DISSERTATION

BACTERIAL DYNAMICS DURING AEROBIC AND ANOXIC BIODEGRADATION OF MTBE AT BENCH AND PILOT-SCALE

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

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Fort Collins, Colorado

Fall, 2007

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COLORADO STATE UNIVERSITY

August 6,2007

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY MARIA E. RAYNAL-GUTIERREZ ENTITLED BACTERIAL DYNAMICS DURING AEROBIC AND ANOXIC BIODEGRADATION OF MTBE AT BENCH AND PILOT-SCALE BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

BACTERIAL DYNAMICS DURING AEROBIC AND ANOXIC BIODEGRADATION OF MTBE AT BENCH AND PILOT-SCALE

Unintentional contamination of groundwater with gasoline oxygenates is a problem that emerged in the 1990s. The fuel oxygenate methyl *tert*-butyl ether (MTBE) and aromatic hydrocarbons benzene, toluene, ethylbenzene and xylenes (BTEX) are major gasoline constituents that co-occur in groundwater when storage tanks leak. Several physical and chemical treatment methods have been implemented for remediating gasoline impacted sites, however they are subject to high operational and construction costs as well as unreliable performance. Bioremediation has emerged as a promising alternative and low cost option, however, an understanding of the microbiology of MTBE remediation is needed in order to optimize this treatment in the field. Three main challenges are addressed in this study: the lack of appropriate microorganisms, anoxic conditions, and the presence of other gasoline components, such as BTEX, that potentially inhibit MTBE bioremediation. Specifically, the objectives of this study are to: *1.)* determine the effect of microbial community and reactor conditions on aerobic MTBE biodegradation in the presence of BTEX; *2.)* explore the potential of wetlands for bioremediating MTBE in the presence of other contaminants under low-oxygen conditions; *3.)* explore the potential of anaerobic permeable reactive barriers (PRBs) for bioremediating MTBE; *4.)* isolate a pure culture capable of anoxic MTBE

biodegradation; and *5.)* characterize bacterial dynamics during biodegradation of MTBE under ferric and sulfate reduction conditions. The overall goal is to support practical treatments for long-term sustainability.

The effect of microbial community and reactor conditions on aerobic MTBE biodegradation in the presence of BTEX was addressed with a batch experiment, where the MTBE removal rates of two aerobic cultures was compared in the presence and the absence of BTEX. Culture MO was previously enriched in MTBE and culture MB was enriched in MTBE and BTEX. A semi-batch reactor was set up using the MO culture. BTEX was added in this reactor. The composition of the microbial consortia was explored using a combination of 16S rRNA gene cloning and quantitative polymerase chain reaction targeting the known MTBE-degrading strain PM1^T. It was observed that the composition and diversity of the microbial community in the reactors played an important role on its ability to remove MTBE and BTEX simultaneously. The removal of these contaminants was feasible in the reactors that had a smaller concentration of PM1^T, but other MTBE degraders were present (*Hydrogenophaga* sp.), and in the reactors that had a more diverse microbial community.

The potential of wetlands for bioremediating MTBE in the presence of other contaminants under low-oxygen conditions was demonstrated with a mesocosm study, using scaled wetlands. The effects of natural attenuation, bioaugmentation and biostimulation were studied and compared. Water coming from the influent of an existing air stripping system in a refinery near Denver, CO. was used as the source water for this experiment. Two mixed cultures were used as the inocula in this test. An anaerobic Fe-reducing MTBE-degrading culture and culture MO (previously enriched in MTBE). High removal rates were observed in the scale wetlands, however, due to a high concentration of PM1^T in the refinery water it was not possible to separate the effect of bioaugmentation or biostimulation from natural attenuation.

The potential of anaerobic permeable reactive barriers (PRBs) for bioremediating MTBE was studied first with a batch experiment in order to determine if the anaerobic Fe-reducing MTBE-degrading culture was able to degrade MTBE under Fe(III) and SO_4 conditions; and then with a column study where two reactive materials were compared. The two reactive materials were selected based on two criteria cost and reactivity. The two slow release electron acceptor sources used in the experiment were $Fe(OH)_3$ and $CaSO_4$. The columns were inoculated with the anaerobic Fe-reducing culture. It was observed that the anaerobic consortia was able to degrade MTBE under both reducing conditions in the batch reactors, however MTBE was not removed in the columns, with the exception of the beginning of the experiment, when the columns were spiked with humic acids. It was assumed that the malfunction of the columns was due to the failure of providing the right conditions for the bacteria to remove MTBE, since the solubility of the reactive media was very low and probably did not release enough electron acceptor to be used for the bacteria.

A pure culture isolate of anoxic MTBE biodegradation was isolated from an anaerobic Fe-reducing culture and an aerobic culture (MO). The purity of the isolates was demonstrated with scanning electron microscopy (SEM), denaturing gel gradient electrophoresis (DGGE) and direct sequencing of the 16S rRNA gene. A phylogenetic three was constructed. The cells were observed to have a rod shape with an approximate length of 1.5 μm and width of 0.5 μm. No flagella were observed. The cells were

determined to be Gram-variable. Based on 16S rRNA sequencing, the closest match for the pure culture was *Desulfosporosinus meridiei* S5 (AF076248). The pure culture was observed to be able to degrade MTBE under Fe(III) and SO_4 reducing conditions.

The characterization of microbial community dynamics during biodegradation of MTBE under ferric and sulfate reduction conditions was studied using semi-batch reactors. A semi-batch reactor was set up using $Fe_2(SO_4)_3$ as the electron acceptor. Once this reactor was actively removing MTBE, its culture was used to seed four sets of batch reactors (nine in total). In the first set, the effect of having one electron acceptor (Fe(III) or SO_4) and sodium sulfide on the removal of MTBE was studied. In the second set, the same conditions were studied but in the absence of sodium sulfide. In the third set the presence of BTEX on the removal of MTBE in the presence and absence of sodium sulfide was determined. In the last set of reactors, the effect of adding two electron acceptors (Fe(III) and SO_4) using a different salt for each electron acceptor (i.e. Na_2SO_4 and $FeCl_3$) was studied. It was observed that the presence of one electron acceptor (vs. both), sodium sulfide and BTEX reduced the removal rate of MTBE. It was also observed that the oxidation-reduction potential also had an effect on the removal of MTBE. Total inhibition or a great reduction in the removal rate was observed in the reactors which source water was more reduced.

Overall it was observed that the removal of MTBE is feasible under aerobic and anaerobic conditions and in the presence of BTEX, and that overall success may be enhanced by several factors. First, it is important to recognize that the characteristics of the microbial consortium do matter. Second, the reactor configuration is also important for controlling the composition and performance of the microbial consortium.

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ACKNOWLEDGMENTS

I will like to thank God for giving me the opportunity to broad my knowledge and to live this experience. I will like to thank all my family and specially my parents, Jose A. Raynal and Maria E. Gutierrez, for their constant support and caring, for enduring with me all the challenges I had. Thanks to Elie El Kassis for helping me with the last step in this process, for keeping his faith on me and for his support. Special thanks also to my friends for their support during hard times and for all the moments and memories that we will share for life. Thanks to the international faith sharing group in John the XXIII for keeping my spirit and my faith high. I will like to thank my advisor, Dr. Amy Pruden, for all her teachings and for guiding me during my Ph D. She was always an inspiration and an example to follow. I will like to thank my co-advisor, Dr. Thomas Sale, for his positivism and for encouraging me to challenge my theories and experiments. I will like to thank the members of Dr. Pruden's research group for being a constant source of help, ideas, constructive discussions, knowledge and comradeship. Thanks to Routing Pei, Hyan Hung, Sage Hiibel, Rachel Hanson, Heather Storteboom, Luciana Pereyra, Chad Mckinney, Matt Hewitt and Ashish Sharma and Barbara Crimi. Thanks to my sponsors CONACYT and Suncor. Thanks to the Gregory Fletcher and the personnel at the refinery in Denver. Thanks to the members of my committee Dr. David Gilbert and Dr. Donald Klein. Thanks to Dr. Terry Engle. Thanks also to all the staff at the Civil and Environmental Engineering Department and at the Office of International Students and Scholars Services. God bless you all.

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A	Cross-sectional area (L^2)
α_L	Dynamic longitudinal dispersivity (L)
α_T	Dynamic transversal dispersivity (L)
Bd	Soil density
BLAST	Basic local alignment search tool
BTEX	Benzene, toluene, ethylbenzene and xylenes
C	Dissolved concentration of the contaminant (M/L^3)
Ce	Dissolved concentration of the contaminant at the effluent (M/L3)
Ci	Dissolved concentration of the contaminant at the influent (M/L3)
D	Diffusion coefficient (L^2/T)
D*	Effective diffusion coefficient
dC/dx	Concentration gradient (M/L^4)
DGGE	Denaturing Gradient Gel Electrophoresis
dh/dl	Hydraulic head gradient
DIPE	Diisopropyl ether
D_L	Longitudinal dispersion (L^2/T)
D_T	Transversal dispersion (L^2/T)
ETBE	Ethyl *tert*-butyl ether
EtOH	Ethanol
F	flux of solute per unit area per unit time (M/ L^2T)
FID	Flame-ionization detector
f_{oc}	Organic carbon fraction
GC	Gas chromatographer
HA	Humic Acids
HIBA	2-hydroxyisobutyric acid
IC	Inhibited control
K	Hydraulic conductivity (L/T)
K_{oc}	Partitioning coefficient with respect to the organic fraction
K_{ow}	Octanol-water partition coefficient
LUST	Liking underground storage tanks
MB	MTBE and BTEX culture
MCL	Maximum Contaminant Level
MeOH	Methanol
MO	MTBE only culture

MPD	2-methyl-1,2-propanediol
MSM	Mineral salt medium
MTBE	Methyl *tert*-butyl
n_e	Effective porosity
PAHs	Polcyclic aromatic hydrocarbons
PBS	Phosphate buffer solution
PCBs	Polychlorinated biphenyls
Pe	Peclet number
PRB	Permeable reactive barrier
θ	Porosity for the saturated media
Q	Volumetric discharge (L^3/T)
Q-PCR	Real-time quantitative polymerase chain reaction
RDP	Ribosomal Database Project
TAME	*Tert*-amyl methyl ether
TBA	*Tert*- butyl alcohol
TBF	*Tert*- butyl formate
TNT	Trinitrotoluene
USEPA	United States Environmental Protection Agency
VOCs	Volatile organic compounds
v_x	Average linear velocity for groundwater (L/T)
ω	Tortuosity coefficient

Chapter 1 Introduction

The addition of oxygenates to gasoline has been a common practice since the late 1970s. The use of fuel oxygenates increased as a consequence of the requirements implemented by the Clean Air Act Amendments in 1990. The United States Environmental Protection Agency (USEPA) requires that gasoline have 2.7% by weight of oxygen during winter and 2% throughout the year in areas that are not contaminated with ozone (USEPA 2006b).

Two classes of fuel oxygenates have been used to replace lead: ethers and alcohols. Ether oxygenates include methyl *tert*-butyl ether (MTBE), ethyl *tert*-butyl ether (ETBE), *tert*-amyl methyl ether (TAME) and diisopropyl ether (DIPE). Alcohol oxygenates include ethanol (EtOH), *tert*-butyl alcohol (TBA) and methanol (MeOH). Of all of these oxygenates MTBE is the most used due to the ability to be produced on-site, its high octane level, low cost, ease of blending with gasoline and ease of transfer and distribution. It has been estimated that four billion gallons of MTBE are used annually in the USA (USEPA 2006b). However, since 2000, many states have banned the use of MTBE. Some of these states include Minnesota, California, Colorado, New York and Michigan (USEPA 2007a).

MTBE has been introduced into the environment due to unintentional gasoline spills and leakage of pipelines and underground storage tanks (LUST) in refineries and gasoline stations, contaminating groundwater and surface water bodies. One monitoring study of drinking water sources showed that between 5 to 10% of all community drinking water wells nationwide had detectable MTBE contamination (Squillace et al. 1999).

Gasoline is a complex mixture of hundreds of hydrocarbons (about 500 compounds) formed mostly by aromatic hydrocarbons, thus, in a gasoline impacted site, other compounds will be also found. Benzene, toluene, ethylbenzene and xylenes (BTEX) are some of the most common constituents of gasoline (Rittmann and McCarty 2001). MTBE and BTEX compounds have high water solubilities, low adsorption coefficients and slow biodegradation rates. These characteristics have contributed to the rapid spread and persistence of these contaminants in the environment, especially MTBE. Besides the concern related to the high mobility of these contaminants, there is also a concern regarding the impact of these contaminants on human health. Although the carcinogenicity of MTBE has not been confirmed, this contaminant is listed as a possible carcinogenic and adds odor and taste to water, thus the EPA has established an advisory drinking water maximum contaminant level of 20 μg/L (USEPA 2006b). The MCLs established for benzene, toluene, ethylbenzene and xylenes are 5μg/L, 0.7 mg/L, 1 mg/L and 10 mg/L, benzene being the most toxic (USEPA 2006a).

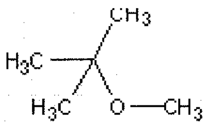
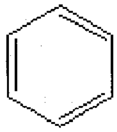
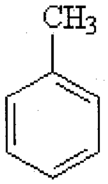
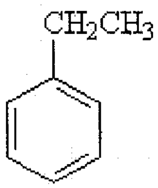
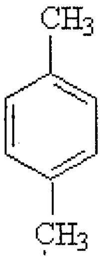
1.1 Fate and Transport of Contaminants in Groundwater

The fate and transport of MTBE and the BTEX compounds in groundwater depends on their physical and chemical properties as well as the hydrogeologic and geological characteristics of the site. As mentioned above, the contamination of groundwater with gasoline derived contaminants occurs due to the leakage of underground storage tanks and pipelines at refineries and gas station, municipal landfills and spills during transport.

Based on fluid mechanics principles, the movement of contaminants in groundwater follows the principle of mass conservation (the Lomonosov-Lavoisier law), which states that in a closed system the mass of a substance will remain constant regardless of all the chemical and physical processes that can take place in the system, thus matter cannot be created nor destroyed, it can only be transformed.

The soil matrix physical composition is complex, since it is formed by solids and pore spaces which may contain water, gas and/or a non-aqueous phase liquid (such as petroleum hydrocarbons). Partitioning of MTBE and BTEX into the gaseous, liquid and solid phases of the soil matrix is directly related to their physical and chemical properties, which are presented in Table 1-1. It can be observed that MTBE and BTEX have high water solubilities, low Henry's Law constants, slow aqueous diffusion coefficients and low octanol-water partition coefficients (K_{ow}). Thus, these contaminants tend to remain dissolved in the aqueous phase and partitioning into the gas and solid phases is minimal. The mass conservation equation for any chemical present in groundwater is described in the following section.

Table 1-1 Physical and Chemical Properties of MTBE and BTEX.

Physical/Chemical Property	**MTBE**	**Benzene**	**Toluene**	**Ethylbenzene**	***p*-Xylene**
Chemical formula	$C_5H_{12}O$	C_6H_6	C_7H_8	C_8H_{10}	C_8H_{10}
Chemical Structure					
Molecular Weight (g/mol)	88.15	78.11	92.14	106.17	106.17
Specific Gravity @25^0C	0.740	0.87	0.87	0.87	0.86
Water Solubility (mg/L)	54,300	1780	534.8	161	156
Dimensionless Henry's Law Constant	0.022	0.22	0.24	0.29	0.31
Diffusion coefficient in water (cm^2/s)$^{(1)}$	1.05E-05	1.10E-05	9.40E-06	8.50E-06	8.50E-06
Biodegradation rate (%/day) $^{(2)}$	0.01-0.1	0.1-1	0.1-1	0.1-1	0.3
Log K_{ow}	1.2	2.13	2.73	3.15	3.15
Log K_{oc}	1.9	1.5	1.56	1.98	2.05

From:

Squillace, P. J., et al. (1997). "Review of the Environmental behavior and fate of Methyl *tert*-Butyl Ether." Environmental Toxicology and Chemistry **16**(9): 1836-1844

(1) ASTM Standard E 1739-1995. *Standard Guide for Risk-Based Corrective Action at Petroleum Release Sites.* Table X1.2, X2.7.

(2) Day, M. J., et al. (2001). "Fate and Transport of Fuel Components Below Slightly Leaking Underground Storage Tanks." Environmental Forensics(2): 21-28

1.1.1 Transport of Contaminants in Groundwater

The transport of any contaminant in groundwater follows the law of conservation of mass. The components of the mass balance equation are: molecular diffusion, advection, sorption and transformation. Each of the parts of the mass balance equation is defined.

Molecular diffusion occurs due to the presence of concentration gradients. It can be expressed as Fick's first law, in one dimension:

$$F = -D\frac{dC}{dx}$$ **Equation 1**

where

F = Mass flux of solute per unit area per unit time (M/L^2T)

D = Diffusion coefficient (L^2/T)

dC/dx = Concentration gradient (M/L^4)

Fick's first law is ruled by the second law of thermodynamics, which states that the movement of fluid occurs from areas of greater concentration to those of lesser concentration, thus, a negative sign is placed in front of the equation.

Diffusion in groundwater is less than what is observed in pure water, due to the presence of solids. Because of this, the solutes must follow longer pathways as they travel around mineral grains. Therefore, in groundwater an effective coefficient must be used:

$$D^* = \omega D$$ **Equation 2**

where ω is the tortuosity coefficient, which has a range from 0 to 1. Diffusion causes the contaminant to spread away from the place where it was introduced.

The movement of water and solute transport in groundwater is described by partial differential equations. The movement of water through a porous medium is governed with Darcy's equation:

$$Q = -KA\frac{dh}{dl}$$ **Equation 3**

where

Q = Volumetric discharge (L^3/T)

K = Hydraulic conductivity (L/T)

A = Cross-sectional area (L^2)

dh/dl = Hydraulic head gradient

Even though Darcy's equation was developed to describe one-dimensional flow, it should be noted that the hydraulic head is a function of space, thus it actually has three dimensional applications.

The hydraulic conductivity can be defined as the ability of a medium to transmit water. It can be measured in the three coordinate directions (X, Y and Z). Its value depends on the matrix configuration and the saturation of the soil. If the value of the hydraulic conductivity is the same in all three directions it is said that the medium is isotropic, otherwise it is referred to anisotropic. Usually, it is assumed that the medium is isotropic. Typical values of the hydraulic conductivity observed for coarse soils range from 10^2 to 10^{-3} cm/s, while in fine grain soils this constant ranges from 10^{-4} to 10^{-10} cm/s.

Advection is the process that causes the movement of contaminants due to the flow of groundwater. Based on Darcy's Law, the average linear velocity is calculated as:

$$v_x = \frac{K}{n_e}\frac{dh}{dl} \qquad \textbf{Equation 4}$$

where n_e is the effective porosity (non-interconnected and dead-end pores are not included). The one dimensional mass flux, Fx, due to advection is given by the following equation:

$$F_x = v_x n_e C \qquad \textbf{Equation 5}$$

where C is the dissolved concentration of the contaminant (M/L^3).

The movement of water through the soil matrix generates mixing of the contaminated groundwater and the pore water. This process is known as mechanical dispersion. Given the nature of the soil matrix, flow channels will form between the soil particles, which force groundwater to move at different rates and to mix with the pore water through diffusion. Since this process is tightly associated with dispersion, both processes are combined to form a parameter called the hydrodynamic dispersion coefficient. The mixing that occurs along the direction of the flow path is called longitudinal dispersion and the mixing that occurs in direction normal to the flow path is called transverse dispersion. Both coefficients are given by the following formulas:

$$\begin{aligned} D_L &= \alpha_L v_x + D^* \\ D_T &= \alpha_T v_x + D^* \end{aligned}$$ **Equation 6**

where

D_L = Hydrodynamic longitudinal dispersion coefficient.

α_L = Dynamic longitudinal dispersivity (L)

D_T= Hydrodynamic transverse dispersion coefficient.

α_T= Dynamic transversal dispersivity (L)

Diffusion and mechanical dispersion can be related with the Peclet number, which is equal to:

$$Pe = \frac{v_x L}{D_L}$$ **Equation 7**

where L refers to the length that the flow travels. It has been observed that when the velocity is very low diffusion will dominate, but when the velocity is very high advection will dominate.

The last term in the mass balance equation is a sorption term. Sorption processes include adsorption, chemisorption, absorption and ion exchange. For organic chemicals only adsorption and absorption occur. Adsorption is the process by which a chemical clings to a solid surface. Absorption occurs when the solute particles diffuse into the pores present in the soil particles and are sorbed onto the interior walls of the pores. Since it is difficult to distinguish the two processes, the term sorption is typically used instead to include both phenomena.

Sorption has a retardation effect on the transport of contaminants in groundwater, which is also known as a chromatographic effect. The organic contaminants dissolved in groundwater tend to partition onto the soil solids due to the presence of organic carbon on the particles surface. The organic carbon content of the soil is called the organic carbon fraction (f_{oc}). The partitioning of contaminants onto the soil surface is directly related to the contaminants' hydrophobicity, which can be quantified using the octanol-water partition coefficient, K_{ow}, of the compound. Consequently, a compound with a high K_{ow} coefficient will have a high tendency to sorb onto the soil. The partitioning coefficient with respect to the organic fraction is referred to as K_{oc}. The value of K_{oc} can be estimated as:

$$K_{oc} = 0.63K_{ow}$$ **Equation 8**

The mass balance equation can be written now that all the terms have been defined. This equation is given by:

$$\frac{\delta C}{\delta t} = D_L \frac{\delta^2 C}{\delta x^2} - v_x \frac{\delta C}{\delta x} - \frac{Bd}{\theta} K_{oc} f_{oc} \frac{\delta C}{\delta t} \pm kC$$ **Equation 9**

where

Bd = Soil density.

θ = Porosity for the saturated media.

The first term on the right side of the equation represents the dispersion of the solute, the second term is the advection of the solute, the third term is the partitioning of the solute into the soil particles and the last term indicates the change of concentration due to biological decay (Fetter 1999).

As the contaminants are released into the subsurface, they will migrate downward, passing through the unsaturated zone until they reach an unconfined aquifer, given that neither MTBE nor BTEX will partition into the gaseous or solid phase. In the case of gasoline contaminants released into groundwater, if the release rate of fuel contaminants to groundwater is greater than the removal rate, accumulation of such contaminant will occur and a plume will be formed.

The chemical and physical characteristics of the contaminants present in groundwater will ultimately determine which transport process will dominate. It can be observed in Table 1-1 that MTBE and the BTEX compounds have a high water solubility, low dimensionless Henry's constants and low affinity to adsorb to organic matter. Therefore, when studying the movement of these contaminants in groundwater, several assumptions can be made to simplify Equation 9. First, sorption can be ignored for most compounds relevant to this study, but for ethylbenzene and *p*-xylene, since these compounds are more hydrophilic (low K_{ow} and low K_{oc}). Second, the dispersion term in Equation 9 can also be ignored for all of the compounds, since the diffusion coefficients for all of the contaminants are very small. Thus, the main two processes that influence the movement of MTBE and the BTEX compounds in groundwater are advection and biodegradation.

1.1.2 Fate

As explained in the previous section, the fate of MTBE and BTEX in groundwater is mainly affected by biodegradation, since no other chemical reactions occur under normal environmental conditions. MTBE ($C_5H_{12}O$) contains two organic functional groups, an ether link and a branched moiety. MTBE is made by reacting methanol, made from natural gas, with isobutylene (2-methyl-1-propene) in the liquid state, using an acidic catalyst at 100°C (USEPA 2006b). Both the ether group and the *tert*-butyl are factors in its resistance to biodegradation. Two abiotic treatments have been used in the past to eliminate ethers, the use of peroxide radicals and acids. However, MTBE is very stable at ambient temperature and pressure. For example, it does not require stabilizers during handling; therefore only strong oxidizers are effective. To chemically destroy the ether bond in the MTBE molecule, the pH typically needs to be reduced to <2. The reaction products are TBA and methanol (Fischer et al. 2005), although further degradation of TBA has been observed (Burbano et al. 2002).

In particular, the *tert*-butyl and the ether groups have been reported to be bio-recalcitrant groups. There are several reasons why biodegradation of MTBE is difficult to achieve. For the bacteria it is difficult to break tertiary carbon molecules and to degrade alkali ethers, due to the steric hindrance for the attacking enzyme(s) (Stocking et al. 2000; Fortin et al. 2001). An example of a natural compound that contains tridimensional ether structure is lignin, which is resistant to biodegradation. The first step in the biodegradation process is the cleavage of the ether ring. The cleavage of ethers in the environment begins with hydroxylation by oxygenases of the tertiary carbon molecules adjacent to the ether bond, and then the ether bond is converted into an unstable hemiacetal. Microorganisms that produce such monoxygenases are: *Pseudomonas putida*, *Nocardia, Streptomyces, Hydrogenophaga flava* ENV735, *Mycobacterium austroafricanum* IFP 2012, *Methylibium petroleiphilum* PM1 (PM1^T), and *Rhodococcus* (Fayolle et al. 2001; Lopes Ferreira et al. 2006). Dioxygenases are produced by *Sphingomonas* (Fayolle et al. 2001).

Once the ether bond in MTBE is broken, TBA and formaldehyde are formed. Formaldehyde is toxic to many bacteria. In addition, MTBE has been reported to be associated with a very low biomass yield (Salanitro et al. 1994; Hanson et al. 1999). Nevertheless, biodegradation of MTBE has been successfully reported in laboratory and field studies (Salanitro et al. 1994; Yeh and Novak 1994; Mo et al. 1997; Hanson et al. 1999; Deeb et al. 2001; Finneran and Lovley 2001; Fortin et al. 2001; Pruden et al. 2001; Pruden et al. 2005).

Gasoline impacted sites have been treated in the past with active and passive treatment methods and more recently with the aid of bioremediation/bioaugmentation. Active remedial methods include groundwater pump-and-treat (air stripping and removal with granular activated carbon), vapor extraction, chemical oxidation and multiphase high-vacuum extraction. However, the success of implementing any of these methods depends greatly on the concentration of the contaminant, the hydrogeology of site, as well as the local MCL and the feasibility of removing the contaminant source. The operational cost associated with these technologies is high and potentially prohibitive, given the cases where the amount of contaminated groundwater is excessive or if the contamination input is constant, which will make the operation time of the system almost infinite (Deeb et al. 2003). Some of the *in situ* alternative treatments that have been previously studied include biodegradation/bioaugmentation (Salinitro et al. 2000; Landmeyer et al. 2001; Smith et al. 2005) and bio(reactive)barriers. These alternative treatments take advantage of the high mobility of the contaminants to intercept them with a treatment zone without having to remove groundwater through pumping. Some of the advantages include long-term operational capacity, and low operational costs, since no energy or is required.

1.2 Alternative MTBE Treatments

1.2.1 MTBE Bioremediation

Bioremediation is considered to be a cost-effective method for treating various contaminants at impacted sites, given that is a treatment method that normally does not require energy, expensive equipment or buildings. Bioremediation is also attractive because the contaminants are transformed to a less toxic form (CO_2) instead of only being transferred from the liquid phase to a solid or gaseous phase. It has been generalized that most organic contaminants found in groundwater are biodegradable as long as the appropriate conditions prevail (pH, temperature, nutrient availability, presence of electron donor/acceptor) (Rittmann and McCarty 2001).

Some of the potential challenges that have been identified when considering bioremediation of MTBE are the co-occurrence of other gasoline constituents, such as BTEX, anaerobic conditions and having an initial high concentration of a culture that can degrade MTBE under the environmental conditions present in the site (Deeb et al. 2001). All these factors result in a reduction of the degradation rate or total inhibition of biodegradation. Two approaches that have been used to overcome the low biodegradation rate of MTBE are biostimulation and/or bioaugmentation (Sylva et al. 2005). Biostimulation refers to the injection of nutrients and/or electron donors/acceptors that are absent at the site to be treated. Bioaugmentation refers to the injection of specialized bacterial cultures to increase the initial density and diversify metabolic capabilities of the microbial community.

1.2.1.1 Aerobic Biodegradation

In the past, the biodegradation pathways of MTBE and BTEX have been determined for some bacterial strains.

Figure 1-1 presents the first step of the aerobic biodegradation of the BTEX compounds, where two hydrogen atoms adjacent to two carbon atoms are replaced with hydroxyl molecules. Then, the benzene ring is broken and converted to catechol, after this biodegradation readily proceeds (Fetter 1999).

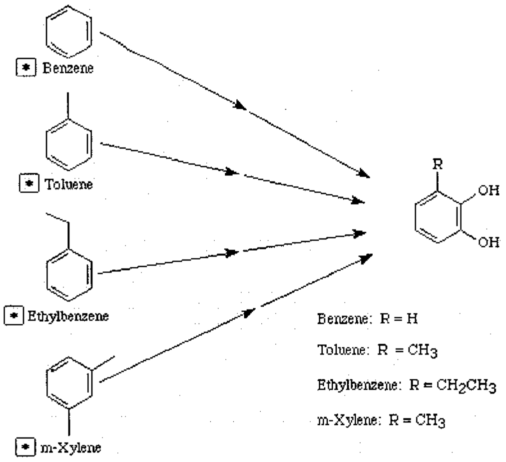


Figure 1-1 Aerobic biodegradation of BTEX. First step of the cleavage of the benzene ring, from http://umbbd.ahc.umn.edu/BTEX/BTEX_map.html for pure cultures. For toluene: Ps. Mendocina, Ps. Pickettii and Ps. Putida. For ethylbenzene: *Pseudomonas putida* 39/D, *Pseudomonas sp.* , *Pseudomonas sp.*, *Pseudomonas sp.* NCIB 10643, NCIB 9816-4 and NCIB 9816-4. For m-xylene: *Sphingomonas yanoikuyae* B1 and *Pseudomonas putida* mt-2.

In the aerobic biodegradation pathway of MTBE, the first transformation is believed to be carried out by a mono-oxygenase enzyme. These enzymes insert one oxygen atom from molecular oxygen into the organic compound being metabolized. The other oxygen atom is reduced to form water. This oxidation step leads to the formation of TBA and either formaldehyde or formic acid; or further oxidation to *tertiary* butyl formate (TBF), which is typically an unstable intermediate. The subsequent hydrolysis of TBF yields TBA and formate. TBA is further biodegraded to 2-methyl-1,2-propanediol (MPD), which most likely first forms an aldehyde intermediate (2-hydroxyisobutanal) before degrading to 2-hydroxyisobutyric acid (HIBA). Downstream intermediates following HIBA may include 2-propanol, acetone, hydroxyacetone (acetol) and others. To date an anaerobic MTBE biodegradation pathway has not been reported (Wilson et al. 2005).

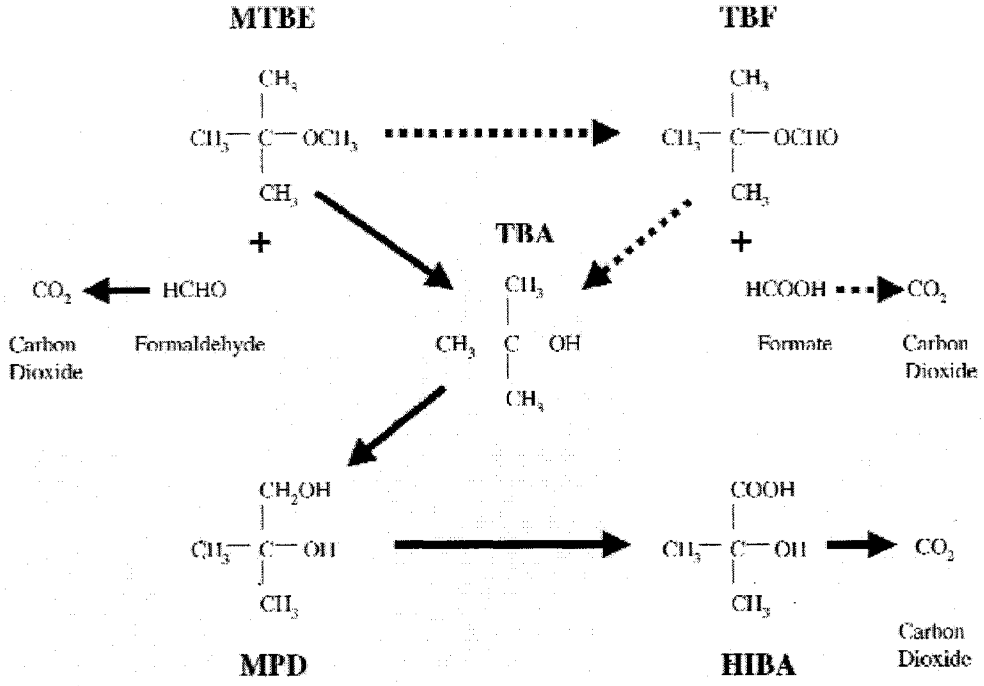


Figure 1-2 Aerobic biodegradation pathway for MTBE. TBF: *tert*-butyl formate, TBA: *tert*-butyl alcohol, MPD: 2-methyl-1,2-propanediol, HIBA: 2-hydroxyisobutyric acid (Hardison et al. 1997; Steffan et al. 1997; Fayolle et al. 2003; Smith et al. 2003a; Smith et al. 2003b).

Several bacterial strains and mixed cultures able to aerobically degrade MTBE have been identified in the laboratory. In 1994, Salanitro et al. were the first to report aerobic bacterial degradation of MTBE (Salanitro et al. 1994). In 1997 Mo et al. were able to isolate three strains from soil, from activated sludge from the Amoco Corporation and from soil near a Gingko tree, that were able to use MTBE as a sole carbon and energy source. However, MTBE was only transformed to TBA. Such isolates were *Methylobacterium mesophilicum, Rhodococcus sp.* and *Arthrobacter ilicis*. The first two were identified as Gram-negative and the third one as Gram-positive (Mo et al. 1997). MTBE degrading genera, including direct metabolizers and cometabolizers, include *Rhodococcus* sp.(Mo et al. 1997), *Mycobacterium austroafricanum* IFP 2012 (Francois et al. 2002), *Rubrivivax gelatinosus* PM1 (Deeb et al. 2001) and *Hydrogenophaga* sp.

(Hatzinger et al. 2001), which are microorganisms that can mineralize MTBE to CO_2 and use this compound as a sole carbon and energy source. PM1^T and *Hydrogenophaga* sp. belong to the β-*Proteobacteria* family and are closely related to *Aquabacterium* and *Leptohrix* (Hristova et al. 2003). There have been other studies that have reported isolates, for example ENV 425, that are able to cometabotabolize MTBE in the presence of other compounds, such as propane (Steffan et al., 1997). In addition, a type of fungi was found to be able to degrade MTBE. Hardison et al. found that the fungal *Graphium sp.* were capable of cometabolizing MTBE in the presence of n-butane (Hardison et al. 1997).

Strain PM1^T is a Gram-negative, rod-shaped, motile, non-pigmented, facultative anaerobe and facultative methylotroph, which has been found to be able to completely mineralize MTBE or benzene as a sole source of carbon and energy. It can also grow on toluene, phenol, ethylbenzene, 3,4-dihydroxybenzoate, 2,5- dihydroxybenzoate, 3,5-dihydroxybenzoate, 2,6-dihydroxybenzoate and 2,3-dihydroxybenzoate. It is also capable of utilizing *tert*-butyl alcohol (TBA), TBF and HIBA, an intermediate of MTBE degradation (Deeb et al. 2001; Nakatsu et al. 2006). The MTBE biodegradation rate of this strain has been used as a parameter to benchmark other aerobic MTBE-degrading strains, given its capability of degrading MTBE at a relatively fast rate (Fortin et al. 2001). The presence of this strain has also been used as a parameter to indicate MTBE bioremediation potential in gasoline impacted sites (Hristova et al. 2003).

MTBE biodegradation has also been demonstrated in the field. In 2000, Salinitro et al. demonstrated in situ MTBE bioremediation in an aquifer after the addition of oxygen and inoculation with a MTBE-degrading mixed culture (MC-100) (Salinitro et al.

2000). Landmeyer et al. and Wilson et al., were able to stimulate native microbial communities with the addition of oxygen (Landmeyer et al. 2001; Wilson et al. 2002). Recently the effect of biostimulation of indigenous bacteria with oxygen and bioaugmentation with the strain PM1^T were observed to enhance MTBE biodegradation (Smith et al. 2005).

Besides the low concentration of oxygen at gasoline impacted sites, there is another challenge that must be overcome when treating such sites with bioremediation/biostimulation. The co-occurrence of MTBE with other contaminants, such as BTEX, is common. The presence of more easily biodegradable hydrocarbons may inhibit the biodegradation of MTBE as a consequence of the preferential use of these hydrocarbons and the depletion of nutrients and electron acceptors (Stocking et al. 2000). To successfully bioremediate these sites, it is necessary to characterize the conditions under which MTBE and the other compounds can be biodegraded simultaneously. In past studies, it was noted that the presence of ethylbenzene, *o*-xylene and *p*-xylene inhibited the biodegradation of MTBE by the pure culture PM1^T in batch reactors, while toluene slightly inhibited the biodegradation of MTBE (Deeb et al. 2001). Results such as these and other reports from the field have discouraged attempts to bioremediate MTBE in the presence of BTEX or other contaminants. On the other hand, other studies have demonstrated that under some conditions MTBE biodegradation can take place in the presence of other compounds such as BTEX in both pure and mixed cultures (Pruden et al. 2001; Sedran et al. 2002; Pruden and Suidan 2004). Thus, there is a need to better understand the conditions under which BTEX inhibits MTBE degradation in order to advance bioremediation of these mixtures in the field.

1.2.1.2 Anaerobic Biodegradation

Since anaerobic/anoxic conditions predominate in groundwater and the cost of injecting oxygen to stimulate aerobic microbial activity may be high, the possibility of inducing anaerobic biodegradation of MTBE is of significant interest. Anaerobic degradation of MTBE was first studied by Yeh et al. in 1994. In this study, it was found that MTBE was only degraded in organic matter-poor soils with a pH around 5.5 (Yeh and Novak 1994). Mormile et al. reported anaerobic degradation of MTBE using river sediments after a 152-day acclimation period (Mormile et al. 1994). However, MTBE was not confirmed to be mineralized in either of these studies. Instead MTBE was converted to TBA, one of the intermediate products formed during MTBE biodegradation.

Mineralization of MTBE under anaerobic conditions has been reported in more recent studies. In a sediment microcosm study conducted by Bradley et al., anaerobic biodegradation of MTBE was studied under methanogenic, denitrifying, sulfate-, manganese- and iron-reducing conditions. These electron acceptors are usually present in groundwater and are only utilized after oxygen has been utilized (Figure 1-3). It was observed that under methanogenic conditions MTBE was transformed to TBA, while under the remaining conditions studied, MTBE was mineralized. The electron acceptor preference of the indigenous bacteria had the following hierarchy: O_2, NO_3, Mn(IV), Fe(III) and SO_4. Mineralization of MTBE was observed after 166 days, ranging in removal percentages from 35 to 100% (Bradley et al. 2001a). In a study conducted by Finneran et al., it was found that MTBE was degraded anaerobically more rapidly in the presence of humic acids (HA) and Fe(III) after 275 days. The addition of Fe(III) was shown to increase mineralization of MTBE and decrease the production of CH_4 (Finneran and Lovley 2001).

Humic acid (HA) is the product of macroanimal and plant decomposition, brown to black in color, very soluble in water at neutral to alkaline pH values and is negatively charged. Humic substances consist of a heterogeneous mixture of compounds for which no single structural formula will suffice. HA are thought to be complex aromatic macromolecules with amino acids, amino sugars, peptides, aliphatic compounds involved in linkages between the aromatic groups. Given that HA is an end product of decomposition of organic matter, it has great resistance to further decomposition. It is a slow-release source of carbon, nitrogen, sulfur and phosphorus for plants and microflora in soil. HA have a great cation exchange capacity (CEC). A substantial fraction of the mass of the humic acids is in carboxylic acid functional groups, which endow these molecules with the ability to chelate positively charged multivalent ions (Mg^{+2}, Ca^{+2}, Fe^{+2}, trace elements to plants and bacteria, as well as other ions that have no positive biological role, such as Cd^{+2} and Pb^{+2}). Nutrients and organic compounds have been observed to adsorb to the HA molecules. The chelating capacity of HA is probably the most important role of HA with respect to living systems. By chelating the ions, they facilitate the uptake of these ions by several mechanisms, one of which is preventing their precipitation, thus increasing their bioavailability (Sylva et al. 2005). The use of HA to enhance the biodegradation of MTBE was proposed by Finneran et al. and it was suggested to act as a electron shuttle between the insoluble electron acceptors (such as ferric iron), the liquid phase and the bacteria (Finneran and Lovley 2001). The positive benefits of using HA to promote the biodegradation of other organic compounds such as PAHs has also been demonstrated (Finneran and Lovley 2001; Van Stempvoort et al. 2002).

A batch-reactor study conducted by Pruden et al. recently characterized anaerobic biodegradation of MTBE under iron-reducing conditions (Pruden et al. 2005). A culture was enriched from mixed inocula from aerobic MTBE-degrading, methanogenic and sulfate-reducing cultures. MTBE degradation occurred after 181 days. In this study, the dominant bacteria in the reactor were identified. It was observed that most of the bacteria present belonged to the δ-Proteobacteria, which is a group that contains sulfate and iron-reducing bacteria (Pruden et al. 2005).

Natural attenuation or biodegradation of MTBE under anoxic conditions has also been reported. Carbon isotope fractionation has been used recently to demonstrate anaerobic degradation of MTBE (Kolhatkar et al. 2002; Kuder et al. 2005; Somsamak et al. 2006). In a study conducted by Kolhatkar et al., the authors monitored the concentration of MTBE in different sampling wells at a contaminated site and observed that the concentration of MTBE decreased on average 30% in three years. In this study the parameter $\delta^{13}C$ was used to differentiate between abiotic and biotic transformation of MTBE. The molecules of each contaminant have a particular $\delta^{13}C$ value, which depends on the source of the contaminant and the release history. This parameter is the ratio of compound specific stable carbon isotopic fractions ($^{13}C/^{12}C$), which increases as MTBE is biodegraded. The formation of TBA was observed, however the microbial community was not studied (Kolhatkar et al. 2002). In a following study, Wilson et al. monitored the concentration of MTBE at a retail gasoline station in Parsippany, New Jersey. The data indicated that the concentration of MTBE decreased over time. The removal varied with distance from the plume. A 53% removal was observed closer to the source and only 13% was observed further from the source in a period of three years. The concentration of TBA was observed to increase in the field. A microcosm study was conducted and it was observed that the concentration of MTBE decreased from 1.46 mg/L to 10 μg/L in 199 days of incubation, which indicated that the reduction in MTBE concentration was due to biological activity. TBA accumulated in the microcosms study. MTBE carboxylation and hydrogenation were the proposed MTBE biodegradation mechanism. The microbial composition of the site was not reported (Wilson et al. 2005).

In another study, the presence of certain metabolites was used as evidence of natural biodegradation of MTBE under anoxic conditions. The site was contaminated with MTBE (up to 130 mg/L), BTEX (up to 150 mg/L) and ammonia (up to 110 mg/L). Methanogenic conditions predominated in of the majority of the plume. Biodegradation by products such as TBA, TBF, acetone, formate, lactate, succinate, pyruvate, propionate and citrate were measured along the plume. It was observed that the concentration of the BTEX compounds decreased with distance from the pollutants source (< 1mg/L). MTBE concentrations also decreased with distance from the source. TBA and TBF were detected downstream. TBA was further transformed to lactate and TBF to formate. It was observed that in zones where the oxygen concentration was > 3 mg/L, TBA accumulated and was degraded only when the oxygen concentration decreased (<1 mg/L). Given that methanogenic conditions predominated in most of the plume, it was suggested by the authors that other electron acceptors such as nitrate, Fe(III) and sulphate were used to oxidize MTBE. The microbial community of the site was not studied (Martienssen et al. 2006).

One of the disadvantages related with anaerobic biodegradation of MTBE is that the biodegradation rate is even slower than under aerobic conditions. The slow anaerobic biodegradation rate of MTBE can be attributed to the low energy yields for bacteria when using other electron acceptors (Table 1-2) and the low availability of such electron acceptors under natural conditions. Typically groundwater has a neutral to basic pH (7-8.5) under these conditions iron is quite insoluble. It is present either as the hydroxo complexes $Fe(OH)_2^+$ and $Fe(OH)_4^-$ or as the solid $Fe(OH)_3$. In order for the bacteria to be able to utilize iron as an electron acceptor, they must invest energy to drive the reaction to the opposite direction to obtain Fe(III). Manganese (IV) solubility is also highly dependant on pH. Sulfate and methane are some of the less energetically favorable electron acceptors and it has been observed that bacteria used them only under highly reduced conditions (Rittmann and McCarty 2001).

Table 1-2 Calculated Free Energy of MTBE for Different Electron Acceptors of the products and reactants, assuming standard conditions (Izqulerdo et al. 1992; Finneran and Lovley 2001). Eh= -nΔG°' (96.485 kJ/volts)

Acceptor	Reactants	Products	ΔG°' (KJ/reaction)
Oxygen[a]	$C_5H_{12}O + 7.5O_2$	$5CO_2 + 6H_2O$	-3348.2
Nitrate	$C_5H_{12}O + 6NO_3^- + H^+$	$5HCO_3^- + 3N_2 + 4H_2O$	-3054.8
Nitrate	$C_5H_{12}O + 3.75NO_3^- + 2.5H^+ + 2.75H_2O$	$5HCO_3^- + 3.75NH_4^+$	-1951.2
Iron	$C_5H_{12}O + 30Fe(OH)_3 + 55H^+$	$5HCO_3^- + 30Fe^{+2} + 76H_2O$	-347.4
Sulfate	$C_5H_{12}O + 3.75SO_4^{-2} + 2.5H^+$	$5HCO_3^- + 3.75H_2S + H_2O$	-275.2
Carbon Dioxide	$C_5H_{12}O + 2.75H_2O$	$3.75CH_4 + 1.25HCO_3^- + 1.25H^+$	-238.7

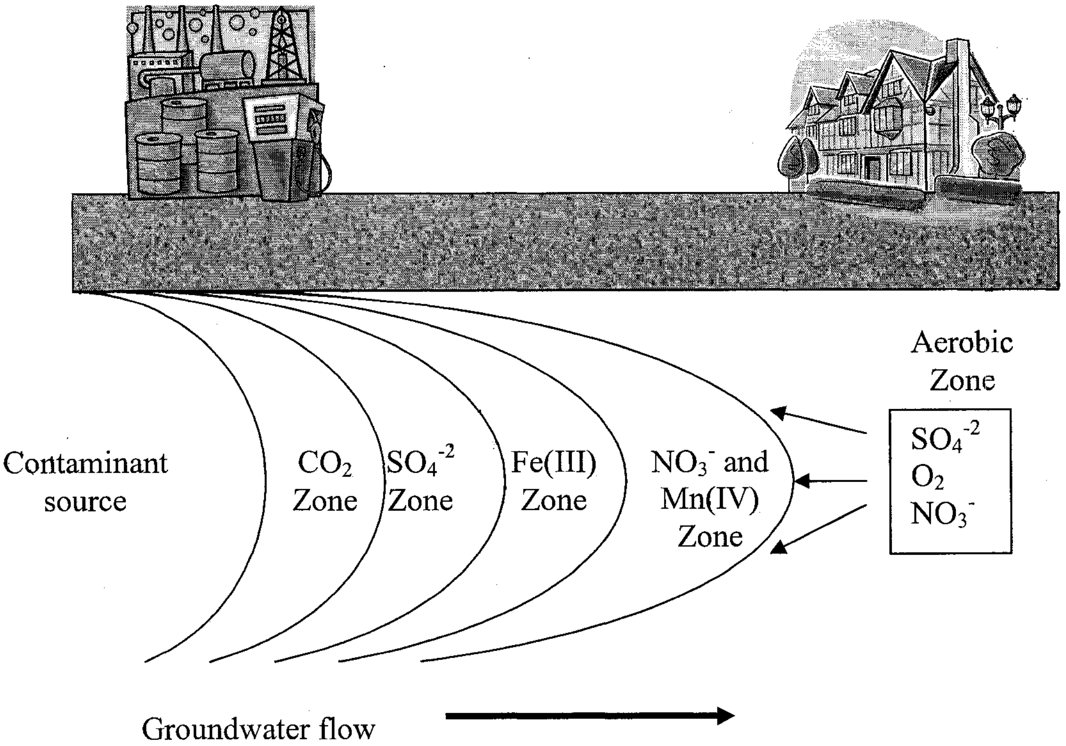


Figure 1-3 Schematic of the idealized distribution of the electron acceptors in the anaerobic zone in groundwater (Finneran et al. 2001).

Reactor	Co-contaminants present	MTBE removal rate	Culture	Observations	Reference
Batch	*Tert-butyl* alcohol, butyl formate, isopropanol, acetone and pyruvate.	4.3-8.6 mg/L-d	Pure cultures: *Methylobacterium* sp. *Rhodococcus* sp. and *Arthrobacter* sp.	Very slow growth, suggested to be a result of metabolites. Other hydrocarbons decreased MTBE degradation rate. MTBE biodegradation by mixed cultures superior to that of pure cultures.	(Mo et al. 1997)
Batch	None	65 mg/L-d	Strain ENV 420 and ENV 425	Cells were grown in propane.	(Steffan et al. 1997)
Batch	n-butane, n-alkalene, acetylene or ethylene	0.35 mg/L-d	*Graphium* sp.	Fungi were grown in n-alkalene. The presence of acetylene and ethylene inhibited MTBE oxidation.	(Hardison et al. 1997)
Batch	BTEX	20-120 mg/L-d	Strain PM1^T	Inhibition of MTBE by some BTEX compounds (ethylbenzene and xylene). Lag phase induced by benzene and toluene compounds.	(Deeb et al. 2001)

Reactor	Co-contaminants present	MTBE removal rate	Culture	Observations	Reference
Continuous flow porous-pot biomass concentrating	Diethyl ether, Diisopropyl ether, Ethanol, or BTEX	10 mg/L-d	Enrichment culture	Performance of the reactors depended greatly on the biomass concentration. Biodegradation of MTBE in the presence of BTEX was observed at a pH range of 7.4-7.6. A high microbial diversity was observed. Each condition studied had a unique microbial consortium composition.	(Pruden et al. 2001)
Biotrickling filters	None	67 mg/L-d	Enrichment culture (F-Consortium)	The biodegradation rate of the F-consortium was similar to the biodegradation rate of strain PM1^T. pH did not affect biodegradation (pH range studied 4 to 8).	(Fortin et al. 2001)
Biotrickling filters	Ethanol, Formate, Pyruvate, Lactate, Acetate, Hydroxylamine, Diethyl ether	20-50 mg/L-d	Enrichment culture (F-Consortium)	MTBE was degraded after depletion of the co-contaminant added. The microbial consortium composition was not characterized.	(Fortin et al. 2001)
Batch	BTEX	26 mg/L-d	Enrichment culture	BTEX reduced TBA degradation rate.	(Sedran et al. 2002)

Reactor	**Co-contaminants present**	**MTBE removal rate**	**Culture**	**Observations**	**Reference**
Continuous flow porous-pot biomass concentrating	BTEX	5 mg/L-d	Enrichment culture	Culture very sensitive to pH.	(Sedran et al. 2002)
Batch	Tolune, *n*-alkanes 1° alcohols, , dextrose, propane, *n*-butane, methane, ethane, TBA or formaldehyde.	3.15-9.97 mg/L-d	*Pseudomonas mendocina* KR1	Cells were grown in n-alkalenes or n-pentane. The cells were unable to further degrade MTBE intermediates (TBA and TBF). The presence of 1 ^{0} alcohols, toluene and dextrose inhibited the oxidation of MTBE in the n-alkalene grown cells. n-alkalenes, propane, butane inhibited the oxidation of MTBE on the cells growth on n-pentane.	(Smith et al. 2003a)
Batch	Acetylene or propane.	6.34 – 57.12 mg/L-d	*Mycobacterium vaccae* JOB5	Cells grown in propane or casein-yeast extract dextrose. TBA and TBF accumulated as byproducts in the batch with cells grown in propane. The presence of acetylene and propane inhibited the oxidation of MTBE.	(Smith et al. 2003b)
Batch	BTEX	2.32-10.58 mg/L-d	Strain UC1	BTEX had no effect on MTBE biodegradation.	(Pruden and Suidan 2004)

Table 1-4 Summary of Past Laboratory Studies of Anaerobic Biodegradation of MTBE.

Reactor	Oxidation-reduction Condition study	MTBE removal rate	Culture	Observations	Reference
Static soil/water microcosm	Sulfate reducing, methanogenic and denitrifying.	0.5 mg/L –d	Indigenous anaerobic mixed culture dominated by denitrifiers and sulfate reducers.	Acidic soil (pH 5-6). MTBE degradation was only observed under methanogenic conditions, low concentration of COD amended with nutrients and molybdate. TBA accumulated.	(Yeh and Novak 1994)
Static soil/water microcosm	Sulfate-, nitrate reducing and methanogenic.	0.07 mg/L-d	Indigenous anaerobic mixed culture.	MTBE was removed only in river sediments under methanogenic conditions at pH 7-7.2. MTBE was transformed to TBA.	(Mormile et al. 1994)

Reactor	Oxidation-reduction Condition study	MTBE removal rate	Culture	Observations	Reference
Static soil/water microcosm	Iron reducing.	0.4 – 1 mg/L-d	Indigenous anaerobic mixed culture.	Humic acid (HS) and anthraquinone-2,6-disulfonate (AQDS) were added. MTBE was only removed when HS or AQDS were present. Mineralization was observed.	(Finneran and Lovley 2001)
Static soil/water microcosm	Oxic, iron-, sulphate-, manganese- and nitrate reducing, and methanogenesis.	0.0017 mg/L-d	Indigenous anaerobic mixed culture.	Absence of methanogenic activity decreased the production of TBA. MTBE mineralization increased as the oxidative potential of the terminal electron acceptor increased in the order of SO4, Fe(III), Mn(IV) < NO3 < O2.	(Bradley et al. 2001a)
Chemostat	Iron reducing.	2.38 mg/L-d	Mixed culture.	Humic acid was added. MTBE was transformed to TBA.	(Pruden et al. 2005)
Batch reactor	Iron reducing.	0.03 mg/L-d	Mixed culture.	Ethanol was added. The presence of ethanol reduced the lag-phase for MTBE biodegradation. MTBE was transformed to TBA.	(Pruden et al. 2005)

1.2.2 Wetlands

Wetland treatment of MTBE may offer several advantages, but has not been well studied. A wetland can be defined as an area saturated by surface water or groundwater. Wetlands include bogs, ponds, swamps, fens, estuaries, and marshes (USEPA 2006c). There are many characteristics that can be used to classify wetlands (flow, vegetation, use, ecology, etc.). However, two main distinctions will be made in this study. The first one has to do with the nature of wetlands, since they can be natural or constructed. The second is related to the water flow in the wetland, which can be exposed to the atmosphere or close to the soil surface. When the water forms a shallow pond, then a wetland is classified as free surface flow wetland. If the water surface is below the ground surface, a wetland is classified as subsurface flow (Campbell and Ogden 1999).

The main functions of wetlands include groundwater recharge, sediment stabilization, nutrient removal/transformation and promotion of aquatic and wildlife diversity. In the past decade there has been an increasing interest in the study of wetlands due to beneficial interactions between water and soil chemistry with plants and bacteria. The potential to treat water at a low cost by using the microbial and plant populations present in wetlands has long been recognized. Wetlands have been used to treat wastewater, storm water and groundwater. Phytoremediation and bioremediation in wetlands have been used to remove nutrients such as ammonium and phosphate, cadmium, uranium, selenium, oil, pesticides, fertilizers, herbicides, TNT (nitrotoluenes), PCBs (polychlorinated biphenyls), VOCs (volatile organic compounds) (Campbell and Ogden 1999).

The main component of wetlands is water. The principal physical treatment mechanisms of contaminants are filtration, sedimentation and adsorption. The water flow rate and chemistry will also determine the biological characteristics of a wetland. The biotic components of wetlands include plants and bacteria. The removal of organic compounds and nutrients is carried out by biological activity (plants and microorganisms). Wetlands provide an ideal environment for microbial activity with anaerobic and aerobic microzones. This enhances nitrification, denitrification and phosphorus removal. Metals in soluble form such as iron, manganese and copper are transformed by microbial oxidation and precipitated in the form of oxides or sulfides (Campbell and Ogden 1999).

To date, the processes that take place in the wetlands are not fully understood. The design of wetlands has been typically based on the design of oxidation ponds (Campbell and Ogden 1999; Rittmann and McCarty 2001).

Example of MTBE Removal in Wetlands:

In May 2003, an engineered radial, insulated and aerated wetland system was implemented by British Petroleum (BP) in Casper, Wyoming. This treatment system treats 3 million gallons per day of gasoline-contaminated groundwater. The surface flow wetland was designed to allow the precipitation of iron, accomplished with the incorporation of a cascade. Without iron removal in these wetlands, precipitation would occur in the aerated subsurface flow wetlands, which could conceivably create a fouling problem. The subsurface flow wetland was designed using an aeration process (US Patents 6,200,469 and 6,406,627) that accelerates the treatment of BTEX and MTBE compounds in the wetland treatment cells. Since startup, the system has been hydraulically loaded at approximately 700,000 gallons per day. Data from this test have not been published and the microbial community has not been studied. Thus, it not possible to determine if the removal of MTBE can be attributed to the activity of the indigenous bacteria (Ferro et al. 2002; Wallace 2004).

The application of constructed wetlands to treat gasoline impacted groundwater is of interest because they are low-cost, may help increase dissolved oxygen, and do not require active maintenance. From a treatment standpoint, wetlands also have several desirable attributes. They provide an ideal complex environment for promoting microbial activity in the rhizosphere due to the presence of oxic and anoxic microzones. Therefore, a wetland may provide ideal conditions for bioremediation of contaminants that require sequential oxic/anoxic conditions.

1.2.3 Permeable Reactive Barriers

Permeable reactive barriers (PRB) are considered to be passive in situ systems with the capacity of active remediation through chemical and biological processes. Underground soil is replaced with a reactive material to create a treatment zone, with the purpose of intercepting and driving reactions within a contaminant plume in groundwater. The reactive material can be installed through the implementation of trenches or injection wells (Damm et al. 2002).

Since the material in the barrier is permeable, a chemical reaction will occur between the barrier material and the ground water or contaminant plume. Advection and dispersion are the mechanisms that control the transport of the contaminants through the wall. The treatment takes places through three mechanisms:

-precipitation

-adsorption

-degradation

The necessary consideration that needs to be done when designing these barriers is that the hydraulic conductivity of the media should be sufficiently large to allow timely and efficient treatment of the contaminated zone, since mineral and biological fouling will occur with time. Other considerations include implementation of an appropriate amount of reactive media to drive reactions and last for a sufficient time period and finally, avoiding the release additional contaminants (Damm et al. 2002).

PRBs have been commonly used to treat organic contaminants in ground water. Zero valent iron (ZVI) has been applied to treat chlorinated solvents (perchloroethylene, PCE, trichloroethylene, TCE). Solid peroxide oxygen releasing compounds (ORC) have been used to enhance biodegradation of gasoline constituents such as MTBE and BTEX, however only toluene has been observed to be removed by this material (Damm et al. 2002). Another study where magnesium peroxide (MgO_2) was used to stimulate indigenous bacteria, MTBE was completely biodegraded after 9 months and only when the BTEX compounds had been removed (Koenigsberg and Mahaffey 2001). Another application of PRBs is the removal metals by increasing the pH of water or by driving reactions that enhance precipitation. For example, ZVI has been used to react and precipitate Cr (VI) (Damm et al. 2002).

PRBs have also been used as bioreactive zones to indirectly remove metals from mine-tailing plumes. In this case, a PRB is constructed with a slow carbon release material (wood chips, alfalfa, spent mushroom compost, whey etc.) and inoculated with sulfate-reducing bacteria (dairy manure). The SO_4 present in the mine-drainage water, is reduced to hydrogen sulfide, which will combine with the metals present to result in metal-sulfide solids that will precipitate. The reduction of sulfate by bacteria increases alkalinity and pH, which further promotes the formation of carbonate and hydroxide containing solids. Similar systems have also been constructed to promote the removal of NO_3^- and phosphate. The barrier is also constructed with an organic carbon source (sawdust, cellulose, straw, etc.) to promote the growth and activity of heterotrophic denitrifying bacteria (Blowes et al. 1995). In particular, hydrogen peroxide and sodium persulfate catalyzed with iron, and potassium and sodium permanganate have been used in PRBs to chemically oxidize MTBE and BTEX (Damm et al. 2002).

The potential application of PRBs to create a bioreactive zone for anaerobic biodegradation of MTBE and BTEX is of interest to this study since this treatment is also a low-cost solution to treat contaminated groundwater. PRBs typically are easy to construct, require relatively inexpensive reactive media, require little active maintenance or energy, and can operate for long periods if they are well designed. By coupling PRBs with bioaugmentation, the bioremediation of MTBE may be enhanced. In this study various reactive media were explored, which served as slow-release electron acceptors.

Chapter 2 Objectives

The overall goal of this research is to investigate potential treatment options for MTBE in the presence of BTEX and with limited oxygen availability. Specifically, the objectives are to:

O1. Determine the effect of culture composition and reactor configuration on MTBE biodegradation in the presence of BTEX

O2. Explore the feasibility of wetlands for MTBE biodegradation in the presence of BTEX and other constituents present in refinery water.

O3. Explore anaerobic MTBE biodegradation in permeable reactive barriers (PRBs) in batch and column reactors.

O4. Isolate and characterize pure cultures capable of anaerobic MTBE degradation.

O5. Explore the effect of electron acceptor, BTEX, and sodium sulfide on anaerobic MTBE degradation.

2.1 O1: Determine the Effect of Culture Composition and Reactor Configuration on MTBE Biodegradation in the Presence of BTEX

A batch test was performed to compare the degradation of MTBE by two distinct enrichment cultures in the presence or absence of BTEX. The two mixed cultures used in this experiment were designated according to the substrate conditions under which they were originally enriched: MTBE only (MO) and MTBE and BTEX (MB). The two cultures were obtained from two previously studied porous pot biomass-concentrating continuous flow reactors (Pruden et al. 2001), from which glycerol freezer stocks were generated upon sacrifice of the reactors in 2002.

The performance of each culture was studied individually and when they were mixed at a 1:1 ratio. The performance of the culture MO was also studied in a semi-batch reactor spiked with MTBE and BTEX. The effect of BTEX on the biodegradation of MTBE was related to the reactor configuration and the microbial community diversity and composition. The source water used in this experiment was made by mixing a mineral salt medium (MSM) with phosphate buffer. The mixture was placed in glass bottles with septa and sterilized in an autoclave. A target initial concentration of 10 mg/L for MTBE and 20 mg/L of BTEX (5 mg/L each) were spiked afterwards in the reactors.

Aqueous samples were taken periodically from each reactor for analysis with purge and trap gas chromatography. The stir plates were stopped and the cells were allowed to settle for 1 hour before 1 mL of the supernatant was sampled at about 2 cm below the water surface. The 1 mL sample was diluted into a 25 mL borosilicate glass sampling vial with a Teflon cap containing 23 mL of DI water with 1 mL of 0.1 M HCl, for sample preservation. The samples were refrigerated and analyzed within one week of sampling.

Two-milliliter samples were collected for microbial community analysis from the batch reactors at the same time as samples were taken for analysis of MTBE and BTEX. Three samples were selected from each reactor at three key MTBE biodegradation time points: onset, middle and end, corresponding approximately to when MTBE was at the initial concentration just before degradation commenced, when MTBE was half of the initial value and just before spiking back (20 µg/L). The composition of the bacterial consortium was explored using a combination of 16S rRNA gene cloning and quantitative polymerase chain reaction targeting the known MTBE-degrading strain PM1^T.

Hypothesis:

- The microbial community composition and the reactor configuration will have a great impact on the simultaneous biodegradation of MTBE and BTEX.
- Reactors having a more diverse community, thus robust, will be able to remove the contaminants simultaneously.
- Higher MTBE removal rate is expected for the semi-batch reactor than in the batch reactors.

2.2 O2: Explore the Feasibility of Wetlands for MTBE Biodegradation in the Presence of BTEX and Other Constituents Present in Refinery Water

The first aim of this study was to determine the effect of bioaugmentation on MTBE and BTEX biodegradation in wetlands. The second aim of this study was to determine whether wetlands can provide suitable conditions for the simultaneous degradation of MTBE and BTEX.

Wetlands at laboratory scale were investigated to build an understanding of their biodegradation potential. Two enrichment cultures were used to seed the wetlands, an anaerobic iron-reducing and an aerobic MTBE-degrading culture. The anaerobic Fe(III)-reducing culture was enriched by mixing the biomass from aerobic MTBE-degrading, methanogenic and sulfate-reducing cultures and providing MTBE as the sole carbon source and Fe(III) as the sole electron acceptor (Pruden et al. 2005). The aerobic culture used was the MO culture described in O1. A schematic of the experimental setup is presented in Figure 2-1.

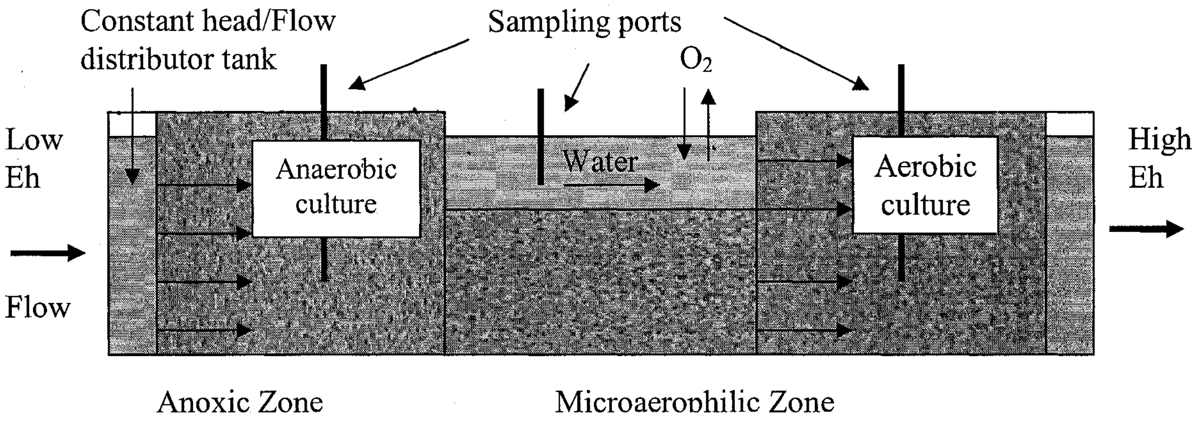


Figure 2-1 Experimental setup of wetlands study.

Commercially available aquaria were used to simulate the wetlands. The experiment was set up in proximity to the air stripping system of a petroleum refinery located near Denver, CO. The water inlet to the existing air-stripper at the refinery was used as the source water for the experiment in order to explore the applicability of the approach for actual treatment of contaminated groundwater. From previous chemical analyses of this water, it was known that the water was contaminated with several organic and inorganic constituents. Contaminants such as acetone, naphthalene, phenol, VOCs (volatile organic compounds), MTBE, benzene, ethylbenzene, toluene xylenes, polycyclic aromatic hydrocarbons (PAHs), nitrate, fluoride and barium were present in the water (data collected by RETEK, Fort Collins, CO). The ammonium concentration was 0.54 mg/L as N, with a C:N ratio of 585 (samples sent to the CSU Soil, Water and Plant Testing Laboratory, Fort Collins, CO). The minimal C/N ratio for bacterial growth is 20 (Sylva et al. 2005). A batch test was conducted, where both aerobic cultures were exposed to the refinery water, in order to confirm that the refinery water did not have a negative effect on the biodegradation of MTBE and BTEX.

Six aquaria with different conditions were studied to determine the most suitable and effective treatment for constructing a wetland at field-scale. The conditions studied are presented Table 2-1.

Table 2-1 Experimental Conditions for the Wetlands Experiment.

Condition	Purpose
No inoculation	Observe the activity of indigenous bacteria
Anaerobic iron-reducing culture + aerobic culture + Fe(III)	Observe effect of biostimulation and bioaugmentation.
Anaerobic iron-reducing culture + Fe(III)	Observe effect of biostimulation and bioaugmentation.
Aerobic culture	Isolate effect of bioaugmentation with aerobic culture.
Anaerobic iron-reducing culture + aerobic culture	Isolate effect of bioaugmentation with both cultures.
No inoculation, bleach killed.	Quantify volatilization.

The tanks were divided into three zones. The first zone was bioaugmented with the anaerobic culture and the last zone was bioaugmented with the aerobic culture (depending on the treatment). Three sampling ports were placed in each zone at three different depths to monitor MTBE and BTEX concentration with space. Aqueous samples were taken weekly at the different sampling points and analyzed by purge and trap gas chromatography. A volume of 25 mL was taken and placed in a glass sampling vial with a Teflon cap and stored at 4^0C until analysis. The samples were stored refrigerated for a period of less than five days.

The pH, temperature, conductivity and oxidation-reduction potential were measured during the sampling events. Samples for bacterial community analysis were taken from the sediments at the beginning of the experiment and at the end to determine the change in composition from the beginning to the end of the experiment. Water samples were also collected at the beginning of the experiment. The relative concentration of $PM1^T$ with respect to the concentration of total bacteria based on the 16S rRNA gene was determined using real time Q-PCR.

Hypothesis:

- Biodegradation of MTBE is possible in wetlands.
- The wetlands that were bioaugmented and/or biostimulated will be enhanced, thus a higher the removal rate will be observed.

2.3 O3: Explore Anaerobic MTBE Biodegradation in Permeable Reactive Barriers (PRBs) in Batch and Column Reactors.

2.3.1 Anaerobic Batch Experiment

Batch experiments were performed to compare Fe(III) and SO_4 as electron acceptors in the anaerobic removal of MTBE, which could potentially be implemented in PRBs. This study also served as a simple way to observe any chemical precipitation reactions that may occur with the different reactive media studied that could have the potential to clog a PRB. The results were used to select the reactive media that was used in the column test (described in section 2.3.2).

Four 1 L amber borosilicate bottles were applied in this experiment in order to test the two electron acceptors. The experimental conditions are presented in Table 2-2. Four controls were set up using gypsum ($CaSO_4{\cdot}2H_2O$) to provide a slow-release source of sulfate as the electron acceptor. Two bottles were killed controls with 0.1 g/L of Mercuric (III) chloride (Sigma-Aldrich, St. Louis, MO).

Table 2-2 Experimental Conditions for the Anaerobic Batch Experiment.

Reactor Name	**Electron Acceptor**	**Concentration of the Electron Acceptor (g/L)**	**Notes**
Na_2SO_4 (A)	Na_2SO_4	0.055	32 mg/L of Na_2S were added. Inoculated.
Na_2SO_4 (B)	Na_2SO_4	0.055	32 mg/L of Na_2S were added. Inoculated.
$FeCl_3$ (A)	$FeCl_3$	0.302	32 mg/L of Na_2S were added. Inoculated.
$FeCl_3$ (B)	$FeCl_3$	0.302	32 mg/L of Na_2S were added. Inoculated.
Control A	$CaSO_4$	6	Uninoculated
Control B	$CaSO_4$	6	Uninoculated
Killed Control A	$CaSO_4$	6	1 mg/L $MgCl_2$ were added.
Killed Control B	$CaSO_4$	6	1 mg/L $MgCl_2$ were added.

Deionized (DI) water amended with a mineral salt medium (MSM) and 10X phosphate buffer solution (PBS) was used as the experiment source water. The MSM composition is described in Pruden et al. 2001 (Pruden et al. 2001).The batch reactors were set up in a N_2 glovebox. A volume of 980 mL of amended DI water was added to each bottle to allow a 20 mL head space for gas production. The bottles were capped with butyl rubber stoppers and screw caps. However, two perforations were made to the rubber stoppers to place two lines of 1/8 inch ID fluorinated ethylene propylene (FEP) tubing. The end of one tube was placed half way into the bottle and the other tube extended only to the bottom of the rubber stopper to enable the controlled release of gases. The outside ends of the tubes were maintained close with a clamp. The bottles were placed under water to reduce the possible diffusion of oxygen into of the batch reactors. Water samples were taken weekly for later GC and molecular analyses. At each sampling event, a 25 mL gastight syringe was flushed three times and subsequently filled with nitrogen gas. The syringe was connected to the gas-release tube. A 1 mL syringe was placed in the liquid sampling tube to collect 0.2 mL aqueous sample for gas chromatography (GC) analysis and a 1 mL samples for molecular analysis, using pressure from the nitrogen-filled syringe to drive the liquid sample out of the bottle. Samples for GC analysis were stored at 4^0C until analysis up to three days and samples collected for molecular analysis were stored at -80^0C until analysis. The bottles were mixed manually by shaking two times a week. The bacterial consortium in of the batch reactors was characterized using a combination of 16S rRNA gene cloning (end of the experiment) and real time quantitative PCR (beginning and end of the experiment).

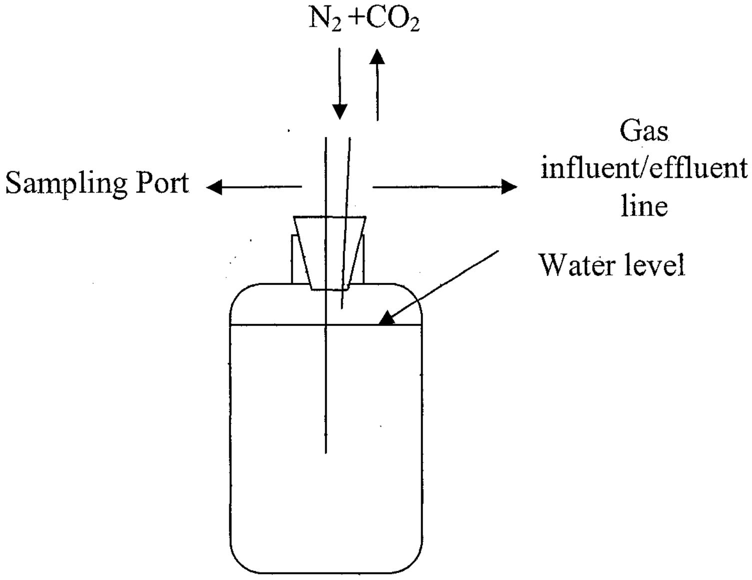


Figure 2-2 Schematic of the anaerobic batch reactors.

Hypothesis:

- The reactors dosed with Fe(III) will yield a higher biomass concentration and a higher MTBE removal rate, than the reactors dosed with SO_4.

2.3.2 Anaerobic Column Test

A column test comparing reactive media in combination with the anaerobic cultures was performed with the purpose of simulating PRBs proposed for implementation at the refinery. Bacterial community analyses such as DGGE, RFLP and real time Q-PCR were carried out on select columns in order to help identify biomarkers associated with anaerobic MTBE biodegradation.

A secondary aim of this experiment was to determine the effect of flow-through treatment on the removal of MTBE under anaerobic conditions. A similar comparison as the one made in O1 was made, where the effect of diversity and the composition of the anaerobic culture were explored on relative performance in terms of MTBE degradation rate.

Four columns were constructed using 45.5 cm long and 11 cm diameter perforated acrylic pipes. The columns had four sampling ports. The sampling ports were located at 3 cm, 9.5 cm, 23 cm and 43 cm (from the influent to the effluent). The columns were packed as follows: 4 cm of sand (particle size ranging from 0.5—0.6 mm), 34 cm of reactive media and 7.5 cm of sand. Three columns were constructed using reactive media mixed with the Fe-reducing anaerobic culture. One column was constructed only with the reactive media, but was not be inoculated. This provided a means to determine the effect of bioaugmentation. A schematic of the columns is presented in Figure 2-3.The experimental conditions are presented in Table 2-3.

Table 2-3 Column Compositions and Purpose for Study

Reactive Medium	Purpose
Gypsum and Ferric Hydroxide	Determine the effect of these two chemicals
Gypsum	Isolate effect of gypsum
Ferric Hydroxide	Isolate effect of ferric hydroxide
Not inoculated Gypsum and Ferric Hydroxide (Control)	Determine biological role of degradation

The flow-rate to the columns was 0.194 L/d, which is equivalent to 0.03 ft/day (assume n= 0.45). The hydraulic retention time of the columns was equal to 10 days. MTBE and concentration was monitored on a weekly basis for nine months. The experiment was repeated for one month to confirm results. In this experiment, amended DI water spiked with MTBE and the MSM used in O1 was used at the beginning of the experiment.

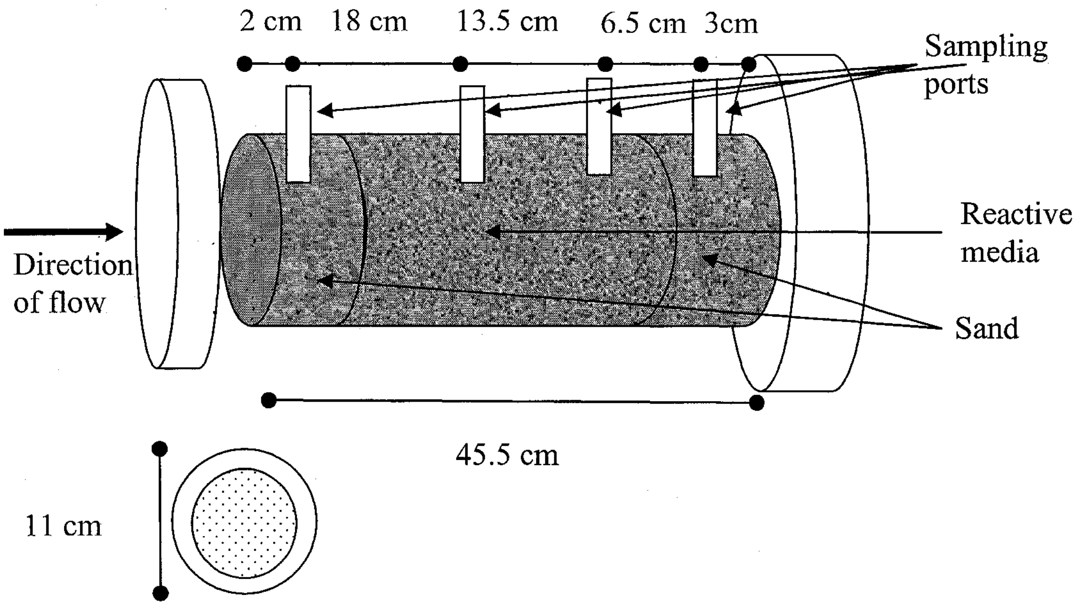


Figure 2-3 Schematic of columns.

Samples for molecular analyses were collected from the reactive media at the beginning and at the end of the experiment. The concentration of total bacteria was determined at the beginning and at the end of the experiment using real time quantitative PCR. The bacterial community was explored using 16S rRNA gene cloning, for the sample taken at the end of the experiment of column that had both electron acceptors.

Hypothesis:

- Since sulfate is more soluble, the column with gypsum only will initially outperform the one with Fe(III). However sulfate yields less energy than Fe(III), thus, in the long term it is expected that Fe(III) + gypsum will provide a higher level of removal.

2.4 O4: Isolate and Characterize Pure Cultures Capable of Anaerobic MTBE Degradation.

Pure cultures were isolated by plating mixed cultures on agarose plates. The mixed cultures include a previously enriched iron-reducing anaerobic culture (Pruden et al. 2005), an aerobic culture previously enriched on MTBE as the sole substrate (MO) (Raynal and Pruden 2007), and the influent from an air stripping system of an petroleum refinery near Denver, CO. The cultures were spread on 4% (v/v) agarose-mineral salt medium (MSM) plates containing 950mg/L of $Fe_2(SO_4)$ (Wako Chemicals USA Inc., Richmond, VA) and placed in an airtight GasPak Jar™ (BD, Sparks, MD) with a BD BBL™ Gas Pak™ Plus gas generator envelope (BD, Sparks, MD) to create anaerobic conditions. An anaerobic indicator (Oxoid, Lenexa, KS) was used to verify that anaerobic conditions were maintained. The MSM contained nutrients and minerals for microbial growth (Pruden et al. 2005). A 5 mL glass vial with a perforated Teflon cap containing MTBE (Arcos, Morris Plane, NJ) was placed inside the chamber to provide the sole carbon substrate.

Each month for eight months, the GasPak Jar™ was opened inside a glovebox with N_2 gas to refill the vial containing MTBE, to replace the gas generator envelope and the anaerobic indicator. Once colonies appeared they were transferred to a new plate and to a 100 mL glass bottle that contained the MSM, 750 mg/L of $Fe_2(SO_4)$ (Wako Chemicals USA Inc., Richmond, VA) and 10 mg/L of MTBE (Arcos, Morris Plane, NJ). The glass bottles were capped with rubber septa and were placed up-side-down in a controlled temperature shaking bed at 25 ^{0}C and 60 rpm. To confirm the ability of the culture to degrade MTBE in liquid, samples were taken biweekly and the concentration of MTBE in the bottles was monitored using gas chromatography. Once MTBE biodegradation was observed (a decrease of 50% from the initial concentration in 35 days) the cultures were serially diluted and 10 µl of the 10^{-2} dilution were plated to isolate pure culture. The colonies growing on the plates and in the liquid cultures were characterized by Gram staining, scanning electron microscopy (SEM), direct sequencing of the 16S rRNA genes and denaturing gel gradient electrophoresis (DGGE). The liquid culture of the 10^{-2} dilution and the colonies growing in the plates from the same dilution were transferred to 250 mL glass bottles caped with rubber septa that contained amended DI water with either Fe(III) or SO_4 as the electron acceptor and 10 mg/L of MTBE. This was done in order to determine the biodegradation rate of MTBE under ferric and sulfate reducing conditions.

Hypothesis:

- A microbial strain exists capable of anaerobic biodegradation of MTBE and that can be isolated in pure culture.

2.5 O5: Explore the effect of electron acceptor, BTEX, and sodium sulfide on anaerobic MTBE degradation.

The main objective of this study was to characterize bacterial community dynamics during biodegradation of MTBE under ferric and sulfate reduction conditions. A second aim of this study was to determine whether MTBE was anaerobically biodegraded in the presence of BTEX.

Anaerobic bioremediation of MTBE was studied under semi-continuous flow and batch conditions comparing two electron acceptors: Fe(II) and SO_4. The effect of the presence of the BTEX compounds was also studied under batch conditions. The diversity and composition of the microbial community was studied in the reactors exhibiting MTBE removal.

A 4L semi-batch reactor with both ferric iron and sulfate was set up. The semi-batch reactor was inoculated with a previously enriched Fe-reducing anaerobic culture obtained from a porous pot biomass-concentrating continuous flow reactor operated under iron-reducing conditions (Pruden et al. 2005) was used as the inoculum. The culture had been stored at -80°C in 20% glycerol since 2002. Once biodegradation of MTBE was observed, nine smaller semi-batch reactors were inoculated from this reactor in order to test the effect of the electron acceptor and sulfide addition

Aqueous samples were taken periodically from each reactor for analysis with purge and trap gas chromatography. Two-milliliter samples were collected for bacterial community analysis from the 4 L semi-batch reactor, at the same time samples were taken for analysis of MTBE and BTEX. Three samples were selected from the semi-batch reactor at three key MTBE biodegradation time points: onset, middle and end, corresponding approximately to when MTBE was at the initial concentration just before degradation commenced, when MTBE was half of the initial value and just before spiking back: to 20 µg/L. Samples for molecular analysis were collected from the smaller reactors at the beginning and at the end of the experiment. The concentration of total bacteria was determined targeting the 16S rRNA gene with real time Q-PCR. The bacterial community of the semi-batch reactor was characterized using 16S rRNA gene cloning.

Hypothesis:

- Higher biomass concentration is expected in the reactors that had Fe(III), since it is thermodynamically more favorable and yields more energy to the cells than sulfate.
- BTEX is expected to inhibit the removal of MTBE as has been observed in aerobic studies.
- Higher microbial diversity in the presence of both electron acceptors compared to the cases where only one electron acceptor was present.

Chapter 3 Materials and Methods

3.1 Monitoring MTBE and BTEX

Five milliliters of aqueous sample was used to measure the concentration of MTBE and BTEX compounds. The concentration of these compounds was determined using a Tekmar-Dohrman (Cincinnati, Ohio) heated purge and trap 2016 and a Hewlett Packard (Rolling Meadows, Illinois) 5890 Series II Gas Chromatograph (GC) with a capillary column and flame-ionization detector (FID). Volatile organic compounds (VOCs) were transferred to the gaseous phase by purging the water sample with an inert gas (ultra-pure Helium) at ambient temperature. The vapor is passed through a trap that sorbs the analytes of interest and is then heated and back-flushed to desorb VOCs into a GC column for separation. The compounds are separated in the column flow to FID for confirmation and quantification. The EPA method 8015 was used (USEPA 2007b).

Eight point calibration curves were generated with concentrations varying from 4 to 2000 μg/L, with R^2 ranging from 98.95% for *p*-xylene to 99.96% for benzene. A calibration curve for TBA, an MTBE intermediate, was also attempted, however the R^2 for the calibration curve of this compound was 87.29%, thus it was determined that the TBA concentration could not be accurately quantified, and its presence or absence in each sample was reported instead.

3.2 Characterization of Microbial Communities

3.2.1 DNA Extraction

The MoBio UltraClean Soil DNA kit (Solana Beach, CA) was used for the extraction of microbial DNA from the aqueous and sediment samples according to manufacturer's protocol. The only modification made to the kit procedure was that the bead-beating time was reduced to 4 minutes instead of 10 minutes to reduce shearing of DNA. This kit was preferred since it includes a solution that removes common PCR inhibitors (humic acids, metals, etc).

3.2.2 Cloning of 16S rRNA Genes

Cloning of 16S rRNA genes was performed to estimate the diversity of the cultures and to identify the dominant microorganisms present. The 16S rRNA genes were polymerase chain reaction (PCR) amplified with the primers 8F and 1492R (Weisburg et al. 1991). The PCR master mix consisted of (per 25 μl reaction): 2.5 μL of 10X buffer, 5 μL of 5X buffer, 0.5 μL of dNTP (10 mM), 0.25 μL of formamide, 1 μM of each primer (5 mM), and 0.35 μL of Taq polymerase (Eppendorf, Westbury, NY). The thermocycler program consisted of an initial denaturing step of 2 minutes at 94°C, followed by 25 cycles of 30 seconds at 94°C, 30 seconds at 50°C and 30 seconds at 72°C. A final extension step was conducted for 10 minutes at 68°C. The resulting PCR products were cloned using the TOPO TA Cloning Kit® (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. Resulting clones were screened for the presence of an insert by PCR using vector-specific M13F and M13R primers. The amplified inserts were digested with the restriction enzyme *MspI* (Promega, Madison, WI.) in order to identify unique inserts by restriction fragment length polymorphism (RFLP). The number of species present in each reactor was determined by using a software developed by Steven Holland: Analytic Rarefaction 1.3 (UGA Stratigraphy Lab, Athens, GA).Unique inserts were purified with ExoSAP-IT (USB, Cleveland, OH) and sequenced by the Macromolecular Resources Facility at Colorado State University (Fort Collins, CO).

3.2.3 Sequence Analysis and Assignment to Functional Groups

Most frequent clones, determined as those which appeared more than four times in the library, were selected from each sample for sequencing. The BLAST tool of the National Institute of Health database was used to search and identify the highest matches for each clone insert. Sequences were also analyzed using the Ribosomal Database Project (RDP) search to aid in phylogenetically classifying the microorganisms represented by clone sequences. A literature review was performed in order to characterize the microorganism corresponding to the highest matches.

3.2.4 Quantification of Total Bacteria and PM1^T by Real-Time Quantitative PCR (Q-PCR)

A TaqMan Q-PCR protocol has been reported recently by (Hristova et al. 2001) for quantifying the known MTBE-degrading strain PM1^T. For PM1^T primers 963F and 1076R and the TaqMan probe PM1 were used. An adaptation of this Q-PCR was used to measure the concentrations of strain PM1^T in the DNA extracts from each experiment. Twenty-four microliters of PCR master mix was mixed with 1 μL of DNA extract. The master mix consisted of 2.5 μL 10X buffer, 5 μL 5X buffer, 0.5 μL dNTP (10 mM), 0.25 μL of each primer (10 mM), 0.375 μL TaqMan probe PM1 (10 mM), 0.35 μL Taq Polymerase (Eppendorf, Westbury, NY), and 1.5 μL Mg^{2+}. All Q-PCR reactions were performed in duplicate using a Cepheid Smart Cycler (Sunnyvale, CA). The amplification program consisted of 2 minutes at 95^{0}C, followed by 50 cycles at 95^{0}C for 15 seconds, 53^{0}C for 60 seconds and 72^{0}C for 20 seconds. The relative concentrations of total bacteria were determined by quantifying total 16S rRNA genes using the TaqMan 16S probe Q-PCR protocol described by Suzuki et al. (Suzuki et al. 2000). The reaction conditions were the same as described above, except that primers 1369F (10 mM) and 1492R (10 mM) and the TaqMan probe 16S were used.

The concentration of the strain PM1^T was normalized by the concentration of total bacteria. A generalized least-square means (GLM) analysis was conducted to obtain p-values in order to determine if the difference the concentration of bacteria observed for each sample at the different times studied was significant. SAS 9.1 software was used to calculate p values (SAS Institute Inc., Cary, NC).

Chapter 4 Results

4.1 Objective 1: Determine the Effect of Culture Composition and Reactor Configuration on MTBE Biodegradation in the Presence of BTEX.

Aerobic MTBE biodegradation in the presence of BTEX by two consortia under batch and semi-batch conditions

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Raynal, M. and Pruden, A. (2007) "Aerobic MTBE biodegradation in the presence of BTEX by two consortia under batch and semi-batch conditions". Biodegradation. In Press.
http://www.springerlink.com/content/a3455v111767255n/?p=d23b8ee2df094718b96a2c7cc08fecf2&pi=3

4.1.1 Abstract

This study explores the effect of microbial consortium composition and reactor configuration on MTBE biodegradation in the presence of BTEX. MTBE biodegradation was monitored in the presence and absence of BTEX in duplicate batch reactors inoculated with distinct enrichment cultures: MTBE only (MO- originally enriched on MTBE) and/or MTBE BTEX (MB- originally enriched on MTBE and BTEX). The MO culture was also applied in a semi-batch reactor which received both MTBE and BTEX periodically in fresh medium after allowing cells to settle. The composition of the microbial consortia was explored using a combination of 16S rRNA gene cloning and quantitative polymerase chain reaction targeting the known MTBE-degrading strain PM1^T. MTBE biodegradation was completely inhibited by BTEX in the batch reactors inoculated with the MB culture, and severely retarded in those inoculated with the MO culture (0.18 ± 0.04 mg/ L-d). In the semi-batch reactor, however, the MTBE biodegradation rate in the presence of BTEX was almost three times as high as in the batch reactors (0.48 ± 0.2 mg/L-d), but still slower than MTBE biodegradation in the absence of BTEX in the MO-inoculated batch reactors (1.47 ± 0.47 mg/L-d). A long lag phase in MTBE biodegradation was observed in batch reactors inoculated with the MB culture (20 days), but the ultimate rate was comparable to the MO culture (0.95 ± 0.44 mg/L-d). Analysis of the cultures revealed that strain PM1^T concentrations were lower in cultures that successfully biodegraded MTBE in the presence of BTEX. Also, other MTBE degraders, such as *Leptothrix* sp. and *Hydrogenophaga* sp. were found in these cultures. These results demonstrate that MTBE bioremediation in the

presence of BTEX is feasible, and that culture composition and reactor configuration are key factors.

4.1.2 Introduction

Unintentional spills at refineries and gas stations from leakage of pipelines and underground storage tanks can lead to serious contamination of aquifers with gasoline constituents. Gasoline oxygenates such as methyl *tert*-butyl ether (MTBE) and ethanol are used in order to reduce the emission of carbon dioxide and ozone forming compounds. MTBE has been used since the late 1970s and became preferred over other gasoline oxygenates in the United States, replacing lead as an anti-knocking compound (USEPA 2006b). The U.S. Geological Survey found that MTBE was the second most common volatile compound present in urban aquifers in the United States (Squillace et al. 1999). MTBE has a high water solubility, low adsorption to soil and low biodegradation rate, and thus MTBE continues to be a prevalent groundwater contaminant (USEPA 2006b). Even though the toxicity and carcinogenicity of MTBE has not been confirmed, the United States Environmental Protection Agency (U.S. EPA) has established a drinking water maximum contaminant level of 20 μg/L, since MTBE adds significant taste and odor (USEPA 2005).

Active plume remediation of gasoline impacted sites is a common treatment technique. Active remedial methods include groundwater pump-and-treat (air stripping and removal with granular activated carbon), vapor extraction, chemical oxidation and multiphase high-vacuum extraction (Deeb et al. 2003). However, the cost associated with the construction, maintenance and operation of these treatments diminishes their feasibility; therefore, bioremediation is a preferred treatment option.

Ever since Salinitro et al. (Salanitro et al. 1994) demonstrated that MTBE was aerobically biodegradable, several efforts have been made to isolate MTBE degraders in pure culture. Aerobic MTBE-degrading bacteria that have been described to date, include: *Rhodococcus* sp. (Mo et al. 1997), *Mycobacterium austroafricanum* IFP 2012 (Francois et al. 2002), ENV 425 (Steffan et al. 1997), *Rubrivivax gelatinosus* strain PM1 (Bruns et al. 2001) and *Hydrogenophaga* sp. (Hatzinger et al. 2001). One common attribute among MTBE degraders is a low yield coefficient, with theoretical to experimental biomass yield ratios (δ_e) ranging from 2.6-5.8 (Salanitro et al. 1994; Hanson et al. 1999; Fortin et al. 2001; Hatzinger et al. 2001; Francois et al. 2002; Fayolle et al. 2003). Energetically, aerobic degradation is favorable over all other electron acceptors (Stocking et al. 2000; Fayolle et al. 2001; Finneran and Lovley 2001; Fortin et al. 2001).

The most well-known aerobic MTBE degrader, strain PM1^T, is capable of complete mineralization of MTBE and can also use benzene as a sole source of carbon and energy (Deeb et al. 2001). Because of its high MTBE biodegradation rate, this strain has been used as a standard to characterize MTBE aerobic biodegradation by other strains (Fortin et al. 2001). In addition, the identification of strain PM1^T in aerobic MTBE-degrading cultures or environmental samples has been used as a parameter to correlate the presence of MTBE degraders with MTBE biodegradation (Hristova et al. 2003).

At gasoline impacted sites, the co-occurrence of MTBE and other organic compounds (such as benzene, toluene, ethylbenzene and xylenes (BTEX)) is a common scenario, especially at refineries. In order to successfully bioremediate these sites, the conditions under which MTBE and the other compounds can biodegrade simultaneously must be characterized. It was noted by Deeb et al. (Deeb et al. 2001) in batch reactors that the presence of ethylbenzene, *o*-xylene, and *p*-xylene inhibited the biodegradation of MTBE by strain PM1^T, while toluene slightly inhibited the biodegradation of MTBE. Results such as these have discouraged attempts to bioremediate MTBE in the presence of BTEX or other contaminants. On the other hand, recent studies have demonstrated that under some conditions MTBE biodegradation can take place in the presence of other compounds such as BTEX in both pure and mixed cultures (Pruden et al. 2001; Sedran et al. 2002; Pruden and Suidan 2004). Thus, a better understanding of the conditions under which BTEX inhibits MTBE biodegradation is needed to advance bioremediation of these mixtures in the field.

Several laboratory experiments have been conducted under different conditions (i.e. various MTBE concentrations, batch or continuous flow tests), implementing different cultures (pure and mixed), and observing the effect of co-contaminants (Table 4-1). However, no study to date has explored the effect of the microbial consortium composition, nor has the effect of batch versus semi-batch conditions been directly compared. The main difference between the semi-batch reactor and the batch reactors in this study was that fresh medium was periodically provided after allowing the cells to settle in the semi-batch reactor. The objective of this study was to investigate the effect of BTEX on MTBE biodegradation on two levels: 1) the composition and overall diversity of the consortium and 2) the reactor configuration. Two distinct enrichment cultures were compared, MO (originally enriched on MTBE-only) and MB (originally enriched on MTBE and BTEX). The effect of batch versus semi-batch reactor configuration was also determined using the MO culture. Analyses of the microbial consortium included: cloning, restriction fragment length polymorphism

(RFLP) screening and sequencing of 16S rRNA genes and quantitative polymerase chain reaction (Q-PCR) quantification of the known MTBE-degrader, strain PM1^T. In addition to its application for addressing an important challenge for MTBE bioremediation, this study is unique in that it explores the relationship between complex microbial consortium dynamics to biodegradation performance.

4.1.3 Materials and Methods

4.1.3.1 Reactor Design and Operation

Fifteen batch reactors (7 in duplicate and 1 uninoculated control) were used to compare the biodegradation of MTBE by two distinct enrichment cultures in the presence or absence of BTEX (benzene, toluene, ethylbenzene and *p*-xylene). The enrichment cultures were designated as the MTBE only culture (MO) and the MTBE and BTEX culture (MB), indicating the substrate conditions under which they were originally enriched. The cultures were obtained from two previously studied porous-pot biomass concentrating continuous flow reactors (Pruden et al. 2001). Glycerol freezer stocks were generated upon sacrifice of the reactors in 2002 and maintained at -80°C until the time of this study. The MO and MB cultures were studied individually and mixed at a 1:1 ratio. A summary of the experimental conditions is presented in Table 4-2.

Batch experiments were carried out in 500 mL borosilicate glass bottles, into which 360 mL of mineral salt medium (MSM) and 40 mL 10X phosphate buffer solution (PBS) were initially aliquotted with a pH of 7.0. The MSM nitrogen source was ammonium and contained trace minerals, as described previously (Pruden et al. 2001). The bottles were sterilized in an autoclave, sealed with butyl rubber septa, and continuously stirred at medium velocity with magnetic stir bars to ensure sufficient dissolved oxygen (DO). The 100 mL headspace provided more than double the requirement for mineralization based on stoichiometry. Opening all bottles for 5 minutes at each sampling provided further assurance that more than sufficient DO was available, while control bottles accounted for any potential losses of MTBE or BTEX due to volatilization. The pH was measured periodically and consistently fell in the range of 7.0 ±0.2 throughout the experiment.

All reactors were inoculated from the same enrichment culture freezer stocks, which allowed direct day by day comparison of the resulting consortia dynamics in different experiments. Enrichment cultures stored at -80°C were warmed initially overnight at -20°C and then placed on ice to melt slowly. The cells were washed three times with MSM by vortexing, centrifuging, and pouring off the supernatant to remove the glycerol used to store the cells. The washed cells were diluted in MSM and 5 mL were used to inoculate each batch reactor, for an initial average concentration of 1 X 10^3 cells/mL. MTBE and BTEX were dissolved in the mixture by adding the appropriate volume with a gastight syringe (Hamilton Company, Reno, NV). A target initial MTBE (Acros, Morris Plane, NJ) aqueous concentration of 10 mg/L and either zero or 20 mg/L (5mg/L each) of BTEX (Fisher, Fair Lawn, NJ) compounds were spiked in the reactors. When biodegradation was observed, the batch reactors were re-spiked periodically with MTBE and/or BTEX. A killed control dosed with 0.1g of sodium azide (Fisher, Fair Lawn, NJ) was also monitored. The batch reactors were maintained at room temperature (~25 ^{0}C), with a total experiment time of 93 days.

A semi-batch reactor was also studied. A 4 L borosilicate glass cylindrical reactor (3,200 mL aqueous phase, 800 mL head-space), closed to the atmosphere and continuously stirred with a magnetic stir bar and stir plate, was sterilized with MSM and PBS, inoculated with the MO culture, and spiked with MTBE and BTEX as described for the batch reactors. The target concentrations of MTBE and BTEX were 10 and 20 mg/L, respectively, as applied in the batch reactors containing BTEX. Instead of spiking the compounds directly when necessary, fresh MSM and PBS containing MTBE and BTEX were provided. Cells were allowed to settle for one hour before removing the spent medium and adding the fresh medium. The performance of the semi-batch reactors was monitored over a period of 105 days. All reactors were named as summarized in Table 4-2.

4.1.3.2 Monitoring MTBE and BTEX

Aqueous samples were taken periodically from each reactor and analyzed within one week. The stir plates were stopped and the cells were allowed to settle for 1 hr before 1 mL of the supernatant was sampled at about 2 cm below the water surface. In a 25 mL borosilicate glass sampling vial with a Teflon cap, the 1 mL sample was diluted into 23 mL of deionized (DI) water with 1 mL of 0.1 M HCl for sample preservation.

Five milliliters of this mixture were used to measure the concentration of MTBE and BTEX compounds. The concentration of these compounds was determined using a Tekmar-Dohrman (Cincinnati, OH) heated purge and trap 2016 and a Hewlett Packard (Rolling Meadows, IL) 5890 Series II Gas Chromatograph (GC) with a capillary column and flame-ionization detector (FID). Eight point calibration curves were generated with concentrations varying from 4 to 2000 μg/L, with R^2 ranging from 98.95% for *p*-xylene to 99.96% for benzene. Because the R^2 value of the calibration curve for *tert* butyl alcohol (TBA), an MTBE intermediate, was 87.29%, it was concluded that the TBA concentration could not be accurately quantified, so its presence or absence in each sample was reported instead. The biodegradation rates for each reactor were calculated from the linear portion of each degradation curve (neglecting lag phase) assuming that the transformations of MTBE to TBA or CO_2 were zero order. Most studies described in a recent review assumed zero order kinetics (Fortin et al., 2001) and its use was further justified based on the relatively high MTBE to cell ratio of this study.

4.1.3.3 Characterization of the Microbial Communities

Two-milliliter samples were collected from the batch reactors for microbial analysis just before the stir plates were turned off and cells settled for MTBE and BTEX sampling. Samples were stored at -80 °C and were later selected for further analysis based on their relationship to three key MTBE biodegradation time points during the second observed degradation event: onset (just before MTBE biodegradation commenced), middle (when MTBE was at ~5 mg/L) and end (just before spiking back to 10 mg/L). Because the MO MBTEX batch reactor biodegraded MTBE at a very slow rate, for comparative purposes it was further analyzed on days when the MO MBTEX semi-batch reactor was sampled.

4.1.3.3.1 DNA Extraction

The MoBio UltraClean Soil DNA kit (Solana Beach, CA) was used for the extraction of microbial DNA according to the manufacturer's protocol. The only modification made to the kit procedure was that the bead-beating time was reduced from ten minutes to four minutes, in order to reduce shearing of DNA.

4.1.3.3.2 Cloning of 16S rRNA Genes

Cloning of 16S rRNA genes was performed to estimate the diversity of the cultures and to identify the dominant microorganisms present in the following samples: MO and MB freezer stocks, one of the MO MBTEX batch reactors and the semi-batch reactor. This provided a means to compare a consortium efficient at biodegrading MTBE in the presence of BTEX, with a BTEX-inhibited consortium, both of which resulted from the same inoculum.

The 16S rRNA genes were polymerase chain reaction (PCR) amplified with the primers 8F and 1492R (Weisburg et al. 1991). The PCR mix consisted of 2.5 μL of 10X buffer, 5 μL of 5X buffer, 0.5 μL of dNTP (10 mM), 0.25 μL of formamide,1 μM of each primer (5 mM), and 0.35 μL of Taq polymerase (Eppendorf, Westbury, NY) for each 25 μL reaction. The thermocycler program consisted of an initial denaturing step for 2 minutes at 94°C, followed by 25 cycles of 30 seconds at 94°C, 30 seconds at 50°C and 30 seconds at 72°C. A final extension step was conducted for 10 minutes at 68°C. The resulting PCR products were cloned using the TOPO TA Cloning Kit® (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Resulting clones were screened for the presence of an insert by PCR using vector-specific M13F and M13R primers. The amplified inserts were digested with the restriction enzyme *MspI* (Promega, Madison, WI) to identify unique inserts by restriction fragment length polymorphism (RFLP). The species richness (number of operational taxonomic units (OTUs) present in each reactor) was estimated using Analytic Rarefaction 1.3 developed by Steven Holland (UGA Stratigraphy Lab, Athens, GA) to determine the average value within 95% and 99% confidence limits. Unique inserts were purified with ExoSAP-IT (USB, Cleveland, OH) and sequenced by the Macromolecular Resources facility at Colorado State University (Fort Collins, CO) using an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

4.1.3.3.3 Quantification of Strain PM1^T by Real-Time Quantitative PCR (Q-PCR)

Hristova et al. (Hristova et al. 2001) recently reported a TaqMan Q-PCR protocol for quantifying the known MTBE-degrading strain PM1^T using primers 963F and 1076R and the corresponding TaqMan probe PM1. An adaptation of this Q-PCR protocol was used to measure the concentrations of strain PM1^T in the enrichment cultures used as inocula, in all of the batch reactors, and in the semi-batch reactor. Twenty-four microliters of PCR master mix was mixed with 1 μl of DNA extract. The master mix consisted of 2.5 μL 10X buffer, 5 μL 5X buffer, 0.5 μL dNTP (10 mM), 0.25 μL of each primer (10 mM), 0.375 μL TaqMan probe PM1 (10 mM), 0.35 μL Taq polymerase (Eppendorf, Westbury, NY), and 1.5 μL Mg^{2+}. All Q-PCR reactions were performed in duplicate using a Cepheid Smart Cycler (Sunnyvale, CA). The amplification program consisted of 2 minutes at 95^0C, followed by 50 cycles at 95^0C for 15 seconds, 53^0C for 60 seconds and 72^0C for 20 seconds. The relative concentrations of total bacteria were determined by quantifying total 16S rRNA genes using the TaqMan Q-PCR protocol described by Suzuki et al. (Suzuki et al. 2000). The reaction conditions were the same as described above, except that primers 1369F (10 mM) and 1492R (10 mM) and the TaqMan probe 16S were used. All Q-PCR reactions were carried out in duplicate.

A generalized least-square means (GLM) procedure was conducted to determine if the difference in strain $PM1^T$ proportions observed for each reactor at the three different times studied was significant. SAS 9.1 software was used to conduct the procedure and determine the p values (SAS Institute Inc., Cary, NC).

4.1.3.3.4 Sequence Analysis and Assignment to Functional Groups

Clones that represented more than 6% of the total in each library were selected for DNA sequencing. The BLAST tool of the National Institute of Health database was used to search and identify the highest matches for each clone insert. Sequences were also analyzed using the Ribosomal Database Project (RDP) Sequence Match Tool to aid in phylogenetically classifying the microorganisms represented by clone inserts. A literature review was performed to further characterize the microorganisms corresponding to the highest matches.

4.1.4 Results and Discussion

4.1.4.1 Reactor Effect

To determine the reactor configuration effect on MTBE biodegradation in the presence of BTEX, concentrations of MTBE and BTEX in the batch and semi-batch reactors were monitored with time (Figure 4-1A, B, C, D and E). It was observed that MTBE biodegraded readily in the presence of BTEX in the semi-batch reactor, but not in most of the batch reactors, even when inoculated with the same MO culture. However, the rate of MTBE biodegradation in the MO MBTEX semi-batch reactor (0.48 ± 0.2 mg/L-d) was about 40% the rate of MTBE biodegradation in the MO MTBE batch reactor (1.47 ± 0.47 mg/L-d); though it was more than three times faster than the MTBE biodegradation rate in the MO MBTEX batch reactor (0.18 ± 0.04 mg/L-d). BTEX compounds were readily biodegraded in all reactors. Based on these results, it is hypothesized that the accumulation of byproducts from the biodegradation of BTEX inhibits biodegradation of MTBE in batch reactors, whereas the semi-batch conditions significantly dilute these compounds. BTEX itself may be responsible for some level of inhibition, but in the semi-batch reactor MTBE biodegradation preceded even with continued additions of BTEX. Inhibition of MTBE biodegradation in the presence of BTEX due to potential build-up of byproducts has been suggested by others (Deeb et al. 2001; Sedran et al. 2002). If MTBE biodegradation pathways differ between the two reactor configurations, then MTBE intermediates could be an alternative cause of inhibition in the batch reactors; however, this kind of inhibition has not been reported previously. TBA was noted to be consistently below detection as a metabolite in all batch reactors and in the semi-batch reactor (data not shown). Thus MTBE biodegradation progressed beyond TBA in all reactors. No abiotic losses were observed in the killed control; therefore removal of MTBE by the cultures could be attributed to biodegradation.

4.1.4.2 Culture Effect

4.1.4.2.1 Overall Effect on Performance

The effect of the culture on performance was determined by monitoring MTBE and BTEX biodegradation in batch reactors inoculated with either the MO or MB culture, or a combination of both. The MB culture appeared to be inferior to the MO culture in terms of two main performance indicators: *1.)* MTBE biodegradation by the MB culture was completely inhibited in the presence of BTEX (Figure 4-1D) even when mixed with the MO culture (data not shown), whereas the MO culture showed some MTBE biodegradation in the presence of BTEX (Figure 1B); *2.)* the lag period for MTBE biodegradation by the MB culture in the absence of BTEX was very long (20 days), whereas there was no apparent lag phase for the MO culture (Figures 4-1A and 1C). However, discounting the lag period, the actual rate of biodegradation in the absence of BTEX was comparable between the MO and MB cultures (1.47 ± 0.47 mg/L-d vs. 0.95 ± 0.44 mg/L-d, respectively). MTBE biodegradation by both cultures was inhibited in the presence of BTEX; however, the MO-inoculated reactors showed less inhibition than the MB-inoculated reactors.

The concentration of MTBE spiked into the MO MTBE and MO MBTEX reactors on Day 22 and Day 10, respectively, was increased to ~20 mg/L. It was observed that the cells in the MO MTBE reactor were not shocked by this change, and that they were able to biodegrade the ~20 mg/L with no apparent rate change. However, the MO MBTEX batch reactor still showed inhibited MTBE biodegradation in the presence of BTEX.

It is interesting to note that the MB culture failed to degrade MTBE in the presence of BTEX, even though this culture was originally collected from a reactor that was simultaneously degrading MTBE and BTEX and had been observed to degrade these compounds simultaneously in batch reactors (Sedran et al. 2002). Thus, storage in the freezer had an apparent effect on the capabilities of the culture. However, this effect did not negatively impact the main purpose of this study, which was to compare the performance of cultures with different compositions. This observation does suggest some caution in using freezer stocks for bioaugmentation purposes.

4.1.4.2.2 Composition of the Microbial Consortia

For a summary of the putative functions of the microorganisms detected in the cultures and the reactors by cloning and the corresponding phylogenetic identities see Table 4-3. It was observed that the majority of the microorganisms belonged to the α, β and γ Proteobacteria classes, which contain several oil-degrading bacteria (Hristova et al. 2001; Feris et al. 2004). The most frequently detected class was β-Proteobacteria, to which most currently known MTBE-degraders belong (Bruns et al. 2001; Hatzinger et al. 2001). Many of the microorganisms identified in the reactors did not have any obvious function related to MTBE or BTEX biodegradation (Table 4-3). While cloning is dependent on PCR, which is known to exhibit biases, this approach was considered to be suitable for comparative purposes, especially with respect to the same culture(s) under different conditions, which would be expected to normalize any potential biases. Also, the PCR cycle number was kept low (25 cycles) to reduce bias as much as possible.

The relative proportions of detected microorganisms are shown in Figure 4-2. This semi-quantitative comparison is based on frequency of occurrence in the clone libraries according to RFLP patterns. Observation of the initial cultures' compositions revealed two different strains of known MTBE-degraders in the MO culture: PM1^T and Leptothrix sp. However, only one known MTBE-degrader, strain PM1^T, was detected in the MB culture. This suggests that initial diversity among MTBE-degraders may play a role in the ability to biodegrade MTBE in the presence of BTEX. Interestingly, the reactor conditions significantly altered the composition of the initial consortia in the inocula, though some populations may also have died during freezer storage. In the case of the MO MBTEX batch reactor, RFLP detected no known MTBE-degraders, which corresponds well to the inhibited MTBE biodegradation observed in this reactor. Furthermore, a BTEX-degrader, Pietermaritzburg bacterium, was found that was not detected in the initial culture (Table 4-3 and Figure 4-2). In the MO MBTEX semi-batch reactor, which was actively biodegrading MTBE, a very different consortium was characterized. Neither strain PM1^T nor *Leptothrix* sp. were found in the semi-batch reactor by RFLP, however, another MTBE-degrader, *Hydrogenophaga* sp., was identified. This microorganism has been studied in other experiments and was characterized as having a slow MTBE biodegradation rate; however, the effect of BTEX has not been reported (Hatzinger et al. 2001). Overall, these results demonstrated that the microbial consortium composition varied between a reactor actively biodegrading MTBE in the presence of BTEX, and a BTEX-inhibited reactor, even when originally inoculated with the same culture. Therefore, reactor configuration had a major influence on the microbial consortium that developed and its ultimate performance.

4.1.4.2.3 Relative Diversity

The results of cloning and RFLP screening of 16S rRNA genes are presented in Figure 4-3. Comparing the number of phylotypes present in the initial cultures revealed that the estimated species richness was higher for the MO culture than for the MB culture (28.5 ± 1 versus 27.8 ± 1, respectively), but that the difference was not significant. However, two different patterns emerged in examining the diversity of the MO BTEX batch and semi-batch reactors with time (Figure 4-3). In the batch reactors, where MTBE biodegradation was inhibited, the microbial diversity decreased with time (species richness = 32.8 ± 1, 18.9± 0.7 and 13.8± 1, respectively). This may have been a result of further enrichment of MTBE degraders, though this is unlikely considering that MTBE biodegradation was poor. Thus, failure of the populations to adapt to the reactor conditions is the more likely scenario. In the semi-batch reactor, species richness increased from Day 14 (21.7 ± 1.1) to Day 18 (45.5 ± 1.2), but then decreased to a moderate value by Day 21 (31.8 ± 1). Notably, the final microbial diversity was higher than the initial microbial diversity in the semi-batch reactor, and about equivalent to the initial diversity in the batch reactor. These results suggest that a diverse microbial consortium may play a role in MTBE biodegradation in the presence of BTEX.

4.1.4.2.4 Quantitative Response of Strain PM1^T

Concentrations of the known MTBE-degrader, strain PM1^T, normalized to total Bacteria were measured by Q-PCR (Figure 4-4). The presence of strain PM1^T in environmental samples has been used as a parameter to demonstrate the presence of MTBE-degraders and thus the likelihood of MTBE biodegradation (Hristova et al. 2003). No changes in total Bacterial concentration, as determined by number of 16S rRNA genes, were detectable in the reactors with time (data not shown). Interestingly, the proportion of strain PM1^T decreased initially in all of the reactors with respect to the original inocula. In the batch reactors inoculated with the MO culture only, the proportion of strain PM1^T remained constant with time. However, in the MB-inoculated batch reactors in the absence of BTEX, a significant increase in strain PM1^T was observed by the time of the MTBE biodegradation endpoint ($p<0.0001$). Though it appears that strain PM1^T in the semi-batch reactor increased and then decreased to the initial level, this change was not statistically significant ($p\sim0.98$).

The fact that strain PM1^T was present in all of the reactors, even when MTBE biodegradation was inhibited by BTEX, indicates that strain PM1^T may not be an optimal indicator of MTBE biodegradation potential when BTEX is present. Deeb et al. noted that MTBE-biodegradation by this strain can be inhibited in the presence of BTEX. The authors attributed this to preference of the MTBE-degrading enzyme for BTEX over MTBE (Deeb et al. 2001). It is also of interest that concentrations of strain PM1^T were the lowest in the semi-batch reactor, which was most effective at biodegrading MTBE in the presence of BTEX. Strain PM1^T was also below the RFLP/cloning 6% detection limit as applied in this study, which is in agreement with the more sensitive Q-PCR results. These observations suggest that other MTBE-degrading microorganisms identified in the semi-batch reactor by cloning, such as *Hydrogenophaga* sp., may play a key role in biodegrading MTBE in the presence of BTEX.

4.1.5 Implications for MTBE Treatment in the Presence of BTEX

A major driver of this study was inconsistent reports of the ability to biodegrade MTBE in the presence of BTEX, which is often a critical factor in bioremediation considering the likelihood of co-contamination of these compounds. This study suggests that MTBE bioremediation in the presence of BTEX is feasible, and that overall success may be enhanced by several factors. First, it is important to recognize that the characteristics of the microbial consortium do matter. MTBE-degraders besides strain PM1^T may be key for overcoming BTEX inhibition of MTBE biodegradation. The consortia that were most successful in biodegrading MTBE in the presence of BTEX actually had the lowest numbers of strain PM1^T, but contained *Hydrogenophaga* sp. There was some evidence that higher diversity, as represented by estimated species richness, was associated with successful MTBE biodegradation in the presence of BTEX. Reactor configuration is also important for controlling the composition and performance of the microbial consortium. Previous studies characterizing BTEX inhibition of MTBE biodegradation have been carried out in batch reactors (Deeb et al. 2001). However, this study demonstrates that a semi-batch reactor provides better performance, probably because BTEX biodegradation intermediates can be flushed out. In other studies using continuous-flow reactors, comparable biodegradation rates of MTBE were observed in the presence and absence of BTEX (Pruden et al. 2001;

Sedran et al. 2002; Pruden and Suidan 2004). Continuous-flow conditions are also more realistic for most remediation scenarios in the field.

In the broader sense, the results of this study also support further research into the possibility of designing microbial communities to achieve desired bioremediation performance. This approach has recently been suggested by Curtis and Sloan (Curtis and Sloan 2006). Implementation of appropriate molecular biological tools and modeling techniques makes this more feasible.

Tables and Figures

Table 4-1 Summary of Previous Aerobic MTBE Biodegradation Studies: Reactor and Culture Conditions.

Reactor	Co-contaminants present	MTBE removal rate	Culture	Observations	Reference
Batch	*Tert-butyl* alcohol, butyl formate, isopropanol, acetone and pyruvate.	4.3-8.6 mg/L-d	Pure cultures: *Methylobacterium* sp. *Rhodococcus* sp. and *Arthrobacter* sp.	Very slow growth, perhaps a result of metabolites. Other hydrocarbons decreased MTBE degradation rate. MTBE biodegradation by mixed cultures superior to that of pure cultures.	(Mo et al. 1997)
Batch	BTEX	2.32-10.58 mg/L-d	strain UC1	BTEX had no effect on MTBE biodegradation.	(Pruden and Suidan 2004)
Batch	BTEX	26 mg/L-d	Enrichment culture	BTEX reduced TBA degradation rate.	(Sedran et al. 2002)
Batch	BTEX	20-120 mg/L-d	strain PM1^{T}	Inhibition of MTBE by some BTEX compounds (ethylbenzene and xylenes). Lag phase induced by benzene and toluene compounds.	(Deeb et al. 2001)
Continuous flow porous-pot biomass concentrating	BTEX	5 mg/L- d	Enrichment culture	Culture very sensitive to pH.	(Sedran et al. 2002)

Reactor	Co-contaminants present	MTBE removal rate	Culture	Observations	Reference
Continuous flow porous-pot biomass concentrating	Diethyl ether, Diisopropyl ether, Ethanol, or BTEX	10 mg/L-d	Enrichment culture	High biomass concentration was key to performance. Biodegradation of MTBE in the presence of BTEX was observed at a pH range of 7.4-7.6. A high microbial diversity was observed. Each condition studied had a unique microbial consortium composition.	(Pruden et al. 2001)
Biotrickling filters	None	67 mg/L-d	Enrichment culture (F-Consortium)	The biodegradation rate of the F-consortium was similar to the biodegradation rate of strain PM1^T. pH did not affect biodegradation (range 4 to 8).	(Fortin et al. 2001)
Biotrickling filters	Ethanol, Formate, Pyruvate, Lactate, Acetate, Hydroxylamine, Diethyl ether	20-50 mg/L-d	Enrichment culture (F-Consortium)	MTBE was degraded after depletion of the co-contaminant added. The microbial consortium composition was not characterized.	(Fortin et al. 2001)

Table 4-2. Experimental Conditions for the Batch Reactors.

Reactor ID	Culture	Initial MTBE conc.(mg/L)	Initial BTEX conc.(mg/L)	Days Operated
MO MTBE a	MO	10	0	51
MO MTBE b	MO	10	0	51
MO MBTEX a	MO	10	20	51
MO MBTEX b	MO	10	20	51
MOMB MTBE a	MO + MB	10	0	51
MOMB MTBE b	MO + MB	10	0	51
MOMB MBTEX a	MO + MB	10	20	51
MOMB MBTEX b	MO + MB	10	20	51
MB MTBE a	MB	10	0	93
MB MTBE b	MB	10	0	93
MB MBTEX a	MB	10	20	51
MB MBTEX b	MB	10	20	51
Killed Control	None	10	20	93

Table 4-3. Summary of Highest Matches and Characteristics of Sequenced Clones.

Highest Match (Score)*+#	Class	Function/Association	Reactor	Reference
MTBE Biodegradation				
Strain PM1: AF176594 (0.96)	β-Proteobacteria	MTBE-degradation	MO & MB cultures	(Bruns et al. 2001)
Leptothrix sp. MBIC3364: Y8; AB015048 (0.90)	β-Proteobacteria	MTBE-degradation	MO culture	(Hamada 1998)
Hydrogenophaga sp. PG-10: AY566583 (0.95)	β-Proteobacteria	MTBE-degradation	Semi-Batch	(Hatzinger et al. 2001)
BTEX/Petroleum Biodegradation				
Bacteroidetes: AY211072 (0.95)	Bacteroidetes	Oil-degrading bacterial consortium	MO culture	(Feris et al. 2004)
Uncultured γ-proteobacterium: AF529326 (0.95)	γ-Proteobacteria	Oil-degrading cyanobacterial consortium	Semi-Batch	(Sanchez et al. 2005)
Rhodobacter sp. TCRI 14: AB017799 (0.94)	α-Proteobacteria	Petroleum-degrading Photosynthetic bacteria	MB culture	(Hristova et al. 2001)
Ralstonia sp. DUT_AHX: DQ40908 (0.95)	β-Proteobacteria	*o*-xylene-degradation	Batch	(Damm et al. 2002)
Comamonas sp. *AV1A*: AF434169 (0.96)	β-Proteobacteria	mcl-PHA synthesizing bacteria, TCE-degradation	Batch	(Ciesielski et al. 2004; Carroll and Zinder 2006)
Pietermaritzburg bacterium Y14-2: AF312216 (0.94)	γ-Proteobacteria	BTX-catabolizing consortium	Batch	(Ralebitso et al. 2001)
Rhodobacter sp.: AF494542 (0.93)	β-Proteobacteria	Benzo[a]pyrene-Mineralizing consortium	Batch	(Thomas et al. 1996)
Alcaligenes sp.: DQ366091 (0.95)	β-Proteobacteria	Oil-degrading Bacterial consortium	Batch & Semi-Batch	(Krooneman et al. 1996)
Unknown Relevance				
Denitrifying β-Proteobacterium: U51102 (0.94)	β-Proteobacteria	Denitrifying Fe-II oxidization	MO & MB cultures	(Straub et al. 1996)
Uncultured bacterium PHOS-HE31: AF314430 (0.97)	Sphingobacteria	Aerobic phosphorus-removal	Semi-Batch	(Dabert et al. 2001)
Uncultured bacterium: AF268998 (0.716)	Genera Incertae Sedis	Putative uncultured phylum	Semi-Batch	(Bond et al. 1995)
Uncultured α-proteobacterium: AB245350 (0.97)	α-Proteobacteria	Unknown	Semi-Batch	(Im and Lee 2005)
Uncultured Flavobacteriales: AY509269 (0.94)	Flavobacteria	Unknown	Semi-Batch	(Eiler and Bertilsson 2004)

*National Center for Biotechnology Information Blast similarity score http://www.ncbi.nlm.nih.gov/BLAST/ .
+Based on 800-1100 bp of 16S rRNA gene sequence information.
#GenBank accession numbers provided following name (eight characters).

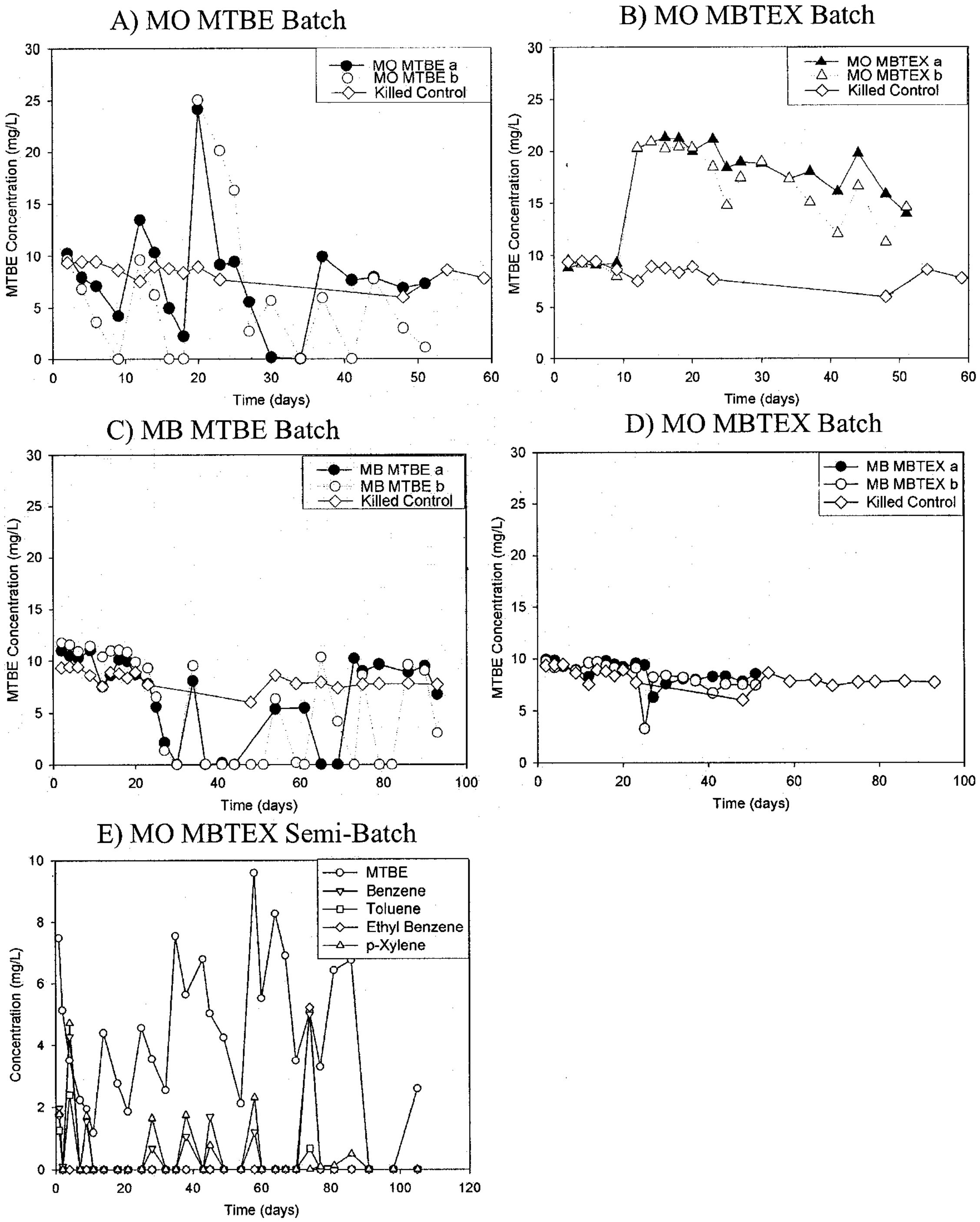


Figure 4-1 Biodegradation of MTBE in batch reactors: (A) MO culture in the absence of BTEX; (B) MO culture in the presence of BTEX; (C) MB culture in the absence of BTEX; (D) MB culture in the presence of BTEX and (E) biodegradation of MTBE and BTEX by the MO culture in the semi-batch reactor. MTBE and BTEX were spiked in periodically at target concentrations 10 and 20 mg/L, respectively, except for Day 12 in the MO MBTEX reactor and Day 20 in the MO MTBE reactor, when MTBE was spiked to ~20 mg

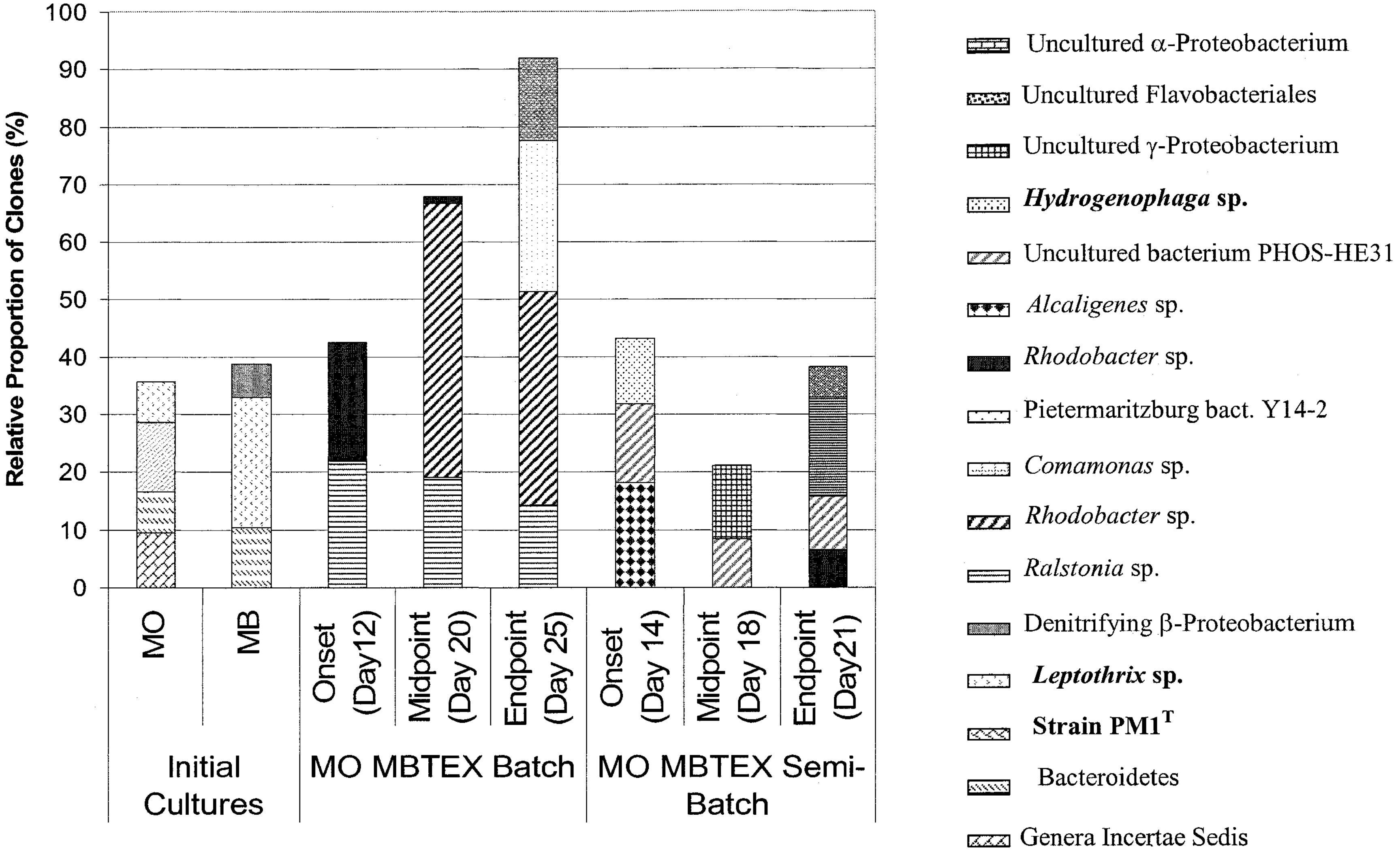
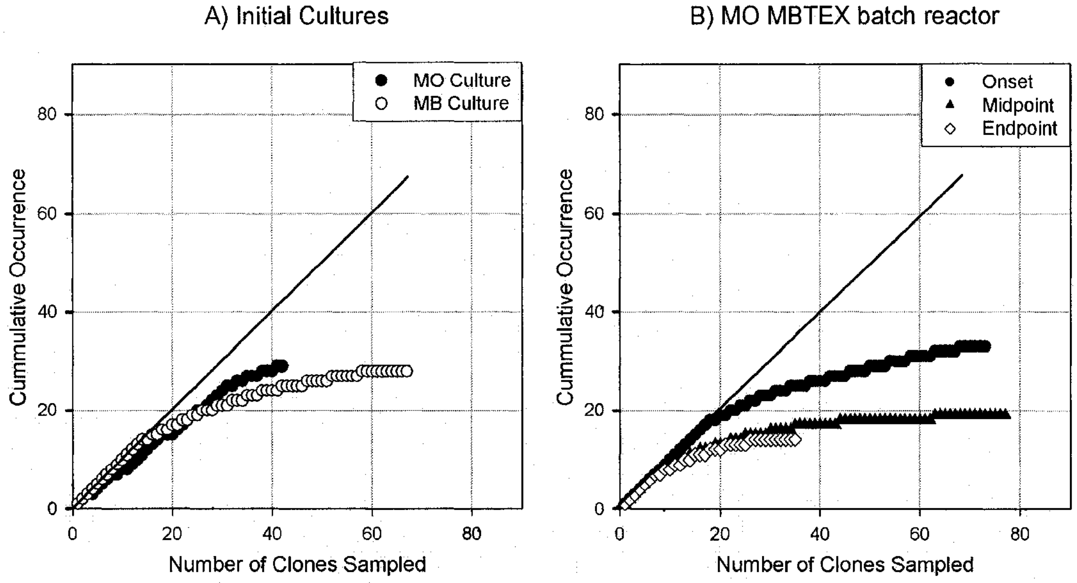


Figure 4-2. Identification of the dominant microorganisms present in the initial cultures (before inoculation), the MO MBTEX batch reactor and the MO MBTEX semi-batch reactor based on cloning, RFLP screening, and sequencing of most common clones (≥6% total library). Three time points were investigated based on the second observed biodegradation event: onset, midpoint and endpoint. Known MTBE-degraders are shown in bold in the legend. Further characterization of the identified strains is presented in Table 4-3.



C) MO MBTEX semi-batch reactor

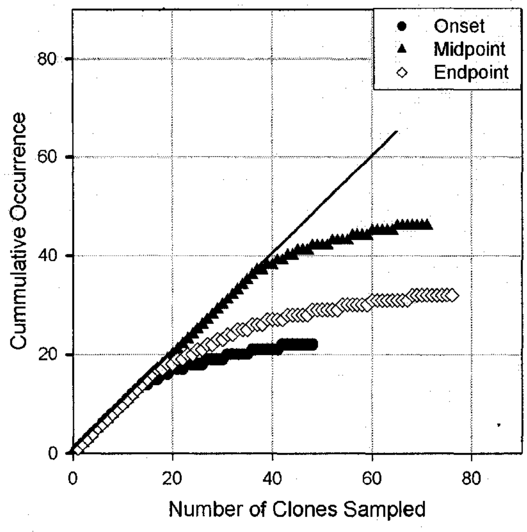


Figure 4-3. Microbial diversity estimated by rarefraction curves generated from cloning and restriction fragment length polymorphism (RFLP) of 16S rRNA genes using *Msp*I restriction enzyme: A) Initial cultures enriched on MTBE only (MO) or MTBE and BTEX (MB); B) Batch reactor inoculated with the MO culture and fed MTBE and BTEX (MO MBTEX); and C) Semi-Batch reactor inoculated with the MO culture and fed MTBE and BTEX (MO MBTEX semi-batch reactor). The three sampling days for the reactors corresponded to the second observed biodegradation event: onset, midpoint, and endpoint. The solid lines represent the hypothetical case that all sampled clones are unique (maximum possible diversity).

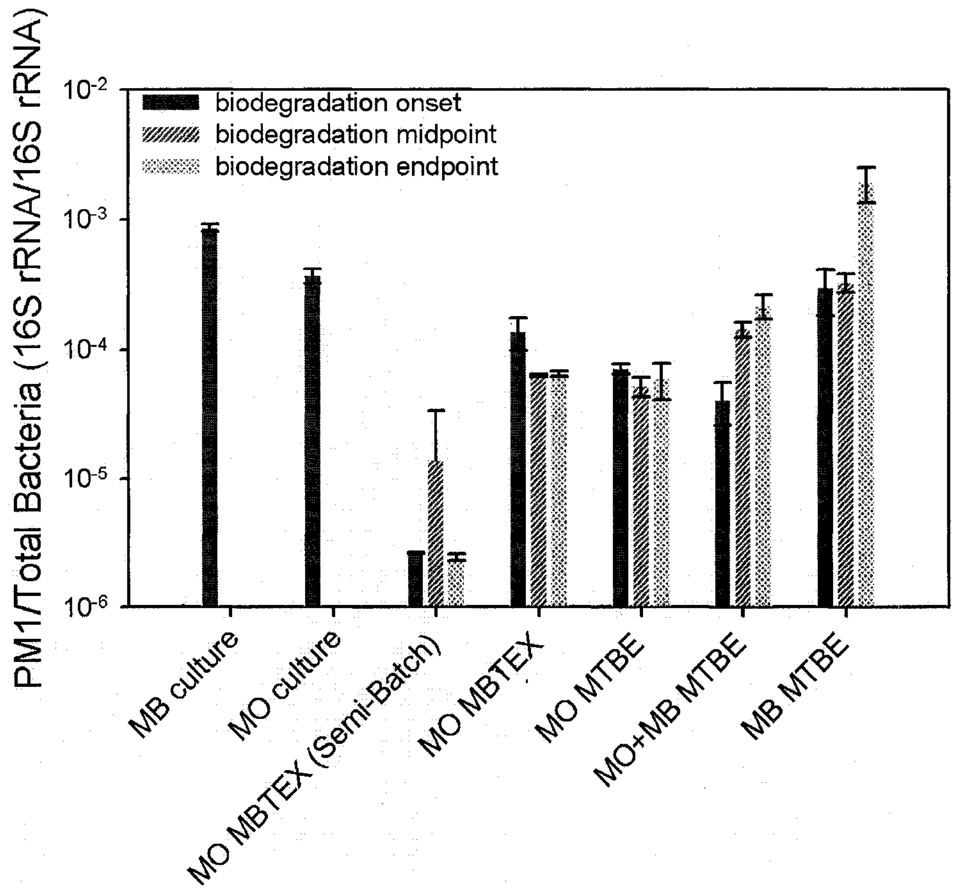


Figure 4-4. Q-PCR analysis of initial (MO and MB), semi-batch reactor (MO MBTEX) and the batch reactor (MO MBTEX, MO MTBE, MO+MB MTBE, MB MTBE) cultures at three experimental time points during the second observed biodegradation event: onset, midpoint and endpoint, except for the MO MBTEX batch experiment, which showed inhibited MTBE degradation and was instead sampled on Day 12, 20, and 25, corresponding approximately to the MO MBTEX semi-batch reactor. Error bars represent the standard error of two replicate Q-PCR measurements.

4.2 Objective 2: Explore the Feasibility of Wetlands for MTBE biodegradation in the Presence of BTEX and Other Constituents Present in Refinery Water

4.2.1 Introduction

From a previous bench experiment conducted in our laboratory comparing the biodegradation of MTBE in batch and semi-batch reactors, one of the main observations made was that simultaneous biodegradation of MTBE and BTEX was observed only in a flow through system (Raynal and Pruden 2007). It had been hypothesized by others that this can be attributed to the accumulation of byproducts from the biodegradation of the BTEX compounds (Deeb et al. 2001; Sedran et al. 2002). Our results also supported the conclusion that BTEX-degraders could be producing substances that inhibit the activity of the MTBE-degraders. Alternatively, based on the concept of competition in microbial ecology, it is possible that the BTEX-degraders compete with MTBE-degraders for nutrients. Given that BTEX compounds are more readily biodegradable than MTBE, BTEX-degraders may grow faster and could be producing antibiotics or other substances that reduce or inhibit the enzymatic activity of the MTBE-degraders (Sylva et al. 2005). However, neither the presence of biodegradation byproducts nor the production or concentration of antibiotics has been measured in previous studies.

Six scaled wetlands were constructed using fish tanks. The influent water was collected from an existing air stripping system at a petroleum refinery near Denver, CO, and the sediments were collected from the river bed of Sand Creek that flanks the refinery. From previous chemical analyses of this water it was known that the water was contaminated with several organic compounds such as: acetone, naphthalene, phenol, VOCs, MTBE, benzene, ethylbenzene, toluene xylene; PAH, inorganic compound such as nitrate, fluoride and barium. Thus, this provided the opportunity to test the capability of scaled-wetlands for treating natural water containing several other contaminants, in addition to MTBE. It was expected that the complexity of the wetland environment would provide a means to degrade MTBE in the presence of other co-contaminants.

The principal physical treatment mechanisms of contaminants in wetlands are filtration, sedimentation and adsorption. The water flow rate and chemistry will also determine the biological characteristics of a wetland. These two characteristics promote the removal of organic compounds and nutrients by microbial activity by providing a niche for different microorganisms. In general, the bacterial community in wetlands is diverse and have a broad capability for degrading a wide range of compounds, this is due to the fact that wetlands provide different environmental conditions by the formation of anoxic and aerobic microzones, thus this either enhances the bioremediation of certain contaminants (i.e. phosphate removal in anoxic section) or inhibits the removal of other contaminants (i.e. inhibition of ammonium intake in anoxic section) (Campbell and Ogden 1999).

The first aim of this experiment was to determine the effect of bacterial composition on MTBE and BTEX biodegradation in wetlands. The second aim was to determine whether wetlands could provide suitable conditions for the simultaneous degradation of MTBE and BTEX. The overall hypothesis was that biodegradation of MTBE is possible in wetlands, since bioaugmentation and/or biostimulation will enhance the process.

4.2.2 Material and Methods

4.2.2.1 Scaled Wetlands Design and Operation

Three conditions were compared in the wetlands: the activity of indigenous bacteria, bioaugmentation and biostimulation. Each scaled wetland was constructed at the same time. Two mixed cultures were used to inoculate the wetlands: an aerobic and an anaerobic culture. Saturated Sand Creek sediments were mixed with the designated inocula in a plastic 15 gallon bucket. The pH of the water from the refinery was spiked with HCl to adjust it approximately to a value of 4 to reduce the activity of the indigenous bacteria in the constant head influent section of the wetlands. The source water was stored in 20 L Tedlar bags (SKC, Fullerton, CA) to reduce the exposure of the water with the atmosphere. The Tedlar bags, containing the acidified source water, were connected to a multi-channel Ismatec peristaltic pump (Ismatec, Glattbrugg, Switzerland). Water samples from the Tedlar bags were withdrawn every time they were refilled with new influent and after a month to ensure that the concentration of the MTBE and BTEX were not changing over time due to microbial activity or other chemical process.

Bleach ($HClO_3^-$) was added to the influent (100 mL, 6%) of the control tank in order to inhibit the activity of bacteria and it was used as a control to quantify volatilization of MTBE and BTEX.

The six conditions studied were:

1. No inoculation.
2. Inoculation with acclimated anaerobic iron-reducing culture and aerobic MTBE and BTEX acclimated culture (Fe(III) added)

3. Inoculation with acclimated anaerobic iron-reducing culture (Fe(III) added).
4. Inoculation with acclimated aerobic MTBE.
5. Inoculation with acclimated anaerobic iron-reducing culture and aerobic MTBE and BTEX acclimated culture.
6. No inoculation, bleach killed.

The amount of ferric iron added was determined according an equation determined by Finneran and Lovley for the anaerobic biodegradation of MTBE using iron as the terminal electron acceptor (Finneran and Lovley 2001):

$$C_5H_{12}O + 30Fe(OH)_3 + 55H^+ \rightarrow 5HCO_3^- + 30Fe^{+2} + H_2O \qquad \textbf{Equation 10}$$

For an initial MTBE concentration of 300 μg/L, 9 mg/L of $Fe(OH)_3$ are required. However, other organic and inorganic compounds present in the source water consume Fe(III), thus a Fe(III) concentration of 27 mg/L was used instead (as $Fe_2(SO_4)_3$; Wako Chemicals USA Inc., Richmond, VA).

The dimensions of the fish tanks were 1.5 ft deep (water depth: 1 ft), 3 ft long (effective length: 2 ft) and 0.5 ft wide. The retention time was 7 days, thus the flow rate was equal to 0.06 ft^3/d. A multi-channel Ismatec peristaltic pump was used to control the flow (Ismatec, Glattbrugg, Switzerland). In order to avoid having preferential flow, constant head sections were allowed to form at the influent and effluent of the tanks. These sections had the following dimensions: 1 ft of water level and 0.5 ft long. These sections were created by placing a perforated stainless steel sheet covered with stainless steel hardware net with 0.5 mm openings to prevent the migration of solids. The same steel sheets were used in the construction of the wetlands section. In the constant head tanks, mineral oil was placed on the top to prevent volatilization and the diffusion of oxygen into water.

The length of the scaled wetlands was divided into three treatment zones (Figure 2-1). The first section was considered to be anoxic, in the second section the water was exposed to the atmosphere in order to increase the oxygen concentration and the last zone was considered to be aerobic/microaerophillic. The first zone was bioaugmented with the anaerobic culture and the last zone was bioaugmented with the aerobic culture. Samples to be analyzed with molecular tools were taken from the sediments at the beginning and at the end of the experiment to determine the change in the bacterial community. The bacterial community of the source water was also characterized to determine the background microbial composition.

Sampling ports were placed in the three zones at three different depths: 3 in., 6 in. and 9 in. below the surface in the anoxic and aerobic zone, and at 1 in., 1.5 in. and 2 in. below the water surface in the central portion. Also, sampling ports were placed at the influent and at the effluent of the tanks. One eighth of an inch ID FEP tubing (Cole-Parmer, Vernon Hills, IL) was used for the sampling ports. At the end of each sampling port Nitex bolting cloth (0.75 mm, Wildlife Supply Company, Buffalo, NY) was wrapped to prevent clogging. A 25 mL syringe was used to withdraw the samples. Water samples were taken every week and analyzed with a GC to determine the concentration of MTBE and BTEX. The pH and the oxidation-reduction potential were measured at each sampling event.

The experiment was set up in proximity to the air stripping system of a petroleum refinery located near Denver, CO. The wetlands were operated on-site for the first three months of the experiment and then were moved to the Engineering Research Center at Fort Collins, CO. The water inlet to the existing air-stripper at the refinery was used as the source water for the experiment in order to explore the applicability of the approach for actual treatment of contaminated groundwater. From previous chemical analyses of this water, it is known that the water is contaminated with several organic and inorganic constituents. Contaminants such as acetone, naphthalene, phenol, VOCs (volatile organic compounds), MTBE, benzene, ethylbenzene, toluene xylenes, polycyclic aromatic hydrocarbons (PAHs), nitrate, fluoride and barium were present in the water (data collected by RETEK, Fort Collins, CO). The ammonium concentration was 0.54 mg/L as N, with a C:N ratio of 585. A minimum C:N ratio of 20 is required for bacterial growth (Sylva et al. 2005). A batch test was conducted, where both aerobic cultures were exposed to the refinery water, in order to confirm that the refinery water did not have a negative effect on the biodegradation of MTBE and BTEX (results not shown).

4.2.2.2 Monitoring MTBE and BTEX

Aqueous samples were taken periodically from each tank and analyzed within one week. In a 25 mL borosilicate glass sampling vial with a Teflon cap, a 1 mL sample was diluted into 23 mL of deionized (DI) water with 1 mL of 0.1 M HCl for sample preservation.

Five milliliters of this mixture were used to measure the concentration of MTBE and BTEX compounds. The concentration of these compounds was determined using a Tekmar-Dohrman (Cincinnati, OH) heated purge and trap 2016 and a Hewlett Packard (Rolling Meadows, IL) 5890 Series II Gas Chromatograph (GC) with a capillary column and flame-ionization detector (FID). Eight point calibration curves were generated with concentrations varying from 4 to 2000 μg/L, with R^2 ranging from 98.95% for *p*-xylene to 99.96% for benzene. Because the R^2 value of the calibration curve for *tert* butyl alcohol

(TBA), an MTBE intermediate, was 87.29%, it was concluded that the TBA concentration could not be accurately quantified, so its presence or absence in each sample was reported instead.

4.2.2.3 Characterization of the Microbial Consortia

Sediment samples were collected at the beginning of the experiment from each treatment zone and each scaled wetland at two time points: the beginning of the experiment and at the end. The samples were stored at -80^{0}C until analysis.

4.2.2.3.1 DNA Extraction

The MoBio UltraClean Soil DNA kit (Solana Beach, CA) was used for the extraction of microbial DNA according to the manufacturer's protocol. The only modification made to the kit procedure was that the bead-beating time was reduced from ten minutes to four minutes, in order to reduce shearing of DNA.

4.2.2.4 Quantification of Strain PM1^T by Real-Time Quantitative PCR (Q-PCR)

Hristova et al. (Hristova et al. 2001) recently reported a TaqMan Q-PCR protocol for quantifying the known MTBE-degrading strain PM1^T using primers 963F and 1076R and the corresponding TaqMan probe PM1. An adaptation of this Q-PCR protocol was used to measure the concentrations of strain PM1^T in the background materials (source water and sediments before inoculation) and in the sediments taken from the scaled wetlands at the beginning and end of the experiment. Twenty-four microliters of PCR master mix was mixed with 1 μl of DNA extract. The master mix consisted of 2.5 μL 10X buffer, 5 μL 5X buffer, 0.5 μL dNTP (10 mM), 0.25 μL of each primer (10 mM), 0.375 μL TaqMan probe PM1 (10 mM), 0.35 μL Taq polymerase (Eppendorf, Westbury, NY), and 1.5 μL Mg^{2+}. All Q-PCR reactions were performed in duplicate using a Cepheid Smart Cycler (Sunnyvale, CA). The amplification program consisted of 2 minutes at 95^{0}C, followed by 50 cycles at 95^{0}C for 15 seconds, 53^{0}C for 60 seconds and 72^{0}C for 20 seconds. The relative concentrations of total bacteria were determined by quantifying total 16S rRNA genes using the TaqMan Q-PCR protocol described by Suzuki et al. (Suzuki et al. 2000). The reaction conditions were the same as described above, except that primers 1369F (10 mM) and 1492R (10 mM) and the TaqMan probe 16S were used. All Q-PCR reactions were carried out in duplicate.

4.2.3 Results

4.2.3.1 Change in MTBE and BTEX Concentration

The total percent removal [(1-Ce/Ci)*100] in each tank with respect to time is presented in Figure 4-6. Ce is the concentration at the effluent and Ci is the concentration at the influent at a given time. One of the problems that were faced during the experiment was that the concentration in the influent constant head tanks of the wetlands was changing over time (data not shown). Several efforts (use of acid, use of mineral oil at the surface to reduce the rate of oxygen diffusion) were made to try to slow down or inhibited the bacterial activity in the influent water, however no effective solution was found. In Figure 4-6 it can also be observed that MTBE removal in the inhibited control was significantly lower than the other wetlands during the first month, but after that time, the removal of MTBE was as high as in the other tanks. The motivation to use bleach instead of mercuric(III) chloride or sodium azide (common salts that are used to killed bacteria), was because it is not as toxic as the other chemicals, which simplified the disposal of the effluent from this wetland and also due to the policy of the refinery to avoid using hazardous chemicals in their installations. Unfortunately, from the results observed, it was not possible to determine if augmentation/biostimulation or natural attenuation were more effective. However, the overall potential of wetlands for removing MTBE in a low oxygen environment was demonstrated. TBA or BTEX were not detected nor at the inlet not at the effluent of the scaled wetlands.

4.2.3.2 Real Time Q-PCR

In order to support the hypothesis that the removal observed at the influent constant head tank was mainly due to microbial activity and not to volatilization, the concentration of the known MTBE degrader (PM1^T) was determined for the samples taken at the beginning of the experiment from the influent water and the sediments. No significant change was observed in the concentration of total bacteria at the two time points. There was also no significant difference between the concentration of total bacteria from the wetlands that were inoculated (bioaugmented) and the inhibited control or uninoculated scaled wetland (data not shown). The relative percentage of this strain in each of the tanks is presented in Figure 5-3. It can be observed that the relative percentage of PM1^T in the influent water was rather high. The high concentration of this known MTBE degrader in the background could explain the rapid removal of MTBE in the influent constant tanks in the wetlands. Also, it was observed that even in cases where $PM1^T$ was initially below-detection, after the 180 day experiment, all tanks contained $PM1^T$, even the inhibited control. The relative concentration of $PM1^T$ was the lowest in the inhibited tank, but this strain was present and might have been responsible for the removal observed in this tank.

4.2.4 Discussion

At the end of this experiment, it was expected that it would be possible to determine which treatment was more effective for the removal of MTBE and BTEX: bioaugmentation or/and biostimulation. It was also expected that this difference was related to differences in the microbial diversity and composition present in each scaled wetland. Sediment samples were collected at the beginning and at the end of the experiments. Water samples for molecular analysis were collected at the beginning of the experiment. DNA was extracted from these samples in order to determine the differences in diversity of the microbial communities of the scaled wetlands.

Some of the problems that were encountered in this experiment were:

- The tanks that were fed with $Fe_2(SO4)_3$ had a very low pH (around 3), which inhibited biodegradation of MTBE by the anaerobic culture. Removal of MTBE in these tanks could be due to the chemical hydrolysis of MTBE at low pH.
- Although several attempts were made to keep the influent anaerobic (Tedlar bags, mineral oil on the constant head tanks), it was observed that the oxidation-reduction

potential in the influent constant head tanks was positive (~30), which indicated that the conditions in this section was microaerophilic and not anoxic.

- The initial design of the wetlands was based on the knowledge that has been gained in the construction of wetlands for treatment of wastewater. A retention time of 7 days was selected, which is the minimum required for wetlands to allow the growth of heterotrophic bacteria and removal of nitrate. But, since the retention time in the constant head tanks was four hours and removal was observed to take place in those sections, a smaller retention time might be required for the diffusion of oxygen and to promote the activity of MTBE degraders.

The following analyses were originally proposed:

- Q-PCR to determine the presence and concentration of the strain PM1^T.
- Cloning/restriction fragment length polymorphism/sequencing to determine the diversity of the microbial community and to identify the most common microorganisms present in the scaled wetlands.

However, considering that there was no clear difference between the wetland treatments, the experiment was ended and only Q-PCR was performed on the samples collected at the beginning and at the end of the experiment. Overall, it could still be concluded that removal of MTBE in the wetlands was high. Therefore, further research into wetlands as an alternative treatment approach for MTBE and BTEX is suggested.

Figures

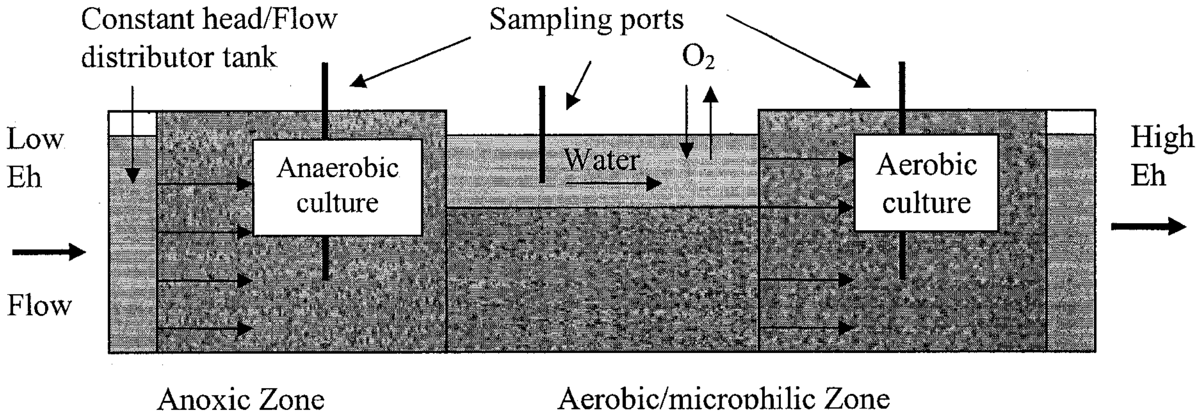


Figure 4-5 Experimental setup of wetlands study.

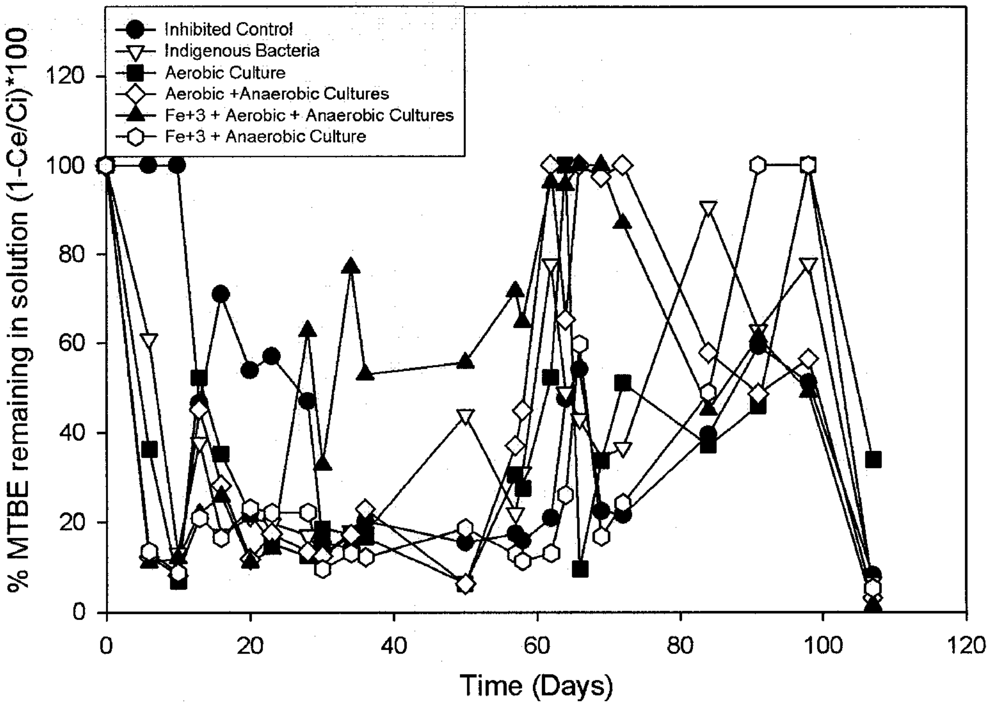


Figure 4-6 MTBE total removal in the scaled wetlands. The influent concentration (Ci) corresponds to the concentration in the Tedlar bags. The effluent concentration (Ce) corresponds to the concentration at the outlet of the wetlands.

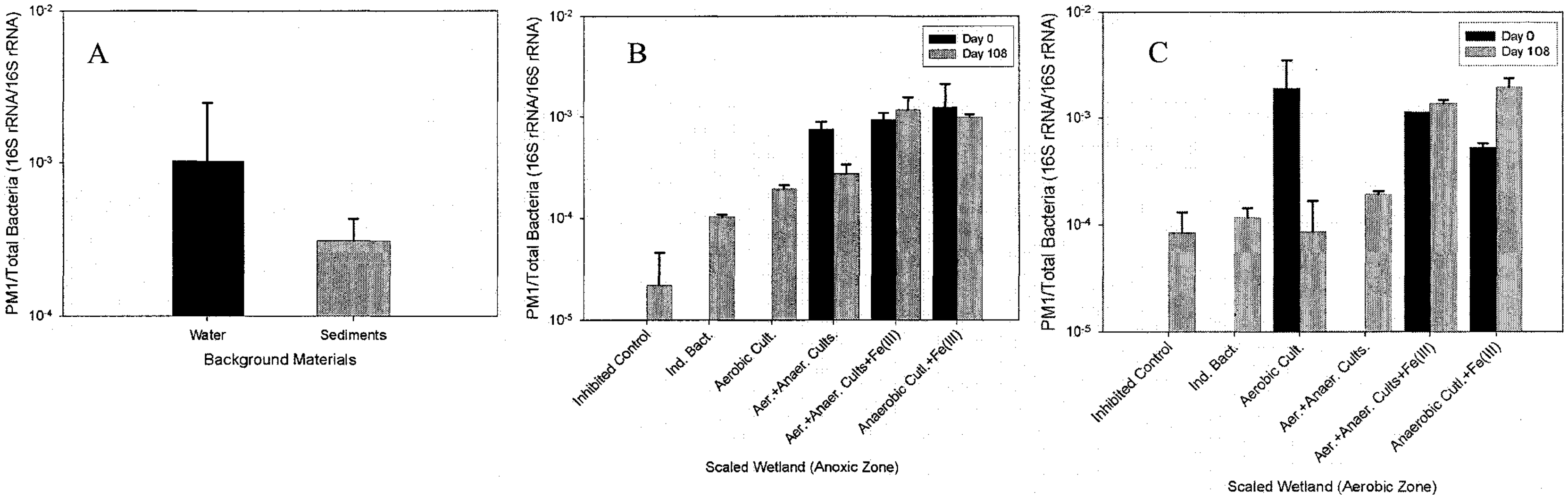


Figure 4-7 Relative concentration of PM1^T In (A) Water and sediments before inoculation, (B) the scaled wetlands in the aerobic zone (first section) and (C) in the anoxic zone (third section). Figures abbreviations: cult(s). is used for culture(s). Ind. Bact. stands for Indigenous Bacteria. Aer. is used for aerobic. Anaer. is used for anaerobic.

4.3 Objective 3: Explore Anaerobic MTBE Biodegradation in Permeable Reactive Barriers (PRBs) in Batch and Column Reactors

4.3.1 Introduction

Methyl *tert*-Butyl Ether (MTBE) is a gasoline oxygenate that has been used in the USA since the late 1970s to meet federal air pollution requirements. MTBE has been released into the environment due to leakage of underground storage tanks and pipelines in refineries and gasoline stations contaminating water supply sources (Deeb et al. 2003; USEPA 2006b).

MTBE has high water solubility, low adsorption to soil particles, low volatilization and a slow biodegradation rate. The chemical stability of MTBE is related to its chemical structure. The compound is formed by four methyl groups surrounding an ether bond. MTBE physico-chemical characteristics have contributed to the mobility and persistence of MTBE in groundwater (USEPA 2006b). In the past few years, the use of MTBE in gasoline blends has been banned in 21 states (USEPA 2007a). Although MTBE is not classified as a carcinogen, it has a very low odor and taste threshold, thus, the US EPA has established an advisory maximum contaminant level (MCL) of 20 µg/L (USEPA 2006a).

Two common clean up strategies of sites impacted with gasoline are pump and treat and vapor extraction. However the feasibility, reliability and cost-effectiveness of these methods have reduced their implementation and other alternative treatments are being considered (Deeb et al. 2003).

Biodegradation is developing as a promising, reliable and cost-effective treatment to active treatment approaches. Biodegradation of MTBE has been studied under different conditions at laboratory scale (aerobic and anaerobic) (Salanitro et al. 1994; Yeh and Novak 1994; Bradley et al. 2001b; Deeb et al. 2001; Finneran and Lovley 2001; Fortin et al. 2001; Landmeyer et al. 2001; Pruden et al. 2001; Pruden et al. 2005). It has been acknowledged that the success of biodegradation greatly depends of the existence of a niche in the environment with the right conditions (nutrients, pH and electron donor/acceptor) (Ellis et al. 2000; Rittmann and McCarty 2001). For MTBE, it has been observed that biodegradation yields a low biomass and it is not environmentally favored due to prevalent anaerobic conditions observed in gasoline impacted sites (Salinitro et al. 2000; Fortin et al. 2001).

The factors that have been observed to positively impact biodegradation as a treatment method are bioaugmentation with an MTBE degrading culture, homogenization of the conditions at the site and sometimes increasing the concentration of nutrients and electron acceptor (usually oxygen) at the site (Salinitro et al. 2000).

Although bioremediation of MTBE under anaerobic conditions has been observed in the laboratory (Mormile et al. 1994; Yeh and Novak 1994; Puig-Grajales et al. 2000; Bradley et al. 2001a; Finneran and Lovley 2001; Pruden et al. 2005), the results obtained are not clear as to under which conditions are required to enhance anaerobic MTBE biodegradation. There are only few reports where natural attenuation has been observed in the field (Kolhatkar et al. 2002; Kuder et al. 2005; Martienssen et al. 2006). In these studies, bioremediation of MTBE was demonstrated by monitoring the concentration of MTBE, metabolites or by performing a compound-specific carbon isotope analysis ($^{13}C/^{12}C$ expressed in $\delta^{13}C$ notation). However, each site had different physical and chemical characteristics.

It was determined by Bradley et al. that MTBE can be degraded under nitrate reducing, manganese reducing, ferric reducing, sulfate reducing and methanogenic conditions (Bradley et al. 2001a), however, in this study it was not established whether different microorganisms were required for each condition or if one type was able to degrade MTBE with various electron acceptors. Thus, the identity of the microorganisms responsible for anaerobic biodegradation of MTBE under each condition remains unknown. In Pruden 2005, it was determined that the bacterial consortium of the reactor was mixed and consisted of sulfate and iron reducing bacteria that belonged to the δ-Proteobacteria class (Pruden et al. 2005).

Permeable reactor barriers (PRBs) offer an alternative low-cost potential treatment option for MTBE in groundwater. PRBs typically are easy to construct, require relatively inexpensive reactive media, require little active maintenance or energy, and if they are well designed can operate for long periods. PRBs are considered to be passive in situ treatment systems with the capacity of active remediation through physical, chemical and/or biological processes, including precipitation, sorption, oxidation/reduction, fixation and/or degradation. Underground soil is replaced with a reactive material to create a treatment zone, with the purpose of intercepting and driving reactions within a contaminant plume in groundwater. The reactive material can be installed through the implementation of trenches or injection wells. Since the material in the barrier is permeable, a chemical reaction will occur between the barrier material and the ground water or contaminant plume. Advection and dispersion are the mechanisms that control the transport of the contaminants through the wall (Damm et al. 2002). PRBs have been commonly used to promote chemical oxidization or reduction of chlorinated solvents, such as perchloroethylene (PCE) and trichloroethylene (TCE), and precipitation of metals. PRBs have also been used to construct a niche to enhance microbiological activity. PRBs have been coupled with bioremediation to enhance the removal of nutrients, such as NO_3^- and PO_4^{-2}, and the remediation of acid mine tailings (Blowes et al. 1995; Damm et al. 2002).

A passive treatment is desirable for the treatment of recalcitrant contaminants such as MTBE, given the long persistence of these contaminants in the environment. A PRB could be used for the treatment of MTBE in situ to promote anaerobic biodegradation. The reactive media used to construct the PRB will consist of a low-cost reactive media that will act as a slow release electron-acceptor source, and will simplify the site characteristics by homogenizing the site conditions. In this study two inexpensive reactive media were explored: ferric hydroxide ($Fe(OH)_3$) and gypsum ($CaSO_4$).

The primary aim of this study was to conduct batch and column experiments to compare reactive media that could later be applied in PRBs. A secondary aim of this study was to determine the effect of the type of electron acceptor used on the removal of MTBE by the anaerobic iron-reducing culture. In order to better understand the role of the bacterial communities involved in anaerobic remediation, the bacterial consortia present in the reactors were characterized.

4.3.2 Materials and Methods

4.3.2.1 Anaerobic Batch Experiment

Four 1 L-amber borosilicate bottles were used for the experiment in order to test two electron acceptor sources in duplicate: $FeCl_3$ and Na_2SO_4. Each batch reactors was inoculated with an anaerobic iron-reducing culture. The anaerobic culture was obtained from a previously studied porous pot biomass-concentrating continuous flow reactor operated under iron-reducing conditions (Pruden et al. 2005), from which glycerol freezer stocks were generated upon sacrifice of the reactor in 2002. Sodium sulfide (Na_2S: 32 mg/L, Fisher, Fair Lawn, NJ) was added to scavenge the oxygen present in solution. Four controls were set up at the same time in 250 mL glass bottles. Gypsum (Grow More Research Farms, Gardena, CA) was used to provide sparingly soluble sulfate as the electron acceptor. Two of the controls were poisoned with 1 mg/L of mercuric chloride ($HgCl_2$, Fisher, Fair Lawn, NJ). The experimental conditions and the names assigned to the eight reactors are presented in Table 4-5.

Even though in theory nitrate is a more thermodynamically favorable option than sulfate, in practice it has two main limitations: 1) nitrate is a negative anion and therefore is easily leached out of the system, reaching surface water bodies; and 2) nitrate is considered a hazardous contaminant in drinking water (MCL: 10 mg/L) (USEPA 2006a). In high concentrations, it can lead to eutrophication and cause health problems such as methemoglobinemia and cancer if it reacts further with secondary amines (Rittmann and McCarty 2001; Sylva et al. 2005). Fe(III) is insoluble at neutral pH while SO_4 tends to adsorb to minerals of various charge (such as permanganate, iron, etc.) (Table 4-4), which ensures the immobility of these ions and make them more suitable for their use in PRBs. The advisory MCLs for Fe(III) and SO_4 are 0.3 mg/L and 250 mg/L, respectively, the sulfate advisory MCL being much higher than that of nitrate (USEPA 2006a).

Table 4-4 Solubility Constant for Fe(III) and SO_4 at 25 ^{0}C (Wikipedia 2007b; Wikipedia 2007a).

Chemical reaction	Kso
$FeCl_3 = Fe^{+3} + 3Cl^-$	1.21 (Fe(III) = 740 g/L)
$Na_2SO_4 = 2Na^+ + SO_4^{-2}$	3.45 (SO_4 = 331.26 g/L)

Amended DI water was used as the source water for the experiment. It contained a mineral salt medium (MSM), 10X phosphate buffer solution (PBS) and a MTBE concentration of 10 mg/L (Acros, Morris Plane, NJ). The MSM contained ammonium as nitrogen source as well as trace minerals, as described in Pruden et al. 2005 (Pruden et al. 2005). The batch reactors were dosed with the appropriate reactive media, then filled up with amended DI water and inoculated with the anaerobic culture. The batch reactors were set up in a N_2 glovebox. A volume of 980 mL of water was added to each bottle. A head space of 20 mL was left for gas production.

The bottles were capped with butyl rubber stoppers and screw caps. Two perforations were made to the rubber stoppers to place two lines of 1/8 inches ID FEP tubing (Cole-Parmer, Vernon Hills, IL). The end of one tube was placed half way into the bottle and the other tube extended only to the bottom of the rubber stopper to enable the controlled release of gases. The outside ends of the tubes were maintained closed with a clamp. The bottles were placed under water to reduce the possible diffusion of oxygen into the batch reactors.

Water samples were taken periodically for GC and molecular analyses. At each sampling event, a 25 mL gastight syringe was flushed three times and subsequently filled with nitrogen gas. The syringe was connected to the gas-release tube and the nitrogen was injected to collect aqueous samples. From the other tube, a 2 mL gastight syringe was used to take a 0.2 mL sample for GC analysis and a 2 mL sample for molecular analysis. The GC-samples were placed in 5 mL vials with Teflon caps containing 4 mL of DI water. The samples were stored at 4^0C until analysis (up to three days), while the samples used for molecular analysis were stored at -80^0C. The bottles were mixed by shaking manually two times a week.

4.3.2.2 Anaerobic Column Test

Four custom made columns were constructed using 45.5 cm long and 11 cm diameter perforated acrylic pipes. The columns had four sampling ports located at 3 cm, 9.5 cm, 23 cm and 43 cm (distance from the influent port). The columns were packed as follows: 4 cm of sand, 34 cm of reactive media and 7.5 cm of sand (Figure 4-8). Three columns were constructed using reactive media mixed with the Fe-reducing anaerobic culture. One column was constructed only with the reactive media, but it was not inoculated. This provided a means to determine the effect of bioaugmentation.

The sand used in the experiment was collected from the river bed of Sand Creek (Commerce City, CO). The sand was baked at 150 ^{0}C for twelve hours before being used to reduce the number of viable bacteria present and to volatilize any VOC adsorbed to the soil particles.

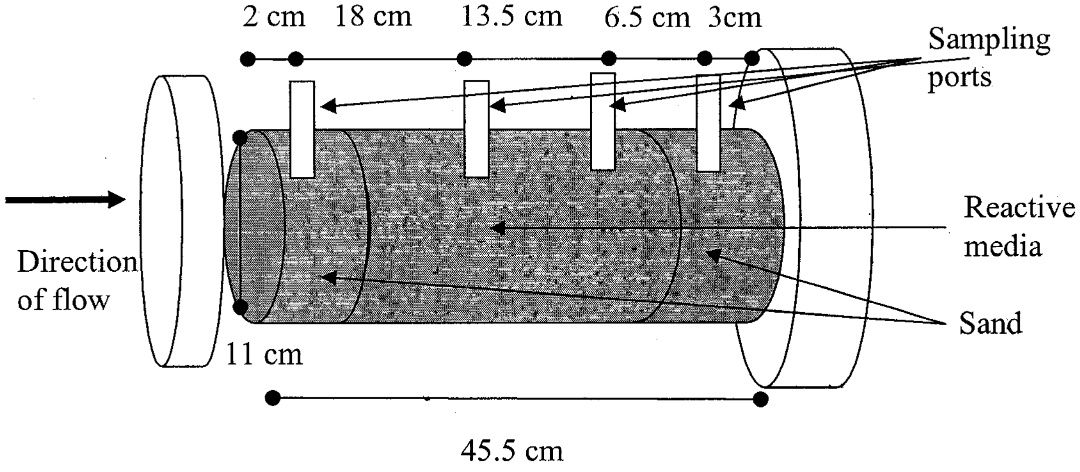


Figure 4-8 Schematic of the columns simulating anaerobic permeable reactive barriers.

The names assigned to the columns as well as the weight of the reactive media used in each column are presented in

Table 4-6. Once the columns were packed and inoculated, they were closed and remained stagnant for 48 hours to allow the bacteria to adapt to the new conditions and to attach to the soil. The flow rate was varied during the experiment. For the first 90 days, the flow rate was kept at 388 mL/d. Then, the flow rate was increased to 194 mL/d for the next 184 days (experiment length 274 days). The hydraulic retention time of the columns was 20 days for the first flow rate and 10 days for the second. Humic acids (Aldrich, St. Louis, MI) were spiked on day 107 to promote the anaerobic biodegradation of MTBE as proposed by Finneran and Lovely (Finneran and Lovley 2001; Finneran et al. 2001).

Humic acid (HA) is the product of macroanimal and plant decomposition, brown to black in color, very soluble in water at neutral to alkaline pH values and is negatively charged. Given that HA is an end product of decomposition of organic matter, it has great resistance to further decomposition, however it is a slow release source of carbon, nitrogen, sulfur and phosphorus for plants and microflora in soil. HA have a great cation exchange capacity (CEC). It has been demonstrated that through the sequestration of cations, nutrients and the absorption of micronutrients and organic compounds, HA stimulates the growth of plants and microorganisms in soil (Sylva et al. 2005). The benefit of using HA to enhance biodegradation of MTBE is that this substances act as an electron shuttle between the sediments, the liquid phase and the bacteria (Finneran and Lovley 2001). The positive benefits of using HA to promote the biodegradation of other organic compounds such as PAHs has also been demonstrated (Finneran and Lovley 2001; Van Stempvoort et al. 2002).

In this study, the concentration of the HA was varied from 10 mg/L to 10 g/L. Sodium sulfide (Na_2S, Fisher, Fair Lawn, NJ) was added in the influent at a concentration of 32 mg/L as an oxygen scavenger. Samples to monitor the concentration of MTBE were taken every week from the influent and the effluent of the columns and stored at 4 ^{0}C until analysis (< three days). The experiment lasted 9 months and was repeated an additional month for repeatability. The samples to be used for molecular analyses were collected from the reactive media in each of the sections of columns at the beginning and at the end of the experiment; and stored at -80 ^{0}C until analysis.

The columns were fed with DI water containing essential nutrients and minerals for biological growth and a MTBE concentration of 10 mg/L. The MSM used in this experiment is described in Pruden et al. 2005 (Pruden et al. 2005). The pH of the source water was adjusted to a value of 7 ± 0.2. The source water was stored in 25 L Tedlar bags (SKC, Fullerton, CA) to reduce the exposure of the water with the atmosphere. The Tedlar bags were connected to a six channel Ismatec peristaltic pump (Ismatec, Glattbrugg, Switzerland).

4.3.2.3 DNA Extraction

The MoBio UltraClean Soil DNA kit (Solana Beach, CA) was used for the extraction of the microbial DNA in the aqueous (batch experiment) and sediment samples (column experiment) according to manufacturer's protocol. The bead-beating time used was 4 minutes, to reduce shearing of the DNA. To the sediments extracted from the columns at the end of the experiment, 50 μl extra of the IRS™ were added to precipitate the high amount of humic acids present in those samples.

4.3.2.4 Cloning of 16S rRNA Genes

Cloning of 16S rRNA genes was performed to estimate the diversity of the cultures and to identify the dominant bacteria present. Samples taken from reactors Na_2SO_4 (B) and $FeCl_3$ (A) at day 82 (MTBE peak removal) were used for this purpose. Also, at day 274 (sacrifice of the columns) a sample from the reactive media of the column gypsum + $Fe(OH)_3$ was used to determine the bacterial consortium composition and diversity. This column was selected since was the most likely to work, given that it had both electron acceptors. The 16S rRNA genes were polymerase chain reaction (PCR) amplified with primers 8F and 1492R (Weisburg et al. 1991). The PCR master mix consisted of (per 25 μL reaction): 2.5 μL of 10X buffer, 5 μL of 5X buffer, 0.5 μL of dNTP (10 mM), 0.25 μL of formamide,1 μM of each primer (5 mM), and 0.35 μL of Taq polymerase (Eppendorf, Westbury, NY). The thermocycler program consisted of an initial denaturing step for 2 minutes at 94°C, followed by 25 cycles of 30 seconds at 94°C, 30 seconds at 50°C and 30 seconds at 72°C. A final extension step was conducted for 7 minutes at 72°C. The resulting PCR products were cloned using the TOPO TA Cloning Kit® (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. Resulting clones were screened for the presence of an insert by PCR using vector-specific M13F and M13R primers. The amplified inserts were digested with the restriction enzyme *MspI* (Promega, Madison, WI.) in order to identify unique inserts by restriction fragment length polymorphism (RFLP). The number of species present in each reactor and the column were determined by using software developed by Steven Holland: Analytic Rarefaction 1.3 (UGA Stratigraphy Lab, Athens, GA). The selected samples for sequencing were purified with ExoSAP-IT (USB, Cleveland, OH) and sequenced by the Macromolecular Resource Facility at Colorado State University (Fort Collins, CO).

4.3.2.5 Sequence Analysis and Assignment to Functional Groups

Each unique clone ribotype present in each sample's clone library was selected for sequencing. The BLAST tool of the National Institute of Health database was used to search and identify the highest matches for each clone insert. Sequences were also analyzed using the Ribosomal Database Project (RDP) search to aid in phylogenetically classifying the microorganisms represented by clone sequences. A literature review was performed in order to characterize the microorganism(s) corresponding to the highest matches.

4.3.2.6 Real-Time Quantitative PCR (Q-PCR)

The relative concentrations of total bacteria were determined by quantifying total 16S rRNA genes following the Q-PCR protocol described by Suzuki et al. (Suzuki et al. 2000). Primers 1369F (10 mM) and 1492R (10 mM), and the TaqMan probe 16S were used. Twenty-four microliters of PCR master mix was mixed with 1 μl of DNA extract. The master mix consisted of 2.5 μL 10X buffer, 5 μL 5X buffer, 0.5 μL dNTP (10 mM), 0.25 μL of each primer (10 mM), 0.375 μL TaqMan probe 16S (10 mM), 0.35 μL Taq Polymerase (Eppendorf, Westbury, NY), and 1.5 μL Mg^{2+}. All Q-PCR reactions were performed in duplicate. A Cepheid Smart Cycler (Sunnyvale, CA) was used to measure the samples. The amplification program consisted of 2 minutes at 95^0C, followed by 50 cycles at 95^0C for 15 seconds, 53^0C for 60 seconds and 72^0C for 20 seconds.

A generalized least-square means (GLM) analysis was conducted to obtain p-values in order to determine if the difference in the concentration of bacteria observed for each sample at the different times studied was significant. SAS 9.1 software was used to calculate p values (SAS Institute Inc., Cary, NC).

4.3.3 Results and Discussion

4.3.3.1 Anaerobic Batch Experiment

4.3.3.1.1 MTBE Removal

The relative concentration of MTBE in the batch reactors is presented in Figure 4-9. MTBE was removed in the presence of both electron acceptors. Ferric chloride seemed to be relatively preferred by the anaerobic Fe-reducing culture over sodium sulfate. However, the removal of MTBE was rather slow in the batch reactors (~60% removal in 82 days). The slow removal could be related to a lag phase of the bacteria adapting to the new environment or could be due to the buildup of byproducts. *Tert*-butyl alcohol (TBA), a common MTBE intermediate, was not observed to form during the experiment. In previous studies it has been observed that bioremediation of MTBE stops in TBA and no further removal of MTBE or TBA is observed (Sulfita and Mormile 1993; Mormile et al. 1994; Yeh and Novak 1994). All of the reactors generated about 1 mL of gas at each sampling event. It can also be observed in Figure 4-9 that the concentration of MTBE did not change significantly in the controls (<20%). However, in the controls that were not poisoned, MTBE was removed to a greater extent than in the controls that contained $HgCl_2$ (16% vs. 7%, respectively).

4.3.3.1.2 Bacterial Consortium Characterization

4.3.3.1.2.1 Bacterial Community Diversity

The rarefraction curves for the batch communities are presented in Figure 4-11. Comparing the number of phylotypes present in both batch reactors the estimated species richness was higher for reactor Na_2SO_4 (B) than for reactor $FeCl_3$ (A) (13.8 ± 0.775 versus 8.8 ± 0.78, respectably).

4.3.3.1.2.2 Functional Groups identified in the reactors

The results of cloning and RFLP screening of 16S rRNA genes are presented in Figure 6-5 and Table 4-7. It can be observed that the microbial communities of both reactors consisted of very different microorganisms, which is attributed to the presence of different electron acceptors. Most of the bacteria present in the batch reactors matched uncultured bacteria of classes Bacteriodetes, Mollicutes, Clostridia, α-, β-, δ- and γ- Proteobacteria (Table 4-7).

The bacteria present in reactor Na_2SO_4 (B) belonged to four main classes: Bacteriodetes, Clostridia, β- and γ-Proteobacteria. The function of the bacteria most closely related to those present in this reactor were associated to iron and sulfate reduction, and denitrification. The bacteria present in reactor $FeCl_3$ (A) matched four classes as well: Bacteriodetes, Clostridia, γ-Proteobacteria and Mollicutes. In this reactor, three types of bacteria were related to bacteria found in sites impacted with gasoline or oil. Both reactors had three common types of bacteria present: Uncultured Eubacterium (belonging to the

Bacteroidetes Class, DQ677015), *Clostridium* sp. (AY532163) and *Shewanella* sp. (AB111109), bacteria related to the reduction of iron or sulfate, but the relative percentages of these groups varied between reactors (Figure 4-12). However, none of the screened clones in either of the reactors was found to match a bacterium able to degrade MTBE or to a bacterium that had been found in a site contaminated with MTBE. One of the microorganisms found in reactor $FeCl_3$ (A) (*Desulfosporosinus* sp.) has been found in gasoline impacted sites and it is able to degrade BTEX (Robertson et al. 2000; Robertson et al. 2001). In Chapter 7, it is demonstrated that an anaerobic microorganism closely related to *Desulfosporosinus* sp. is capable of degrading MTBE.

4.3.3.1.3 Real time Q-PCR

The concentration of total bacteria based on real time Q-PCR using the 16S rRNA gene in both reactors is presented in Figure 4-13A. Day 0 represents the beginning of the experiment and day 82 represents the point where a peak in MTBE removal was observed. It can be noted that the concentration of bacteria greatly increased in both reactors from the beginning of the experiment to day 82. The initial low biomass present in the batch reactors possibly had a detrimental effect on the removal of MTBE and thus may have accounted for the long lag phase observed in this experiment. As expected, the reactors that contained $FeCl_3$ had a higher biomass concentration than the reactors that were amended with Na_2SO_4. This was expected based on the higher energy that Fe(III) yields to the cells. The difference in the total concentration of bacteria on day 82 between reactors Na_2SO_4 (A) and reactors $FeCl_3$ (A) and (B) was found to be significant ($p<0.0001$). While the difference in concentration of total bacteria for reactor (day 82) Na_2SO_4 (B) was significant only when compared to reactor $FeCl_3$ (A). However, when the rate of MTBE biodegradation versus concentration of 16S rRNA genes was plotted, no correlation was found (data not shown). Therefore, the concentration of total bacteria did not have an apparent effect on the rate at which MTBE was removed in the four reactors.

4.3.3.2 Anaerobic Column Test

4.3.3.2.1 MTBE Removal

The total removal, defined as 1 –Ce/Ci (Ce effluent concentration, Ci influent concentration), of MTBE in the columns during the experiment is presented in Figure 4-10. It can be observed that MTBE was not removed until humic acids (HA) were added, however only 30% of the influent MTBE concentration was removed. Once HA were washed out from the system MTBE removal could not be consistently replicated. Several attempts were made thereafter by adding HA at different concentrations to the influent, but it was not possible to stimulate the bacteria again. One possible cause is that due to the low solubility of the electron acceptors used, the columns did not provide a favorable environment for the bacteria, thus the bacteria were unable to grow fast enough to colonize the columns and reach a significant biomass concentration to remove MTBE at the rate studied (1 mg/L-d). The columns that removed MTBE at the beginning of the experiment were the inoculated columns that had $Fe(OH)_3$ (Figure 4-10). The other two columns, gypsum only and the uninoculated control, removed MTBE at only one time point. The removal in the control is attributed to bacteria present in the indigenous soil that could have been stimulated by the nutrients and the presence of HA. Again, the removal in the inoculated columns that had Fe(III), could be attributed to the higher energy yield of this electron acceptor compared to SO_4.

Even though HA have been used previously to enhance anaerobic MTBE biodegradation (Finneran and Lovley 2001; Pruden et al. 2005), the exact function and the required concentration have not been determined. In our case, it appeared to stimulate the bacteria at the beginning of the experiment, but thereafter the presence of HA seemed to have a detrimental effect on the bacteria. It is possible that HA had chelated the nutrients that were added at the influent. It is also possible that HA could have adsorbed and coated the reactive media, decreasing the availability of the electron acceptor.

4.3.3.2.2 Bacterial Consortium Characterization

4.3.3.2.2.1 Bacterial Community Diversity

The microbial community diversity calculated from cloning and RFLP screening of 16S rRNA genes are presented in Figure 4-11. Comparing the number of phylotypes present in column gypsum + $Fe(OH)_3$ with the microbial diversity of the batch reactors, it can be observed that the diversity in the column was smaller (7.89 ± 0.62 versus 13.8 ± 0.775 and 8.8 ± 0.78), even though the reactors and the column were inoculated with the same anaerobic culture. A greater diversity was initially expected in the columns, given that they provided a more complex environment for the bacteria (two electron acceptors and flow through conditions). It is possible that this low diversity could have affected the performance of the bacterial community to remove MTBE, likewise, the inability of MTBE degrading microbes to adapt to the unsuitable conditions in the column could have decreased diversity and hindered performance.

4.3.3.2.2.2 Functional Groups identified in the columns

A summary of the different phylotypes present in the columns is presented in Figure 4-12 and Table 4-7. As well as what was observed in the batch reactors, it can be observed that most of the bacteria present in the columns matched uncultured types. The bacteria found in the column belonged to five classes: Bacteroidetes, Clostridia, α-, δ- and β-Proteobacteria. The bacteria present in the column appeared to have a more diverse range of functions than the bacteria present in the batch reactors (TCE, hydrocarbon, fuel and methane degradation, denitrification and thiosulfate-oxidation). Even though the column had microorganisms that have been found in fuel contaminated sites, these bacteria were not reported to degrade MTBE. This is not surprising, since no anaerobic MTBE degrader has been reported previously.

4.3.3.2.3 Real time Q-PCR

The results for the concentration of total bacteria based on real time Q-PCR using the 16S rRNA gene are presented in Figure 4-13B. It can be observed that at the beginning of the experiment the concentration of total bacteria in the $Fe(OH)_3$ and gypsum + $Fe(OH)_3$

columns was very low (~ 1 copy/g), while in the gypsum and the uninoculated control columns the concentration of bacteria was below the detection limit. The concentration of bacteria in the sand was also very small in comparison. At day 274 (when the columns were sacrificed), the concentration of total bacteria increased in all of the columns, including the control, but not in the gypsum column. The change in concentration of total bacteria in gypsum + $Fe(OH)_3$ column was found to be significant ($p < 0.0001$). Although an increase in concentration of total bacteria was observed in most columns by the end of the experiment, the bacteria present were unable to remove MTBE at the required rate to match the loading to the column (1mg/L-d). However, the increase does suggest that the populations were degrading MTBE, even if it was not detectable.

Comparing the biomass concentration in the columns with those in the batch reactors, the biomass concentration was higher for the batch reactors than for the columns, which could mean that the bacteria present in the columns were unable to use the insoluble form of the electron acceptors to remove MTBE at a significant level, but since an increase in biomass was observed, probably the bacteria were using the organic matter present in the HA as carbon source.

4.3.4 Conclusions

A batch experiment was performed to compare the effect of the type of electron acceptor used on the removal of MTBE. A batch study was performed to select the reactive media to be used in a column study, which in turn could later be applied in PRBs. The microbial communities of both experiments were characterized to determine whether there was a difference in composition depending on the electron acceptor present (Fe(III) vs. SO4) and the reactor configuration (batch reactors vs. columns).

From the results, it was concluded that the culture was able to use both electron acceptors (Fe(III) and SO4) under batch conditions to biodegrade MTBE. However, the removal of MTBE was observed to be rather slow (6mg/L in 82 days).

In the column study, MTBE was biodegraded in the inoculated columns that had $Fe(OH)_3$ only when 10 g/L of humic acids were spiked into the system, but when the humic acids were washed out from the system, the bacteria could not remove MTBE anymore. It was not possible to re-stimulate the community with repeated addition of HA.

The concentration of total bacteria was determined for both experiments. No significant difference was observed between the biomass concentrations in the batch reactors, but a significant difference was observed with respect to the biomass concentration in the columns, which was much smaller than those observed in the batch reactors. Although the concentration of total bacteria did not have an effect on the removal rates observed in the batch reactors, it did seemed to have a detrimental effect on the removal of MTBE in the column.

When the bacterial communities of two of the batch reactors (Fe_2SO_4 (B) and $FeCl_3$ (A)) were compared to the column gypsum + $Fe(OH)_3$, it was observed that each microbial community was distinct in composition. The batch reactors shared three types of bacteria, while the reactors only shared one type of bacteria with the column. No known MTBE degraders were found in the reactors or the column, although some phylotypes present in the column were related to bacteria found in fuel contaminated sites. Future work should focus on isolating and characterizing anaerobic MTBE degraders so that the conditions under which they thrive can be better understood. This can help improve implementation of anaerobic MTBE remediation in the field.

Tables and Figures

Table 4-5 Experimental conditions for the anaerobic batch reactors.

Reactor Name	**Electron Acceptor**	**Concentration of the Electron Acceptor (g/L)**	**Notes**
Na_2SO_4 (A)	Na_2SO_4	0.055	32 mg/L of Na_2S were added. Inoculated.
Na_2SO_4 (B)	Na_2SO_4	0.055	32 mg/L of Na_2S were added. Inoculated.
$FeCl_3$ (A)	$FeCl_3$	0.302	32 mg/L of Na_2S were added. Inoculated.
$FeCl_3$ (B)	$FeCl_3$	0.302	32 mg/L of Na_2S were added. Inoculated.
Control A	$CaSO_4$	6	Uninoculated
Control B	$CaSO_4$	6	Uninoculated
Killed Control A	$CaSO_4$	6	1 mg/L $HgCl_3$were added.
Killed Control B	$CaSO_4$	6	1 mg/L $HgCl_3$ were added.

Table 4-6 Experimental conditions for the anaerobic columns.

Column Name	**$Fe(OH)_3$ (Kg)**	**$CaSO_4$ (Kg)**	**Inoculum**
$Fe(OH)_3$	5	0	Anaerobic Fe-reducing culture
Gypsum ($CaSO_4$)	0	5	Anaerobic Fe-reducing culture
Gypsum ($CaSO_4$) + $Fe(OH)_3$	2.5	2.5	Anaerobic Fe-reducing culture
Control Gyp+ $Fe(OH)_3$	2.5	2.5	NA

Table 4-7 Summary of Highest Matches and Characteristics of Sequenced Clones.

Highest Match (Score)$^{*+\#}$	**Class**	**Function/ Association**	**Reactor**
Unc. Eubacterium DQ677015 (98)	Bacteroidetes	Sulfate Red.	Na_2SO_4 (B) and $FeCl_3$ (A)
Clostridium sp. PPf35E4 AY548782 (98)	Clostridia	Sulfate Red.	Na_2SO_4 (B)
Comamonas testosteroni AY653219 (99)	β–Proteobacteria	Activated Sludge	Na_2SO_4 (B)
Uncultured Eubacterium AJ412670 (98)	β–Proteobacteria	Denitrifying Bact.	Na_2SO_4 (B)

Highest Match (Score)*$^{+\#}$	Class	Function/ Association	Reactor
Clostridium sp. AY532163 (97)	Clostridia	Iron Red.	Na_2SO_4 (B), $FeCl_3$ (A) and Col. Gyp+$Fe(OH)_3$
Shewanella sp. AB111109 (98)	γ-Proteobacteria	Iron Red.	Na_2SO_4 (B) and $FeCl_3$ (A)
Desulfosporosinus sp. AF076244 (96)	Clostridia	Gasoline impact. Site	$FeCl_3$ (A)
Uncultured low G+C Gram-positive AY128088 (96)	Mollicutes	Acidified Waste Water	$FeCl_3$ (A)
Gracilibacter thermotolerans strain JW/YJL-S1 (90)	Clostridia	Acid-sulfate water	$FeCl_3$ (A)
Unc. Bacterium AY770939 (99)	γ-Proteobacteria	Oil-cont. Field	$FeCl_3$ (A)
Uncultured Bacterium DQ266900 (99)	Bacteroidetes	Alkaline soil	$FeCl_3$ (A)
Uncultured Bacterium DQ443901 (99)	Clostridia	NA	$FeCl_3$ (A)
Uncultured Firmicutes EF420229 (98)	Clostridia	Oil sands tailings	$FeCl_3$ (A)
Uncultured Bacterium AY662035 (98)	β–Proteobacteria	Groundwater	Col. Gyp+$Fe(OH)_3$
Sedimentibacter sp. EF059533 (94)	Clostridia	Dechlorination	Col. Gyp+$Fe(OH)_3$
Uncultured δ–Proteobacteria AF529133 (91)	δ–Proteobacteria	TCE-cont.	Col. Gyp+$Fe(OH)_3$
Uncultured Bacterium DQ202142 (98)	Bacteroidetes	Denitrifying Bact.	Col. Gyp+$Fe(OH)_3$
Uncultured Bacterium AY548933 (96)	β–Proteobacteria	ANNAMOX reactor	Col. Gyp+$Fe(OH)_3$
Uncultured Bacterium AB273804 (97)	Clostridia	Methane-degrading	Col. Gyp+$Fe(OH)_3$
Azospirillum sp. AF411852 (98)	α-Proteobacteria	Fuel-Cont.	Col. Gyp+$Fe(OH)_3$
Uncultured α-Proteobacteria emb|AJ318184.1|UAL318184 (97)	α-Proteobacteria	Methane-degrading	Col. Gyp+$Fe(OH)_3$
Uncultured soil bacterium DQ297948.1 (97)	α-Proteobacteria	Hydrocarbon Cont. soil	Col. Gyp+$Fe(OH)_3$
Uncultured β–Proteobacterium AJ224618 (97)	β–Proteobacteria	Thiosulfate-ox.	Col. Gyp+$Fe(OH)_3$

*National Center for Biotechnology Information Blast similarity score http://www.ncbi.nlm.nih.gov/BLAST/ .
$^{+}$Based on 1000-1100 bp of 16S rRNA gene sequence information.
$^{\#}$GenBank accession numbers provided following name (eight characters).

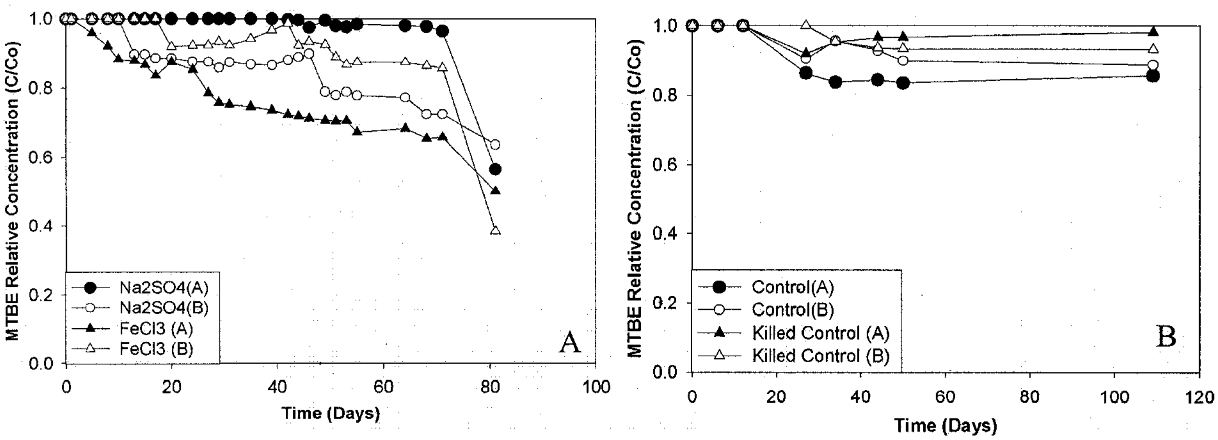


Figure 4-9 Change in concentration of MTBE using DI water with nutrients in the batch reactors comparing Fe(III) and SO4 only. The target initial MTBE concentration was 10 mg/L. All of the controls contained gypsum as the electron acceptor and 1 mg/L of $HgCl_3$ was added to the killed controls.

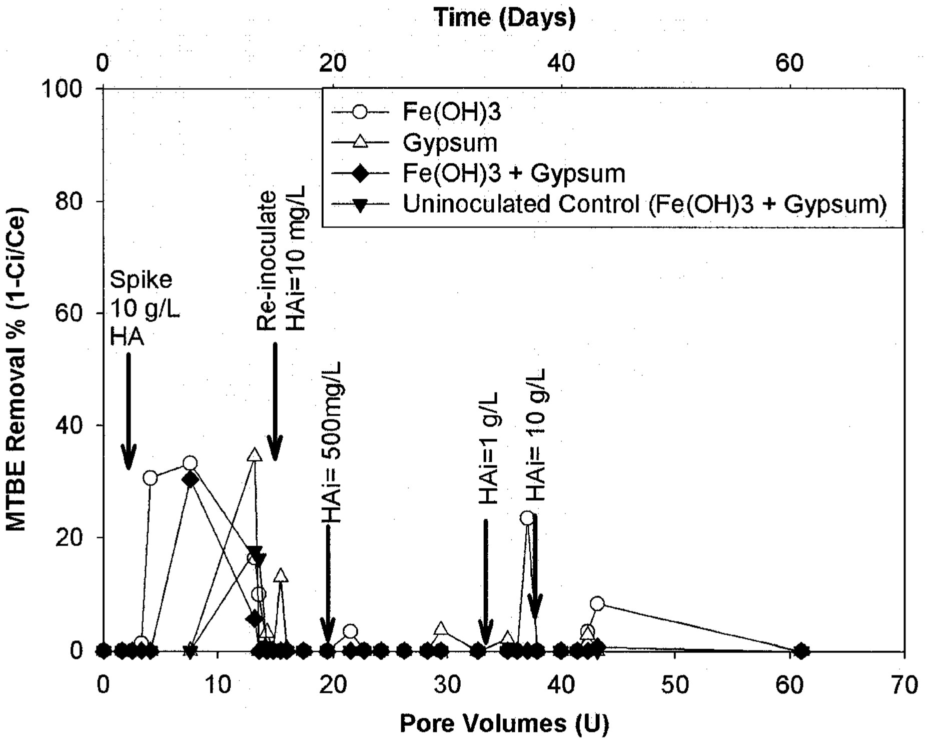


Figure 4-10 Total % removal of MTBE from the influent to the effluent in the columns. The target initial MTBE concentration was 10 mg/L. Humic acids (HA) were spiked four weeks after the experiment was started, then it was added to the influent water at various concentrations. The hydraulic retention time was 20 days the first two months (7 pore volumes) and then it was decreased to 10 days for the remainder of the experiment. The control was not inoculated. Length of the experiment: 274 days (61 pore volumes).

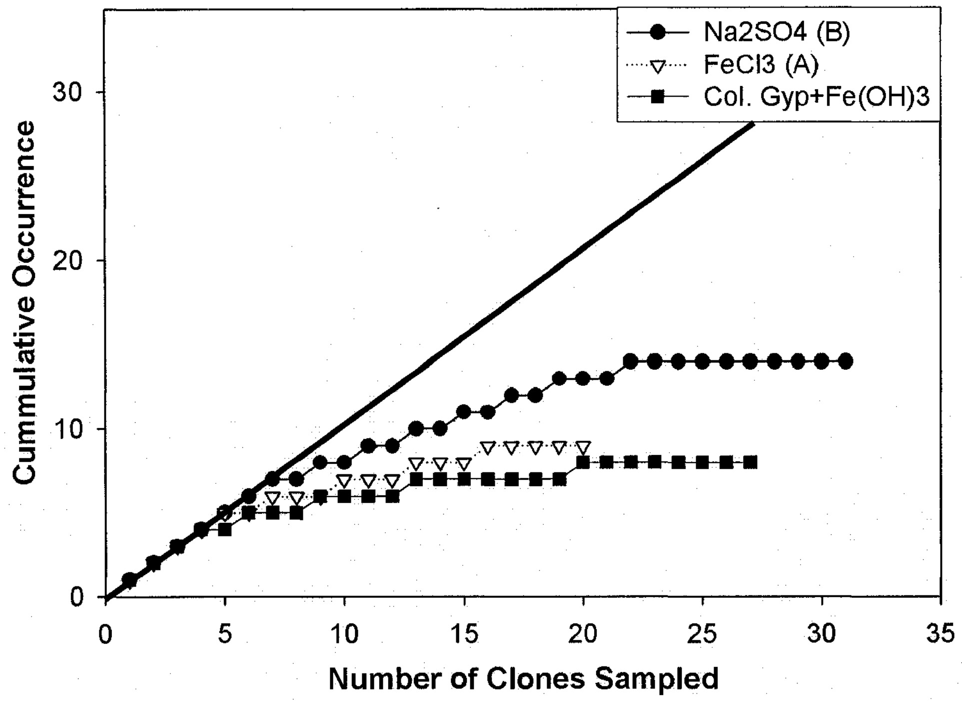


Figure 4-11 Rarefraction curves generated from cloning and restriction fragment length polymorphism (RFLP) of 16S rRNA genes using *Msp*I restriction enzyme. Samples from the batch reactors Na_2SO_4(B) and $FeCl_3$ were taken at day 82 (peak MTBE removal), while the sample taken from the column Gyp+$Fe(OH)_3$ was taken at day 274 (column sacrifice). The solid lines represent the hypothetical case that all sampled clones are unique (maximum possible diversity).

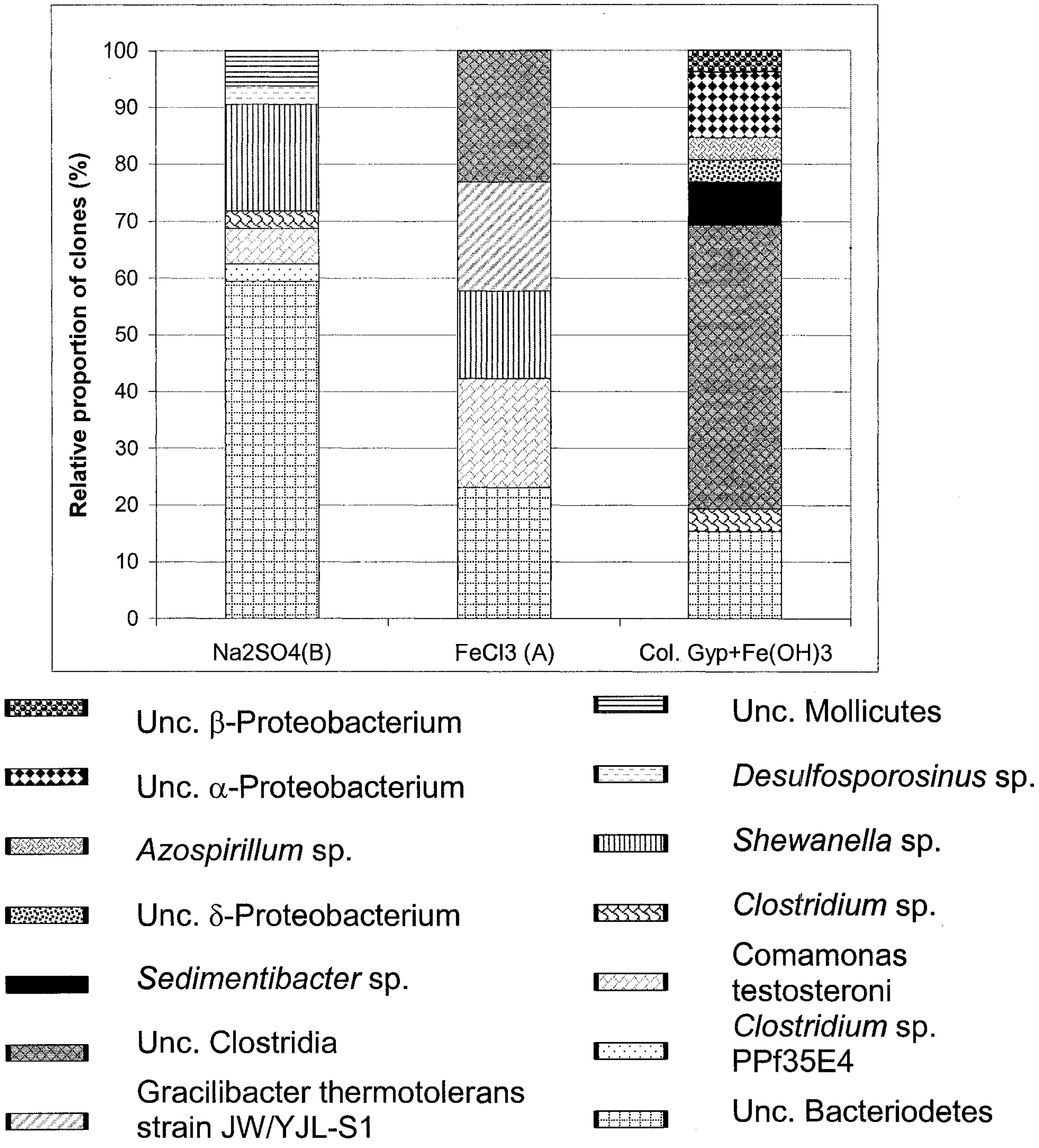


Figure 4-12 Identification of the dominant microorganisms present in the batch reactors on day 82 and in the Gypsum + $Fe(OH)_3$ column on day 274 based on cloning, RFLP screening, and sequencing of most every type of clone present.

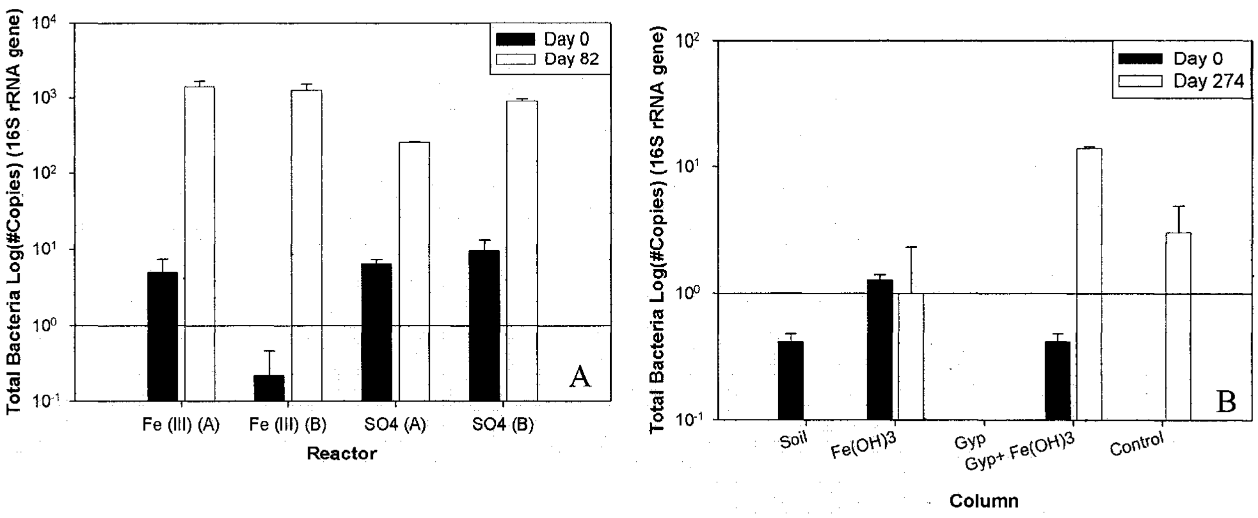


Figure 4-13 Concentration of total bacteria in (A) the batch reactors and (B) in the columns at the beginning and at the end of the experiments.

4.4 Objective 4: Isolate and Characterize Pure Cultures Capable of Anaerobic MTBE Degradation.

4.4.1 Introduction

Methyl *tert*-butyl ether (MTBE) is a gasoline oxygenate added in an effort to comply with the regulations of the 1990 Clean Air Act. MTBE is introduced into the environment due to leakage of underground storage tanks and pipelines at refineries and gas stations. The wide use of MTBE since the late 1970s and its physico-chemical characteristics, such as high water solubility, low adsorption capacity and slow biodegradation, have contributed to the persistence of this contaminant in the environment. Treatment of MTBE by traditional methods, such as pump and treat and GAC adsorption, are challenging and in most cases ineffective, expensive and not sustainable (Deeb et al. 2003). Thus bioremediation has developed as a promising low cost and sustainable treatment. Even though bioremediation of MTBE has been demonstrated as a viable treatment, most of the studies have been conducted under aerobic conditions and it has been reported that it does not degrade readily in many environments. Some of the conditions that have been observed to slow down or inhibit biodegradation of MTBE include: absence of oxygen; which is common at sites impacted by gasoline, the presence of other gasoline constituents, such as benzene, toluene, ethyl benzene and xylenes (BTEX); and the microbial community composition (Deeb et al. 2001; Deeb et al. 2003; Fayolle et al. 2003; Raynal and Pruden 2007).

Given that anaerobic conditions prevail in groundwater, the feasibility of promoting anaerobic biodegradation of MTBE is of particular interest (Mormile et al. 1994; Yeh and

Novak 1994; Finneran and Lovley 2001; Pruden et al. 2005). In recent studies, natural attenuation has been attributed to anaerobic biodegradation using the change of carbon isotope fractionation as an indicator of microbial activity. Microorganisms preferentially degrade the lighter isotopes of carbon and hydrogen (^{12}C and ^{1}H), thus with biodegradation the heavier molecules (^{13}C and ^{2}H) accumulate in the environment. This fractionation increases the isotopic ratio of ^{13}C to ^{12}C and ^{2}H to ^{1}H (Moreels et al. 2004; Kuder et al. 2005; Wilson et al. 2005; Somsamak et al. 2006). Although some mixed cultures have been identified to be able to anaerobically degrade MTBE (Mormile et al. 1994; Yeh and Novak 1994; Bradley et al. 2001b; Finneran and Lovley 2001; Pruden et al. 2005), to our knowledge, no pure cultures have been isolated. The purpose of this study was therefore to isolate and characterize a pure culture capable of anaerobic biodegradation of MTBE. Understanding the characteristics of such an organism can help guide anaerobic bioremediation in the field and also provide a target for DNA probes for identifying and quantifying anaerobic MTBE degrading bacteria.

4.4.2 Materials and Methods

4.4.2.1 Isolation Procedure

Pure cultures were isolated by plating a previously enriched anaerobic iron-reducing culture (Pruden et al. 2005), an aerobic culture previously enriched on MTBE as the sole substrate (MO) (Raynal and Pruden 2007) and the influent from an air striping system of a petroleum refinery near Denver, CO. The cultures were spread on 4% (v/v) agarose-MSM (mineral salt medium) plates containing 950 mg/L of $Fe_2(SO_4)$ (Wako Chemicals USA Inc., Richmond, VA) and placed in an airtight GasPak Jar™ (BD, Sparks, MD) with a BD BBL™ Gas Pak™ Plus gas generator envelope (BD, Sparks, MD) to create anaerobic conditions. An anaerobic indicator (Oxoid, Lenexa, KS) was used to verify that anaerobic conditions were maintained. The mineral salt medium (MSM) contained nutrients and minerals for microbial growth, as described in Pruden et al. 2005 (Pruden et al. 2005). A 5 mL glass vial with a perforated Teflon cap containing MTBE (Arcos, Morris Plane, NJ) was placed inside the chamber to provide the sole carbon substrate.

Each month for eight months, the GasPak Jar™ was opened inside a glovebox with N_2 gas to refill the vial containing MTBE, to replace the gas generator envelope and the anaerobic indicator. Once colonies appeared they were transferred to a new plate and to a 100 mL glass bottle that contained MSM, 750 mg/L of $Fe_2(SO_4)$ (Wako Chemicals USA Inc., Richmond, VA) and 10 mg/L of MTBE (Arcos, Morris Plane, NJ). The glass bottles were capped with rubber septa and were placed up-side-down in a controlled temperature shaking bed at 25 ^{0}C and 60 rpm. To confirm the ability of the culture to degrade MTBE in liquid, samples were taken biweekly and the concentration of MTBE in the bottles was monitored using by Gas Chromatography (described in section 4.4.2.3). Once MTBE biodegradation was observed (a decrease of 50% from the initial concentration in 35 days) the cultures were serially diluted and 10 μl of the 10^{-2} dilution were plated to verify the purity of the culture. The purity of the colonies growing on the plates and in the liquid cultures was further verified by microscopy, direct sequencing of the 16S rRNA genes and denaturing gel gradient electrophoresis (DGGE).

4.4.2.2 Growth Media

Once a pure culture was isolated in liquid medium, the culture was placed in three different growth media in triplicate in order to obtain the biodegradation rate under iron and sulfate reducing conditions. The experimental conditions for these batch studies are summarized in Table 4-8. Two of the growth media contained the same MSM applied in the plates, the only difference was that either Fe(III) or SO_4 were added, instead of using both (i.e. $Fe_2(SO_4)_3$). Ferric chloride (Fisher, Fair Lawn, NJ) was used as the source of Fe(III) and sodium sulfate (Fisher, Fair Lawn, NJ) was used as the source of SO_4. The third medium used was DSMZ 320 described by Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ 2007), where ammonium sulfate (Fisher, Fair Lawn, NJ) was used as the source of SO_4. Rezasurin (1mg/L, Fisher, Fair Lawn, NJ), sodium sulfide (32 mg/L, Fisher, Fair Lawn, NJ) and MTBE (10 mg/L, Arcos, Morris Plane, NJ) were added to all of the growth media. Nine 250 mL glass bottles, sealed with butyl rubber stoppers and aluminum crimps, were operated as batch reactors. The bottles were set up in a N_2 glovebox. A headspace of 10 mL was left in each bottle to allow gas generation. The bottles were placed in a shaking bed upside-down, to maintain the septa moist and prevent entry of oxygen, and agitated at 60 rpm. The bottles were kept at room temperature (~25 ^{0}C). Two milliliter samples for molecular analysis were taken at the beginning and at the end of the experiment to determine the change in biomass concentration.

The experiment was repeated on a smaller scale with a higher inoculum concentration in order to observe the effect on the biodegradation rate. A second set of three batch reactors were set up in 25 mL volume glass bottles sealed with butyl rubber stoppers and aluminum crimps. Cells were scraped directly from Petri dishes in order to obtain a higher initial cell density in these reactors. The growth medium contained the same MSM applied in the plates, the only difference between the reactors was the electron donor; either $FeCl_3$, Na_2SO_4 or $Fe_2(SO_4)_3$ were added. The target initial MTBE (Arcos, Morris Plane, NJ) concentration was 10 mg/L. The reactors were operated in the same way as the first set. Two milliliter samples were taken at the beginning of the experiment and at the end. The pH and the oxidation-reduction potential were measured at the beginning and at the end of the experiments.

Table 4-8 Summary of the experimental conditions for both sets of batch reactors.

Reactor Name	$FeCl_3$ (mg/L)	Na_2SO_4 (mg/L)	$Fe_2(SO_4)_3$ (mg/L)	Na_2S (mg/L)	Medium	Inoculum
$FeCl_3$ (A)	604	0	0	32	Pruden et al. 2005	Liquid culture
$FeCl_3$ (B)	604	0	0	32	Pruden et al. 2005	Liquid culture
$FeCl_3$ (C)	604	0	0	32	Pruden et al. 2005	Liquid culture
Na_2SO_4 (A)	0	110	0	32	Pruden et al. 2005	Liquid culture
Na_2SO_4 (B)	0	110	0	32	Pruden et al. 2005	Liquid culture
Na_2SO_4 (C)	0	110	0	32	Pruden et al. 2005	Liquid culture
M320 (A)*	0	240 as $(NH_4)_2SO4$	0	32	DSMZ 320	Liquid culture
M320 (B)	0	240 as $(NH_4)_2SO4$	0	32	DSMZ 320	Liquid culture
M320 (C)	0	240 as $(NH_4)_2SO_4$	0	32	DSMZ 320	Liquid culture
Fe (III)	604	0	0	0	Pruden et al. 2005	Colonies
SO_4	0	110	0	0	Pruden et al. 2005	Colonies
$Fe_2(SO_4)_3$	0	0	750	0	Pruden et al. 2005	Colonies

* DSMZ: Deutsche SammLung von Mikroorganismen und Zellkulturen GmbH (DSMZ 2007)

4.4.2.3 Monitoring MTBE concentration in the liquid cultures

Aqueous samples were taken periodically by opening the bottles inside an N_2 glovebox. Two hundred microliters were taken from each reactor and diluted into 5 mL borosilicate glass sampling vial with a Teflon cap containing 4.8 mL of DI water. The samples were analyzed immediately; otherwise, they were stored at 4 ^{0}C for less than one day. EPA method 8015 was used (USEPA 2007b) to measure MTBE concentrations by gas chromatography using a Tekmar-Dohrman (Cincinnati, OH) heated purge and trap 2016 and a Hewlett Packard (Rolling Meadows, IL) 5890 Series II Gas Chromatograph (GC) with a capillary column and flame-ionization detector (FID).

Eight point calibration curves were generated with concentrations varying from 4 to 2000 μg/L, with an R^2 of 98% for MTBE. Because the R^2 value of the calibration curve for *tert* butyl alcohol (TBA), an MTBE intermediate, was 87.29%, it was concluded that the TBA concentration could not be accurately quantified, so its presence or absence in each sample was reported instead.

4.4.2.4 Morphology of the Pure culture

4.4.2.4.1 White Light Microscopy

Gram-staining was used to observe the morphology of the cells present in the plates and in the liquid cultures, using a Gram-staining kit (Fisher, Fair Lawn, NJ). The cells were heat-fixed in glass slides and stained. A drop of oil was then placed on top of the slides and the cells were observed at 100x with a Nikon Eclipse E200 microscope (Melville, NY).

4.4.2.4.2 Electron Microscopy

Cell morphology was characterized with the aid of an electron microscope (EM) (JEOL JSM 6500F). The cells were fixed by resuspending the cell pellet in the 2.5% glutaraldehyde for 24-48 hours at 4°C. Then, the cell pellet was washed three times in 0.1 M phosphate buffer. The cells were fixed again by resuspending the cell pellet in osmium oxide (OsO_4). The samples were incubated for 30 minutes at 4°C. The resulting cell pellet was washed three times in 0.1 M phosphate buffer and then dehydrated by resuspending the cells in increasing ethanol concentrations (20, 40, 60, 70, 80, 90, 100 %) for 10 minutes at 4°C at each concentration. The cell pellet was then re-suspended in less than 0.1 mL of 100% ethanol. About 0.5 µL of the samples was applied to a silicon wafer of slide for EM. The slides were dried in a desiccator for 20 minutes. A 5 µm gold coating was applied to the slides before scanning. Samples were visualized using a JEOL JSM 6500F at Colorado State University Scanning Electron Microscopy Laboratory (Fort Collins, CO).

4.4.2.5 DNA Extraction

DNA was extracted from the liquid cultures present in the glass bottles. The MoBio UltraClean Soil DNA kit (Solana Beach, CA) was used for the extraction of the microbial DNA according to manufacturer's protocol using a bead-beating time of 4 minutes, to reduce shearing of the DNA.

4.4.2.6 Amplification of the 16S Region

The 16S rRNA gene was amplified using primers 8F and 1492R (Weisburg et al. 1991). The PCR products were then purified and sequenced directly (without cloning). The PCR master mix included 13.4 μL of purified water, 2.5 μL of 10X buffer, 5 μL of 5X buffer, 0.5 μL of dNTP (10 mM), 1 μL of each primer, 8F and 1492R (5 μM), 0.25 μL of formamide, and 0.35 μL of Taq polymerase (Eppendorf, Westbury, NY) per 25 μL PCR reaction. The thermocycler program consisted of an initial denaturing step for 2 minutes at 94°C, followed by 25 cycles of 30 seconds at 94°C, 30 seconds at 50°C and 30 seconds at 72°C. A final extension step was conducted for 10 minutes occurred at 68°C. The PCR products were purified with ExoSAP-IT (USB, Cleveland, OH) and sequenced by the Colorado State University Macromolecular Resources Facility (Fort Collins, CO).

4.4.2.7 Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) was used to identify polymorphisms among the 16S rRNA genes of the cultures growing on liquid and solid media. DNA was amplified directly from colonies picked from the plates or DNA extract from the liquid cultures using primers I-341F (with a GC clamp) and I-533R (Watanabe et al. 2001). The PCR master mix contained 12.9 μL purified water, 2.5 μL 10X buffer, 1 μL Mg^{2+}, 5 μL 5X buffer, 1 μL dNTP (10 mM), 1.25 μL of each primer (5mM), 0.25 μL formamide and 0.35 μL Taq polymerase (Eppendorf, Westbury, NY) per 25 μL reaction. The thermocycler program consisted of an initial denaturing step at 94°C for two minutes, denaturing at 94°C for 15 seconds, primer annealing for 15 seconds beginning at 52°Cand reducing by 1°C every two cycles until it reaching 47°C, extension at 72°C for 20 seconds. Amplification consisted of 35 cycles after reaching the final temperature. A final extension step at 72°C took place for 7 minutes and after completion the program was held at 4°C.

The amplified DNA was loaded on acrylamide gels with a denaturing gradient of 30%-50% (100% defined as 7M urea, 40% vol/vol formamide in 1X TAE buffer and 8% acrylamide/bis solution). The gel was run at 55 volts and 57^0C for 18 hours. Gels were stained using SybrGold nucleic acid stain diluted in 1:10,000 1X TAE buffer for 15 minutes before being visualized. Central portions of the bands were excised and placed in a 0.5 mL tube with 36 µl of sterile DI water, which was used as a template for a second PCR using the same primers. PCR products were purified with ExoSAP-IT (USB, Cleveland, OH) and sequenced by the Colorado State University Macromolecular Resource Facility (Fort Collins, CO).

4.4.2.8 Real time Q-PCR

The relative concentrations of total bacteria present in the liquid cultures were determined at the beginning and at the end of the experiment by quantifying total 16S rRNA genes using a previously described TaqMan Q-PCR method (Suzuki et al. 2000). Twenty-four microliters of PCR master mix was mixed with 1 µl of DNA extract. The master mix consisted of 2.5 μL 10X buffer, 5 μL 5X buffer, 0.5 μL dNTP (10 mM), 0.25 μL of each primer (primers 1369F (10 mM) and 1492R (10 mM)), 0.375 μL TaqMan probe 16S (10 mM), 0.35 μL Taq polymerase (Eppendorf, Westbury, NY), and 1.5 μL Mg^{2+}. All Q-PCR reactions were analyzed using a Cepheid Smart Cycler (Sunnyvale, CA). The amplification program consisted of 2 minutes at 95°C, followed by 50 cycles at 95°C for 15 seconds, 53°C for 60 seconds and 72°C for 20 seconds. All Q-PCR reactions were carried out in duplicate and three independent runs were done for each sample.

A generalized least-square means (GLM) procedure was conducted to determine if the differences between concentrations of total bacteria as determined by Q-PCR were significant. SAS 9.1 software (SAS Institute Inc., Cary, NC) was used to conduct the procedure and determine the p values.

4.4.2.9 Sequences Analysis

The 16S rRNA genes from the liquid cultures and the excised DGGE-bands from the colonies growing on the plates and in the liquid cultures were sequenced to determine the phylogenetic identity of the microorganisms present. The BLAST tool of the National Institute of Health database was used to search and identify the highest matches for the 16S rRNA gene PCR products and the DGGE bands. Sequences were also analyzed using the Ribosomal Database Project (RDP) sequence match tool to aid in phylogenetic classification. A literature review was performed in order to characterize the microorganism corresponding to the highest matches.

4.4.2.10 Phylogenetic Analysis

DNA sequences were aligned using ClustalX (Thompson et al. 1997) and edited using Proseq v2.91 (Filatov 2002). Sequences from the most closely related bacterial strains obtained from the BLAST database were included in the alignment. A tree was constructed using ClustalX (Thompson et al. 1997) and TreeViewX Win 32 (Page 2001) was used to visualize the tree. The 16S rRNA sequences of the following organisms (Genbank accession no. in brackets) were used: *Desulfotomaculum auripigmentum* (U85624), *Desulfosporosinus meridiei* S5 (AF076248), *Desulfosporosinus orientis* (AJ493052), *Desulfitobacterium dehalogenans* (L28946), *Desulfovibrio ferrireducens* (AJ582758), *Desulfitobacterium chlororespirans* (U68528), *Desulfosporosinus orientis* DSM 8344 (Y11571), *Desulfosporosinus youngii* JW/YJL-B18 (DQ11747); and *Chlamydia suis* (AY661794) as the outgroup (tree root).

4.4.3 Results

4.4.3.1 Biodegradation of MTBE

MTBE was observed to degrade in the three different media tested, without accumulation of TBA. It was observed that the removal rate in each reactor was different and independent of the growth medium (Figure 4-14A). There was not a common trend between the triplicates, which implies that other factors could have affected the removal of MTBE, such as the initial concentration of active bacteria. Considering the low initial inoculum density applied in this study, and the slow rate at which anaerobic bacteria grow, it is not surprising that the level of replication was low.

An average 25% removal (reactors FeCl3 (B) and M320 (B)) was observed in 20 days for an initial concentration of MTBE of 10 mg/L (0.125 mg/L-d). After this time, the liquid cultures reached a plateau after which no further removal was observed (Figure 4-14). Two of the reactors that contained Na_2SO_4 (reactor B and C) did not show evidence of MTBE removal over the duration of the experiment; however reactor Na_2SO_4 (A) removed about 23% of MTBE in this period. Poor reproducibility between duplicates have been previously observed in studies related to anaerobic biodegradation of MTBE (Mormile et al. 1994; Yeh and Novak 1994; Finneran and Lovley 2001). Yeh and Novak attributed the variability in the results of replicates as a consequence of different microbial densities in each reactor (Yeh and Novak 1994).

4.4.3.2 Comparison of the MTBE Biodegradation Rates under Iron and Sulfate reducing conditions

The change of the relative MTBE concentration in the batch reactors containing $FeCl_3$, $Fe_2(SO_4)_3$ and Na_2SO_4 is presented in Figure 4-14B. In this case, the greatest removal was observed in the reactor containing Na_2SO_4. A 40% removal in this reactor was observed in 28 days. It should be noted that reactors $FeCl_3$ A, B and C and reactor Fe(III) (Figure 4-14A and B) had the same growth medium and electron acceptor, just as reactors Na_2SO_4 A, B and C and reactor SO_4, however there were three differences: 1) the cells used to inoculated these reactors came from the plates, 2) a higher concentration of cells was used and 3) the second set of reactors did not contain Na_2S. The difference in the performance (faster biodegradation rate observed in the second set of batch reactors for Na_2SO_4) could be due to an inhibitory effect of Na_2S (added only to the first set of batch reactors) or due to a difference in biomass concentration, being higher for the second set than the first.

4.4.3.3 Isolation and enrichment of MTBE degrading culture

It was found that the same strain was isolated from two of the mixed cultures, while no culture could be isolated from the refinery water. The pure culture obtained did not form well-separated colonies, instead it tended to form a brown biofilm on the surface of the agarose plates. Small black particles were also observed to form on the surface of the plates, presumably ferrous iron. The isolate grew well when the liquid cultures were plated onto the agarose plates, but not when transferred from plate to plate. It appeared that the culture preferred to grow at a liquid-air interface, which was available in the liquid culture, and when the liquid was plated, but not when the colonies were transferred from plate to plate.

4.4.3.4 Sequences Analysis

Legible sequences were obtained for liquid cultures with the 16S PCR products and DGGE bands, but not for the DGGE bands excised from the gel were the colonies were used as the PCR template. The images taken from the DGGEs performed for the two sample sets are presented in Figure 7-2 A and B. A clear difference in the composition of the colonies on the plates (Figure 7-2 A) and the liquid cultures (Figure 7-2 B) was observed. It was observed that the colonies on the plates were formed from three possible species and the liquid cultures by two. It should be noted that most microorganisms have on average four copies of the 16S genes (Ninet et al. 1996; Conville and Witebsky 2007), thus, it is possible that the bands came from one bacterium if there is any slight variation among these copies. The sequences obtained for the 16S rRNA products and DGGE bands of the liquid cultures, which came from the two initial cultures: the aerobic MO culture and the anaerobic iron-reducing culture matched the same microorganism: *Desulfosporosinus meridiei* S5 (96% identity, Score 896, AF076248). This type of microorganism has been previously reported to grow in sites impacted with gasoline. *Desulfosporosinus meridiei* S5 is a Gram positive organism characterized by its ability to reduce sulfate and to form spores (Robertson et al. 2001).

4.4.3.5 Phylogenetic Analysis

A phylogenetic tree of the pure culture in comparison to other members of the *Desulfoporosinus* genus is presented in Figure 4-17. It can be observed that even though the closest match for the pure culture was *Desulfosporosinus meridiei* S5 (96% similarity, AF076248) from the tree the pure culture is also related to *Desulfosporosinus orientis* DSM 8344 (Y11571), thus this organism most likely belongs to the genus *Desulfosporosinus* (i.e. identity 96%< 98%).

4.4.3.6 Morphology of the pure culture

It was observed that the isolate did not stain blue-purple or red-pink, but rather pinkish purple and orange (results not shown). Therefore, it was characterized as Gram-variable. It has been reported that other genera, such as *Actinomyces, Arthobacter, Corynebacterium, Mycobacterium* and *Propionibacterium*, stain pink instead of purple, even though all of these genera are Gram-positive cells. Other factors such as the growth stage of the cells also affect the accuracy of this method. Cultures of *Bacillus*, *Butyrivibrio* and *Clostridium* stain Gram-negative during growth phase (Beveridge 1990). Therefore, these results are consistent with the Gram positive characterization based on 16S rRNA sequencing.

The image taken for the pure culture with SEM is presented in Figure 4-18. It can be observed that the cells have a rod shape with an approximate length of 1.5 μm and width of 0.5 μm. No flagella were detected with this method.

4.4.3.7 Real time Q-PCR

The initial concentrations of total bacteria for the batch reactors are presented in Figure 4-16. It can be observed that the concentration of total bacteria in the first set of batch reactors was rather low compared to the concentration of second set of batch reactors. This is logical, since the second set of batch reactors were inoculated with a higher concentration of cells.

4.4.4 Conclusions

The same microorganism was isolated from two different mixed consortia, the MO aerobic culture (Raynal and Pruden 2007) and the anaerobic Fe-reducing culture (Pruden et al. 2005). The pure culture was capable of growing on agarose plates under Fe(III) reducing conditions using MTBE as the sole carbon source. The pure culture was also able biodegrade MTBE in liquid media containing Fe(III) or SO_4 with no observable difference in rate. The average removal rate was equal to 0.125 mg/L-d (0.121.mg-MTBE/g-cells-d), however, only 25% removal from an initial 10 mg/L was observed. When the study was repeated at a higher initial cell density, a slightly higher rate of 0.45 mg/L-d was observed. The morphology of the pure culture was determined with SEM. The cells had a rod shape with an approximate length of 1.5 μm and width of 0.5 μm. No flagella were observed. The cells were Gram-variable. Based on 16S rRNA sequencing, the closest match for the pure culture was *Desulfosporosinus meridiei* S5 (AF076248), however when a phylogenetic tree was constructed using the sequences of other microorganisms of the *Desulfosporosinus* genus, it was observed that the pure culture was not very closely related to any of the members selected from this genus.

Figures

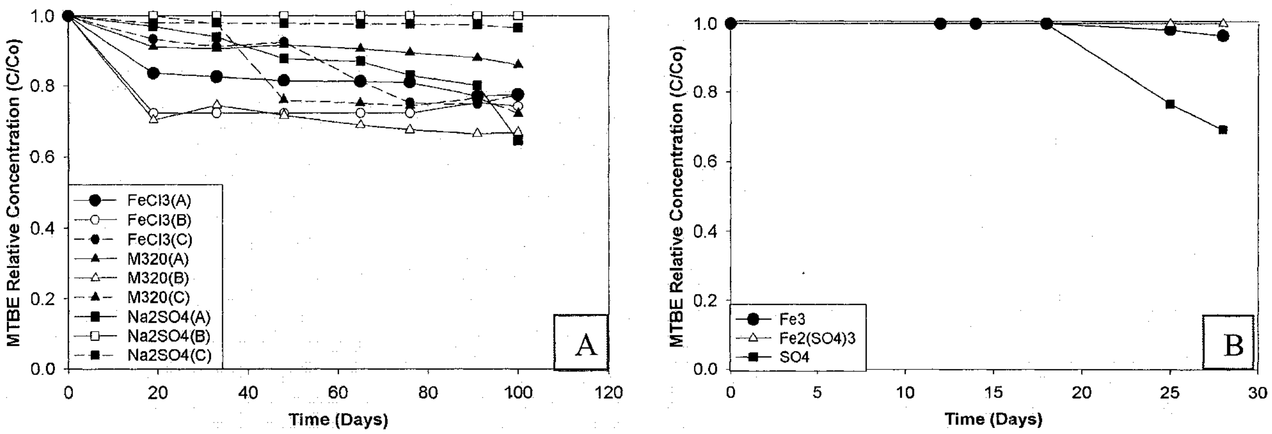


Figure 4-14 MTBE removal in the batch reactors containing the isolated pure culture and an initial MTBE concentration of 10 mg/L. The bottles were kept at room temperature (~25 ^{0}C) with an average pH of 7. A) Comparison of the biodegradation of MTBE by the pure culture in different media. B) Comparison of the biodegradation of MTBE in the presence of two electron acceptors: Fe(III) (as $FeCl_3$) and SO_4 (as Na_2SO_4) and both electron acceptors: $Fe_2(SO_4)_3$.

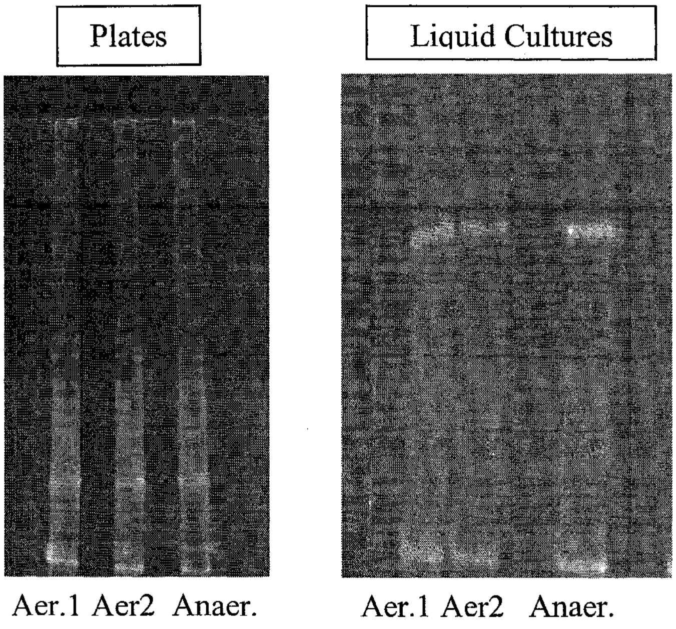


Figure 4-15 DGGE images for Plates) direct PCR on cells extracted from the plates for the aerobic and anaerobic inocula and Liquid) for PCR conducted on the DNA extractions of the liquid cultures for the aerobic and the anaerobic inocula. Aer. 1 and 2 indicates that the original inoculum was the aerobic culture (MO). Anaer. indicates that the original inoculum was from the Fe-reducing culture.

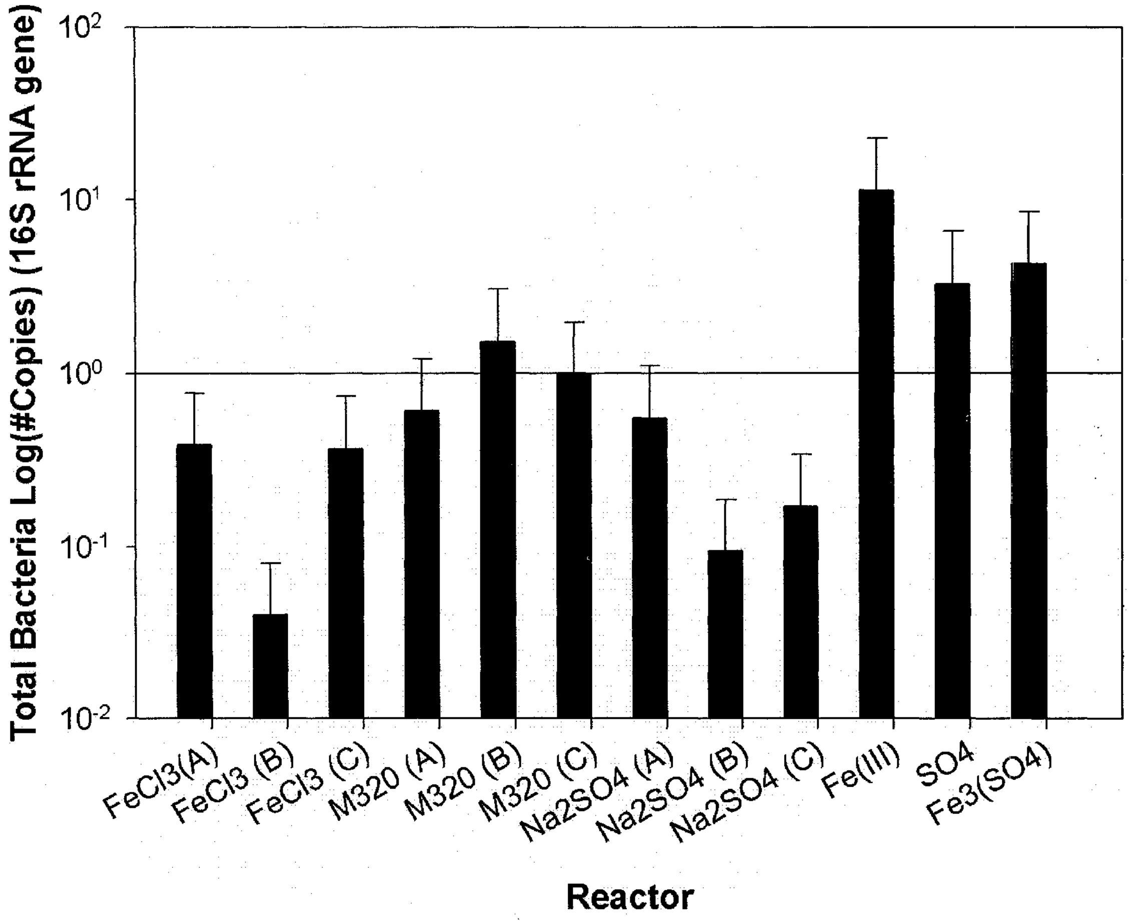


Figure 4-16 Concentration of total bacteria determined by real time Q-PCR using the 16S rRNA gene for the batch reactors that contained different growth media and the comparison of the electron acceptors.

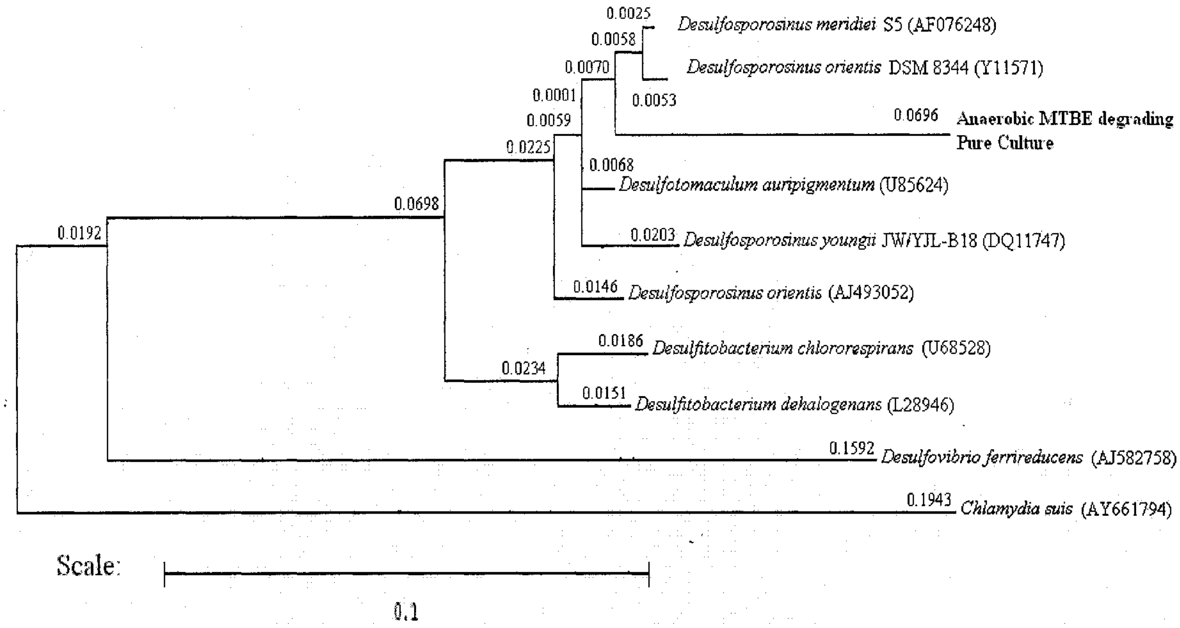


Figure 4-17 Phylogenetic position of isolated MTBE-degrading culture among other types of Gram positive bacteria from the genus *Deulfosporosinus*. Bar, 0.1 substitution per nucleotide position in 16S rRNA gene sequences. Names of the microorganisms followed by the Genbank accession no. in brackets.

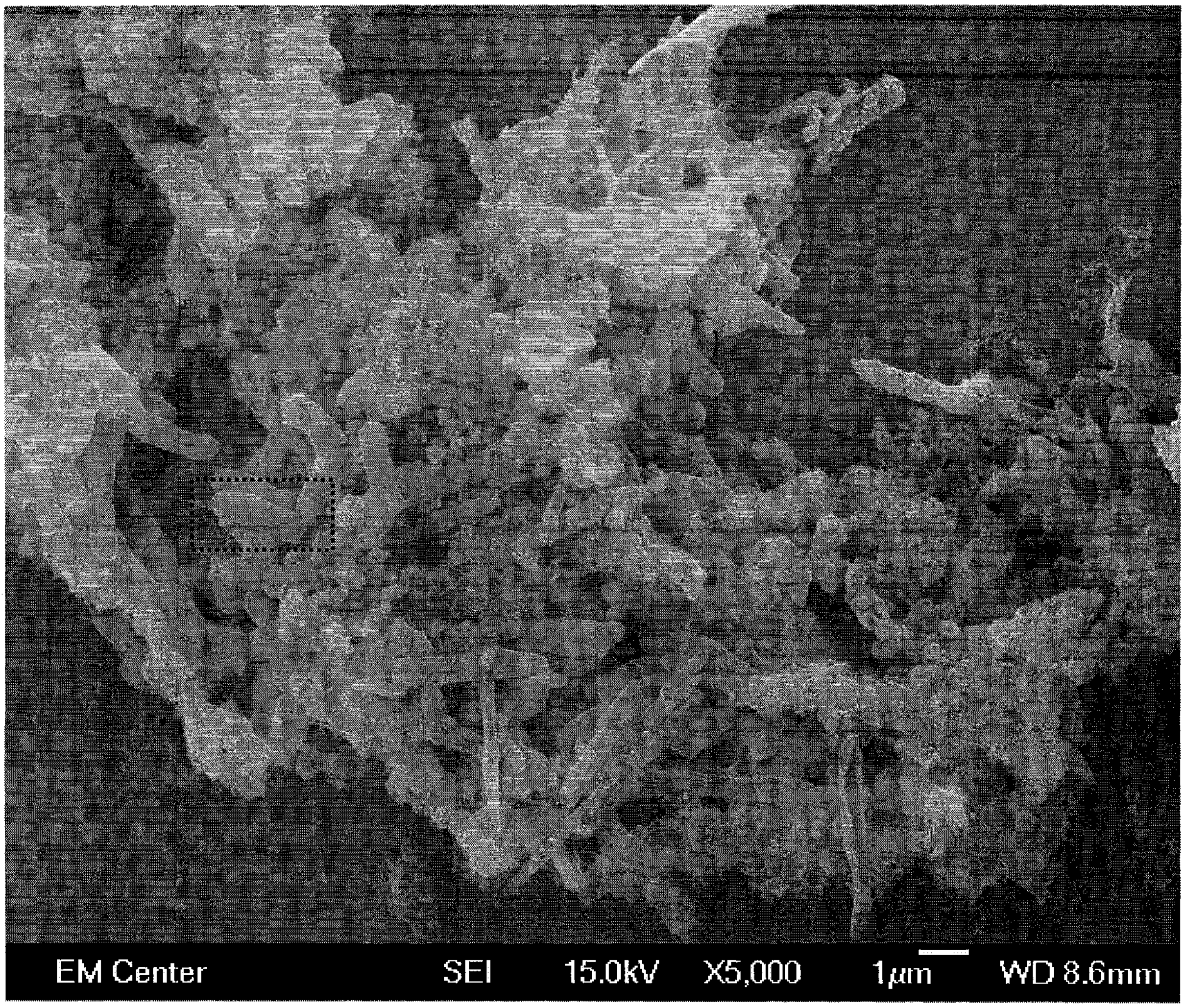


Figure 4-18 Image obtained with scanning electron microscopy (SEM). The dashed box indicates one of the rod-shaped cells.

4.5 Objective 5: Explore the Effect of Electron Acceptor, BTEX, and Sodium Sulfide on Anaerobic MTBE Degradation.

4.5.1 Introduction

Methyl *tert*-butyl ether (MTBE) is a gasoline additive that has been used since the late 1970s to increase the octane level and solve air pollution problems. MTBE enters the environment due to leakage of underground storage tanks and unintentional spills. It has been reported as one of the most common organic contaminants in groundwater in the USA (Squillace et al. 1999; USEPA 2006b). Some of the physico-chemical characteristics of this compound that have contributed to its persistence in the environment are its high water solubility, low adsorption to soil particles and low biodegradation rate. The natural state of groundwater tends to be anoxic to anaerobic. This factor can further decrease the biodegradation rate of MTBE (Deeb et al. 2003). Although MTBE is not listed as a carcinogenic, it has a very low odor and taste threshold, the main reason why the US EPA established an advisory maximum contaminant level (MCL) of 20 μg/L (USEPA 2006b; USEPA 2006a).

There are several studies were anaerobic biodegradation of MTBE has been studied for different electron acceptors (Mormile et al. 1994; Yeh and Novak 1994; Bradley et al. 2001a; Bradley et al. 2001b; Finneran and Lovley 2001; Pruden et al. 2005). However, in just a few of these studies removal of MTBE had been observed.

In a sediment microcosm study conducted by Bradley et al. MTBE was mineralized under sulfate-, manganese-, nitrate- and iron-reducing conditions. MTBE was transformed to TBA under methanogenic conditions. The authors suggested that the bacteria present in the sediments followed the subsequent electron acceptor hierarchy: oxygen > nitrate > manganese (IV) > iron III> sulfate. Mineralization of MTBE was observed after 166 days, ranging in removal percentages from 35 to 100% (Bradley et al. 2001a). MTBE biodegradation was studied under ferric conditions in two later studies (Finneran and Lovley 2001; Pruden et al. 2005). In the study conducted by Finneran et al., it was found that MTBE was degraded anaerobically more rapidly in the presence of humic acids (HA) and Fe(III) after 275 days. The addition of Fe(III) was shown to increase mineralization of MTBE and decrease the production of CH_4 (Finneran and Lovley 2001). A batch-reactor study conducted by Pruden et al. recently characterized anaerobic biodegradation of MTBE under iron-reducing conditions. A culture was enriched from mixed inoculum from aerobic MTBE-degrading, methanogenic and sulfate-reducing cultures. MTBE degradation was observed to proceed after 181 days (Pruden et al. 2005).

One of the gaps in the current knowledge regarding anaerobic bioremediation of MTBE is that the microbial community in the prior mentioned studies has not been well-characterized. In the study conducted by Yeh and Novak the bacterial densities of denitrifiers, lactate-using and acetate-using sulfate reducing bacteria (SRB) were determined. This analysis explained the high variability in removal between triplicates (Yeh and Novak 1994). However no specific phylogenetic groups were characterized. In the study conducted by Pruden et al. the dominant bacteria in the reactor were profiled using DGGE. It was observed that most of the bacteria present belonged to the δ-Proteobacteria, which is a group that contains sulfate and iron-reducing bacteria (Pruden et al. 2005).

Most of the studies conducted up to date have demonstrated MTBE biodegradation by incubation of aquifer sediments (Sufilta and Mormile 1993; Sulfita and Mormile 1993; Mormile et al. 1994; Yeh and Novak 1994; Bradley et al. 2001a; Bradley et al. 2001b; Finneran and Lovley 2001; Moreels et al. 2004), in batch reactors (Pruden et al. 2005) and continuous flow reactors (Pruden et al. 2005).

In the present study, anaerobic bioremediation of MTBE was studied under semi-continuous flow conditions and batch reactors, comparing two electron acceptors: Fe(III) and SO_4. The effect of the presence of the BTEX (benzene, toluene, ethylbenzene and *p*-xylene) compounds was also studied. The diversity and composition of the microbial community was studied in the reactors exhibiting MTBE removal. The main objective of this study was to characterize microbial community dynamics during biodegradation of MTBE under ferric and sulfate reduction conditions. A second aim of this study was to determine whether MTBE was anaerobically biodegraded in the presence of BTEX.

4.5.2 Materials and Methods

4.5.2.1 Reactor Design

Ones semi-batch reactor and ten batch reactors were operated in parallel to compare anaerobic biodegradation of MTBE with different electron acceptors. First a 4 L reactor was set up in a borosilicate flask (3,950 mL aqueous phase, 50 mL head-space), closed to the atmosphere and continuously stirred with a magnetic stir bar and stir plate. The source water was amended with a mineral salt medium (MSM) and a 10x phosphate buffer solution (PBS). The MSM nitrogen source was ammonium and contained trace minerals, as described previously (Pruden et al. 2005). The bottle and the liquid medium were sterilized in an autoclave before inoculation. The electron acceptor used in for this semi-batch reactor was $Fe_2(SO_4)_3$ (Wako Chemicals USA Inc., Richmond, VA,750 mg/L). Na_2S (32 mg/L) was added to scavenge any trace oxygen that may have remained solution. The pH was adjusted to 7.0 with NaOH.

A previously enriched Fe-reducing anaerobic culture obtained from a porous pot biomass-concentrating continuous flow reactor operated under iron-reducing conditions (Pruden et al. 2005) was used as the inoculum. The culture had been stored at -80°C in 20% glycerol since 2002.The glycerol freezer stocks were warmed initially overnight at -20°C and then placed on ice to melt slowly. The cells were washed three times with MSM by vortexing, centrifuging, and pouring off the supernatant to remove the glycerol used to store the cells. The washed cells were diluted in MSM and 5 mL were used to inoculate the 4 L reactor.

Once MTBE biodegradation was observed in the 4L bioreactor (day 100), ten smaller batch reactors were set up in 500 mL borosilicate glass serum bottles, into which 450 mL of mineral salt medium (MSM) and 40 mL 10X phosphate buffer solution (PBS) were initially aliquotted with an initial pH of 7.0. The bottles were sterilized in an autoclave, sealed with butyl rubber septa, and continuously stirred at medium velocity with magnetic stir bars. About 10 mL of headspace were left for gas generation. This headspace was purged with nitrogen gas. The electron acceptors tested were $FeCl_3$ (Fisher, Fair Lawn, NJ), Na_2SO_4

(Fisher, Fair Lawn, NJ) and $Fe_2(SO_4)_3$ (Wako Chemicals USA Inc., Richmond, VA). Sodium sulfide (32 mg/L; Fisher, Fair Lawn, NJ) was added to three of the reactors to scavenge any oxygen present in solution. Ten reactors were inoculated with the active culture growing in the 4 L reactor. Liquid samples (10 mL) were taken from this reactor, washed with MSM three times to remove any trace chemicals (electron acceptor), centrifuge and then diluted in MSM. The average initial cell concentration in the reactors was 5 cells/mL (determine with real time Q-PCR). One of the batch reactors was not inoculated and was used as a control to measure any possible abiotic losses (volatilization). The experimental conditions are summarized in Table 4-9. Four conditions were tested to observe changes in the removal rate of MTBE: 1) presence of one electron acceptor, 2) presence of sodium sulfide, 3) presence of BTEX and 4) presence of both electron acceptors.

Table 4-9 Configuration of the semi-batch reactors.

Reactor Name	$FeCl_3$ (mg/L)	Na_2SO_4 (mg/L)	$Fe_2(SO_4)_3$ (mg/L)	BTEX (mg/L)	Na_2S (mg/L)
$FeCl_3$ + (Na_2S)	604	0	0	0	32
$FeCl_3$	604	0	0	0	0
Na_2SO_4 + (Na_2S)	0	110	0	0	32
Na_2SO_4	0	110	0	0	0
$Fe_2(SO_4)_3$ + (Na_2S)	0	0	750	20	32
$Fe_2(SO_4)_3$	0	0	750	20	0
Na_2SO_4 + $Fe_2(SO_4)_3$	0	110	160	0	0
Na_2SO_4 + $FeCl_3$	160	110	0	0	0
$Fe_2(SO_4)_3$+ $FeCl_3$	160	0	160	0	0
Uninoculated Control	0	0	0	0	0

MTBE and BTEX were dissolved in the mixture by adding the appropriate volume with a gastight syringe (Hamilton Company, Reno, NV). A target initial MTBE (Acros, Morris Plane, NJ) aqueous concentration of 10 mg/L and either zero or 20 mg/L (5mg/L each) of BTEX (Fisher, Fair Lawn, NJ) compounds were spiked in the reactors. BTEX consisted of benzene, toluene, ethylbenzene and *p*-xylene. For the semi-batch reactor, when biodegradation was observed (MTBE concentration felt below detection limit), the cells were allowed to settle and the medium was replaced with fresh medium containing MTBE and the MSM.

All of the reactors were set up inside a N_2 glovebox. The septa were perforated with two holes to place two lines of 1/8 inches ID FEP tubing (Cole-Parmer, Vernon Hills, IL). The end of one tube was placed half way into the bottle and the other tube extended only to the bottom of the rubber stopper to enable the controlled release of gases. The outside ends of the tubes were maintained closed with a clamp. Water samples were taken weekly for later GC and molecular analyses. At each sampling event, a 25 mL gastight syringe was flushed three times and subsequently filled with nitrogen gas. The syringe was connected to the gas-release tube and the nitrogen was injected to collect aqueous samples. From the other tube, a 2 mL gastight syringe was used to take a 0.2 mL sample for GC analysis and a 2 mL sample for molecular analysis. The samples were stored at 4^0C prior to analysis by GC, up to three days, while the samples used for the molecular analysis were stored at -80^0C.

4.5.2.2 DNA Extraction

The MoBio UltraClean Soil DNA kit (Solana Beach, CA) was used for the extraction of the microbial DNA in the aqueous samples according to manufacturer's protocol. The only modification made to the kit procedure was that the bead-beating time was reduced to 4 minutes instead of 10 minutes, to reduce shearing of the DNA.

4.5.2.3 Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) was used to compare the profiles of the cultures present in each reactor and to determine the effect of electron acceptor on the microbial community. 16S rRNA genes were amplified from the DNA extracts by polymerase chain reaction (PCR) using universal primers I-341F (with a GC clamp) and I-533R (Watanabe et al. 2001). The PCR master mix contained 12.9 μL purified water, 2.5 μL 10X buffer, 1 μL Mg^{2+}, 5 μL 5X buffer, 1 μL dNTP (10 mM), 1.25 μL of each primer (5mM), 0.25 μL formamide and 0.35 μL Taq polymerase (Eppendorf, Westbury, NY) per 25 μL reaction. The thermocycler program consisted of an initial denaturing step at 94°C for two minutes, denaturing occurred at 94°C for 15 seconds, primer annealing began at 52°C, which was reduced by 1°C every two cycles until it reached 47°C, and lasted for 15 seconds, extension occurred at 72°C for 20 seconds. Amplification consisted of 35 cycles after the final temperature was reached. A final extension step at 72°C took place for 7 minutes and after completion the program was held at 4°C.

The amplified DNA was loaded on acrylamide gels consisting of a 30%-50% of denaturant linear gradient (100% 7M urea, 40% vol/vol formamide in 1X TAE buffer and 8% acrylamide/bis solution). The gels were run at 55 volts and 57^{0}C for 18 hours. Gels were stained using SybrGold nucleic acid stain diluted in 1:10,000 1X TAE buffer for 15 minutes before being visualized. Central portions of the bands were excised and placed in a 0.5 mL tube with 36 μl of sterile DI water, which was used as a template for a second PCR using the same primers.

4.5.2.4 Real Time Q-PCR

The relative concentrations of total bacteria were determined by quantifying total 16S rRNA genes using the TaqMan 16S probe Q-PCR protocol described by Suzuki et al. (Suzuki et al. 2000). Primers 1369F (10 mM) and 1492R (10 mM) and the 16S TaqMan probe were used. Twenty-four microliters of PCR master mix was mixed with 1 μl of DNA extract. The master mix consisted of 2.5 μL 10X buffer, 5 μL 5X buffer, 0.5 μL dNTP (10 mM), 0.25 μL of each primer (10 mM), 0.375 μL TaqMan probe (10 mM), 0.35 μL Taq Polymerase (Eppendorf, Westbury, NY), and 1.5 μL Mg^{2+}. All Q-PCR reactions were analyzed using a Cepheid Smart Cycler (Sunnyvale, CA). The amplification program consisted of 2 minutes at 95^{0}C, followed by 50 cycles at 95^{0}C for 15 seconds, 53^{0}C for 60 seconds and 72^{0}C for 20 seconds. All samples were run in duplicate and three independent runs were performed for each sample.

4.5.3 Results

4.5.3.1 Effect of Electron Acceptor on MTBE removal rate

The removal of MTBE with time in the 4L semi-batch reactor with both ferric iron and sulfate available as electron acceptors is presented in Figure 4-20. A lag phase of 20 days was observed before MTBE removal was observed. After this lag phase, removal became consistent and 10 mg/L of MTBE were removed in almost 10 days with repeated spiking of MTBE and fresh medium. The average rate of MTBE removal was 0.319±0.14 mg/L-d (for an average biomass concentration of 188 cells/mL, 59.99 mg-MTBE/ g-cells-d).

Several smaller semi-batch reactors were inoculated from the 4L semi-batch reactors in order to test the effect of the electron acceptor and sulfide addition. Figure 4-20A and B provide a comparison of the MTBE removal in the presence of different forms of the electron acceptor, with and without sodium sulfide addition. A shorter lag phase was observed in all reactors, about 12 days with sodium sulfide addition and no detectable lag without sulfide addition. The shorter lag was likely because the cells had been pre-acclimated to the 4L semi-batch reactor. MTBE removal showed a highly similar pattern in the presence of either $FeCl_3$ or Na_2SO_4, both with and without sodium sulfide addition. The rates were 0.150 and 0.156 mg/L-d for $FeCl_3$ and Na_2SO_4, respectively, in the presence of sulfide and 0.546 and 0.533 mg/L-d, respectively, in the absence of sulfide. Thus, in addition to extending the lag phase, the presence of sodium sulfide significantly reduced the rate of biodegradation. However, MTBE removal plateaued after only 5 days in the absence of sodium sulfide at just above 75% removal for both $FeCl_3$ and Na_2SO_4, whereas MTBE removal was still taking place in the presence of sulfide by the end of this study (52 days).

In the smaller batch reactors that had both electron acceptors available, MTBE removal appeared inhibited (Figure 4-20B). In reactors $Na_2SO_4 + Fe_2(SO_4)_3$ and $Fe_2(SO_4)_3 + FeCl_3$, no significant removal of MTBE was observed after 30 days. A 20% removal was observed in reactor $Na_2SO_4 + FeCl_3$ after a 27 day lag phase. Considering that highly consistent removal of MTBE was observed in the presence of both electron acceptors in the 4L batch reactor to which $Fe_2(SO_4)_3$ was added, it may be that ionic strength was the cause of inhibition, rather than the presence of both electron acceptors. When the oxidation-reduction potential was measured for these reactors at the end of the experiment, the oxidation-reduction potential in the 4 L semi-batch reactor was equal to 0 mV, while the oxidation-reduction potential in reactor $Na_2SO_4 + Fe_2(SO_4)_3$ was -265 mV, -249 mV in the $Na_2SO_4 + FeCl_3$ reactor and -241 mV in the $Fe_2(SO_4)_3 + FeCl_3$ reactor. The oxygen concentration in all of the reactors ranged from 0.6 to 0 mg/L. Also, the addition of sodium sulfide did not have a negative effect on the 4L semi-batch reactor. A comparison between the removal rate and the ionic strength calculated for each reactor is presented in Table 4-10. It was observed that the reactors that had the highest ionic strength were also the reactors that had the highest removal rates, this was specially true for the semi-batch reactor and the batch reactors that contained both electron acceptors. The low removal rate in the batch reactors was attributed to a deficiency of the electron acceptor.

4.5.3.2 Anaerobic Biodegradation of MTBE in the presence of the BTEX compounds

Interestingly, MTBE removal was observed in the presence of BTEX when sodium sulfide was absent, but not when it was present (Figure 4-20C). However, less MTBE removal was observed in the presence of BTEX, plateauing at about 50% removal. This demonstrates that as has been reported for aerobic biodegradation (Deeb et al. 2001), BTEX also inhibits anaerobic biodegradation. Furthermore, the presence of sodium sulfide appeared to enhance this inhibitory effect. Even though the inoculum for this study was not previously exposed to BTEX, a small removal of these compounds was observed. However, the BTEX compounds were not completely removed and the removal reached a plateau just as observed for MTBE (Figure 4-21 A and B).

4.5.3.3 Denaturing Gradient Gel Electrophoresis (DGGE)

The images taken from the DGGEs of the batch reactors are presented in Figure 4-22. It can be observed that the communities in the smaller reactors are rather simple and seem to be formed only by four bacteria. The same pattern was observed in all of the batch reactors, regardless of the electron acceptor present. It was also observed that the bacterial community of the 4 L semi-batch was distinct from the other batch reactors. This may be because the batch reactors were inoculated from the 4L semi-batch reactor on days 100, and 110, whereas the DGGE analysis of this reactor was conducted on Days 80-90. Therefore, there may have been further enrichment of the culture in the 4L reactor by the time the smaller reactors were inoculated.

4.5.3.4 Real Time Q-PCR

The concentrations of total bacteria were determined by Q-PCR quantification of 16S rRNA genes at the beginning and end of each experiment, and during biodegradation of MTBE after the second spiking event in the 4L semi-batch reactor (Figure 4-23A, B and C). In all reactors, except the $FeCl_3$ batch reactor, a decrease in the number of cells was observed from the beginning to the end of the experiment. For example, the concentration of total bacteria was observed to decrease in the semi-batch reactor from 5 X 10^2 cells/mL to 50 cell/mL after 80 days (assuming two 16S rRNA copies per mL). The decrease observed in the batch reactors was even higher. This is likely because of a failure of much of the inoculum to adapt to the initial conditions. Also, total bacteria in the batch reactors was quantified 25 days after MTBE removal stopped in the reactors where removal was observed, thus a significant portion of the bacteria may have died (Figure 4-23B and C). A build-up of toxic biodegradation intermediates may have also played a role on the observed decrease of biomass. During monitoring of the total bacteria in the semi-batch reactor during biodegradation of the second MTBE spike, it was possible to detect an initial increase in cells, followed by a decrease (Figure 4-23A). This suggests that cells increase in response to initial addition of MTBE, but then decrease as MTBE decreases in availability.

Although overall the concentration of total bacteria decreased in all of the batch reactors, one interesting trend was that the batch reactors containing ferric iron as an electron acceptor decreased to a lesser extent than those containing only sulfate, and an intermediate decrease was observed in the reactors containing both. This suggests that ferric iron is associated with a higher yield (or smaller death coefficient) than sulfate, which would be expected based on difference in the thermodynamic potential of these two electron acceptors.

4.5.4 Conclusions

Anaerobic bioremediation of MTBE was studied under semi-continuous flow and batch conditions comparing two electron acceptors: Fe(II) and SO_4. The effect of the presence of the BTEX compounds was also studied under batch conditions. The diversity and composition of the microbial community was studied in the reactors exhibiting MTBE removal. The main objective of this study was to characterize the microbial community dynamics during biodegradation of MTBE under ferric and sulfate reduction conditions and to gain insight into the behavior of possible anaerobic MTBE degraders.

The removal rate in a 4L semi-batch reactor with both ferric iron and sulfate was equal to 0.319 ± 0.14 mg/L-d. A lag phase of 20 days was observed in this reactor. Nine smaller batch reactors were inoculated from the 4L semi-batch reactors in order to test the effect of the electron acceptor and sulfide addition. A shorter lag phase was observed in all reactors, about 12 days with sodium sulfide addition and no detectable lag without sulfide addition. MTBE removal showed a highly similar pattern in the presence of either $FeCl_3$ or Na_2SO_4, both with and without sodium sulfide addition. The rates were 0.150 and 0.156 mg/L-d for $FeCl_3$ and Na_2SO_4, respectively, in the presence of sulfide and 0.546 and 0.533 mg/L-d, respectively, in the absence of sulfide. Thus, in addition to extending the lag phase, the presence of sodium sulfide significantly reduced the rate of biodegradation. However, MTBE removal plateaued after only 5 days in the absence of sodium sulfide, whereas MTBE removal was still taking place in the presence of sulfide by the end of this study (52 days).

In the smaller batch reactors that had both electron acceptors available, MTBE removal appeared inhibited. In reactors Na_2SO_4 + $Fe_2(SO_4)_3$ and $Fe_2(SO_4)_3$ + $FeCl_3$, no significant removal of MTBE was observed after 30 days. A 20% removal was observed in reactor Na_2SO_4 + $FeCl_3$ after a 27 day lag phase. The inhibition on MTBE removal observed in these reactors was attributed to a difference in ionic strength, compared to the 4L semi-batch reactor. The ionic strength of these reactors was calculated using only the electron acceptor and sodium sulfide (added only to the 4 L semi-batch reactor), since the source water had the same chemical composition for all of the reactors. The ionic strength in the 4 L semi-batch reactor was equal to 2.2 M, 0.93 M for reactor Na_2SO_4 + $Fe_2(SO_4)_3$, 0.72 M for reactor Na_2SO_4 + $FeCl_3$, and 0.95 M for reactor $Fe_2(SO_4)_3$ + $FeCl_3$. The oxidation-reduction potential for the 4 L semi-batch reactor was equal to 0 mV, while the oxidation-reduction potential in reactor Na_2SO_4 + $Fe_2(SO_4)_3$ was -265 mV, -249 mV in the Na_2SO_4 + $FeCl_3$ reactor and -241 mV in the $Fe_2(SO_4)_3$ + $FeCl_3$ reactor. Also, the addition of sodium sulfide did not have a negative effect on the 4L semi-batch reactor. It is clear that there was a difference in ionic strength and oxidation-reduction conditions. It should be noted that the concentration of $Fe_2(SO_4)_3$ was much higher in the 4 L semi-batch reactor than in the smaller reactors (750 vs. 160 mg/L). Also, the oxidation-reduction potential was the smallest in this reactor, which indicates that the bacteria need to have the electron donor in much higher excess that the one used here (3 times higher the required to biodegrade 10 mg/L according to (Finneran and Lovley 2001).

In the reactors that contained BTEX and $Fe_2(SO_4)_3$ as the electron acceptor source, MTBE removal was observed in absence of sodium sulfide, but not when it was present. However, less MTBE removal was observed in the presence of BTEX, plateauing at about 50% removal. The presence of BTEX seemed to inhibit MTBE removal, which was further inhibited in the presence of sodium sulfide. It is interesting to note that the inoculum for this study was not previously exposed to BTEX, however it was able to remove a small percentage of these compounds was observed. Nevertheless, the BTEX compounds were not completely removed and the removal reached a plateau just as observed for MTBE.

When the bacterial composition of the different reactors was compared by DGGE, it was observed that the communities in the smaller batch reactors were rather simple and seemed to be formed only by four microorganisms. The same pattern was observed in all of the batch reactors. It was also observed that the microbial community of the 4 L semi-batch was distinct from the other batch reactors. This may be because the batch reactors were inoculated from the 4L semi-batch reactor on days 100, and 110, whereas the DGGE analysis of this reactor was conducted on Days 80-90. Therefore, there may have been further enrichment of the culture in the 4L reactor by the time the smaller reactors were inoculated. The fact that the microbial communities of the batch reactors were nearly identical indicates that differences in performance is a result of the conditions (electron acceptor, BTEX, and/or presence of sulfide), rather than the composition of the cultures.

The concentration of total bacteria was determined by Q-PCR quantification of 16S rRNA genes at the beginning and end of each experiment, and during biodegradation of MTBE after the second spiking event in the 4L semi-batch reactor; and at the beginning and at the end in the smaller reactors. In all reactors, except the $FeCl_3$ batch reactor, a decrease in the number of cells was observed from the beginning to the end of the experiment. Even though the concentration of total bacteria decreased in all of the batch reactors, one interesting trend was that the batch reactors containing ferric iron as an electron acceptor decreased to a lesser extent than those containing only sulfate, and an intermediate decrease was observed in the reactors containing both. This suggests that ferric iron is associated with a higher yield (or smaller death coefficient) than sulfate, which would be expected based on difference in the thermodynamic potential of these two electron acceptors. Future work would include studying the effect of ionic strength on the anaerobic MTBE removal in greater detail.

Tables and Figures

Table 4-10 Comparison of removal rate, concentration of the electron acceptors and the ionic strength in the semi-batch reactor and the batch reactors.

Reactor	Removal Rate (mg/L-d)	Fe(III) (M)	SO_4 (M)	I (M)
$FeCl_3$ + (Na_2S)	0.150	0.21	0	1.32
$FeCl_3$	0.546	0.21	0	1.26
Na_2SO_4 + (Na_2S)	0.156	0	0.47	1.16
Na_2SO_4	0.533	0	0.47	1.10
Semi-Batch Reactor	0.319±0.14	0.28	0.47	2.22
$Fe_2(SO_4)_3$+ $FeCl_3$	0	0.14	0.13	0.95
Na_2SO_4 + $Fe_2(SO_4)_3$	0	0.07	0.27	0.93
Na_2SO_4 + $FeCl_3$	0.07	0.07	0.15	0.72
BTEX+ (Na_2S)	0	0.15	0.25	1.20
BTEX	0.5	0.15	0.25	1.14

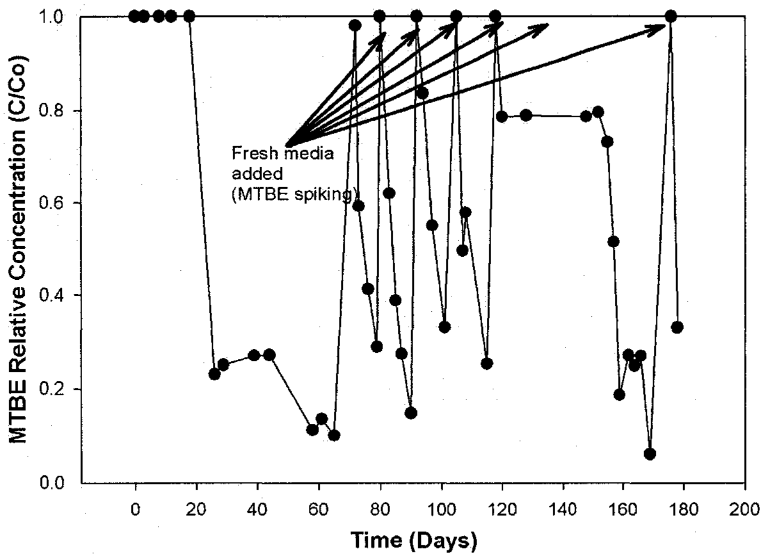


Figure 4-19 MTBE removal in the 4 L semi-batch reactor that contained $Fe_2(SO_4)_3$. The target initial MTBE concentration was 10 mg/L. Na_2S was added.

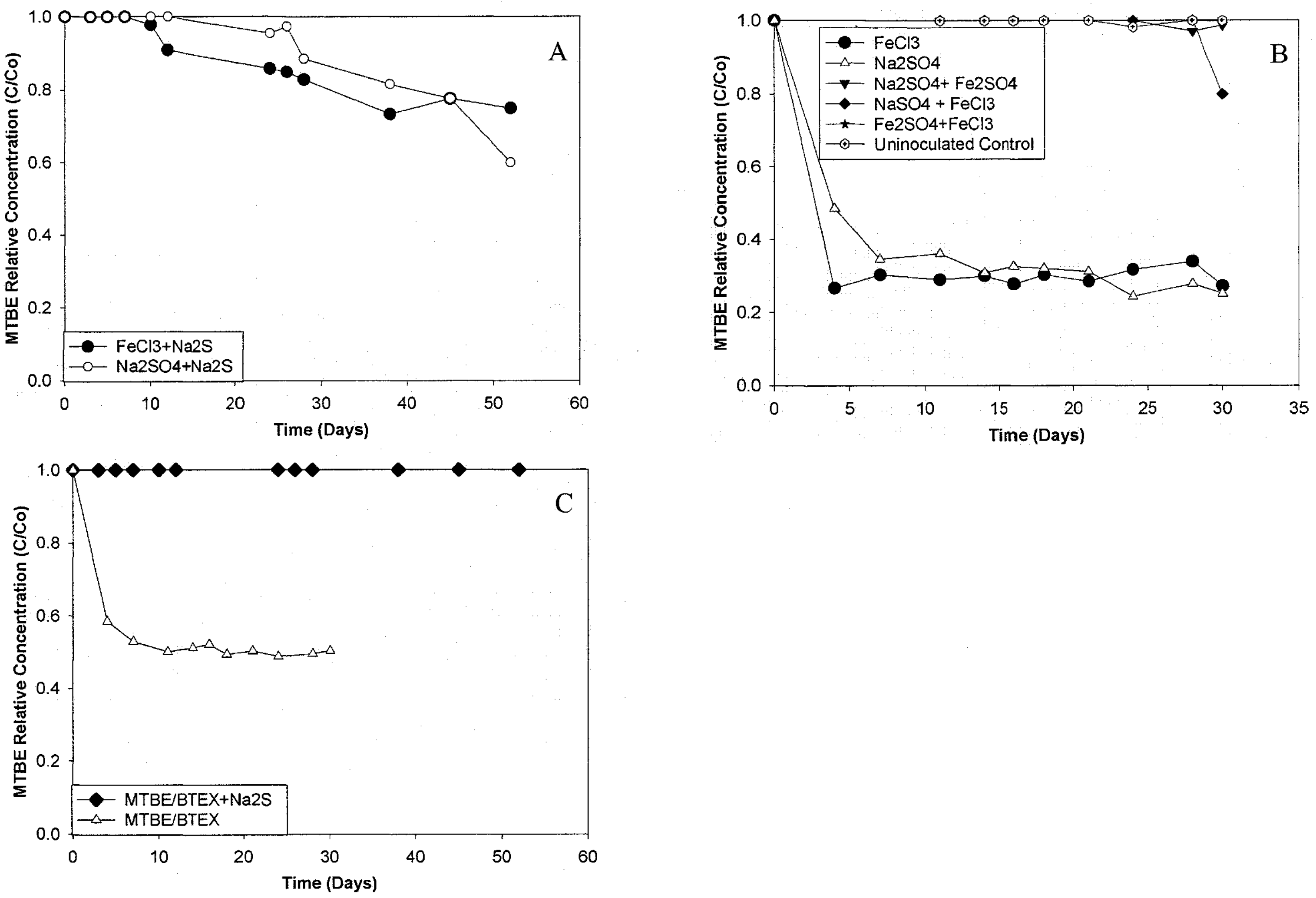


Figure 4-20 MTBE removal in: (A) the semi-batch reactors where the effect of having one of two electron acceptors (Fe^{+3} and SO$_4^{-2}$) in the presence of Na$_2$S; (B);the batch reactors where the presence of having one or both of the two electron acceptors(Fe^{+3} and SO$_4^{-2}$) in the absence of Na$_2$S; (C) in the batch reactors that contained BTEX in the presence and absence of Na$_2$S.

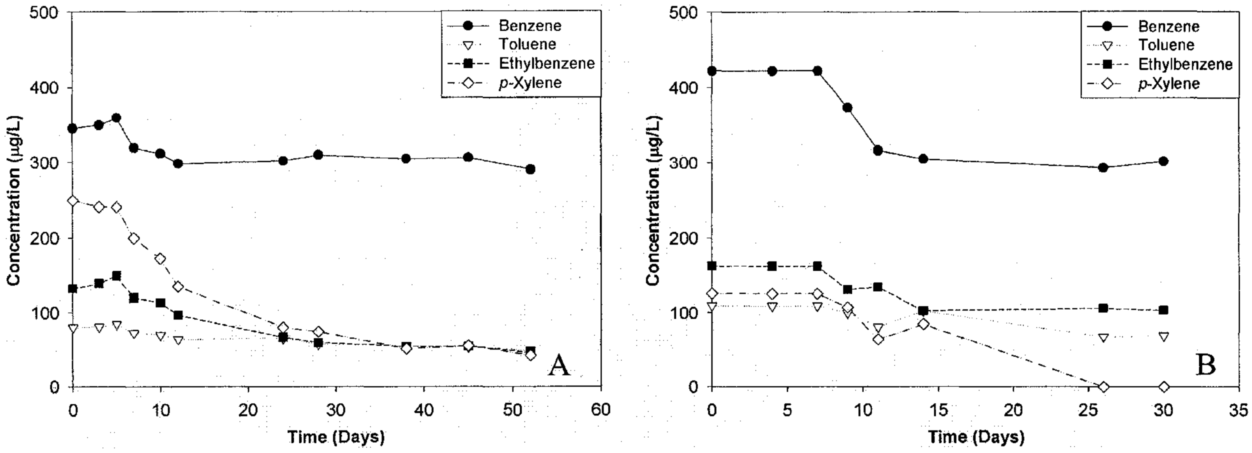


Figure 4-21 Change in concentration of the BTEX (benzene, toluene, ethylbenzene, *p*-xylene) compounds in reactors (A) MTBE/BTEX ($Fe_2(SO_4)_3 + Na_2S$) and (B) MTBE/BTEX ($Fe_2(SO_4)_3$).

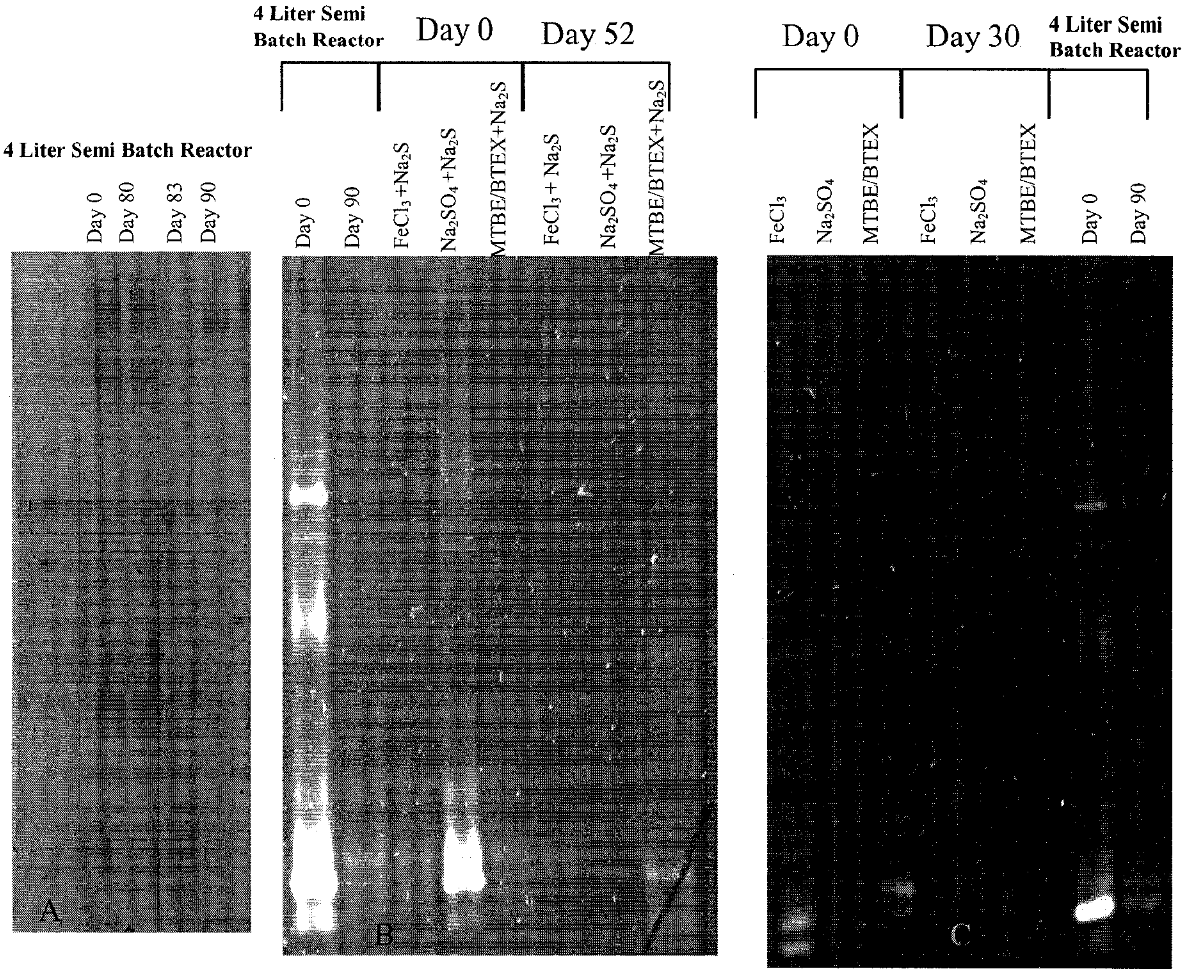


Figure 4-22 Image of the DGGEs for the Semi-batch reactors. (A) reactor $Fe_2(SO_4)_3$ + Na_2S (4L semi-batch reactor) at day 0, day 80, day 83 and day 90. (B) $Fe_2(SO_4)_3$ + Na_2S reactor at day 0, $Fe_2(SO_4)_3$ + Na_2S reactor at day 93, $FeCl_3$ +Na_2S reactor at day 0, Na_2SO_4 +Na_2S reactor at day 0, MTBE/BTEX+Na_2S ($Fe_2(SO_4)_3$) reactor at day 0, $FeCl_3$ + Na_2S reactor at day 52, Na_2SO_4 + Na_2S reactor at day 52, MTBE/BTEX+Na_2S ($Fe_2(SO_4)_3$) reactor at day 52. (C) $FeCl_3$ reactor at day 0, Na_2SO_4 reactor at day 0, MTBE/BTEX($Fe_2(SO_4)_3$) reactor at day 0, $FeCl_3$ reactor at day 30, Na_2SO_4 reactor at day30, MTBE/BTEX($Fe_2(SO_4)_3$) reactor at day 30, $Fe_2(SO_4)_3$ + Na_2S reactor at day 0.

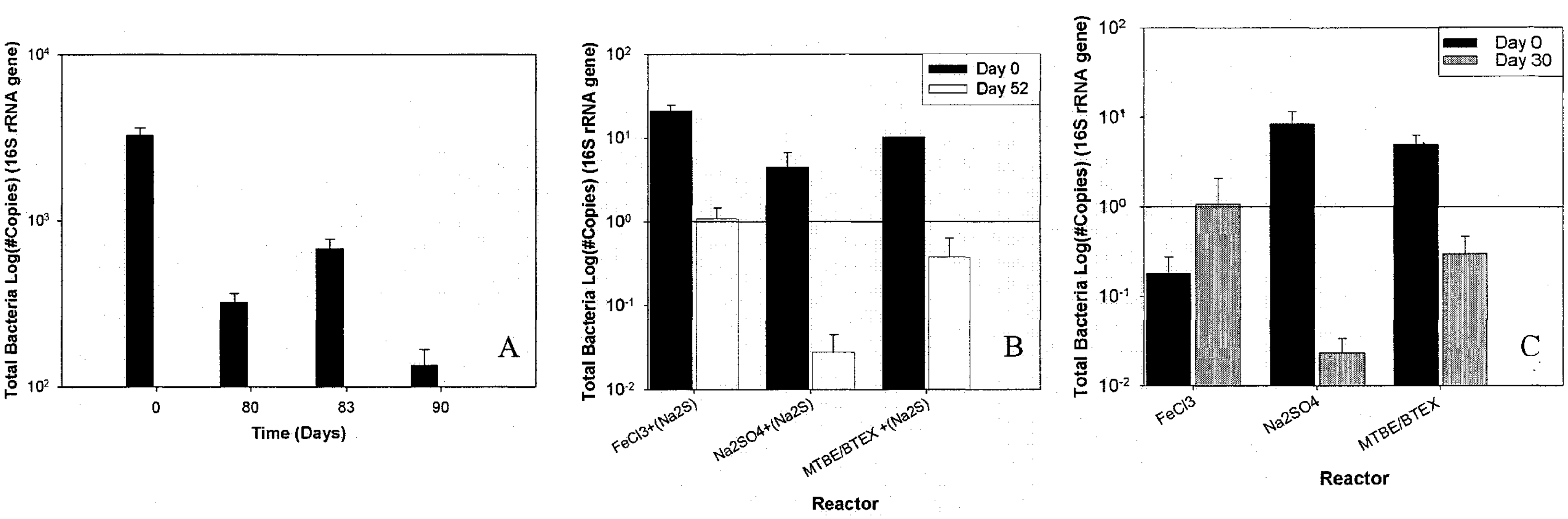


Figure 4-23 Concentration of total bacteria in: (A) the 4 L reactor amended with $Fe_2(SO_4)_3$ at the beginning of the experiment and at three different times. The samples taken at these days correspond to three key points in the removal of MTBE: beginning (day 80), middle (day 83) and end (day 90). (A) The semi-batch reactors where the effect of the electron acceptors (Fe^{+3} and SO_4) and the presence of BTEX on the removal of MTBE was studied in the presence and (B) in the absence of Na_2S.

Chapter 5 Conclusions and Recommendations

The overall goal of this research was to investigate potential treatment options for MTBE in the presence of BTEX and with limited oxygen availability. Specifically, the objectives were to:

5.1 Determine the effect of culture composition and reactor configuration on MTBE biodegradation in the presence of BTEX

A batch test was performed to compare the degradation of MTBE by two distinct enrichment cultures in the presence or absence of BTEX. The two mixed cultures used in this experiment were designated according to the substrate conditions under which they were originally enriched: MTBE only (MO) and MTBE and BTEX (MB). The two cultures were obtained from two previously studied porous pot biomass-concentrating continuous flow reactors (Pruden et al. 2001). The performance of each culture was studied individually and when they were mixed at a 1:1 ratio. The performance of the MO culture was also studied in a semi-batch reactor spiked with MTBE and BTEX. The effect of BTEX on the biodegradation of MTBE was related to the reactor configuration and the bacterial community diversity and composition.

5.1.1 Expected Results

It was expected that:

- The microbial community composition and the reactor configuration will have a great impact on the simultaneous biodegradation of MTBE and BTEX.
- Reactors having a more diverse community, thus robust, will be able to remove the contaminants simultaneously.
- Higher MTBE removal rate is expected for the semi-batch reactor than in the batch reactors.

5.1.2 Results

- This study suggested that MTBE bioremediation in the presence of BTEX is feasible, and that overall success may be enhanced by several factors. First, it is important to recognize that the characteristics of the microbial consortium do matter. The presence of other MTBE-degraders besides strain PM1^T, the most well-known MTBE degrader, may be key for overcoming BTEX inhibition of MTBE biodegradation.
- The consortia that were most successful in biodegrading MTBE in the presence of BTEX actually had the lowest numbers of strain PM1^T, but contained *Hydrogenophaga* sp., which has also been reported to degrade MTBE. There was some evidence that higher diversity, as represented by

estimated species richness, was associated with successful MTBE biodegradation in the presence of BTEX.

- Reactor configuration is also important for controlling the composition and performance of the microbial consortium. Previous studies characterizing BTEX inhibition of MTBE biodegradation have been carried out in batch reactors (Deeb et al. 2001). However, this study demonstrates that a semi-batch reactor provides better performance, probably because BTEX biodegradation intermediates can be flushed out. In other studies using continuous-flow reactors, comparable biodegradation rates of MTBE were observed in the presence and absence of BTEX (Pruden et al. 2001; Sedran et al. 2002; Pruden and Suidan 2004). Continuous-flow conditions are also more realistic for most remediation scenarios in the field.

5.1.3 Future Studies and Recommendations

- Further analysis needs to be done to design primers and Taqman probes to detect and to quantify other aerobic MTBE degraders, in addition to $PM1^T$, such as *Hydrogenophaga* sp.
- The formation of byproducts should be monitored when monitoring the biodegradation of MTBE with the aid of a GC-MS (gas chromatographer and mass spectrometry), given that this instrument is more sensitive to detect compounds with the same retention time.

- The field application of the findings of this experiment will be that in a aerobic environment MTBE will be degraded in the presence of BTEX if flow through conditions exist and if the bacterial community is diverse and have various MTBE degraders, not only PM1^T.

5.2 Explore the feasibility of wetlands for MTBE biodegradation in the presence of BTEX and other constituents present in refinery water .

The first aim of this study was to determine the effect of bioaugmentation on MTBE and BTEX biodegradation in wetlands. The second aim of this study was to determine whether wetlands can provide suitable conditions for the simultaneous degradation of MTBE and BTEX.

Wetlands at laboratory scale were investigated to build an understanding of their biodegradation potential. Two enrichment cultures were used to seed the wetlands, an anaerobic iron-reducing and an aerobic MTBE-degrading culture. The anaerobic Fe^{+3}-reducing culture was enriched by mixing the biomass from aerobic MTBE-degrading, methanogenic and sulfate-reducing cultures and providing MTBE as the sole carbon source and Fe(III) as the sole electron acceptor (Pruden et al. 2005).

Commercially available aquaria were used simulate the wetlands. The water inlet to an existing air-stripper at a refinery near Denver, CO was used as the source water for the experiment in order to explore the applicability of the approach for actual treatment of contaminated groundwater. From previous chemical analyses of this water, it was known that the water was contaminated with several organic and inorganic constituents (data collected by RETEK, Fort Collins, CO). A batch test was conducted where both cultures were exposed to the refinery water, which confirmed that the refinery water did not have a negative effect on the biodegradation of MTBE and BTEX.

The conditions studied were: activity of indigenous bacteria, bioaugmentation with the aerobic culture, bioaugmentation with the anaerobic culture, biostimulation with Fe(III) and bioaugmentation with the anaerobic culture; and biostimulation with Fe(III) and bioaugmentation with the aerobic and the anaerobic cultures.

The tanks were divided into three zones. The first zone was bioaugmented with the anaerobic culture and the last zone was bioaugmented with the aerobic culture. Three sampling ports were placed in each zone at three different depths to monitor MTBE and BTEX concentration with space. Aqueous samples were taken weekly at the different sampling points and analyzed by purge and trap and gas chromatography. The pH, temperature, conductivity and oxidation-reduction potential were measured during the sampling events. Samples for microbial community analysis were taken from the sediments at the beginning of the experiment and at the end to determine the change in composition from the beginning to the end of the experiment. Water samples were also collected at the beginning of the experiment.

5.2.1 Expected Results

The hypotheses drawn at the beginning of the experiment were:

- Biodegradation of MTBE is possible in wetlands.

- The wetlands that were bioaugmented and/or biostimulated will be enhanced, thus a higher the removal rate will be observed.

5.2.2 Results

- It was observed that MTBE and the BTEX compounds were readily removed in all of the wetlands, including the inhibited control. Thus, it was not possible to isolate the effect of biostimulation and bioaugmentation.
- The reason of the high performance of the scaled wetlands was attributed to a high concentration of PM1^T present in the influent water (detected with Q-PCR). This strain has been observed to be a facultative anaerobe and able to grow and degrade MTBE under iron reducing conditions (Nakatsu et al. 2006). As described in this dissertation, this strain has a very high MTBE removal rate (Fortin et al. 2001). Therefore, further research into wetlands as an alternative treatment approach for MTBE and BTEX is suggested.

5.2.3 Future Studies and Recommendations

- As a recommendation, this experiment should be repeated inoculating the wetlands with the above mentioned cultures and using deionized water amended with nutrients and spiked with MTBE and BTEX, to determine whether the cultures benefit from the presence of Fe(III) and whether there is a benefit from using two cultures, one aerobic and one anaerobic.

- The design of the experiment had several flaws, which were discovered during the process of analyzing data. In the constant head tanks gravel should be added to reduce the contact of water with the atmosphere. Also, instead of designing the scaled wetlands as free surface flow, the next experiment should be designed using subsurface flow wetlands. This will reduce the loss of contaminants due to volatilization.
- The bacterial community in the refinery water should be characterized in order to determine if other MTBE degraders besides $PM1^T$ are also present.
- Wetlands could be applied in the field to create aerobic and flow through conditions. Studies should be made to evaluate the indigenous bacterial community in order to determine whether the community needs to be biostimulated with nutrients or an alternative electron acceptor (besides oxygen) or whether biaugmentation is required, in the case that the bacterial community is not able to biodegrade MTBE.

5.3 Explore anaerobic MTBE biodegradation in permeable reactive barriers (PRBs) in both batch and column reactors.

This experiment consisted of two parts: a batch experiment and a column study. The batch experiment was performed to compare the performance of an anaerobic culture under Fe(III) and SO_4 reducing conditions, added as $FeCl_3$ and Na_2SO_4 respectively. Electron acceptors that could potentially be implemented as slow-release electron acceptor sources in PRBs in the form of $Fe(OH)_3$ (ferric hydroxide) and $CaSO_4$ (gypsum) were investigated in the column studies.

In the first part of the experiment four 1 L-amber borosilicate bottles were used to test the two electron acceptors in duplicate. Na_2SO_4 (Fisher, Fair Lawn, NJ) was used as the source of SO_4 at a concentration of 5.5 mg/L. $FeCl_3$ Fisher, Fair Lawn, NJ) was added as the source of Fe(III) in a concentration of 302 mg/L. Four controls were set up using gypsum (6g/L; Grow More Research Farms, Gardena, CA) as the electron acceptor. Two of which were killed with 0.1 g/L of mercuric (III) chloride (Sigma-Aldrich, St. Louis, MO).

Deionized (DI) water amended with a mineral salt medium (MSM) and 10X phosphate buffer solution (PBS) was used as the experiment source water. The MSM composition is described in Pruden et al. (Pruden et al. 2001).The batch reactors were set up in a N_2 glovebox. A volume of 980 mL of amended DI water was added to each bottle to have a 20 mL head space to allow for gas production. The bottles were capped with butyl rubber stoppers and screw caps. Two perforations were made to the rubber stoppers to place two lines of 1/8 inch ID FEP tubing. The end of one tube was placed half way into the bottle and the other tube extended only to the bottom of the rubber stopper to enable the controlled release of gases. The outside ends of the tubes were maintained closed with a clamp. The bottles were placed under water to reduce the possible diffusion of oxygen into of the batch reactors. Water samples were taken weekly for later GC and molecular analyses. The bottles were mixed manually by shaking two times a week. It was hypothesized that the batch reactors that contain $FeCl_3$ will yield a higher biomass and MTBE removal rate. This is because Fe(III) is thermodynamically more favorable and thus yields more energy to the cells.

The second part of the experiment consisted of a column study. Four custom made columns were constructed using 45.5 cm long and 11 cm diameter perforated acrylic pipes. The columns had four sampling ports. The ends of the columns were packed with sand and the middle section was packed with a mixture of sand and the reactive media ($Fe(OH)_3$ and/or $CaSO_4$). Three columns were inoculated with the Fe-reducing anaerobic culture. The fourth column was not inoculated. This provided means to determine the effect of bioaugmentation. The sand used in the experiment was collected from the river bank of Sand Creek (Denver, CO). The sand was baked at 150 ^{0}C for twelve hours before being used to reduce the number of viable bacteria present and to volatilize any VOC adsorbed to the soil particles.

The flow rate was varied during the experiment. For the first 90 days, the flow rate was kept at 227 mL/d, until removal of MTBE was observed. Then, the flow rate was increased to 500 mL/d for the next 184 days (experiment length 274 days). The hydraulic retention time of the columns was 20 days for the first flow rate and 10 days for the second. Humic acids (Aldrich, St. Louis, M) were spiked on day 107 to promote anaerobic biodegradation of MTBE as proposed by Finneran and Lovely as an essential component in anaerobic biodegradation of MTBE, since it works as an electron shuttle between the electron donor and the bacteria (Finneran and Lovley 2001; Finneran et al. 2001). In this study, the concentration of the HA was varied from 10 mg/L to 10 g/L. Sodium sulfide (Na_2S, Fisher, Fair Lawn, New Jersey) was added in the influent at a concentration of 32 mg/L as an oxygen scavenger. Samples to monitor the concentration of MTBE were taken every week from the influent and the effluent. The experiment lasted 9 months and was repeated an additional month to verify the results. The samples to be used for the molecular analyses were collected from the reactive media in each of the three columns' sections at the beginning and at the end of the experiment.

The columns were fed with amended DI water, containing essential nutrients and minerals for biological growth and a MTBE concentration of 10 mg/L. The pH of the source water was adjusted to a value of 7 ± 0.2. The source water was stored in 25 L Tedlar bags (SKC, Fullerton, CA) to reduce the exposure of the water with the atmosphere. The Tedlar bags were connected to a six channel Ismatec peristaltic pump (Ismatec, Glattbrugg, Switzerland).

The microbial communities from both experiments were characterized to determine the differences in composition depending on the electron acceptor present (Fe(III) vs. SO_4) and the reactor configuration (batch reactors vs. columns).

5.3.1 Results: Batch Experiment

For the batch reactors it was expected that:

- The reactors dosed with Fe(III) will yield a higher biomass concentration and a higher MTBE removal rate, than the reactors dosed with SO_4.

From the results it was concluded that the culture was able to use both electron acceptors (Fe(III) and SO_4) under batch conditions to biodegrade MTBE. However, the removal of MTBE was observed to be rather slow (6mg/L in 82 days). The concentration of total bacteria between the reactors amended with $FeCl_3$ and the reactors amended with Na_2SO_4 was not found to be significant ($p<0.0001$).

5.3.2 Results: Column Study

For the column study it was expected that:

- Since sulfate is more soluble, the column with gypsum only will initially outperform the one with Fe(III). However sulfate yields less energy than Fe(III), thus, in the long term it is expected that Fe(III) + gypsum will provide a higher level of removal.
- In the column study, MTBE was biodegraded only when 10 g/L of humic acids were spiked into the system at the beginning of the experiment, but when the humic acids were washed out from the system, the bacteria could not remove MTBE anymore. It was not possible to re-stimulate the community with repeated addition of HA. The columns that removed MTBE at the beginning of the experiment were the inoculated columns that had $Fe(OH)_3$
- Although, no significant difference was observed between the biomass concentrations in the reactors, a significant difference was observed with respect to the biomass concentration in the columns, which was much smaller that those observed in the batch reactors.
- When the bacterial communities of two of the batch reactors (Fe_2SO_4 (B) and $FeCl_3$ (A)) were compared to the gypsum + $Fe(OH)_3$ column it was observed

that each microbial community was distinct in composition. The batch reactors shared three types of bacteria, and only one bacterial type was present in both the column and the batch reactors. No known MTBE degraders were found in the reactors or the column, though no anaerobic MTBE degraders have been isolated and reported to date. However, some phylotypes present in the column were related to bacteria found in fuel contaminated sites.

5.3.3 Future Studies and Recommendations

- Future work should focus on isolating and characterizing anaerobic MTBE degraders so that the conditions under which they thrive can be better understood. This can help improve implementation of anaerobic MTBE remediation in the field.
- Different retention times should be tested in the column to determine the bioremediation rate at which the anaerobic Fe-reducing culture is able to degrade MTBE under the studied conditions. Also, higher biomass concentrations should be used in anaerobic experiments in an attempt to accelerate the removal rate and to decrease the initial lag phase.
- PRBs could be applied in the field, although in this study the bacterial consortium was able to biodegrade MTBE under Fe(III) and SO4 reducing conditions, there was also evidence that Fe(III) yielded more biomass. In this

experiment, the initial cell concentration played an important role, higher than the electron acceptor used.

5.4 Isolate and characterize a pure culture capable of anaerobic MTBE degradation.

Pure cultures were isolated by plating mixed cultures present in an previously enriched anaerobic iron-reducing culture (Pruden et al. 2005), an aerobic culture previously enriched on MTBE as the sole substrate (MO) (Raynal and Pruden 2007) and the influent from an air striping system of a petroleum refinery near Denver, CO. The cultures were spread on 4% (v/v) agarose-MSM (mineral salt medium) plates containing 950 mg/L of $Fe_2(SO_4)$ (Wako Chemicals USA Inc., Richmond, VA) and placed in an airtight GasPak Jar™ (BD, Sparks, MD) with a BD BBL™ Gas Pak™ Plus gas generator envelope (BD, Sparks, MD) to create anaerobic conditions. An anaerobic indicator (Oxoid, Lenexa, KS) was used to verify that anaerobic conditions were maintained. The mineral salt medium contained nutrients and minerals for microbial growth, in Pruden et al. 2001. A 5 mL glass vial with a perforated Teflon cap containing MTBE (Arcos, Morris Plane, NJ) was placed inside the chamber to provide the sole carbon substrate.

Each month for eight months, the GasPak Jar™ was opened inside a glovebox with N_2 gas to refill the vial containing MTBE, to replace the gas generator envelope and the anaerobic indicator. Colonies grew only from the iron-reducing anaerobic and the MO aerobic culture. Once colonies appeared they were transferred to a new plate and to a 100 mL glass bottle that contained the MSM, 750 mg/L of $Fe_2(SO_4)$ (Wako Chemicals USA Inc., Richmond, VA) and 10 mg/L of MTBE (Arcos, Morris Plane, NJ). The glass bottles were capped with rubber septa and were placed up-side-down in a controlled temperature shaking bed at 25 ^{0}C and 60 rpm. To confirm the ability of the culture to degrade MTBE in liquid, samples were taken biweekly and the concentration of MTBE in the bottles was monitored using by Gas Chromatography. Once MTBE biodegradation was observed (a decrease of 50% from the initial concentration in 35 days) the cultures were serially diluted and 10 µl of the 10^{-2} dilution were plated to isolate a pure culture. Cells were also placed in a more selective liquid media containing either $FeCl_3$ or Na_2SO_4. The morphology of the colonies growing on the plates and in the liquid cultures was characterized by scanning electron microscopy (SEM) and Gram staining. The properties of the 16S rRNA gene were determined by direct sequencing and denaturing gel gradient electrophoresis (DGGE).

5.4.1 Expected Results

At the beginning of the experiment it was hypothesized that:

- A microbial strain exists capable of anaerobic biodegradation of MTBE and that can be isolated in pure culture.

5.4.2 Results

- With the aid of molecular methods and microscopy, it was possible to demonstrate that a pure culture was isolated from both cultures. These methods also revealed that the same microorganism was isolated from both sources of inoculum. The isolated bacterium was found to be able to grow under ferric and sulfate reducing conditions with no observable difference in rate, was Gram-variable, and was rod shaped with dimensions: 1.5 μm (length) and 0.5 μm (width). No flagella were observed by SEM. The average removal MTBE rate was equal to 0.125 mg/L-d.

5.4.3 Future Studies and Recommendations

- The sequenced obtained of the 16S rRNA gene for the pure culture should be used to design primers and Taqman probes to identify and to quantify this microorganism in environmental samples and anaerobic mixed cultures.

5.5 Characterize microbial community dynamics during biodegradation of MTBE under ferric and sulfate reduction conditions.

The main objective of this study was to characterize the microbial community dynamics during biodegradation of MTBE under ferric and sulfate reduction conditions and to gain insight into the behavior of possible anaerobic MTBE degraders. A second aim of this study was to determine whether MTBE was anaerobically biodegraded in the presence of BTEX.

The anaerobic bioremediation of MTBE was studied under semi-continuous flow and batch conditions comparing two electron acceptors: Fe(II) and SO_4. The effect of the presence of the BTEX compounds was also studied under batch conditions. The diversity and composition of the microbial community was studied with the aid of denaturing gradient gel electrophoresis (DGGE) in the reactors exhibiting MTBE removal.

A 4L semi-batch reactor with both ferric iron and sulfate was set up. Once biodegradation of MTBE was observed, nine smaller semi-batch reactors were inoculated from this reactor in order to test the effect of the electron acceptor and sulfide addition. MTBE removal showed a highly similar pattern in the presence of either $FeCl_3$ or Na_2SO_4, both with and without sodium sulfide addition. However, the presence of sodium sulfide increased the initial lag phase and reduced the biodegradation of MTBE.

In the smaller batch reactors that had both electron acceptors available, MTBE removal appeared inhibited. In reactors Na_2SO_4 + $Fe_2(SO_4)_3$ and $Fe_2(SO_4)_3$ + $FeCl_3$, no significant removal of MTBE was observed after 30 days. A 20% removal was observed in reactor Na_2SO_4 + $FeCl_3$ after a 27 day lag phase. The inhibition on MTBE removal observed in these reactors was attributed to a difference in ionic strength and the oxidation-reduction potential, compared to the 4L semi-batch reactor. The ionic strength in the 4 L reactor was equal to 2.2 M, while the ionic strength ranged from 0.45 to 0.77 M in the batch reactors. It seemed that the anaerobic culture required a higher concentration of the electron acceptor, which in turn would increase the ionic strength and the oxidation-reduction potential.

In the reactors that contained BTEX and $Fe_2(SO_4)_3$ as the electron acceptor source, MTBE removal was observed in absence of sodium sulfide, but not when it was present. However, the presence of BTEX seemed to inhibit MTBE removal, which was further inhibited in the presence of sodium sulfide. It is interesting to note that the inoculum for this study was not previously exposed to BTEX, however it was able to remove a small percentage of these compounds was observed. Nevertheless, the BTEX compounds were not completely removed and the removal reached a plateau just as observed for MTBE.

When the microbial composition of the different reactors was compared by DGGE, it was observed that the communities in the smaller batch reactors were rather simple and seemed to be formed only by four microorganisms. The same pattern was observed in all of the batch reactors. It was also observed that the microbial community of the 4 L semi-batch was distinct from the other batch reactors. This may be because the batch reactors were inoculated from the 4L semi-batch reactor on days 100, and 110, whereas the DGGE analysis of this reactor was conducted on Days 80-90. Therefore, there may have been further enrichment of the culture in the 4L reactor by the time the smaller reactors were inoculated. The fact that the microbial communities of the batch reactors were nearly identical indicates that differences in performance is a result of the conditions (electron acceptor, BTEX, and/or presence of sulfide), rather than the composition of the cultures.

The concentration of total bacteria was determined for each reactor by Q-PCR quantification of 16S rRNA genes at the beginning and end of each experiment, and during biodegradation of MTBE after the second spiking event in the 4L semi-batch reactor. In all reactors, except the $FeCl_3$ batch reactor, a decrease in the number of cells was observed from the beginning to the end of the experiment. Even though the concentration of total bacteria decreased in all of the batch reactors, one interesting trend was that the batch reactors containing ferric iron as an electron acceptor decreased to a lesser extent than those containing only sulfate, and an intermediate decrease was observed in the reactors containing both. This suggests that ferric iron is associated with a higher yield (or smaller death coefficient) than sulfate, which would be expected based on difference in the thermodynamic potential of these two electron acceptors.

5.5.1 Expected Results

At the beginning of the experiment it was expected to observe:

- Higher biomass concentration is expected in the reactors that had Fe(III), since it is thermodynamically more favorable and yields more energy to the cells.
- Inhibition of MTBE removal in the presence of BTEX as it has been observed in aerobic studies.
- Higher microbial diversity in the presence of both electron acceptors compared to the cases where only one electron acceptor was present.

5.5.2 Results

- It was observed that the presence of one electron acceptor (vs. both), sodium sulfide and BTEX reduced the removal rate of MTBE. It was also observed that the ionic strength and oxidation-reduction potential also had an effect on the removal of MTBE. Total inhibition or a great reduction in the removal rate was observed in the reactors which source water had a smaller ionic strength and was more reduced.

- The highest biomass concentration was observed in the 4 L semi-batch reactor. In all reactors, except the $FeCl_3$ batch reactor, a decrease in the number of cells was observed from the beginning to the end of the experiment. Even though the concentration of total bacteria decreased in all of the batch reactors, one interesting trend was that the batch reactors containing ferric iron as an electron acceptor decreased to a lesser extent than those containing only sulfate, and an intermediate decrease was observed in the reactors containing both. This suggests that ferric iron is associated with a higher yield (or smaller death coefficient) than sulfate, which would be expected based on difference in the thermodynamic potential of these two electron acceptors.
- Also the highest microbial diversity was observed in the 4 L semi-batch reactor (according to DGGE analysis). The microbial community in the batch reactors was observed to be formed by four microorganisms and was identical in all of the smaller reactors. However, this could result from inoculating the reactors at day 100 of the 4 L semi-batch reactor, which could lead to further enrichment of the culture. The fact that the microbial communities of the batch reactors were nearly identical indicates that differences in performance is a result of the conditions (electron acceptor, BTEX, and/or presence of sulfide), rather than the composition of the cultures.

5.5.3 Future Studies and Recommendations

- The composition of the bacterial community is important in the removal of MTBE, thus the bacterial community of each reactor should be profiled with a more sensitive molecular tool, like cloning instead of DGGE to determine whether the success or failure of the reactors was due to the chemical composition of the source water or to the bacterial community composition.
- Different values for the ionic strength need to be investigated by varying the concentration of the electron acceptor to determine the effect of the ionic strength in more detail.
- The information obtained from this experiment could be used in practice by determining if under anaerobic conditions the electron donor is available for the bacteria (concentration, precipitation due to presence of sulfide).

Appendix A Anaerobic Batch Experiment to test different reactive media and the removal of MTBE at different pH values.

Twenty four 1 L-amber borosilicate bottles were used for the experiment in order to test eight conditions. The experimental conditions are presented in Table A-1. For each condition, three batch reactors were prepared, two of which were inoculated with an anaerobic iron-reducing culture and the third one was used as a killed control, which was poisoned with 0.1 g/L of Mercuric (III) chloride (Sigma-Aldrich, St. Louis, MO). The anaerobic culture was obtained from a previously studied porous pot biomass-concentrating continuous flow reactor operated under iron-reducing conditions (Pruden et al. 2005), from which glycerol freezer stocks were generated upon sacrifice of the reactor in 2002.

Table A-1 Experimental Conditions for the Anaerobic Batch Reactors. Initial pH 7.5.

Reactive Media	Concentration	Purpose
Gypsum (Grow More Research Farms, Gardena, CA)	0.06 g/L	Determine the effect of Gypsum (inexpensive slow-release SO_4^{-2} source) on MTBE abiotic and biotic removal.
$Fe(OH)_3$ (Blue Grass Chemical Specialities, New Albany, IN)	0.3 g/L	Determine the effect of e^- acceptor Fe (III) (a thermodynamically favorable but relatively insoluble e^--source) on biodegradation.
Gypsum + $Fe(OH)_3$	0.06 gr/L + 0.3 g/L	Identify potential synergy between Fe(III) and SO_4^{-2}, a combination being considered at the refinery.
$Fe_2(SO_4)_3$ (Wako Chemicals USA, Richmond, VA)	0.35 g/L	Compare the effect of adding Fe (III) and SO_4^{-2} as electron acceptors in a soluble form.
Na_2SO_4 (Fisher, Fair Lawn, NJ)	0.06 g/L	Compare the effect of adding soluble SO_4^{-2} as electron acceptors.

Table A-2 Experimental Conditions to observe pH effect. The reactors were amended with $Fe_2(SO_4)_3$. The pH was adjusted at the beginning of the experiment and measured at the end.

pH	Purpose
2	Effect of acidic pH on biodegradation
6.5	Effect of near neutral pH on biodegradation
8	Effect of basic pH on biodegradation

Even though in theory nitrate is a better option than sulfate, thermodynamically in practice it has two main limitations: 1) nitrate is a negative anion and therefore is easily leached out of the systems, reaching surface water bodies; and 2) nitrate is considered a hazardous contaminant in surface waters (MCL: 10 mg/L) (USEPA 2006a). In high concentrations, it can lead to eutrophication and cause health problems such as methemoglobinemia and cancer if it further reacts with secondary amines (Rittmann and McCarty 2001; Sylva et al. 2005). Fe(III) is insoluble at neutral pH while SO_4 tends to adsorb to variable charge minerals, which ensures the immobility of these ions and make them more suitable for their use in PRBs. The advisory MCLs for Fe(III) and SO_4 are 0.3 mg/L and 250 mg/L, respectively, being the sulfate advisory MCL much higher than that of nitrate (USEPA 2006a).

Table A-3 Solubility Constant for Fe^{+3} and SO_4 at 25 ^{0}C (Snoeyink and Jenkins 1980) .

Chemical reaction	Log Kso
$Fe(OH)_3 + 3\ H^+ = Fe^{+3} + 3\ H_2O$	3.2 ($Fe^{+3} = 4.8\ X10^{-8}$ g/L)
$CaSO_4 \cdot 2\ H_2O = Ca^{+2} + SO_4^{-2} + 2\ H_2O$	4.59 ($SO_4 = 2.04$ g/L)

The conditions were tested using two different source waters at two different times. During the first part of the experiment the air sparging system influent of the refinery was used as the source water. Then amended DI water was used as the source water. The amended DI water contained a mineral salt medium (MSM), 10X phosphate buffer solution (PBS) and a MTBE concentration of 10 mg/L (Acros, Morris Plane, NJ). The MSM contained ammonium as nitrogen source as well as trace minerals, as described in Pruden et al. 2001. The batch reactors were dosed with the appropriate reactive media, then filled up with the source water and inoculated with the anaerobic culture. No nutrients were added to the water coming from the refinery. The batch reactors were set up in a N_2 glove box. A volume of 980 ml of water was added to each bottle. A head space of 20 ml was left for gas production. The bottles were capped with butyl rubber stoppers and screw caps. Two perforations were made to the rubber stoppers to place two lines of 1/8 inches ID FEP tubing (Cole-Parmer, Vernon Hills, IL). The end of one tube was placed half way into the bottle and the other tube extended only to the bottom of the rubber stopper to enable the controlled release of gases. The outside ends of the tubes were maintained closed with a clamp. The bottles were placed under water to reduce the possible diffusion of oxygen into of the batch reactors. Water samples were taken weekly for later GC and molecular analyses. At each sampling event, a 25 ml gastight syringe was flushed three times and subsequently filled with nitrogen gas. The syringe was connected to the gas-release tube and the nitrogen was injected to collect aqueous samples. From the other tube, a 2 ml gastight syringe was used to take a 0.2 ml sample for GC analysis and a 2 ml sample for molecular analysis. The GC-samples were stored at 4^0C until analysis, up to three days, while the samples used for the molecular analysis were stored at -80^0C. The bottles were mixed by shaking manually two times a week.

The results obtained for the first part of the experiment, where the water from the refinery was used as the source water, is presented in Figure A-1. The results where amended

DI water was used as the source water are presented in Figure A-2. At the end of each stage, the pH, redox potential and dissolved oxygen were measured. The pH varied ±0.2 units from the original pH 7. The redox potential was negative for all of the reactors. The redox potential varied from 42.8 to -37.4 milivolts for the reactors that had Fe^{+3} as the electron acceptor, in the range for Fe^{+3} [100 to 0] milivolts. While the redox potential ranged from -134 to -237 in the reactors that had SO_4^{-2} as the electron acceptor, also in the range for SO_4^{-2} [-200 to -300] milivolts .

When comparing soluble electron acceptor sources (Figure A-1A and Figure A-2A), it can be observed that MTBE was removed under both conditions (ferric and sulfate reduction), however for the experiment where the water from the refinery was used, the culture preferred sulfate as the electron acceptor rather than ferric iron (Figure A-1A). In the experiment where amended DI water was used as the source water, no the effect of the electron donor was not appreciable.

When comparing insoluble electron acceptor sources (Figure A-1B and Figure A-2B), MTBE was observed biodegrade in the conditions studied. For the experiment where the water from the refinery was used as the source water, there seems to be a clear trend where the bacteria preferred gypsum over ferric hydroxide, given that the greater removal of MTBE took place in the reactors that had only gypsum or had both source, but MTBE was not removed in the reactors that had only ferric hydroxide. In the case where amended DI water was used as the source water, it was more difficult to see a clear preference of the bacteria over one electron source over the other.

The comparison made between the removal of MTBE at different pH values is presented in Figure A-1, Figure A1C and Figure A-2C. Although MTBE was removed at pH

2, it was not possible to determine whether the removal was hydrolysis of MTBE due to the acidic conditions or bioremediation. The removal of MTBE in each reactor seemed to be independent of pH, since no repeatability was observed between duplicates.

Given that there was a difference on the removal rate between the reactors that had the refinery water and the amended water, it is possible that the source water also had an effect. One of the main problems encountered in this experiment was that it was not possible to inactivate the controls. It can be observed that for some cases the removal in the controls is much higher that in the duplicated reactors, which made difficult the analysis of the results to determine whether the observed removal was due to biodegradation or abiotic effects, such as hydrolysis.

Figures

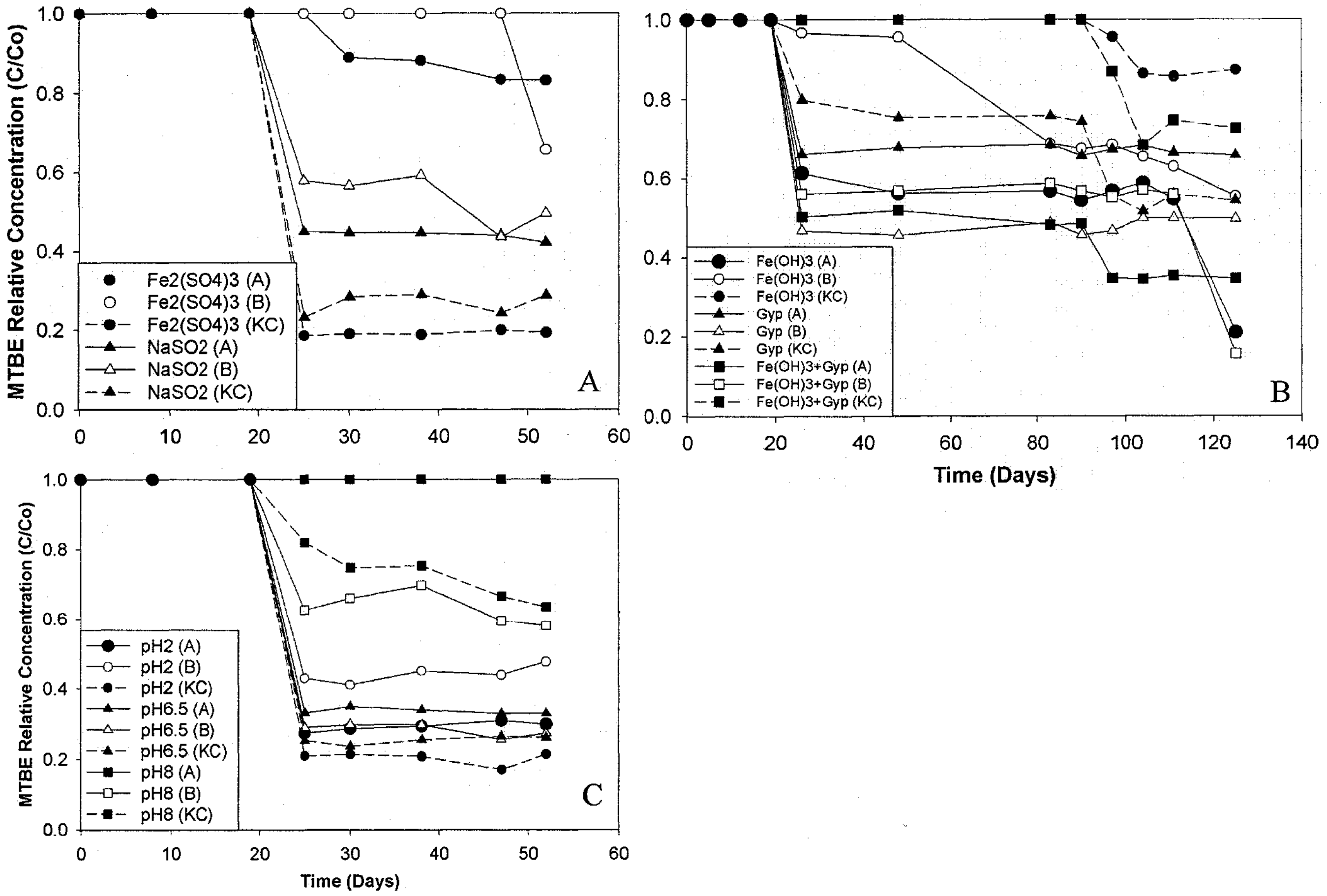


Figure A-1 Change in MTBE concentration in the batch reactors when the water from the refinery was used. No nutrients were added. The target initial MTBE concentration was 10 mg/L.

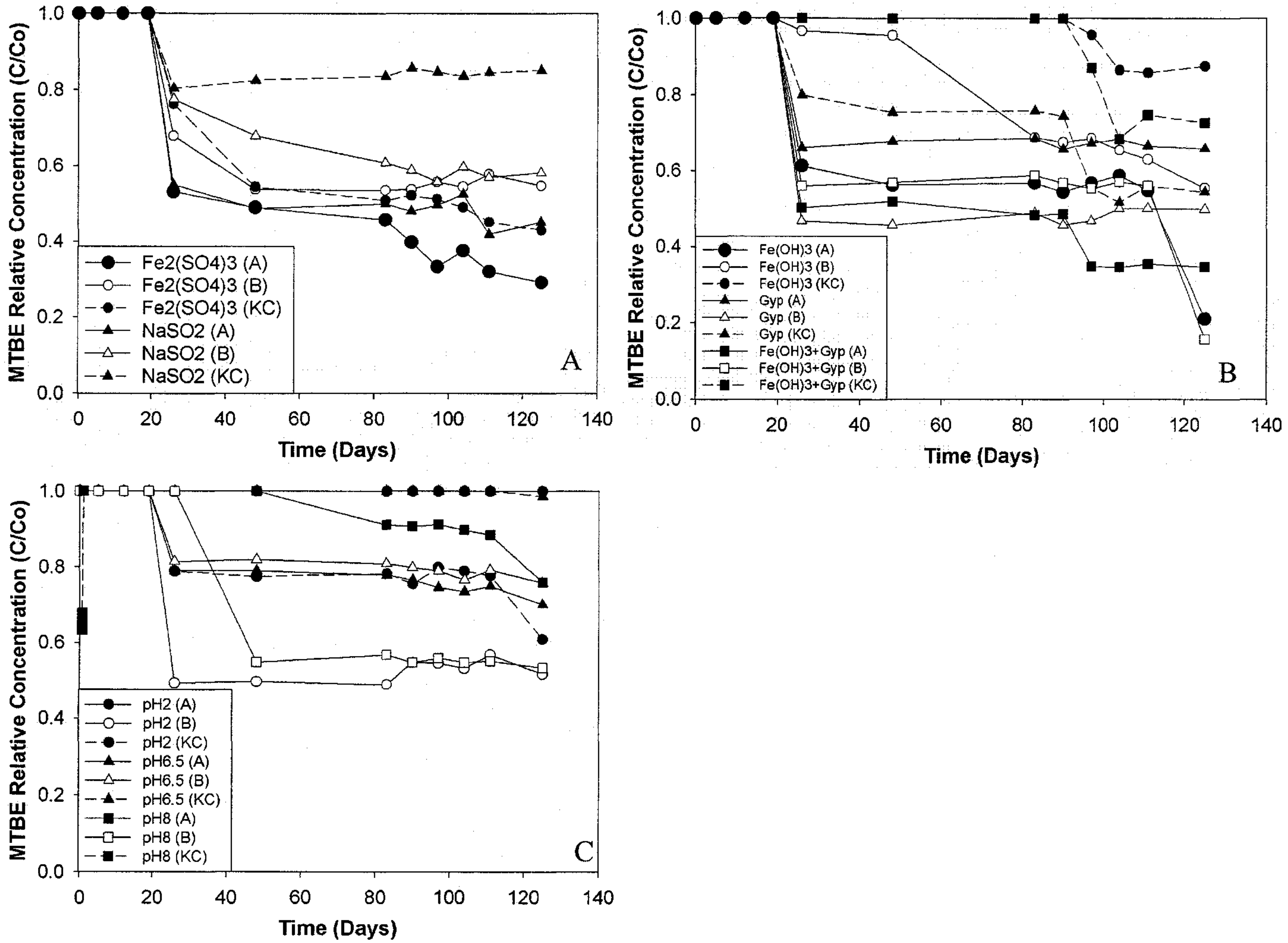


Figure A-2 Change in concentration of MTBE using DI water with nutrients. The target initial MTBE concentration was 10 mg/L.

Appendix B Batch Experiment to Determine if the Anaerobic Fe-reducing culture could be enhanced with the addition of other organics.

A batch experiment was conducted to determine if the performance Fe-reducing culture could be enhanced by adding organic substances. The experimental conditions are presented in Table B-1. Two hundred and fifty milliliter glass bottles were used for this experiment. The glass bottles were caped with rubber butyl septas. The initial pH was adjusted to 8. $Fe_2(SO_4)_3$ was used as the electron acceptor. The target initial MTBE concentration was 20 mg/L. The inoculum for the columns was taken from the 4 L anaerobic semi-batch reactor.

Table B-1 Experimental conditions for the chemical batch experiment.

Composition	Concentration (mg/L)	Abbreviation
Ammonium Acetate	200	Acetate
Lactic Acid	5	Lactate
Propionic Acid	5	Piruvate
Urea	250	Urea
Sodium Citrate	200	Citrate
Humic acid	500	HA
Dextrose + Cellulose	41 and 36	Dextrose + Cellulose
Dextrose + Cellulose + Propionic Acid	41, 36 and 5	Dex+Cell+ Propionic Acid
Ethanol + Humic acid	1 and 500	EtOH+ HA
Na_2S +Humic acid (Control)	32 and 500	Control

The results for this experiment are presented in Figure B-1. After 38 days, the removal of MTBE was about 35% in most of the reactors. It was observed that the presence of the organic chemicals added did not have a significant effect on the removal of MTBE by the Fe-reducing culture. When comparing the removal of MTBE in the reactors with the control, it can be observed that the removal was similar in all of the reactors. The presence of lactic acid seemed to have an inhibitory effect, since 15% of MTBE was removed in the period studied.

Figures

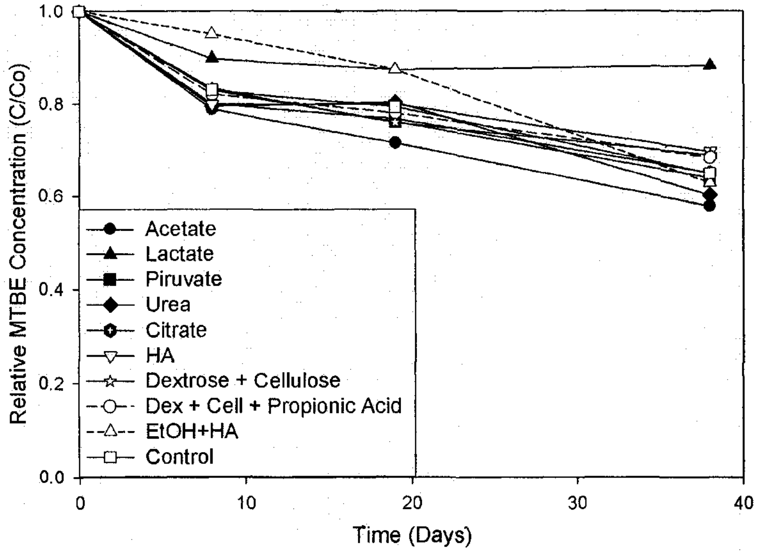


Figure B-1 Relative concentration of MTBE in the batch reactors with time. The initial MTBE concentration was 20 mg/L. $Fe_2(SO_4)_3$ was used as the electron acceptor.

Appendix C Primer and Taqman probes design for the detection and quantification of the pure culture in environmental samples.

Using the 16S sequence obtained for the anaerobic MTBE-degrading pure culture and the software Primer3 v 0.4.0 (Skaletsky 2000) Taqman probes and primers were designed. The first approach was to design a Taqman probe and primers that were able to target most of the microorganism contain in the Desulfosporosinus class. The second step was to design a Taqman probe and primers that could detect only the isolated pure culture. The sequence and the information of the primers and probes are presented in Table C-1.

Table C-1 Desulfosporosinus class or Desulfosporosinus meredie specific 16S rRNA gene targeting PCR primers and Taqman probes.

Primer/Taqman Probe	Annealing Temp. ^{0}C	Sequence 5'-3'	Specificity
Desulfosp. Probe	54.2	CTAAATACAGGGGTTGCG	Desulfosporosinus Genus
Desulfo-Left	59.99	CTTCTGTTGTACCGGCCATT	Desulfosporosinus Genus
Desulfo-Right	60.05	CAGCTCGTGTCGTGAGATGT	Desulfosporosinus Genus
MTBEAnPureCult Probe	59.96	ATACCGTCATCGTCGGACTC	Desulfosporosinus meridiei S5 (AF076248).
MTBEAnPureCult-Left	59.95	GTCGAGCCCTGATCTCTCAC	Desulfosporosinus meridiei S5 (AF076248).
MTBEAnPureCult-Right	59.92	CCCGATATGATGCAGTCCTT	Desulfosporosinus meridiei S5 (AF076248).

PCR on the extracted DNA from the pure culture was performed in 25 μL volume samples to determine the annealing temperature of the primers, before determining the program for Q-PCR. The primers sets to detect the pure culture and the Desulfosporosinus genus of the 16S rRNA gene and their corresponding annealing temperatures are reported in Table C-1. Each PCR mixture contained 1 μL template DNA, 13.4 μL sterilized DI water, 5 μL 5X buffer solution, 2.5 μL 10X buffer solution, 1.5 μL Mg (10 μM), 0.5 μL dNTP, 0.25 μL of each primer (10 μM), 0.25 μL Formamide and 0.35 μL Taq polymerase. Thermal cycling was carried out by using an initial denaturation step of 94°C for 4 min, followed by 25 cycles of denaturation at 94°C for 30 sec, annealing for 40 sec (60°C Desulfosporosinus genus and 56°C for the culture specific primers) and elongation at 72°C for 2 min. Cycling was completed by a final elongations step of 72°C for 7 min.

The PCR products were cloned using TOPO-TA cloning vector kit (Invitrogen, Carlsbad, CA) to determine the specificity of the primers. Positive clones were PCR amplified with primers M13. Unique inserts were purified with ExoSAP-IT (USB, Cleveland, OH) and sequenced by the Macromolecular Resources Facility at Colorado State University (Fort Collins, CO).

Several attempts were made to try to determine the annealing temperature of the TaqMan probes for the Desulfosporosinus genus and the pure culture, however it was not possible. Amplification with the primers using Q-PCR was observed using SybrGreen. The sequences obtained from the amplified 16S rRNA gene fragments, did not match any of the Desulfosporosinus genus members nor the pure culture. A less variable fragment should be selected to design the primers and the probes in the future.

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