. (1); b: *E. coli* 16S rRNA numbering (NCBI accession number J01859).

checked with the Blast program at the National Center for Biotechnology Information (NCBI) and the Check_Probe program supported by the Ribosomal Database Project (RDP). Table 3 lists the 16S rRNA probes used in this research. Probe S-*-Tarom-0162-a-A-24 (Tarom162)

targets the strict anoxic toluene-degraders including *T. aromatica* strains K172 and T1 and *Azoarcus* sp. mXyN1. Probes S-St-PpaW-0816-a-A-21 (PpPaW816), S-St-PpF1-0865-a-A-21 (PpF865), and S-St-GM1-0997-a-A-21 (GM997) are strain-specific probes targeting *P. putida* PaW1, *P. putida* F1, and isolate GM1, respectively. Probe S-S-Atol-0484-a-A-18 (Atol484) targets *A. tolulyticus* strains Tol-4, Td-1, Td-2, Td-3, Td-15, Td-17, Td-19, and Td-21. Bacteria domain-specific probe S-D-Bact-0338-a-A-18 (EUB338) serves as a universal bacterial probe in this research. All of the probes were labeled with digoxygenin (DIG) at the 3' end using the oligonucleotide 3'-end labeling kit (Roche Molecular Biochemicals, Indianapolis, IN). Check_Probe program indicated that none of the probes have secondary structures except S-*-Tarom-0162-a-A-24 which has a potential for development of secondary structures.

The half dissociation temperature (T_d) of a probe is defined as the temperature at which 50% of the probe-target duplex remains intact during a specified washing period (40). A T_d of each probe, whether it was previously or newly designed, was experimentally determined. RNA samples from target organisms were applied on positively charged nylon membranes (Roche Molecular Biochemicals, Indianapolis, IN) and were hybridized according to the method described below. After hybridization, the membranes were submerged in 1×SSC solution (0.15 M NaCl and 0.015 M sodium citrate) and were cut into individual strips. Each strip was washed three times for ten minutes in 20 ml 2×SSC washing solution (0.3 M NaCl, 0.03 M sodium citrate, and 3.5 mM sodium dodecyl sulfate) following by three additional washings in 0.1×SSC washing solution (0.015 M NaCl, 0.0015 M sodium citrate, and 3.5 mM sodium dodecyl sulfate) at the desired temperature. As shown in Figure 3, this washing step was repeated 12 times over a range of temperatures (30°C, 33°C, 36°C, 39°C, 42°C, 45°C, 48°C, 51°C, 54°C, 57°C, 60°C, 68°C). The amount of probe that remained attached to the strip after washing was detected and quantified following the methods described below.

Table 3. Probe data

Binding position[a]		Sequence 5'-3'	Specificity	T_d (ºC)	Reference
0338-0355	Probe: S-D-Bact-0338-a-A-18 (EUB338)	GCTGCCTCCCGTAGGAGT	Domain *Bacteria*	59	(2)
	E. coli JM109[b]	ACUCCUACGGGAGGCAGC			
0162-0185	Probe: S-*-Tarom-0162-a-A-24 (Tarom162)	GACGTATGCGGTATTAGCGTACC T	*T. aromatica* strain T1 and	47	This study
	T. aromatica T1[b]	AGGUACGCUAAUACCGCAUACGUC	K172, and *Azoarcus* sp.		
	P. putida PaW1[c]	· · · A ·	mXyN1		
0484-501	Probe: S-S-Atol-0484-a-A-18 (Atol484)	GCTTCTTC TGACAGTACC	*A. tolulyticus* strains Tol-4,	41.5	(47)
	A. tolulyticus Tol-4[b]	GGUACUGUCAGAAGAAGC	Td-1, Td-2, Td-3, Td-15,		
	A. indigens VB32[c]	· · · · · C· · A · · · · · · · · ·	Td-17, Td-19, Td-21		
0816-836	Probe: S-St-PpPaW-0816-a-A-21 (PpPaW816)	TCC ATCGGCTAGTTGACATCG	Strain *P. putida* PaW1	45	This study
	P. putida PaW1[b]	CGAUGUCAACUAGCCGAUGGA			
	P. putida F1[c]	· · · · · · · · · · · · · · · · · U · · · ·			
0865-0885	Probe: S-St-PpF1-0865-a-A-21 (PpF865)	CCAGGGGGTCAACTTAATGCG	Strain *P. putida* F1	45	This study
	P. putida F1[b]	CGCAUUAAGUUGACCCCUGG			
	P. putida PaW1[c]	· · · · · · · · · · · · · · · G· · · · ·			
0997-1017	Probe: S-St-GM1-0997-a-A-21 (GM997)	CATCTCTGGCAGGTTCTCAGC	Isolate GM1	47	This study
	Isolate GM1[b]	GCUGAGAACCUGCCAGAGAUG			
	P. aeruginosa[c]	· · · · · · · · U · U· · · · · · · · · ·			

a: *E. coli* 16S rRNA sequence numbering; b: positive control; c: negative control. Dot indicates the same nucleotide as the above

sequence.

Preparation of standard blots.

Standard blots for each probe were prepared by using known amount of 16S rRNA extracted from target organisms and non-target organisms. The mass of RNA was determined using a mass ladder approach (8). In this approach, the RNA extraction was quantified by 1.5 % agarose gel electrophoresis using a series dilution of precision molecular DNA mass standards (Bio-Rad Laboratories, Hercules, CA) and staining with vistra green nucleic acid stain (Amersham Pharmacia Biotech, Piscataway, NJ). The gel was scanned with a Storm$^\circledR$ 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and the intensity of bands was quantified using ImageQuant$^\circledR$ (Molecular Dynamics, Sunnyvale, CA). The intensities of the series dilution of precision molecular mass standard were used to construct a standard curve. Only the samples that had intensities falling within the standard curve were used to determine the mass of the RNA extraction. To minimize the degradation of RNA, the RNA extractions from target and non-target organisms were blotted onto positively charged nylon membranes as described below immediately after standard curves were determined. The mass of RNA loaded on the membranes ranged from 400pg to 5pg (mass determined by precision molecular DNA mass standard approach).

Dot blot hybridization and probe-target detection.

RNA was denatured by addition of 3 volumes of 2 % glutaraldehyde immediately before making series dilutions in 1µg/ml poly(A) (Sigma, St. Louis, MO). 10 µl of each dilution was applied on positively charged nylon membranes by using a dot blot device (Bio-Rad Laboratories, Hercules, CA) under slight vacuum. The RNA was fixed to the membranes with a UV crosslinker (Fisher Scientific, Pittsburgh, PA). The membranes were prehybridized in 20 ml/100 cm^2 of hybridization solution (5×SSC, 0.1% N-lauroylsarkosine, 0.02% sodium dodecyl sulfate, 1% blocking reagent (Roche Molecular Biochemicals, Indianapolis, IN)) at 68°C for an hour. Hybridization was carried out by adding labeled probes (10× the amount of RNA) in 5 ml/100 cm^2 of the hybridization solution at 30°C for 12-16 hours. The probes were denatured at 68°C for 5 minutes, except S-*-Tarom-0162-a-A-24 (Tarom162) at 90°C was denatured to disrupt any secondary structures before being added to the hybridization tubes. Hybridized membranes were subsequently washed at previously determined T_d temperatures three times for 10 minutes with 20 ml 2×SSC and three additional washings with 0.1×SSC washing solution.

The probe-target duplex was detected with anti-DIG alkaline phosphatase and a chemiluminescent substrate CSPD (Roche Molecular Biochemicals, Indianapolis, IN) according to the procedure provided by the manufacturer with the following modifications: membranes were incubated at 37°C for 20 minutes and at room temperature for an additional 2 hours. The luminescent light emission was then recorded on X-ray films (Roche Molecular Biochemicals, Indianapolis, IN) for 3 hours. Signal intensity was quantified directly from films with a dual-wavelength flying-spot scanner (Shimadzu Corporation, Japan) using a transmissive photo mode at a wavelength of 600nm.

When hybridizing reactor samples, standard blots (see below) including target and non-target organisms were hybridized, washed, and detected for each probe simultaneously with reactor samples. The intensities of the standard blot for each probe were used to generate a standard curve. Only the dilution of reactor samples that had an intensity within the linear range of the standard curve were used for quantification. Probe S-D-Bact-0338-a-A-18 hybridizes with the domain Bacteria; therefore, it served as a universal bacterial probe. The specific microbial activity based on our probing technique is defined as follows:

$$\text{specific microbial activity using probe A (\%)} = \frac{\dfrac{\text{RNA mass for target organism in sample determined by probe A standard}}{\text{spectrophotometric mass of sample RNA loaded}}}{\dfrac{\text{RNA mass for all bacteria in sample determined by universal standard}}{\text{spectrophotometric mass of sample RNA loaded}}} \times 100$$

RESULTS AND DISCUSSIONS

Identification of the isolate GM1.

Initial efforts were made to isolate colonies on m-R2A plates (14) either in the presence or in the absence of toluene vapor. However, the isolates obtained were not able to biodegrade toluene under denitrifying conditions. Figure 1 shows the utilization of each of the BTX compounds as a single substrate by the isolate GM1 under aerobic conditions. Toluene, *m*-, and *p*-xylene were used as sole carbon and energy sources. Sulfate concentrations in the tests remained constant (data not shown), indicating sulfate is not related to toluene, *m*-, and *p*-xylene biodegradation. Tests conducted under anoxic conditions showed that the isolate GM1 did not biodegrade toluene using nitrate as an electron acceptor (data not shown).
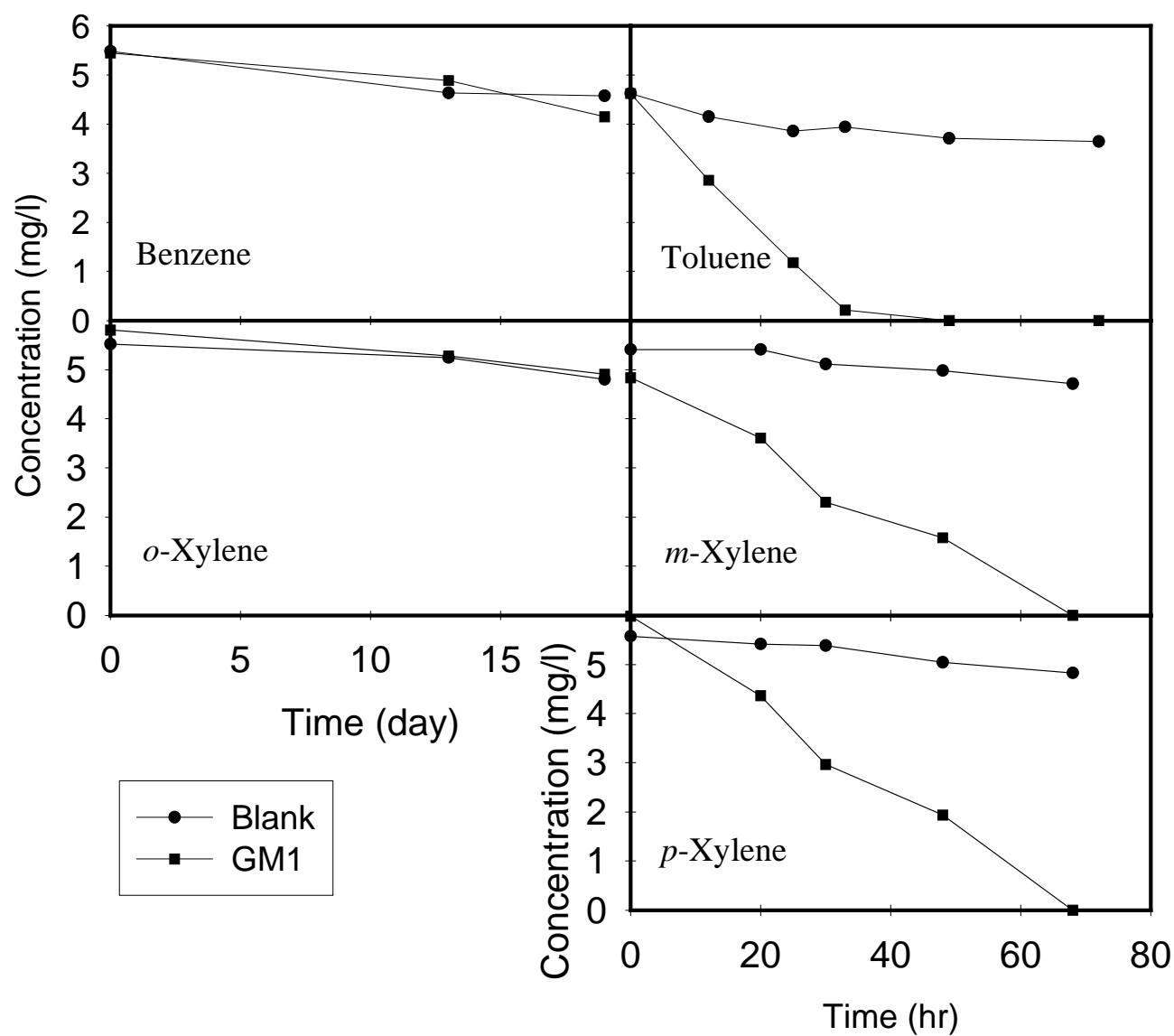
Figure 1. The utilization of BTX compounds as single substrates by isolate GM1 under aerobic conditions.

The isolate GM1 was determined to be a gram negative rod. The Blast program in NCBI was used to compare the Lasergene assembled 16S rRNA gene sequence of GM1 with other sequences in the Genebank. The results indicated that the sequence of GM1 has over 99% similarity with *Pseudomonas stutzeri*. *P. stutzeri* strains have been reported to biodegrade naphthalene (15, 31) and *o*-xylene (6). Further investigation is needed to determine whether the isolate GM1 is a new strain.

Phylogenetic tree.

A phylogenetic tree of all of the selected target toluene-degraders and their negative controls is illustrated in Figure 2. *A. tolulyticus*, a group of facultative toluene-degraders, is more closely related to *T. aromatica* strains T1, *T. aromatica* K172 and *Azoarcus* sp. mXyN1 than to the aerobic toluene-degraders including *P. putida* strains F1, *P. putida* PaW1, and isolate GM1. *Azoarcus* sp. mXyN1 is genetically closely related to *Thauera* genus although it is currently classified as *Azoarcus* genus (NCBI accession number X83533). Isolate GM1, currently identified as a strain of *P. stutzeri*, is located within the cluster of *Pseudomonas* genus.

Probe specificity.

The specificities of the probes designed in this research were checked with the Blast program in NCBI and Check_Probe program in RDP; no exact matching complements were found. Non-target organisms were selected to serve as negative controls and have at least one mismatch in the sequences. However, the probe S-S-Atol-0484-a-A-18 (Atol484) targeting *A. tolulyticus* strains Tol-4, Td-1, Td-2, Td-3, Td-15, Td-17, Td-19, and Td-21, obtained from Zhou et al.(47), has a perfect match with *A. evansii* KB740, a benzoate-degrading denitrifier (4). Consequently, in dot blot experiments with reactor samples or any other environmental samples, it is possible that this or other closely related nontarget organisms hybridized to probe S-S-Atol-0484-a-A-18 (Atol484). Two different oligonucleotide probes targeting the same *A. tolulyticus* group were proposed by Hess et al.(18); however, these probes also detect *A. evansii* KB740.
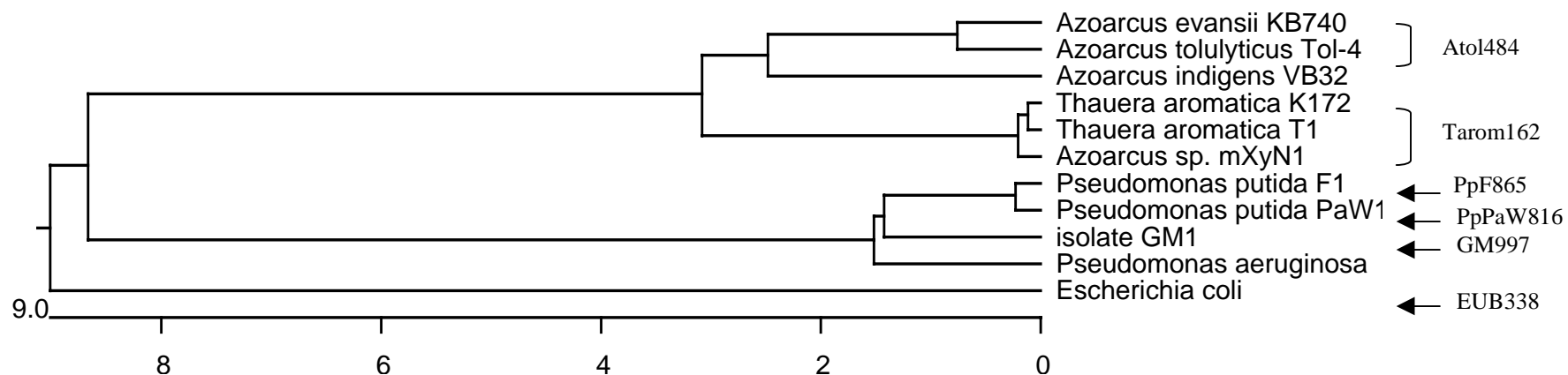
Figure 2. Phylogenetic relationship among the investigated toluene-degraders and their negative controls. The scale beneath the tree measures the distance between sequences; units indicate the number of substitution events as defined by Lasergene (20).

A. indigens VB32 serves as a negative control for probe Atol484;

P. putida PaW1 serves a as a negative control for probes Tarom162 and PpF865;

P. putida F1 serves as a negative control for probe PpPaW816;

P. aeruginosa serves as a negative control for probe GM997.

Optimum washing temperature determination.

Figure 3 shows the optimum washing temperature determined for each probe used in this research. The optimum washing temperature of probe S-D-Bact-0338-a-A-18 (EUB338) was determined as 59 ºC by coworkers in the same laboratory using the same approach (data not shown). The dissociation temperatures of probes S-*-Tarom-0162-a-A-21 (Tarom162), S-S-Atol-0484-a-A-18 (Atol484), S-St-PpPaW-0816-a-A-21 (PpPaW816), and S-St-GM1-0997-a-A-21 (GM997) were determined as 47ºC, 41.5ºC, 45ºC, and 47ºC, respectively. The optimum washing temperature was adjusted to 45ºC for the probe S-St-PpF1-0865-a-A-21 (PpF865) in order to exclude the contribution of probe retention by non-target organisms. Although negative controls were not included in the experiments to determine T_d for probes Tarom162, Atol484, and PpPaW816, a negative control for each probe was used in the dot blot hybridization with all reactor samples (the amount of RNA blotted on the membranes was the same as the positive control). These negative controls did not show detectable hybridization signals at the determined optimum washing temperatures.

Standard blots.

A standard blot serves as a standard for the hybridization of the respective probe with environmental samples. Taking the preparation of standard blots for the probe of S-D-Bact-0338-a-A-18 (EUB338) as an example, Figure 4 shows the agarose gel image of *E. coli* RNA and the molecular DNA mass ladder standards to quantify an *E. coli* RNA extraction.

Although many studies have been conducted on using oligonucleotide probes to investigate microbial activities in environmental samples, the methodology of quantification remains unclear. It was reported that RNA extractions from reference organisms were used to generate standard curves, thus making it possible to relate the specific probes to the universal probe (29, 29, 30, 46, 46). RNA extractions from the reference organisms were measured by a spectrometer, assuming that 1 mg of RNA per ml is equal to 20 optical density units at a wavelength of 260 nm (30). Although considered as a conventional method, this practice is questionable since the presence of DNA and large-subunit rRNA will contribute to the spectrometric readings. The employment of the mass ladder approach eliminated the problems associated with the spectrometric quantification method in that only 16S rRNA bands were
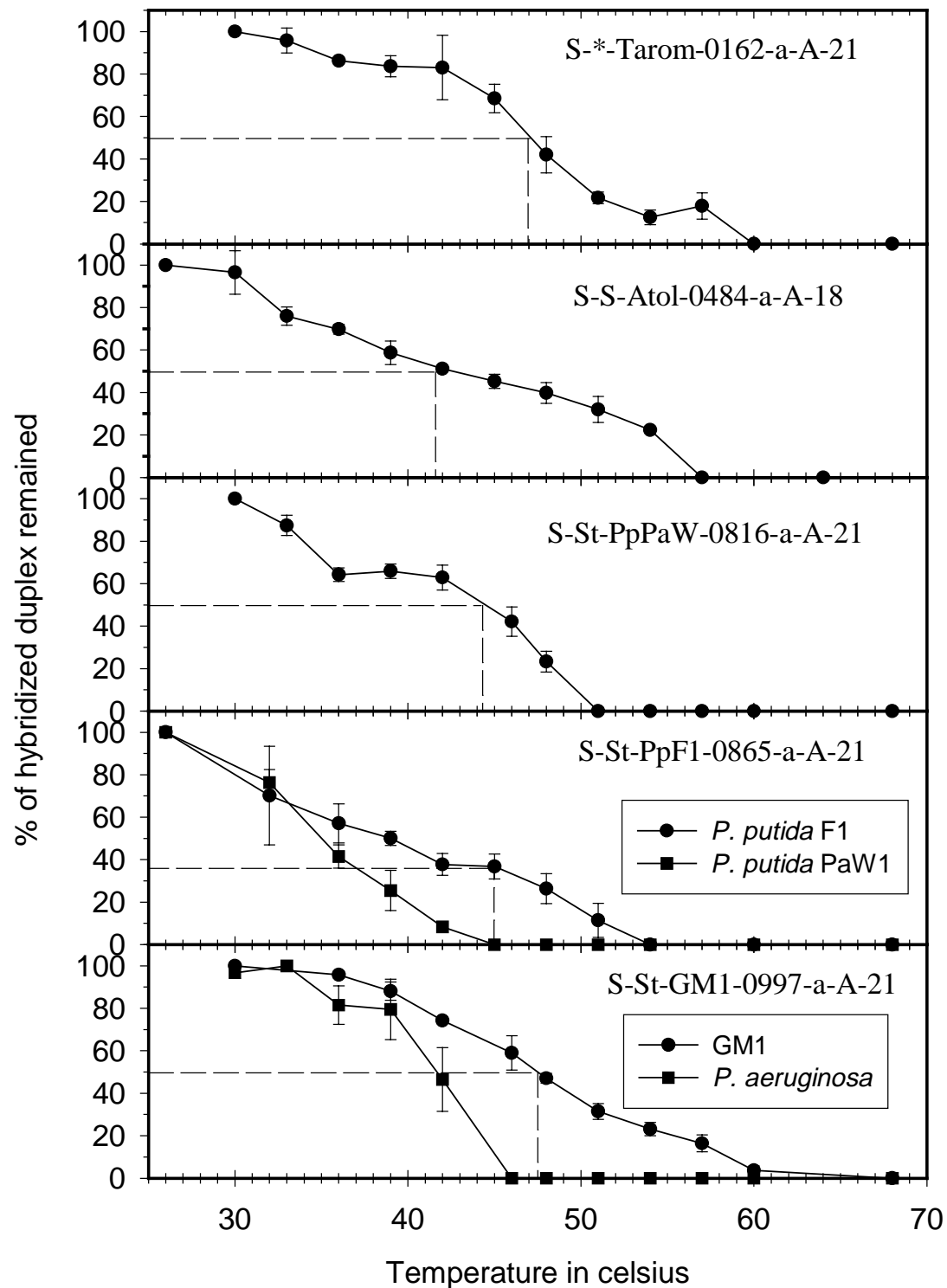
Figure 3. Probe T_d determination for five 16S rRNA probes used. T_d was selected to be the point at which 50% of hybridized duplexes remained, except for S-St-PpF1-0865-a-A-21 where a higher T_d was selected to improve probe specificity.

Figure 4. Vistra Green-stained gel with mass ladder and *E. coli* extractions

Lane 1-6: *E. coli* RNA extraction, 34ng, 20ng, 16ng, 12ng, 8ng, 4ng (per mass ladder)

Lane 7-11: Mass ladder, 40ng, 30ng, 20ng, 10ng, 5ng, (mass for 1000bp band)

Imaged by Storm 860 PhosphorImager

23S rRNA bands

16S rRNA bands

1000bp mass ladder standard
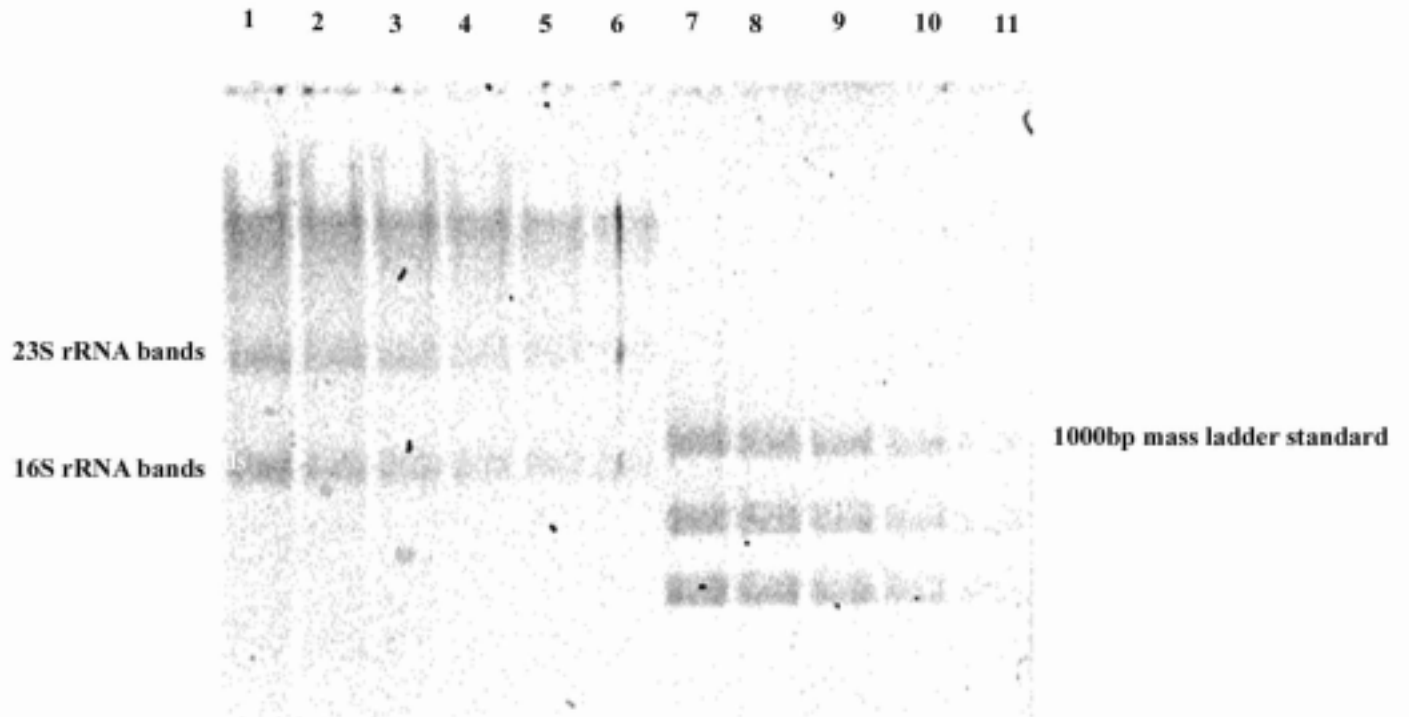
quantified after the separation of 16S rRNA from other forms of nucleic acids by gel electrophoresis.

Dot blot hybridization with reactor samples.

　　Anoxic sequencing batch reactor (ANX SBR). Figures 5A, B, C, D and E show the microbial activities of reactor samples associated with probes Tarom162 (targeting *T. aromatica* strains K172, T1, and *Azoarcus* sp. mXyN1), Atol484 (targeting *A. tolulyticus*), PpPaW816 (targeting *P. putida* PaW1), PpF865 (targeting *P. putida* F1), and GM997 (targeting isolate GM1), respectively. The microbial activities exhibited by probe Tarom162 in Figure 5A corresponded to the profiles of toluene and *m*-xylene biodegradation and enzyme activities shown in Figure 1 in our previous paper (24). This suggests that the group of anoxic toluene-degraders contributed to the biodegradation of toluene and *m*-xylene. The probe PpPaW816 targeting the strict aerobe, *P. putida* PaW1, did not produce quantifiable hybridization signals as shown in Figure 1D. On the other hand, both *A. tolulyticus* and GM1 are able to consume biogenic substrates via anoxic metabolic pathways; therefore, it is not surprising that probes targeting these organisms detected activity in the anoxic SBR, although their activities appear low (see Figures 5B and 5E). Unlike *P. putida* PaW1, the probe PpF865 targeting strict aerobe *P. putida* F1 showed sufficient hybridization signal for detection in the anoxic SBR as shown in Figure 5C. This may be due to the nonspecific binding of this probe with nontarget organisms, although the optimum washing temperature was adjusted as discussed above to exclude false positive signals. It is also possible that the probe is capturing the signal of a facultative variant that is closely related to *P. putida* F1.

　　Anoxic/Microaerobic/Aerobic sequencing batch reactor (ANX/MA/AER SBR). The microbial activities of toluene-degraders in the ANX/MA/AER SBR were summarized in Figure 6. In general, the microbial activities in the ANX/MA/AER SBR were higher than the activities in the ANX SBR, although the activities generated by probe Atol484 in Figure 6B remained lower compared to the rest. PpPaW816, which did not show activity in the ANX SBR, was detected in the ANX/MA/AER SBR.
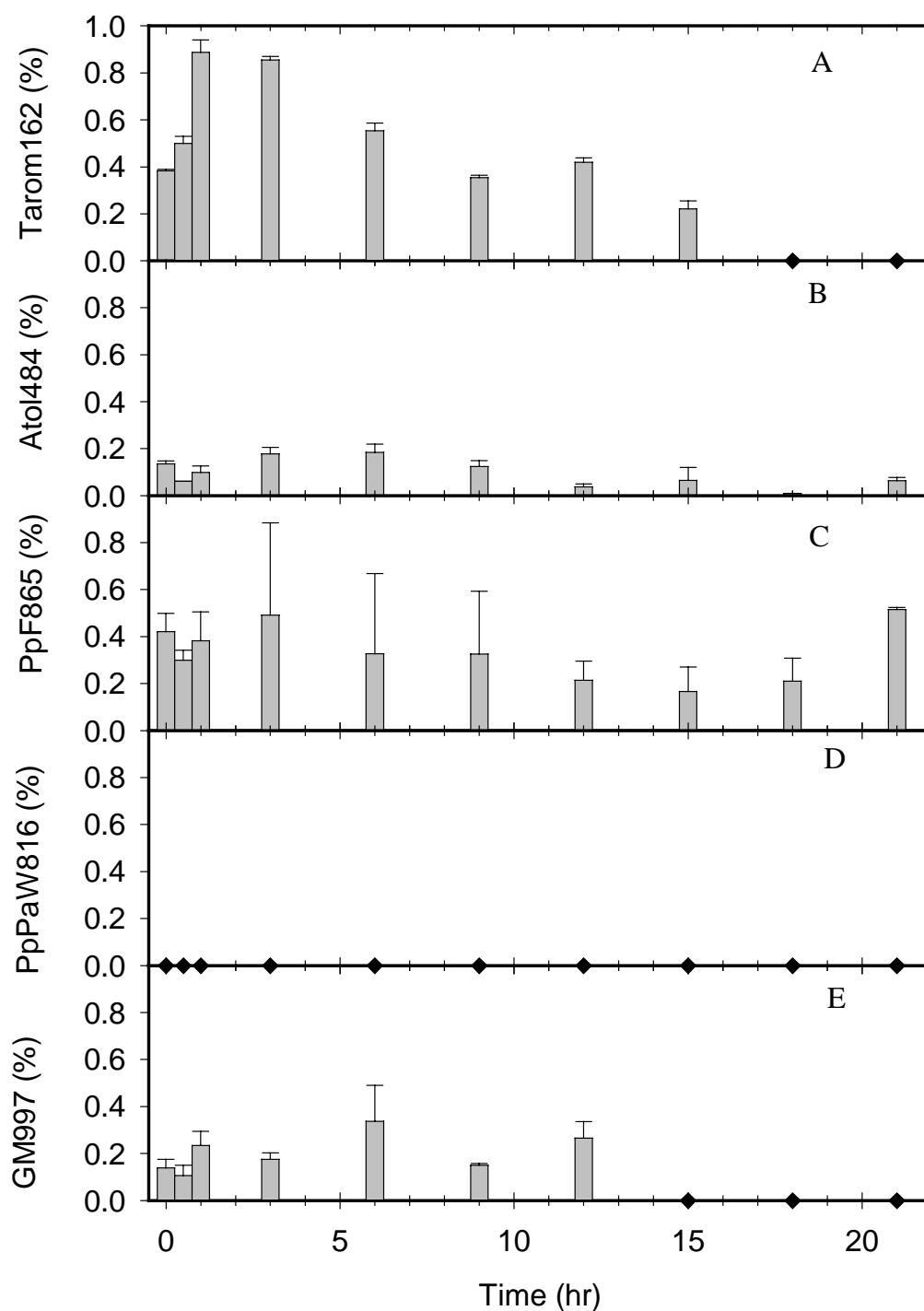
Figure 5. The distribution of toluene-degraders in ANX SBR, (A) Probe Trom162, (B) Probe Atol484, (C) Probe PpF865, (D) Probe PpPaW816, (E) Probe GM997 Diamond symbols indicate non-detectable hybridization signals. Error bars represent ± one standard deviation and are not visible for some samples due to low variability.

Unlike in the ANX SBR, the activities associated with probe Tarom162 (shown in Figure 6A) did not diminish over the course of the reaction cycle. The targeting organisms, *T. aromatica* T1 and K172 (4, 37) and *Azoarcus* sp. mXyN1 (27), have been shown to be capable of aerobically biodegrading benzoate and other oxygen-dependent BTX biodegradation intermediates. These anoxic toluene-degraders in the ANX/MA/AER SBR may scavenge such intermediates generated by the oxygen-dependent BTX biodegradation pathways as well as biogenic substrates; therefore, they managed to retain high rRNA contents under microaerobic and aerobic conditions. *P. putida* strains PaW1 and F1 are strict aerobic organisms. In addition to biodegrading biogenic substrates, they are able to biodegrade benzene, *o*-, and *p*-xylene, the three BTX compounds available under aerobic conditions. Probes PpPaW816 and PpF865 show detectable hybridization signals under aerobic conditions (Figures 6C and 6D). However, activity levels remained high during the ANX phase. Target organisms may retain rRNA after settling and into the ANX zone, even though they are not growing. Alternatively, the probe PpF865 may target closely related organisms that are facultative on biogenic substrates. Additionally, GM1 can utilize nitrate as an electron acceptor to uptake biogenic substrates under anoxic conditions while it biodegrades *p*-xylene under aerobic conditions. This may contribute to the observation of the relatively constant activities shown in Figure 6E under anoxic, microaerobic, and aerobic conditions.

Anoxic/Microaerobic sequencing batch reactor (ANX/MA SBR). Figure 7 summarizes the activities of the investigated bacteria group in the ANA/MA SBR. As in the ANX/MA/AER SBR, the bacteria in the ANX/MA SBR showed higher activities than in the ANX SBR. In addition, microbial activities associated with probes Tarom162, Atol484, PpPaW816, and PpF865 showed similar activity patterns (Figures 7A, 7B, 7C and 7D, respectively) as in the ANX/MA/AER SBR (Figures 6A, B, C, and D). Probe GM997 showed higher hybridization signals in the ANX/MA SBR (Figure 7E) than in the ANX/MA/AER SBR (Figure 6E). As under aerobic conditions, the ability of maintaining high rRNA contents of the anoxic toluene-degraders under microaerobic conditions may be due to the consumption of BTX biodegradation intermediates formed by oxygen-dependent processes as well as biogenic substrates. Under microaerobic conditions, the activities associated with probes PpPaW816 and PpF865 (Figures 7C and D, respectively) are comparable to the levels observed under aerobic conditions (Figures 6C and D).
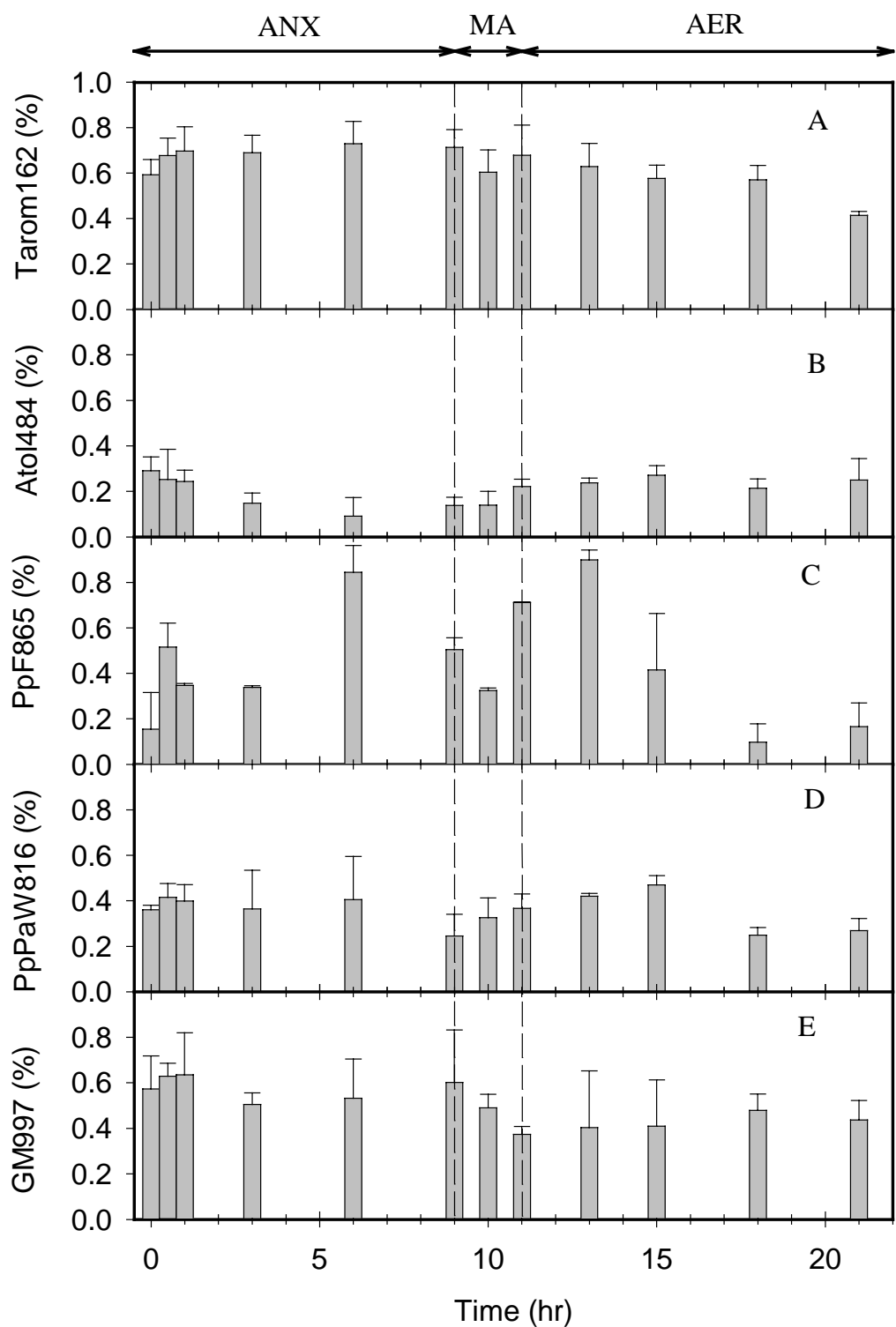
Figure 6. The distribution of toluene-degraders in ANX/MA/AER SBR, (A) Probe Trom162, (B) Probe Atol484, (C) Probe PpF865, (D) Probe PpPaW816, (E) Probe GM997. Error bars represent ± one standard deviation and are not visible for some samples due to low variability.
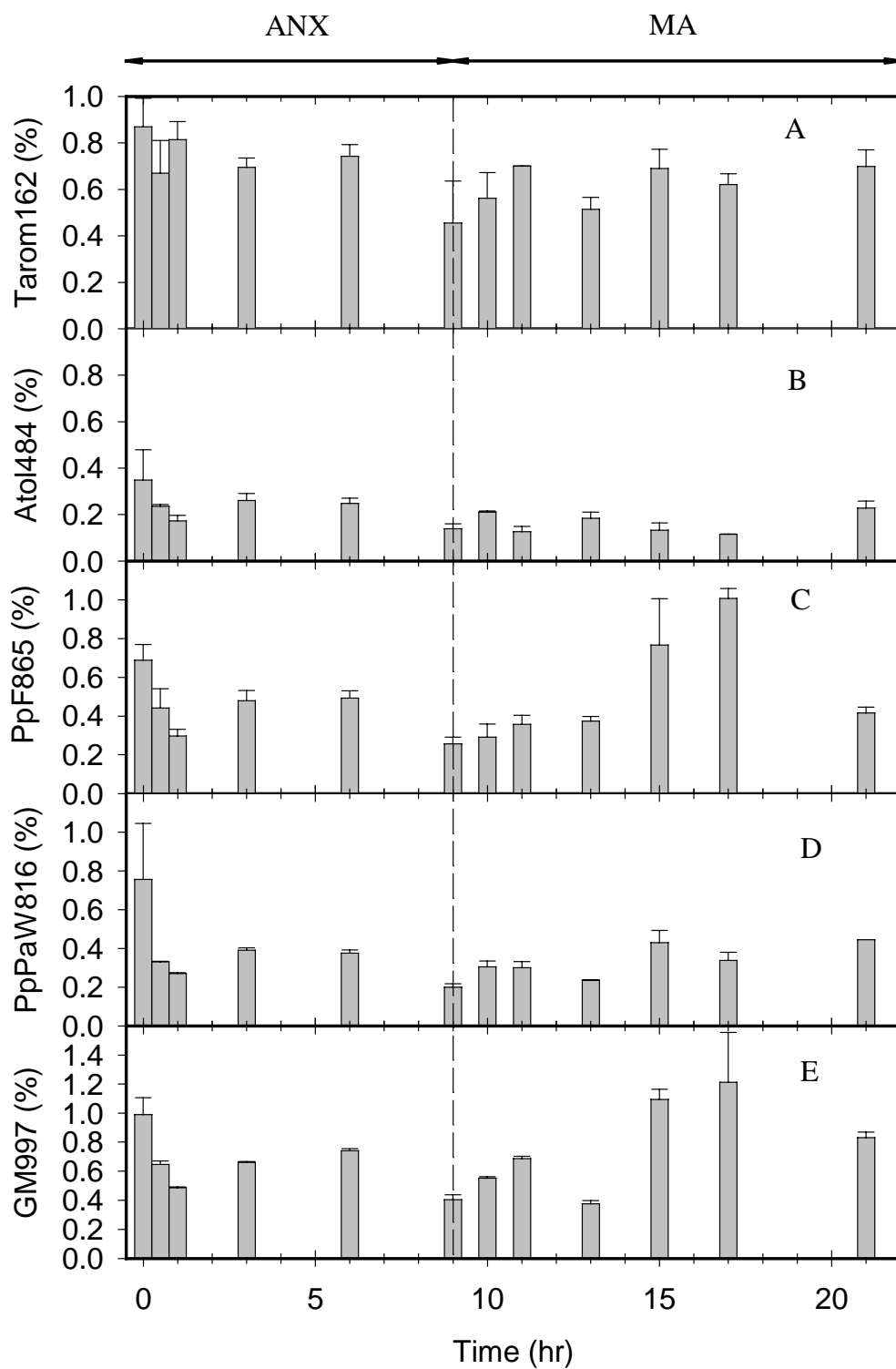
Figure 7. The distribution of toluene-degraders in ANX/MA SBR, (A) Probe Trom162, (B) Probe Atol484, (C) Probe PpF865, (D) Probe PpPaW816, (E) Probe GM997. Error bars represent ± one standard deviation and are not visible for some samples due to low variability.

The hybridization data obtained from the ANX, ANX/MA/AER, and ANX/MA SBRs show that multiple redox environments retained more bacteria which functioned at higher metabolic levels based on the bacterial subset targeted by the probes. The probes used showed higher hybridization signals in the ANX/MA/AER and ANX/MA SBRs. A more diverse bacteria consortium is beneficial in that one bacterial group can biodegrade the metabolic intermediates generated by other bacterial groups, even if the bacterial group lacks the ability to biodegrade the substrates from the starting point. This will prevent the formation of possible dead end intermediates in biological treatment processes. Although *T. aromatica* strains T1 and K172, and *Azoarcus* sp. mXyN1 are strict anoxic toluene-degraders, they also have the ability to participate in the aerobic biodegradation of intermediates formed by oxygen-dependent BTX biodegradation pathways as well as biogenic substrates.

The biodegradation of biogenic substrates are believed to contribute significantly to the microbial activities detected by 16S rRNA hybridization; therefore, the activities shown in Figure 5-7 cannot be solely credited to BTX biodegradation. The comparable activity levels of strict aerobic toluene-degraders under microaerobic conditions in ANX/MA SBR and aerobic conditions in ANX/MA/AER SBR imply that the oxygen levels under the microaerobic conditions may be sufficient for BTX biodegradation. Although both strains of *P. putida* are strict aerobes, their activities under anoxic conditions in SBRs containing multiredox environments may be due to retained activities under aerobic or microaerobic conditions after settling. These activities cannot be related to BTX biodegradation under anoxic conditions. The retained rRNA contents are presumably associated with housekeeping enzyme synthesis since the levels of catechol 1,2-dioxygenase and catechol 2,3-dioxygenase were low under anoxic conditions (24). This may also be true for GM1, which was undoubtedly also influenced by the presence of biogenic substrates since GM1 is a general denitrifier.

Atol484, a probe which targets a group of facultative toluene-degraders, showed low hybridization responses in all three reactor configurations. In a three-membered chemostat microbial culture, Lu and Grady (23) found that specialists (degrade a limited number of xenobiotic compounds) outcompeted generalists (degrade a broader range of xenobiotic compounds). Slater and Godwin (35) also suggested that a generalist may be at a competitive disadvantage because it carries unnecessary genetic information. This may help to explain why

this probe showed lower activities than the probes targeting strict anoxic and strict aerobic toluene-degraders in the reactor configurations studies here.

The activities shown in Figures 5-7 are around 1% which may appear low. However, in a study on soil bacterial communities by using quantitative reverse transcription-PCR and temperature gradient gel electrophoresis (TGGE), Felske et al. (12) reported that the 16S rRNA contents of 10 out of the 20 predominant ribotypes were around 1%. Snaidr et al. (36) also reported that the abundance of the predominant genera ranged from 2-9% in an activated sludge treatment plant.

Efforts were made to isolate anoxic toluene-degraders from the anoxic enrichment SBR as described previously. Modified R2A plates (14) were used to isolate bacterial colonies. However, none of the isolates obtained exhibited an ability to biodegrade toluene under denitrifying conditions, although hybridization techniques showed positive signals by using probes specific for anoxic toluene-degraders. This is consistent with other studies which showed that culture-dependent methods do not produce representative results when studying microbial ecologies predominated by slow-growing bacteria (42).

Hybridization techniques have been applied in environmental matrices including activated sludge (e.g. (25, 30, 42, 44)) and biofilm (e.g. (3, 17, 22, 26, 34, 44)) wastewater treatment systems for a decade. However, these studies utilized organisms with simple substrate profiles (3, 17, 25, 30, 34, 44), single substrate systems (22, 26) ,or genus-specific probes (42) in mixed substrates systems. The study we presented here attempted to look at the microbial activities of bacterial groups with the ability to biodegrade BTX compounds in systems fed with mixed substrates. The probes used here target much narrower bacterial groups and, in some cases, strain-specific probes were used (based on current databases). These unique characteristics require special cautions in interpreting the data. The contribution of other substrates on the activities of the organisms carrying the BTX biodegradation abilities should not be excluded. A complex picture of the substrate utilization profiles of the target organisms would help in interpreting the results. Nevertheless, our data suggested that a multiple redox environment in a BTX-fed single-sludge activated sludge system favored a more diverse microbial ecology, and also enhanced the microbial activities of the targeted BTX-degrading organisms.

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CHAPTER 5. ENGINEERING SIGNIFICANCE

Using conventional aerobic biological treatment processes to treat wastewaters containing benzene, toluene, o-, m-, and p-xylene (BTX), as is commonly practiced in industries, is debatable since the volatile nature of these compounds can lead to stripping of these compounds from the aqueous phase into the atmosphere. These processes violate National Emissions Standards for Hazardous Air Pollutants (NESHAPs) which regulate volatile organic compounds emanating from various sources, including wastewater streams. Consequently, cost-effective and environmentally friendly approaches for removing BTX contaminants from wastewaters are desirable.

This research investigated a treatment alternative which involves a sequential anoxic/microaerobic single sludge system to treat BTX-containing wastewater. Toluene and m-xylene were consistently biodegradable under anoxic conditions while benzene, o-, and p-xylene were biodegradable under microaerobic conditions with and without nitrate. Microaerobic conditions can be used to replace aerobic conditions in treating BTX-containing wastewater in that the kinetics of benzene, o-, and p-xylene biodegradation measured under microaerobic conditions in the presence of nitrate or nitrite are comparable to the aerobic biodegradation rates. Under microaerobic conditions, the activities of enzymes associated with oxygen-dependent BTX biodegradation can be induced to the levels expressed under aerobic conditions by supplementing nitrate or nitrite as alternative electron acceptors. Although the BTX biodegradation potential under microaerobic conditions has been studied previously, the methods used to control microaerobic conditions were not standardized. This research also suggested that oxidation-reduction potential can provide a reliable and consistent measurement for microaerobic conditions.

The experimental system studied here was operated in a way that allowed NO_x to bleed through into the microaerobic phases. A more typical condition would be NO_x limitation at some point along the reactor cycle in an engineered system assuming that nitrification is used to sustain denitrification. Without supplemental NO_x, the BTX biodegradation kinetics under microaerobic conditions would be slower, which would translate into larger and more expensive basins. This would be partially compensated by a smaller air handling system and lower

operating costs. Further work is needed to optimize the cost associated with supplementing with NO_x versus constructing a larger treatment system.

Another contribution of this research is to use molecular biology tools to investigate engineering systems. The microbial community in a biological wastewater treatment system largely defines the system's treatment efficiency; however, knowledge about the effect of reactor configurations on the microbial ecology in an engineered system is very limited. By using 16S rRNA probes targeting representative toluene-degraders, this research showed that an alternating reactor with multiple redox conditions can foster a more diverse microbial community which facilitates the biodegradation of contaminants in wastewater streams. Additionally, target organisms showed higher metabolic activities in the multiple redox activated sludge systems. The results obtained provide some insights in understanding an engineered biological wastewater treatment system at a molecular level, which can ultimately lead to optimizing the system's reactor configuration in order to improve treatment efficiency.

It is anticipated that microaerobic metabolism will play a role in the treatment of BTX or other volatile organic compounds (VOC) containing wastewater. In particular, this study shows the utility of using NO_x supplemented microaerobic conditions to treat wastewater containing aromatic hydrocarbons. Bubbleless membrane technologies are continually under development, and this research demonstrates a possible application for the technology, which may be used to provide aerobic and microaerobic conditions to treat VOC-contaminated wastewaters which are regulated by NESHAP guidelines.