The oxygen dependence of the degradation rate of Methyl tert-Butyl Ether by a bacterial isolate
by Elsa Emilie Meiser

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Civil Engineering
Montana State University

Abstract:
Methyl tert-Butyl Ether (MTBE) is a fuel oxygenate added to gasoline to boost octane and reduce emissions of volatile organics, nitrogen oxides, and toxics such as benzene. Bacterial degradation represents a viable means of remediating MTBE that is contaminating the environment. The aerobic degradation of MTBE is catalyzed by the enzyme monooxygenase. This enzyme is dependent upon molecular oxygen. The Michaelis-Menton model is a mathematical model that describes the enzyme-mediated dependence of reaction rate on a given substrate. The Hill model further describes enzyme mediated reaction kinetics when cooperativity among enzymes has been introduced.

This study investigated the behavior of MTBE Degradation rate as a function of dissolved oxygen concentration by a bacterial isolate, PMl. Three experiments were run. In each experiment, batch microcosms containing aqueous nutrient media, MTBE, PMl, and oxygen were monitored over a period of 7 days. MTBE and oxygen concentration were determined at each sampling event. These values were used to correlate MTBE degradation rate to dissolved oxygen concentration. This data was fit to the Michaelis-Menton and Hill models using a non-linear regression algorithm.

This study shows that the biodegradation rate of MTBE by PMl is highly dependent on dissolved oxygen concentrations, specifically at concentrations of dissolved oxygen below 2 mg/L. This sensitivity to dissolved oxygen concentration may be an important factor to consider in the design and operation of treatment and remediation systems. Furthermore, PMl was capable of complete mineralization of up to 20 mg/L of MTBE in 7 days with sufficient oxygen present. Both the Hill model and the Michaelis-Menton model represent adequate predictors of the behavior of the data.

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APPROVAL

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Elsa Emilie Meiser

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission of the College of Graduate Studies.

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ABSTRACT

Methyl *tert*-Butyl Ether (MTBE) is a fuel oxygenate added to gasoline to boost octane and reduce emissions of volatile organics, nitrogen oxides, and toxics such as benzene. Bacterial degradation represents a viable means of remediating MTBE that is contaminating the environment. The aerobic degradation of MTBE is catalyzed by the enzyme monooxygenase. This enzyme is dependent upon molecular oxygen. The Michaelis-Menton model is a mathematical model that describes the enzyme-mediated dependence of reaction rate on a given substrate. The Hill model further describes enzyme mediated reaction kinetics when cooperativity among enzymes has been introduced.

This study investigated the behavior of MTBE Degradation rate as a function of dissolved oxygen concentration by a bacterial isolate, PM1. Three experiments were run. In each experiment, batch microcosms containing aqueous nutrient media, MTBE, PM1, and oxygen were monitored over a period of 7 days. MTBE and oxygen concentration were determined at each sampling event. These values were used to correlate MTBE degradation rate to dissolved oxygen concentration. This data was fit to the Michaelis-Menton and Hill models using a non-linear regression algorithm.

This study shows that the biodegradation rate of MTBE by PM1 is highly dependent on dissolved oxygen concentrations, specifically at concentrations of dissolved oxygen below 2 mg/L. This sensitivity to dissolved oxygen concentration may be an important factor to consider in the design and operation of treatment and remediation systems. Furthermore, PM1 was capable of complete mineralization of up to 20 mg/L of MTBE in 7 days with sufficient oxygen present. Both the Hill model and the Michaelis-Menton model represent adequate predictors of the behavior of the data.

CHAPTER 1

INTRODUCTION

Methyl *tert*-Butyl Ether (MTBE) has been used in gasoline since the 1970s to boost octane and reduce emissions of volatile organics, nitrogen oxides, and toxics such as benzene. Over the past decade, MTBE has been detected with increasing frequency in surface and drinking water supplies across the nation, often as a result of gasoline leaks and spills. Traditional techniques that are effective for treatment of other constituents of gasoline are not as effective for remediation of MTBE (EPA 2000). As a result, alternative techniques are currently being explored.

Bioaugmentation and natural attenuation represent two remediation techniques in which bacteria are used to degrade MTBE. Often, low levels of dissolved oxygen characterize aquifer environments contaminated by MTBE. In these cases the addition of oxygen would stimulate aerobic degradation activity, and thus enhance the remediation effort.

MTBE can be difficult to degrade due to its chemical characteristics. While other gasoline constituents such as the BTEX compounds (benzene, toluene, ethylbenzene, and the xylenes) will readily sorb to soils and are quite amenable to microbial degradation, MTBE resists retardation and decay in natural systems. MTBE is also more soluble than the BTEX compounds. As a result, MTBE plumes at contaminated sites extend farther down gradient than BTEX plumes. MTBE travels at virtually the speed of groundwater due to its solubility and low sorption coefficient, thus increasing its potential to impact domestic water supplies (US EPA Office of Pollution Prevention and Toxics 1994).

MTBE is used almost exclusively as a fuel additive for motor gasoline (US EPA 1997). MTBE is classified as an "oxygenate" because it raises the oxygen content of gasoline, helping it to burn more completely and thus reducing harmful tailpipe emissions. Oxygenates are helpful in reducing emissions in two ways. Firstly, the oxygen that they contain dilutes or displaces gasoline components such as aromatics and sulfur. Secondly, additional oxygen increases oxidation during the combustion of gasoline, thus making the gasoline burn more cleanly.

Under the Clean Air Act Amendments of 1990, Congress mandated the use of reformulated gasoline (RFG) in areas of the country with the worst ozone or smog problems. The Act required that RFG must contain a minimum of 2 percent oxygen by weight for the Year-round Reformulated Gasoline Program and 2.7 percent oxygen by weight for the Winter Oxyfuel Program (US EPA 2000).

Reformulated gasoline has been effective in the United States since 1995. The use of RFG as compared to conventional gasoline has resulted in annual reductions of smog forming pollutants (volatile organic compounds and nitrogen oxides) and toxics (such as benzene). With the second phase of the RFG program, which began in January 2000, the EPA estimates that smog-forming pollutants are being reduced annually by at least 105 thousand tons, and toxics by at least 24 thousand tons. RFG accounts for approximately 30% of gasoline nationwide (US EPA 1999).

The Clean Air Act Amendments of 1990 do not specifically require the use of MTBE. Refiners may choose to use other oxygenates such as ethanol or *tert*-amyl ether (TAME). However, MTBE is popular because it can be produced within existing

refineries, it blends well with gasoline without phase separation, and can be transferred through existing pipelines (Suflita and Mormile 1993). MTBE is produced in very large quantities in the United States. In 1999, over 200,000 barrels per day of MTBE were produced. This is not surprising, since as of 1997 MTBE was used in over 84% of reformulated gasoline supplies (US EPA 1998).

MTBE has been found in urban water supplies, in surface waters such as recreational lakes, and in drinking water wells (Squillace 1998; Delzer 1996). However, the question of whether or not MTBE is harmful when ingested by humans remains unanswered. The EPA has classified MTBE as a possible human carcinogen based on laboratory experiments in which MTBE was found to cause cancer in lab rats (US EPA 1997). MTBE is also generally unpleasant in taste and odor. Studies conducted to determine the concentrations at which individuals can detect the taste and odor of the chemical led to the establishment of an EPA Advisory recommendation of acceptable MTBE levels at or below 20 to 40 μg/L (US EPA 1997). However, this level is not an enforceable standard.

Some states have chosen to mandate their own enforceable MTBE standards. The most notable of these is California. Currently, California has a secondary maximum contaminant level of 13 μg/L and a primary MCL of 5 μg/L. Montana has adopted a Human Health Standard of 30 μg/L (MT DEQ 2002).

While it has been instrumental in reducing smog-forming and toxic air pollutants, MTBE is undesirable in water supplies. In fact, MTBE was included in The Clean Air Act's lists of hazardous air pollutants. Leaking Underground Storage Tanks (LUSTs) are

a major source of environmental contamination that directly contribute to the introduction of MTBE into water supplies. As of September 30, 2001 state and local UST programs had submitted information to the EPA confirming 418,918 UST releases, prompting 379,243 cleanup initiatives. Nearly all USTs contain petroleum (US EPA 2002).

The Federal Government is currently considering legislation to limit or even eliminate MTBE from reformulated gasoline supplies. California, where high population density has exacerbated widespread MTBE contamination, has already mandated the complete phase-out of MTBE by the year 2010.

Bioaugmentation and natural attenuation are attractive remediation alternatives because of their use of microorganisms to completely degrade MTBE to inert compounds. It is now widely accepted that aerobic degradation of MTBE involves oxidation by the monooxygenase (MO) enzyme. The MO enzyme is dependent on molecular oxygen.

The Michaelis-Menton kinetic model describes enzyme mediated reaction kinetics when the behavior of the reaction rate is dominated by a single substrate such as oxygen. Figure 1.1 is a schematic of the characteristics of the Michaelis-Menton model. Michaelis-Menton plots contain three distinct regions that correspond to different reaction orders. At low substrate concentrations, the reaction accelerates as more substrate is added, reflecting first order kinetics. At high substrate concentrations, the concentration of enzyme becomes limiting, and additional substrate will not accelerate the reaction. This is known as zero order kinetics. The third region is a transition period between first order and zero order where kinetics are mixed.

The Michaelis-Menton model is given as follows:

$$\mu = \frac{\mu_{max} \circ [S]}{K_m + [S]}$$

where μ = reaction rate

μ_{max} = maximum reaction rate

[S] = substrate concentration

K_m = half saturation coefficient

The term K_m represents the amount of substrate required to bind one half of the available enzyme. If K_m is small, then tight binding occurs and the enzyme has a high affinity for the substrate. A large K_m represents weak binding and low substrate affinity.

For the purpose of fitting the data in for the experiments described in this thesis, the model parameters are described as follows:

μ = rate of MTBE degradation [mg L^{-1} MTBE $days^{-1}$] or

adjusted MTBE degradation rate [$days^{-1}$]

μ_{max} = maximum rate of MTBE degradation [mg L^{-1} MTBE $days^{-1}$] or

adjusted maximum rate of MTBE degradation [$days^{-1}$]

[S] = concentration of dissolved oxygen, [mg L^{-1}]

K_m = half saturation coefficient

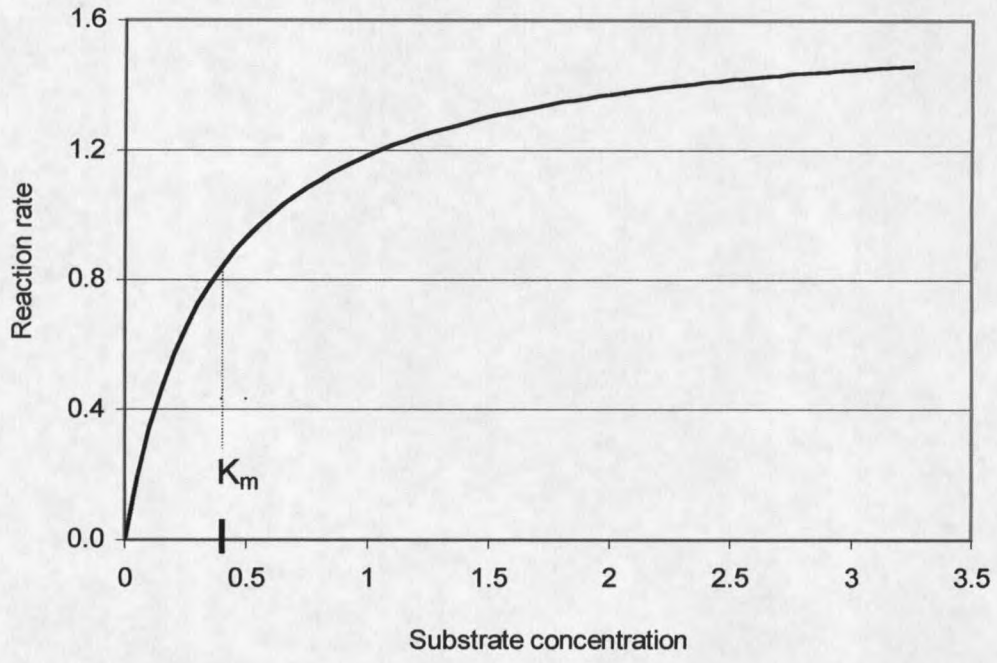

Figure 1.1 Schematic of the Michaelis-Menton model.
$\mu_{max} = 1.628$, $K_m = 0.373$.

The Hill model describes enzyme mediated reaction kinetics in which cooperativity occurs. Cooperativity refers to the phenomenon by which an enzyme can bind several substrate molecules at any given time. The binding of substrate at a given site leads to a conformational change in shape of the enzyme as a whole, so that the catalytic activity of other sites is changed. Thus, initial enzyme activity increases the activity of subsequent enzymes.

Hill kinetics produce characteristically sigmoidal curves that include a lag time for enzyme activation. A schematic of the Hill model is shown in Figure 1.2. After this lag period, the Hill model displays an initial region of first order kinetics followed by a region of zero order kinetics, similar to the Michaelis-Menton model. This model is given as:

$$\mu = \frac{\mu_{max} \bullet [S]^n}{(K_m)^n + [S]^n}$$

The parameter n in the Hill equation can be thought of mechanistically as the number of molecules that bind to a receptor. Recall that the Hill model describes enzyme activity in which one enzyme can bind several substrate molecules. Values of the parameter n greater than one indicate positive cooperativity, in which initial enzyme activity increases subsequent activity. Similarly, values of n less than one indicate negative cooperativity, in which initial enzyme activity decreases subsequent activity.

A significant study by Park and Cowan (1997) found that the biodegradation rate of MTBE was highly dependent on dissolved oxygen concentration when degraded by an aerobic mixed culture. Specifically, MTBE degradation was inhibited at oxygen concentrations below 2 mg L^{-1}. Furthermore, the study found the half saturation coefficient, K_m, to be 0.9 mg DO L^{-1}. Typical K_m concentrations for heterotrophs are on the order of 0.1 to 0.2 mg DO L^{-1}. The authors state that this reveals that the MTBE degrading mixed culture which they studied is more sensitive to the concentration of DO that normal heterotrophs.

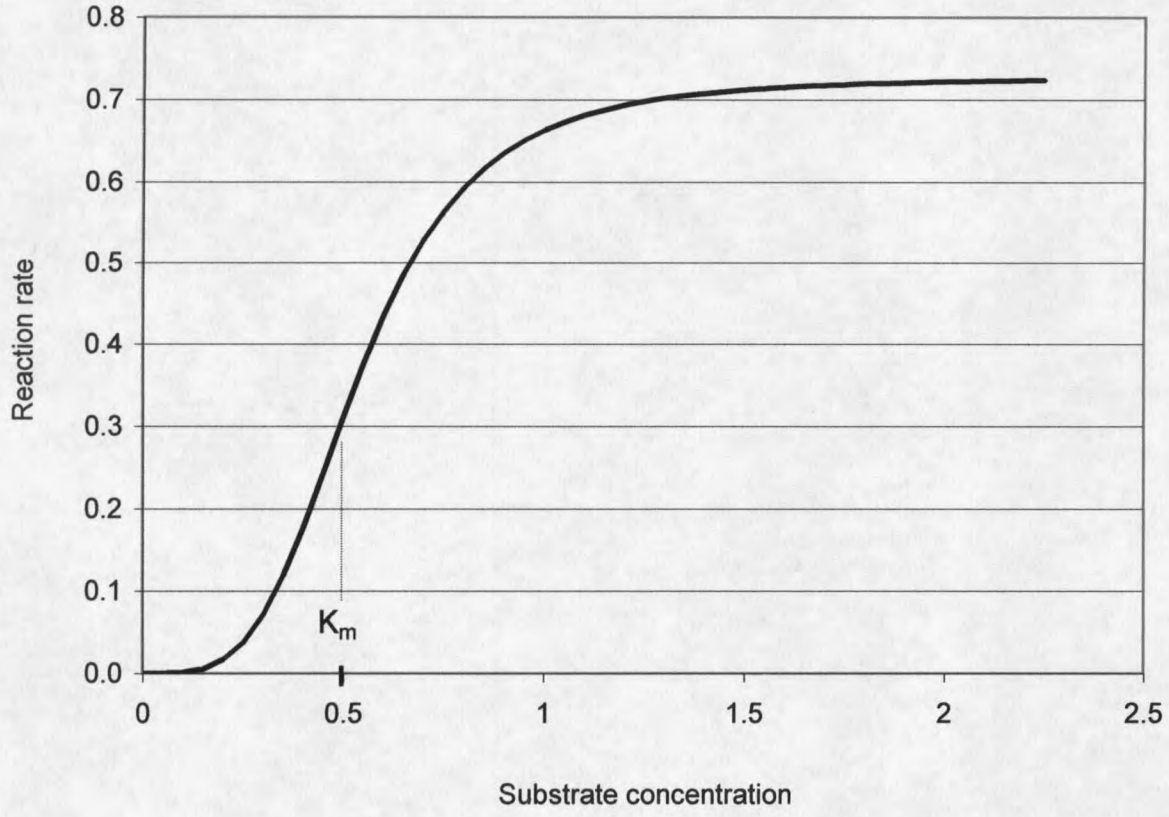

Figure 1.2 Schematic of the Hill model.
$\mu_{max} = 0.73$, $K_m = 0.54$, $n = 3.78$.

This study investigates the dependence of MTBE biodegradation rate on dissolved oxygen concentration when degraded by an aerobic bacterial isolate. The Michaelis-Menton and Hill model are fit to the data to determine the best model for predicting the behavior of this system. These findings are compared to the findings of Park and Cowan in which an aerobic mixed culture was investigated.

CHAPTER 2

LITERATURE REVIEW

Chemical Characteristics

MTBE is an aliphatic ether with the chemical formula $C_5H_{12}O$ (Figure 2.1). Table 2.1 shows the physical and chemical characteristics of MTBE and the BTEX compounds. MTBE has a molecular weight of 88.15 g $mole^{-1}$. It is a flammable colorless liquid at room temperature. The solubility of MTBE is approximately 50 g L^{-1} at 25°C. The Henry's Law Constant of MTBE ranges from 0.018 to 0.24 in the reported literature. MTBE has a log octanol-water partitioning coefficient (log K_{ow}) of 1.20.

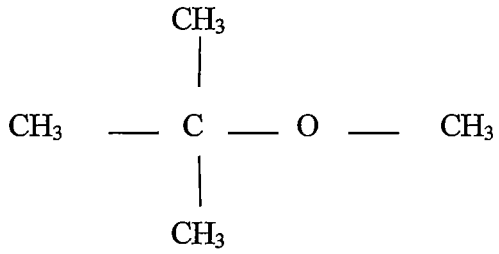

Figure 2.1 Chemical Structure of MTBE

MTBE is the most widely used fuel oxygenate. It is commonly found in the environment with the BTEX (benzene, toluene, ethyl-benzene, xylene) compounds.

Table 2.1 Physical and Chemical Properties of MTBE, Benzene, Toluene, Ethyl-benzene, and O-xylene.

Physical and chemical properties	Benzene	Toluene	Ethyl-benzene	O-xylene	MTBE
Molecular weight [g $mole^{-1}$]	78.11	92.14	106.17	106.17	88.15
Vapor Density @ 1 atm; 10°C (Air = 1)	3.36	3.97	4.57	4.57	3.8
Specific gravity @ 25°C	0.88 (1)	0.8669 (1)	0.867 (1)	0.8802 (1)	0.744 (1)
Water solubility [mg L^{-1}]	1730 (1)	534.8 (1)	161 (1)	175 (1)	43,000-54,300 50,000 (1)
Henry's Law constant	0.23 (2)	0.272 (2)	0.336 (2)	0.212 (2)	0.2399 (1) 0.04496(1) 0.05722(1) 0.1226 (1) 0.026 (1) 0.018 @ 20°C (1)
Vapor pressure [mm Hg] (@ 25°C)	76, 95.19 (1)	28.4 (1)	9.53 (1)	6.6 (1)	245-276 (1)
Log K_{ow}	2.36 (2)	2.73 (2)	3.24 (2)	3.10 (2)	1.20 (1)

(1) OSTP, 1997. (2) Crittenden et al, 1997.

Compared to the BTEX compounds, MTBE is more soluble, less volatile, and less sorptive (Table 2.1). Although MTBE is classified as a volatile organic compound, MTBE will partition more strongly that benzene from the gas phase to the water phase. Based on its chemical and physical characteristics, MTBE moves virtually at the speed of groundwater.

MTBE Detection in the Environment

During the past decade, MTBE has been detected with increasing frequency in US water sources. Three studies are discussed in the following section. The first two studies, conducted by the United States Geological Survey (USGS), look at both urban and agricultural well water contamination by MTBE as well as MTBE detection in urban storm water runoff. A third study investigates the occurrence of MTBE in a multiple use lake in the Sierra Nevada Mountains. These three studies highlight the affinity of MTBE for partitioning into the water phase as well as the high occurrence of MTBE in these water sources.

As part of the National Water Quality Assessment Program of the USGS, an assessment of 60 volatile organic compounds (VOCs) in untreated, ambient groundwater in the US was conducted from 1985 to 1995. MTBE was the second most frequently detected chemical, contaminating 27% of the 210 urban wells sampled, 1.3% of the 549 agricultural wells sampled, and 1% of the 412 wells in deeper groundwater from major aquifers. No MTBE was detected in drinking water wells. Only 3% of the shallow wells sampled in urban areas had concentrations of MTBE that exceeded 20 $\mu g\ L^{-1}$. The

reporting level for MTBE was 0.2 $\mu g\ L^{-1}$. In shallow urban groundwater, MTBE was generally not found with the BTEX compounds, likely due to its high mobility and low sorption coefficient (Squillace et al. 1996).

Another study conducted by the USGS in 1991 through 1995 sampled 16 cities with populations of over 100,000 persons that were required by the Clean Water Act to obtain permits for stormwater discharged from municipal separate storm sewer systems into surface waters. The study encompassed 11 states. MTBE was the seventh most frequently detected VOC in urban storm water, following toluene, total xylene, chloroform, total trimethylbenzene, tetrachloroethene, and naphthalene. MTBE was detected in 6.9% of the 592 storm water samples collected. When detected, MTBE concentrations ranged from 0.2 to 8.7 $\mu g\ L^{-1}$. Sixty-six percent of all occurrences of MTBE detection occurred with BTEX compounds, with a proportionate increase in concentrations when these compounds occurred together. Eighty-three percent of all detections of MTBE were in samples collected during October through March of each year, which corresponds with the expected seasonal use of oxygenated gasoline (Delzer et al. 1996).

A study conducted by Reuter et al. (1998) sampled Donner Lake in California to determine, among other things, the relative contribution of motorized watercraft as a source of MTBE. Donner Lake is located in the Sierra Nevada Mountains and is classified as a multiple-use lake. MTBE measurements were made at 9 individual depths from surface to bottom on 16 dates. The study found that 86% of the change in MTBE concentration between the respective dates was explained by motorized watercraft use.

Neither highway runoff nor precipitation contributed significantly. MTBE concentrations ranged from <0.1 $\mu g\ L^{-1}$ to a high of 12 $\mu g\ L^{-1}$. Between July 1 and July 7, 1997, MTBE content of the lake rose from 115 to 365 kg. By January, levels had declined to 15 kg, suggesting a strong correlation between increases in MTBE contamination and motorized watercraft use.

Health Effects

Studies performed on laboratory rats show that MTBE is capable of causing adverse health effects including tumors. MTBE exposure in these studies is primarily of two types: exposure through inhalation or direct injection.

The USEPA in its MTBE drinking water advisory of 1997 reviewed three separate studies related to the carcinogenicity of MTBE. The only oral cancer exposure study was conducted by Belpoggi et al (1995). In this study, Sprague-Dawley rats were given MTBE in olive oil, at doses up to 1000 mg kg^{-1} four days per week for two years. The study claims that exposure caused a dose-related increase in the incidence of combined leukemias and lymphomas in female rats and an increase in Leydig cell adenomas (benign testicular tumors) in the high dose male rats. The EPA Advisory warns against using this study to quantitatively assess risks from drinking water exposure. It cites as reasons both the potential differences in the method of delivery (bolus vs. drinking), as well as the possibility of vehicle (olive oil) interactions (US EPA 1997).

The USEPA reviewed two studies on the potential carcinogenicity of MTBE after inhalation exposure. Chun et al. (1992) administered MTBE to F344 rats at concentrations up to 8,000 ppm for 2 years. Exposure to MTBE caused an increase in the incidence of combined renal tubular adenomas and carcinomas, as well as Leydig cell adenomas of the testes in the male rats. The increase of Leydig cell adenomas of the male rats in this study was not significantly different from the historical control value, although the difference from the concurrent control was significant.

In the other inhalation study, Burleigh-Flayer et al. (1992) gave MTBE to CD-1 mice at concentrations up to 8,000 ppm for 18 months. This exposure was associated with a statistically significant increase in the incidence of hepatocellular carcinomas in male mice and of hepatocellular adenomas in female mice. The EPA Advisory again proposes that the data from the above two studies could not be used to develop adequate health advisory values since there is "no well developed pharmacokinetic model for converting a chronic inhalation exposure of MTBE to an equivalent oral exposure" (US EPA 1997).

The USEPA Drinking Water Advisory concludes that data support the conclusion that MTBE poses a potential for carcinogenicity to humans at high doses. The data do not support confident, quantitative estimation of risk at low exposure.

A public/private partnership was established in 1997, under the administrative oversight of the American Petroleum Institute (API), to develop aquatic toxicity data sufficient to calculate ambient water quality criteria for MTBE. Commercial laboratories conducted nineteen freshwater and marine tests. Data were developed and reported under

the oversight of industry study monitors and Good Laboratory Practice standards auditors, with USEPA scientists participating in advisory and critical review roles. The study determined the preliminary freshwater criteria for acute (*Criterion Maximum Concentration*) and chronic (*Criterion Continuous Concentration*) exposure effect protection to be 151 and 51 mg MTBE L^{-1}, respectively. Calculated, preliminary marine criteria for acute and chronic exposure effect protection were found to be 53 and 18 mg MTBE L^{-1}, respectively. These values indicated that ambient MTBE concentrations documented in US surface waters to date do not constitute a risk to aquatic organisms (Mancini et al. 2002).

Regulations

The USEPA has established a recommended health advisory for MTBE of 20 to 40 $\mu g\ L^{-1}$ (US EPA 1997). These values are based on taste and odor thresholds and are aimed to protect the majority of the population from adverse health effects, recognizing that some persons may exhibit symptoms of MTBE exposure at levels below 20 $\mu g\ L^{-1}$.

Some states have adopted their own standards based on EPA guidelines and perceived health threats. California has set the most stringent standards, adopting a secondary Maximum Contaminant Level (MCL) of 5 $\mu g\ L^{-1}$. Legislation is pending in California to set a primary MCL of 13 $\mu g\ L^{-1}$. However, secondary MCLs are enforceable in the state of California. Other states have likewise adopted their own standards. Montana has approved a WQB-7 Health Based Standard of 30 $\mu g\ L^{-1}$ (MT DEQ 2002).

Biological Remediation of MTBE

Biological organisms are capable of MTBE degradation, as is evidenced by a number of separate studies. The mineralization of MTBE has been investigated and demonstrated under both anaerobic and aerobic conditions, substantiated with data from field and laboratory experiments. Indigenous organisms as well as isolated organisms have been found capable of MTBE degradation.

Degradation rates by indigenous organisms are usually slower than degradation rates by isolated MTBE degrading organisms. Degradation by indigenous organisms most often follows an acclimation period that may last as long as six months. In some instances the presence of molecular oxygen will stimulate the mineralization of MTBE. The sections below describe studies investigating MTBE mineralization by biological organisms.

Anaerobic Mineralization

Oxygen transport in surface water and groundwater systems is often limited. The studies discussed below show that certain indigenous bacteria are capable of MTBE degradation under anoxic conditions. In the first three studies, laboratory microcosm experiments were conducted using site sediments containing soil bacteria. The last study looks at in-situ MTBE degradation, again by indigenous bacteria.

A study by Mormille et al. (1993) tested a series of alcohol, ketone, ester, and ether oxygenates for their susceptibility to anaerobic decay in samples from four chronically contaminated sedimentary environments. The ether oxygenates tested were

generally not degraded by various inocula, regardless of electron acceptor status. The exceptions included methyl butyl ether, which was depleted in sulfate reducing and methanogenic incubations, as well as the partial transformation of methyl *tert*-butyl ether to *tert*-butanol after a 152-day acclimation period in a single replicate from Ohio River sediment chronically contaminated with fuel (Suflita and Mormile 1993).

Yeh and Novak (1994) conducted an evaluation of the biodegradation of MTBE, ethyl tertiary butyl ether (ETBE), and tertiary butyl alcohol (TBA) by bacteria in soils of different origin and varying characteristics. The biodegradation under sulfate reducing, methanogenic, and denitrifying conditions was measured in static soil and water microcosms. Three site soils were examined. TBA was degraded in all three site soils in unamended microcosms in which oxygen and nitrate were excluded while sulfate and carbon dioxide were abundant. Degradation proceeded more slowly in the Site 1 soil that was characterized by lower amounts of nutrients and organic matter. ETBE was degraded in the Site 1 soils, but in neither of the other site soils for both the unamended as well as denitrifying treatments. MTBE was not degraded in any of the three unamended site soils tested over the 250-day study period, nor was any MTBE degradation evidenced under denitrifying conditions. Degradation of MTBE did occur under methanogenic conditions in oligotrophic soils with low organic matter and at a pH between 5.0 and 6.0. ETBE was degraded under these conditions as well. The co-existence of ethanol and other easily degradable organics inhibited MTBE and ETBE degradation. The data indicate that TBA was the easiest compound to degrade under the conditions tested, whereas MTBE was the most recalcitrant.

Surface water-sediment microcosms were investigated for MTBE degradation by Bradley et. al (1999). Sediments were collected from three surface water sites including two streams and one lake. No detectable oxygen was found at any of the three sampling sites. The study found that mineralization of [U-^{14}C]MTBE to $^{14}CO_2$ generally increased as the oxidative potential of the predominant terminal electron acceptor increased in the order of SO_4, Fe(III), Mn(IV) < NO_3 < O_2. In the presence of substantial methanogenic activity, TBA was accumulated. However, this phenomenon decreased in the absence of significant methanogenic activity. The authors note that microbial mineralization of MTBE to CO_2 under Mn(IV)- or SO_4- reducing conditions had not been previously reported. From these findings, it is possible to determine that microorganisms inhabiting lakes and streams can degrade MTBE effectively under a range of anaerobic terminal electron-accepting conditions.

A field investigation conducted by Hurt et al. (1999) revealed convincing in-situ evidence of anaerobic biodegradation of MTBE. A high-resolution characterization of a fuel contaminated aquifer was accomplished by means of eleven temporary, direct-push wells. Sample water from these wells was passed through a flow cell to determine dissolved oxygen, oxidation-reduction potential, conductivity, pH and temperature. Water samples were removed and analyzed for BTEX, MTBE, and TBA. Areas that contained high amounts of MTBE at the first sampling event in 1996 showed a noticeable lack of MTBE in 1997, coupled with an occurrence of TBA in relatively high concentrations. Accumulation of TBA indicates that biodegradation of MTBE was occurring.

Aerobic Mineralization

Numerous studies performed over the past decade demonstrated aerobic biodegradation of MTBE, by both indigenous and isolated microorganisms (Salanitro et al. 1994; Park and Cowan 1997; Mo et al. 1997; Hardison et al. 1997; Steffan et al. 1997; Eweis et al. 1997; Bradley et al. 1999; Hanson et al. 1999; Hyman et al. 1998; Pruden et al. 2001; Stringfellow et al. 2000; Deeb et al. 2001). MTBE degrading microorganisms include mixed cultures as well as individual isolated organisms. These microorganisms represent in part the genera Methylobacterium, Thodococcus, Arthrobacter, Nocardia, Pseudomonas, Alcaligenes, and Rhizobium, as well as a Graphium species of filamentous fungus (Mo et al. 1997; Pruden et al. 2001; Hardison et al. 1997). These cultures and organisms were isolated from such diverse media as biotreatment sludges, the fruit of the gingko tree, biofilters and activated carbon, as well as contaminated and uncontaminated soils and sediments.

The presence of easily degradable organics apparently inhibits the degradation of MTBE. Organic matter, hydrocarbons such as isopropanol, acetylene, ethylene, methanol and the BTEX compounds, keytones such as acetone, acids such as pyruvate, t-butanol, butyl formate, as well as TCE have all been found to inhibit the mineralization of MTBE (Mo et al. 1997; Hardison et al. 1997; Bradley et al. 1999; Pruden et al. 2001; Stringfellow et al. 2000). A study by Eweis et al. (1997) suggests that MTBE inhibition by toluene may be a result of nitrogen limitations at excessive carbon loading rates. This hypothesis is supported by the fact that an equivalent MTBE loading caused the same inhibition as did toluene. Deeb et al. (2001) found that addition of benzene or toluene to

an MTBE degrading system caused inhibition of MTBE mineralization with a concurrent lag in benzene or toluene degradation. After the initial lag period, degradation of benzene and toluene proceeded rapidly. However, a study by Pruden et al. (2001) in which Diethyl ether, diisopropyl ether, ethanol, and the BTEX compounds were provided as carbon sources in addition to MTBE provided no evidence of inhibition. In fact, complete removal of both MTBE as well as the additional hydrocarbon species was seen in each case.

Enzyme Mediation

Data from Hyman (1998) indicate that MTBE will be cometabolically oxidized by monooxygenase (MO) enzyme activity that is induced by oxidation of n-alkanes under aerobic conditions. Cometabolism refers to a process in which organisms are able to degrade a substrate without gaining energy or cell mass. It has been found that cell mass yields from MTBE mineralization are often low, further suggesting that MTBE degradation occurs cometabolically. The MO enzyme requires molecular oxygen to oxidize the target chemical. Steffan et al. (1997) analytically confirmed that MO enzyme is utilized by propanotrophs in cometabolically degrading MTBE.

Aerobic cometabolic degradation of MTBE depends strongly on concentrations of surrounding molecular oxygen. The following data strongly suggests that enhanced biodegradation strategies may successfully remove MTBE from the subsurface.

A study by Park and Cowan (1997) investigated the effect of dissolved oxygen on the biodegradability of MTBE by MTBE acclimated cultures. All experiments were conducted in a 16-place Comput-Ox respirometer. Oxygen uptake data was regressed

against time at different concentrations of dissolved oxygen. Initial MTBE concentrations in the experiments were 36.7 mg/L.

Park and Cowen (1997) showed that when dissolved oxygen (DO) concentrations were greater than 2 mg L^{-1}, the MTBE degradation rate was independent of DO; however, when DO fell below 2 mg L^{-1}, which is common in the vicinity of a gasoline release, the MTBE degradation rate decreased dramatically. Furthermore, Park and Cowen concluded that the microbial population capable of degrading MTBE is much more sensitive to DO concentrations than typical organic-degrading microbial populations.

Mo et al. (1997) also found that as oxygen availability increased, the MTBE degradation rate increased. This study found in a laboratory experiment that the MTBE degradation rate in an oxygen rich serum bottle was at least twice as fast as that in an oxygen-limited bottle. Furthermore, biodegradation activities due to oxygen-enhanced air sparging increased significantly over standard air sparging in a field trial. Salanitro et al. (1998) concludes that "the ability to transport O_2 and sustain adequate dissolved O_2 levels . . . is critically important to the success of stimulating the aerobic bioremediation of MTBE."

Each of these studies indicates that MO enzyme activity coupled with the introduction of high levels of molecular oxygen into the subsurface will result in successful MTBE biodegradation. If MTBE is biodegraded as the sole carbon and energy source it is likely that other, more easily degraded compounds are preferentially utilized or competitively inhibit the biodegradation of MTBE. Consequently, the introduction of

molecular oxygen will facilitate the rapid aerobic degradation of these easily degraded compounds, which will then allow the microbes to biodegrade MTBE. Alternatively, if MTBE is biodegraded cometabolically with other aromatics and/or alkanes, then stimulation of MO enzyme activity by addition of molecular oxygen will facilitate enhanced cometabolic biodegradation of MTBE along with these compounds. Under both scenarios, a strategy of enhanced aerobic biodegradation will result in the effective removal of MTBE from the subsurface.

Oxygen is often a limiting factor in field biodegradation. Both field and lab experiments showed that increasing oxygen concentrations positively stimulates the aerobic degradation of MTBE. Field degradation rates will likely be slower than those seen in the lab. This is due to the presence of easily degradable organics as well as temperature effects. Temperature is of specific importance in Montana, where average soil temperatures are often significantly lower than the temperatures at which many lab experiments are run. These factors must be kept in mind when extrapolating laboratory finding to field situations.

CHAPTER 3

EXPERIMENTAL DESIGN AND METHODS

Overview

The purpose of this research was to determine the dependence of the MTBE degradation rate by a bacterial isolate on the concentration of dissolved oxygen. This was accomplished by conducting three microcosm experiments in which a bacterial population was given MTBE as a sole carbon and energy source under different treatments of dissolved oxygen concentration. The disappearance of MTBE over time at the different dissolved oxygen concentrations was measured and analyzed.

Experiments One and Two were identical to each other in methodology. Experiment Two was a repeat of Experiment One with the intention of verifying the results of Experiment One. In Experiments One and Two, a bacterial population was grown in a carbon rich medium and then transferred into microcosm jars where it was given MTBE as a sole carbon source for the first time. The purpose of Experiment Three was to observe the behavior of the MTBE Degradation rate when the bacterial population had previously been sustained on MTBE as a sole carbon and energy source. Thus, the methodology of Experiment Three differed from that of Experiments One and Two only in the manner in which the bacterial culture was grown before being introduced into the microcosms.

The following sections detail the design of the experiments and the methods used.

Bacterial Isolate

The microorganism used in these experiments was PM1. This microorganism was originally isolated at the University of California-Davis from a compost biofilter (Hanson et al. 1999). Strain PM-1 is a gram-negative, uniflagellated rod that produces an extracellular matrix. The bacteria form white pinpoint colonies when grown on R2A agar. Strain PM-1 was found to be a member of the β1 subgroup of *Proteobacteria* by 16S rDNA analysis.

PM1 was obtained on an agar plate of 0.5x R2A directly from the University of California-Davis. Stock cultures of PM1 were prepared from isolated colonies inoculated into R2A broth (Table 3.1). The vials of stock cultures were placed in a -70 °C freezer until they were removed for use. Cultures used in the described experiments were started from the same frozen stock.

Table 3.1 Nutrient Composition of R2A Broth.

Constituent	Concentration [g L^{-1}]
yeast extract	0.5
proteose peptone	0.5
casamino acids	0.5
dextrose	0.5
soluble starch	0.5
sodium pyruvate	0.3
dipotassium hydrogenphosphate	0.3
magnesium sulfate	0.05

Experimental Methods Used in All Experiments Prior to Addition of PM1

Experiments were run in batch format. Microcosms were developed in 120-mL glass serum vials containing aqueous nutrient media, microorganisms, MTBE, and a gaseous headspace.

The glass serum vials used were washed with dish soap and then baked in an oven at 500°C for 3 hours. To ensure the sterility of the vials, the jars were capped with foil that was not removed until the microcosms were assembled in an aseptic environment.

The aqueous media used was a basal salts media (BSM) composed primarily of phosphate and sulfate salts (Table 3.2). Each serum vial received 70 milliliters of fresh basal salts media. Serum vials were then capped using Teflon lined silica septa and aluminum crimp seals.

Table 3.2 Nutrient Composition of Basal Salts Media.

Constituent	Concentration [mg L^{-1}]
KH_2PO_4	17
K_2HPO_4	44
$Na_2HPO_4 \bullet 7H_20$	101
$MgSO_4 \bullet 7H_20$	23
NH_4Cl	3.4
$(NH_4)_2SO_4$	40
$FeCl_3 \bullet 6H_20$	1

For each experiment, a 100-mL stock of R2A was aseptically inoculated from a frozen stock culture. Freshly inoculated cultures of PM1 took 3 to 4 days to reach a culture density of 10^9 CFU mL^{-1}. Cultures were then centrifuged at 6000 rpm for 20 minutes to separate supernatant from cells.

Growth of PM1 Culture: Experiments One and Two

After removing the supernatant, the cells were suspended in fresh BSM containing no extraneous carbon sources. Centrifugation was repeated, with subsequent removal of the supernatant. The culture was finally suspended in fresh BSM to an optical density as shown in Table 3.3. Centrifugation steps were performed to remove all residual carbon sources from the cells.

The suspension was vortexed for 30 seconds to disassociate clumped cells. Three milliliters of the culture was removed using a sterile pipette and added to each microcosm.

Table 3.3 Experiments One and Two: Initial Culture Optical Density.

Experiment #	Initial Culture Optical Density
1	0.63
2	0.68

Growth of PM1 Culture: Experiment Three

After removing the supernatant, the cells were suspended in fresh BSM containing no extraneous carbon sources. Centrifugation was repeated, with subsequent removal of the supernatant. The culture was finally suspended in 100 milliliters of BSM.

A sterile stock solution of 1000 mg L^{-1} MTBE was prepared. From this stock, 2 milliliters were added to the PM1 suspension, for an approximate MTBE concentration of 20 mg L^{-1}. After 4 days, a sample was removed and analyzed for MTBE concentration. The MTBE concentration in the culture was found to be zero. A fresh stock culture of 1000 mg L^{-1} MTBE was prepared, from which 2 milliliters was again added to the suspension.

After 6 days, a sample was again removed and analyzed. The MTBE concentration was found to be zero. The culture was then centrifuged and washed with BSM twice, as had been done previously. The final suspension had an optical density as shown in Table 3.4. From this point forward, the methods used in all three experiments were the same.

Table 3.4 Experiment Three: Initial Culture Optical Density.

Experiment #	Initial Culture Optical Density
3	0.7

Experimental Methods Used in All Experiments After Addition of PM1

The headspace of each serum vial was purged with UHP grade helium. Gas was fed through the septum of the jar using a 20-gauge stainless steel needle connected to tygon tubing, with a separate needle for venting. Each jar was purged to remove ambient gases from the headspace while maintaining atmospheric pressure.

Sufficient helium was removed and oxygen added to achieve three targeted headspace concentrations. Microcosms receiving 'high' treatments had a total of 10 milliliters of oxygen added to a headspace of 50 milliliters, for an initial headspace concentration of approximately 20% oxygen by volume. To achieve a 'medium' treatment, 2.5 milliliters of oxygen were added, for an initial headspace concentration of approximately 5% oxygen by volume. The 'low' treatment received 0.5 milliliters of oxygen for an approximate headspace concentration of 1% oxygen by volume. Target dissolved oxygen concentrations were calculated from target headspace concentrations using Henry's Law.

Sterile controls were prepared by adding 1% sodium azide to the BSM media solution. The purpose of these controls was to determine if any losses in MTBE were occurring due to causes other than biological degradation. The sterile controls were given a target headspace concentration of 20%.

A sterile stock of MTBE at a concentration of 1000 mg L^{-1} was prepared. Each microcosm jar received one milliliter of the MTBE stock solution, for an approximate initial concentration of 14 mg L^{-1}. The microcosms were covered in foil to avoid

photodegradation and placed on a shaking table at 150 rpm and room temperature. The experimental matrix is shown in Table 3.5.

Table 3.5 Experimental Microcosm Matrix.

Microcosm ID	Approximate Dissolved Oxygen Concentration (mg L^{-1})	Approximate MTBE Concentration (mg L^{-1})	Approximate Cell Density (CFU mL^{-1})
low1	0.5	14	10^7
low2	0.5	14	10^7
low3	0.5	14	10^7
medium1	3	14	10^7
medium2	3	14	10^7
medium3	3	14	10^7
high1	7	14	10^7
high2	7	14	10^7
high3	7	14	10^7

Sampling and Analysis Methods

Microcosms were sampled every other day over a seven-day period, for a total of 4 sampling events creating three intervals. Days are numbered starting at day 0, so that interval one is day 0 through day 2, and so forth. At each event, aqueous microcosm samples were removed and measured for MTBE and TBA concentrations and optical density. Gaseous samples were removed from the headspace and measured for nitrogen, oxygen, and carbon dioxide. Six milliliters of aqueous volume was removed from 70 milliliters, amounting to 8.6% of the total volume.

At every sampling event, each microcosm jar was vortexed for 30 seconds to disassociate and resuspend flocked cells. The septum of the jar was sterilized with ethanol. A 1.5-mL aqueous sample was removed using a sterile syringe and needle, from which one milliliter was dispensed to a cuvette for an optical density measurement. The remaining volume was dispensed to a GC sample vial fitted with a 400-μL conical glass insert. The insert allowed for reliable measurement of MTBE at small sample volumes.

Aqueous samples were run on an HP 5890 Gas Chromatograph fitted with a carbon-packed 15-foot stainless steel column. Samples were analyzed for MTBE and TBA concentration. Quantification limits were 500 $\mu g\ L^{-1}$ for MTBE and 1 $mg\ L^{-1}$ for TBA.

MTBE standards of 1, 5, 10 and 20 $mg\ L^{-1}$ were prepared from a fresh 1000 mg L^{-1} stock solution. Standards were prepared in larger vials, and then transferred to 2-mL GC vials with inserts in the same manner in which the microcosm samples were collected. This was to ensure that the standards received the same treatment as the samples.

A headspace sample of 300 microliters was removed from each microcosm, with subsequent manual direct injection into an HP5890 Gas Chromatograph. The column used was stainless steel, packed with 80/120 Carbopack™ B/3% SP™-1500. Samples were analyzed for oxygen, nitrogen, and carbon dioxide. Monitoring nitrogen levels allowed for determination of air contamination in the microcosms. Carbon dioxide levels were monitored to verify that biodegradation was occurring.

All volume removed was replaced from a gaseous stock at the initial headspace concentration. This was done at each sampling event after both the aqueous and the gaseous samples had been removed. Covered microcosms were placed on the shaker table at room temperature between sampling events.

Bacterial Quantification Methods

In order to determine accurate culture density measurements for Experiments One and Two, a bacterial suspension was prepared in the same manner as in Experiments One and Two. After the culture had reached acceptable turbidity, a dilution series was prepared. The optical density of a number of dilutions from the series was measured and recorded. Samples from these selected dilutions were streaked onto agar plates.

The plates were allowed to sit for one week. After one week, the colonies on each plate were counted. Original culture densities were calculated in CFU mL^{-1} and correlated to the optical density measurements. The regression of culture density to optical density for Experiments One and Two is shown in Figure 3.1.

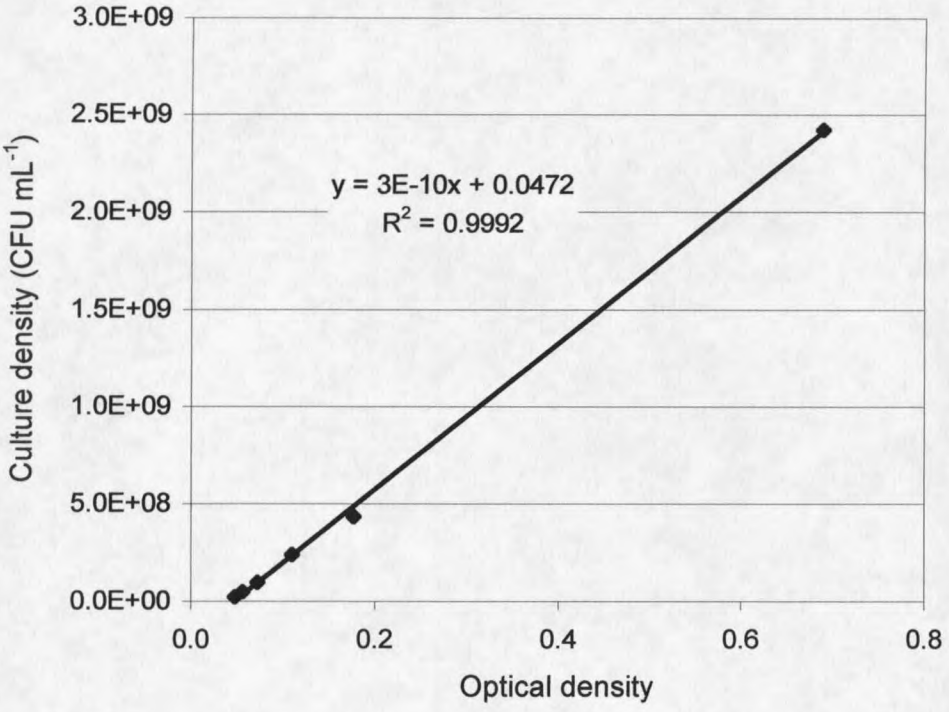

Figure 3.1 Experiments One and Two: Culture Density Versus Optical Density Measurements.

To determine accurate culture densities in Experiment Three, optical density measurements were made of samples taken from each microcosm at the conclusion of the experiment. From these samples, a dilution series was prepared and sampled. Samples were streaked onto agar plates. After one week, the colonies that had grown on the appropriate plates were counted. Three of the plates did not grow colonies and thus could not be counted. From these counts, original culture densities in CFU mL^{-1} were calculated. Culture density was correlated to optical density measurement as shown in Figure 3.2.

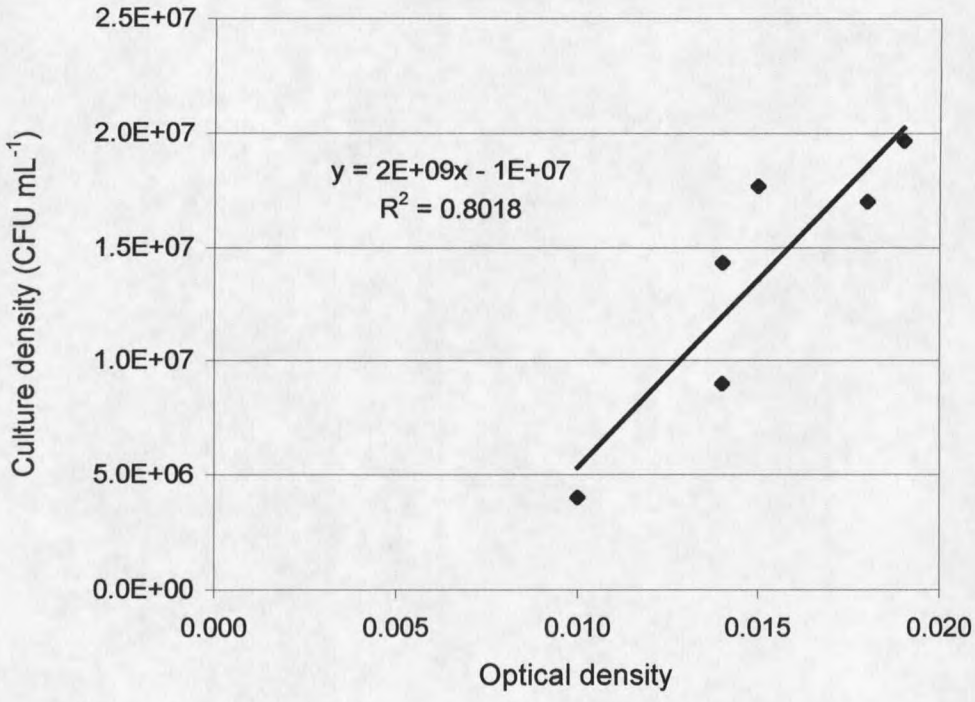

Figure 3.2 Experiment Three: Culture Density Versus Optical Density Measurements.

CHAPTER 4

RESULTS

Determination of the Effect of Increasing Dissolved Oxygen Concentration on the MTBE Degradation Rate

The purpose of Experiments One and Two was to determine the mathematical relationship between dissolved oxygen concentration and MTBE degradation rate, μ. This was achieved by measuring the concentration of MTBE at given time intervals. From these measurements, the degradation rate of MTBE was calculated over a range of dissolved oxygen concentrations. Experiment Two was a repeat of experiment One with the intention of corroborating results.

Initial MTBE concentrations in the microcosms ranged from 10 to 20 mg L^{-1}. This range was chosen as it was known to not be toxic to the microorganisms, and because it was well within the range of detection with the available equipment. Samples were collected every two days over a six-day period, for a total of three intervals per experiment. For each experiment, day 0-2 is referred to as the 'first interval', day 2-4 as the 'second interval', and day 4-6 as the 'third interval'.

Two separate analyses of the data were performed. The first compared MTBE degradation rate to dissolved oxygen concentration. Data analyzed in this manner are referred to as 'unadjusted' data. The units for unadjusted data are mg L^{-1}MTBE $days^{-1}$. The second analysis took the MTBE concentration in each microcosm at the beginning of each interval into account. The purpose of the second analysis was to determine if

MTBE concentration in the microcosms was significant to the mathematical relationship describing the dependence of μ on dissolved oxygen concentration. This was done by dividing MTBE degradation rate by the concentration of MTBE in each microcosm at the start of each interval. This data is referred to as 'adjusted' data. The units for the adjusted data are mg L^{-1}MTBE/mg L^{-1}MTBE•day, or just $days^{-1}$. In the following sections, the units for the adjusted data are given as $days^{-1}$ with the understanding that this is indicative of a degradation rate divided by a concentration. The Michaelis-Menton model fit to both the unadjusted data and adjusted data are shown below. The Hill model can be similarly determined.

Michaelis-Menton: Unadjusted

$$\mu = \frac{\mu_{max} \bullet [O_2]}{K_m + [O_2]}$$

where μ = MTBE degradation rate [mg L^{-1}MTBE]

μ_{max} = maximum MTBE degradation rate [mg L^{-1}MTBE $days^{-1}$]

$[O_2]$ = dissolved oxygen concentration [mg DO L^{-1}]

K_m = half saturation coefficient

Michaelis-Menton: Adjusted

$$\mu = \frac{\mu_{max} \bullet [O_2]}{K_m + [O_2]} \bullet [MTBE]$$

where μ = adjusted MTBE degradation rate [$days^{-1}$]

μ_{max} = maximum adjusted MTBE degradation rate [$days^{-1}$]

$[O_2]$ = dissolved oxygen concentration [mg DO L^{-1}]

K_m = half saturation coefficient

[MTBE] = MTBE concentration at the beginning of the interval [mg MTBE L^{-1}]

In some cases, measured MTBE values would increase from one sample event to the next. This is due to GC response sensitivity over the course of the experiment. GC responses for the standards varied by up to 10%. This means that there was a 10% range of variation for any given data point. This phenomenon was apparent in the control responses, in which MTBE concentration would appear to increase from one sample event to the next. Data points indicating negative changes in MTBE were not included in the regression calculations. In these cases, calculated MTBE degradation rates would be negative. Thus, changes in MTBE concentration of this type are referred to as negative changes.

Carbon dioxide was observed in the medium and high treatment gas samples during the third and in some cases second intervals of each experiment. Carbon dioxide was also observed in some samples of the low treatments during the third interval of each experiment. TBA was detected in the medium and high treatment microcosms, but did not accumulate. These findings indicate that active biodegradation was occurring in the microcosms.

Samples removed from each microcosm at the end of each experiment were streaked onto agar plates. No colonies grew from the samples of the killed controls. The colonies that grew from all other microcosms were white pinpoint colonies, homogenous in nature and identical to the colonies plated from the original stock culture.

Experiment One

Microcosms were adjusted to three treatments of headspace oxygen concentration The dissolved oxygen concentration was then calculated using Henry's Law. The three treatments are referred to as the low, medium, and high treatments. The dissolved oxygen concentration ranges for each treatment over all three intervals are as follows: low (0.30 to 1.2 mg L^{-1} O_2), medium (1.8 to 2.2 mg L^{-1} O_2), and high (5.6 to 7.4 mg L^{-1} O_2). Figure 4.1 shows dissolved oxygen concentrations in each microcosm at the end of each interval over the course of Experiment One. Cell densities for each microcosm are given in Table 4.1. Cell densities are given for the beginning of each interval.

Table 4.1 Experiment One: Cell Density in Each Microcosm at the Beginning of Each Interval.

sample ID	Cell Density CFU mL^{-1}		
	day 0-2	day 2-4	day 4-6
low1	4.5E+07	6.0E+07	3.7E+07
low2	4.5E+07	4.1E+07	4.1E+07
low3	6.0E+07	5.6E+07	3.7E+07
medium1	4.5E+07	4.5E+07	4.8E+07
medium2	5.2E+07	4.5E+07	4.5E+07
medium3	4.8E+07	7.1E+07	5.2E+07
high1	4.5E+07	4.8E+07	5.2E+07
high2	4.1E+07	4.1E+07	6.0E+07
high3	4.1E+07	4.5E+07	5.2E+07

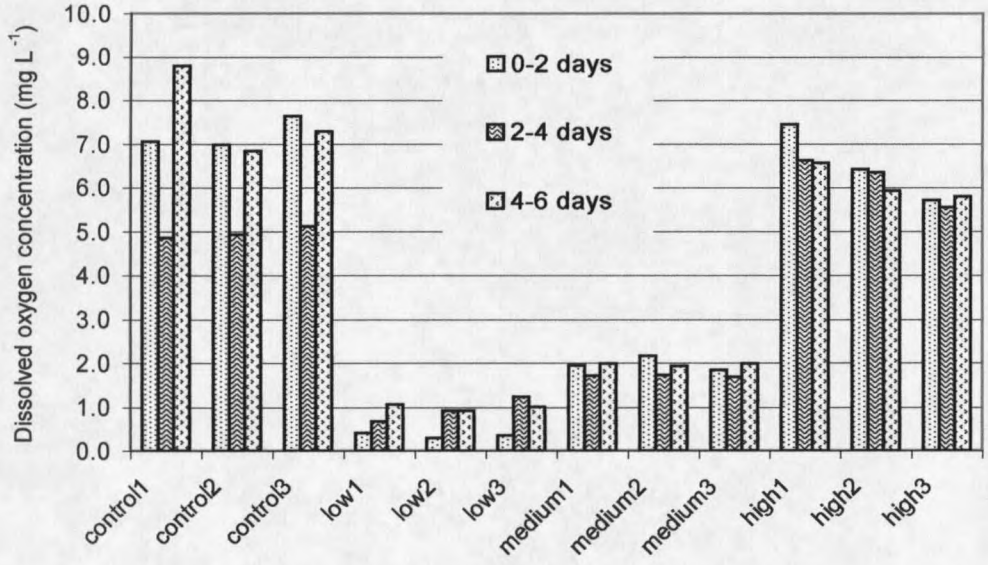

Figure 4.1 Experiment One: Dissolved Oxygen Concentration at the End of Each Interval.

Experiment One: Unadjusted Data

Figure 4.2 shows MTBE degradation rate, μ, as a function of dissolved oxygen concentration for all three intervals of Experiment One. During the first interval, MTBE degradation rates increased with increasing dissolved oxygen concentration. During the second interval, μ initially increases between the low and medium treatments, and then remains fairly constant between the medium and high treatments. During the third interval, μ increases between the low and medium treatments.

The range of MTBE degradation rates for the low treatment was from 0.43 to 0.52 mg L^{-1} MTBE $days^{-1}$ for the first interval, 0.02 to 0.41 mg L^{-1} MTBE $days^{-1}$ for the second

interval, and 0.58 to 1.14 mg L^{-1} MTBE $days^{-1}$ for the third interval. For the medium treatment, MTBE degradation rates for the first interval ranged from 1.56 to 2.11 mg L^{-1} MTBE $days^{-1}$, from 2.08 to 2.49 mg L^{-1} MTBE $days^{-1}$ for the second interval, and from 1.4 to 1.6 mg L^{-1} MTBE $days^{-1}$ for the third interval. At the high treatment, the MTBE degradation rates varied from 2.62 to 2.81 mg L^{-1} MTBE $days^{-1}$ during the first interval, from 1.96 to 2.50 mg L^{-1} MTBE $days^{-1}$ during the second interval, and dropped to 1.46 mg L^{-1} MTBE $days^{-1}$ for the third interval. The MTBE degradation rate for two of the microcosms at the high treatment for the third interval could not be determined as the MTBE concentration in these microcosms decreased to below the detection limit at some undetermined point during the interval. Thus only one data point is reported for this interval.

Note the high amount of variability in the control data, shown in the open symbols. This is most likely due to variation of GC response within a 10% range. In the two subsequent experiments, control samples showed the same wide variation in 'disappearance rate'. The MTBE disappearance in the controls is attributable to MTBE removal from the controls in all manners besides biodegradation. The most likely cause for this variation was identified as variation in response due to a lack of GC sensitivity. A statistical analysis is later performed to determine if the treatment data is significantly different than the control data.

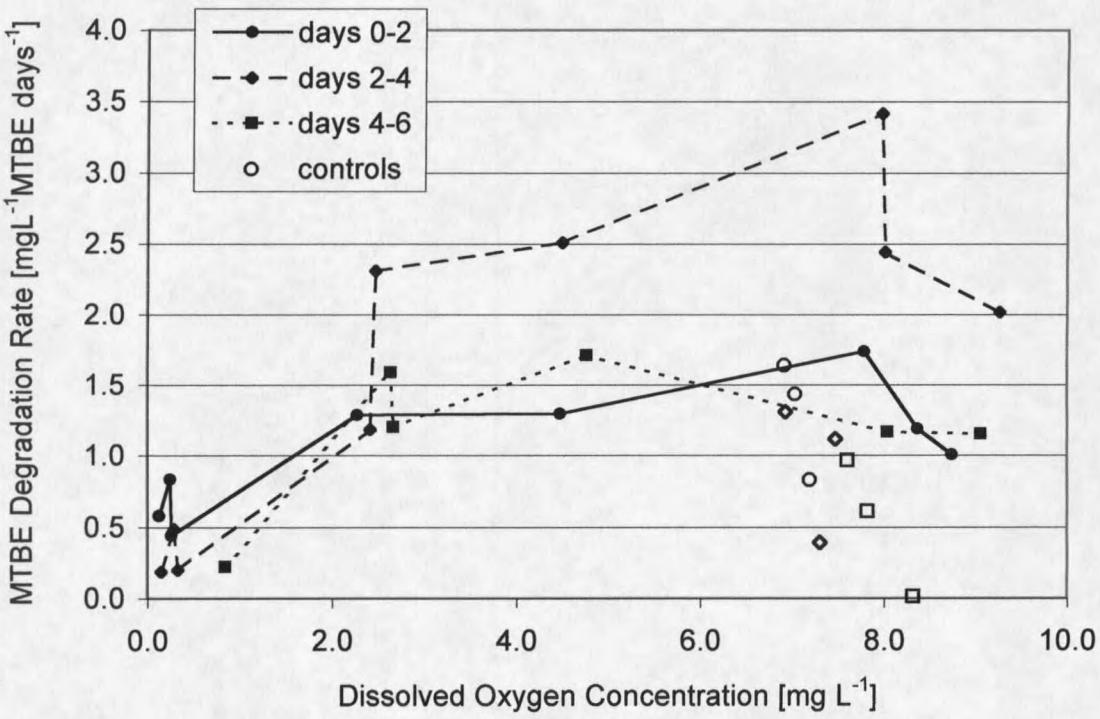

Figure 4.2 Experiment One: Unadjusted Data. MTBE Degradation Rate Versus Dissolved Oxygen Concentration.

Experiment One: Adjusted Data

In this analysis, the data show an increasing trend in adjusted MTBE degradation rates with dissolved oxygen concentration. Figure 4.3 shows $\mu_{adjusted}$ as a function of dissolved oxygen concentration. The first two intervals are characterized by accelerating μ between the low and medium treatments. For these intervals, MTBE degradation rate remains fairly constant between the medium and high treatments. During the third interval, $\mu_{adjusted}$ consistently increases with increasing dissolved oxygen concentration.

The range of adjusted MTBE degradation rates for the low treatment was from 0.04 to 0.06 $days^{-1}$ during the first interval, from 0.0018 to 0.04 $days^{-1}$ for the second

interval, and from 0.07 to 0.11 $days^{-1}$ for the third interval. For the medium treatment, the range of adjusted MTBE degradation rates varied from 0.15 to 0.19 $days^{-1}$ during the first interval, 0.29 to 0.37 $days^{-1}$ over the second interval, and from 0.65 to 0.66 $days^{-1}$ for the third interval. Adjusted MTBE degradation rates for the high treatment varied from 0.23 to 0.24 $days^{-1}$ during the first interval, from 0.36 to 0.40 $days^{-1}$ during the second interval, and was 0.72 $days^{-1}$ for the third interval.

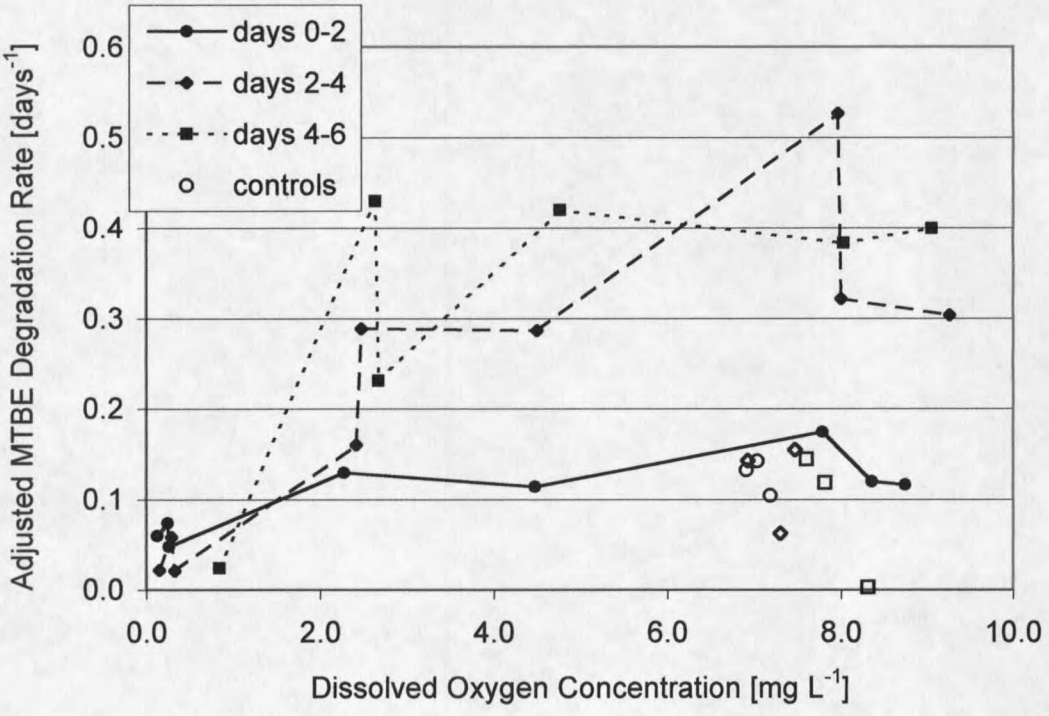

Figure 4.3 Experiment One: Adjusted Data. MTBE Degradation Rate Versus Dissolved Oxygen Concentration .

Experiment Two

Microcosms in Experiment Two were adjusted to three treatments of oxygen concentration. The dissolved oxygen concentration ranges for each microcosm over the course of Experiment Two are shown graphically in Figure 4.4. The ranges for each treatment over all three intervals are as follows: low (0.13 to 0.84 mg L^{-1} O_2), medium (2.3 to 4.8 mg L^{-1} O_2), and high (7.8 to 9.3 mg L^{-1} O_2). Table 4.2 gives cell densities for each microcosm over the course of Experiment Two.

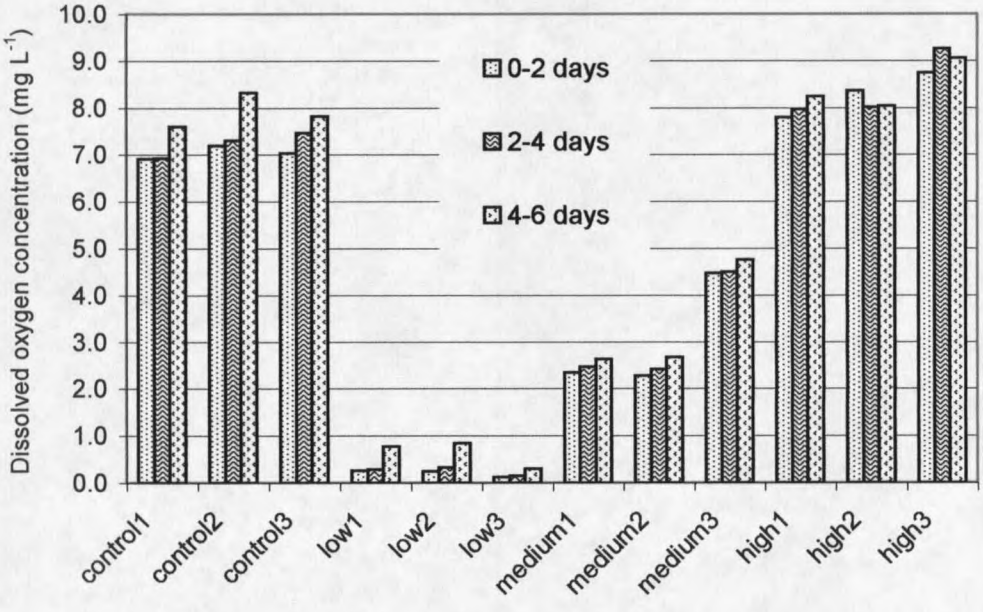

Figure 4.4 Experiment Two: Dissolved Oxygen Concentration at the End of Each Interval.

Table 4.2 Experiment Two: Cell Density in Each Microcosm at the Beginning of Each Interval.

sample ID	Cell Density CFU mL^{-1}		
	day 0-2	day 2-4	day 4-6
low1	2.6E+07	2.2E+07	3.0E+07
low2	3.0E+07	2.2E+07	1.8E+07
low3	2.6E+07	2.2E+07	2.6E+07
medium1	3.0E+07	3.3E+07	4.5E+07
medium2	3.0E+07	3.0E+07	3.3E+07
medium3	2.6E+07	3.3E+07	4.5E+07
high1	3.3E+07	3.7E+07	4.1E+07
high2	2.6E+07	3.0E+07	4.1E+07
high3	3.0E+07	3.7E+07	3.3E+07

Experiment Two: Unadjusted Data

In this experiment, μ increased between the low and high treatments of dissolved oxygen for all three intervals. Values of μ remained fairly constant between the medium and high dissolved oxygen treatments for all three intervals. Figure 4.5 is a graph of μ as a function of dissolved oxygen concentration. The degradation rate does appear to be more dependent on dissolved oxygen concentration at lower concentrations. At higher concentrations of DO (above approximately 2 mg L^{-1}) MTBE degradation rate is much less dependent on oxygen concentration and in fact becomes fairly constant.

MTBE degradation rates for the low treatment varied from 0.44 to 0.58 mg L^{-1} MTBE $days^{-1}$ during the first interval, and from 0.19 to 0.49 mg L^{-1} MTBE $days^{-1}$ during the second interval. The value of μ for the only measurable data point in the third interval was 0.22 mg L^{-1} MTBE $days^{-1}$. At the medium treatment, the only point for which μ could be calculated during the first interval had a μ of 1.29 mg L^{-1} MTBE $days^{-1}$.

Degradation rates ranged from 1.18 to 2.51 mg L^{-1} MTBE $days^{-1}$ during the second interval, and from 1.2 to 1.71 mg L^{-1} MTBE $days^{-1}$ during the third interval. The MTBE degradation rates for the high treatment varied from 1.0 to 1.74 mg L^{-1} MTBE $days^{-1}$ for the first interval, 2.02 to 3.42 mg L^{-1} MTBE $days^{-1}$ during the second interval, and 1.15 to 1.16 mg L^{-1} MTBE $days^{-1}$ during the third interval.

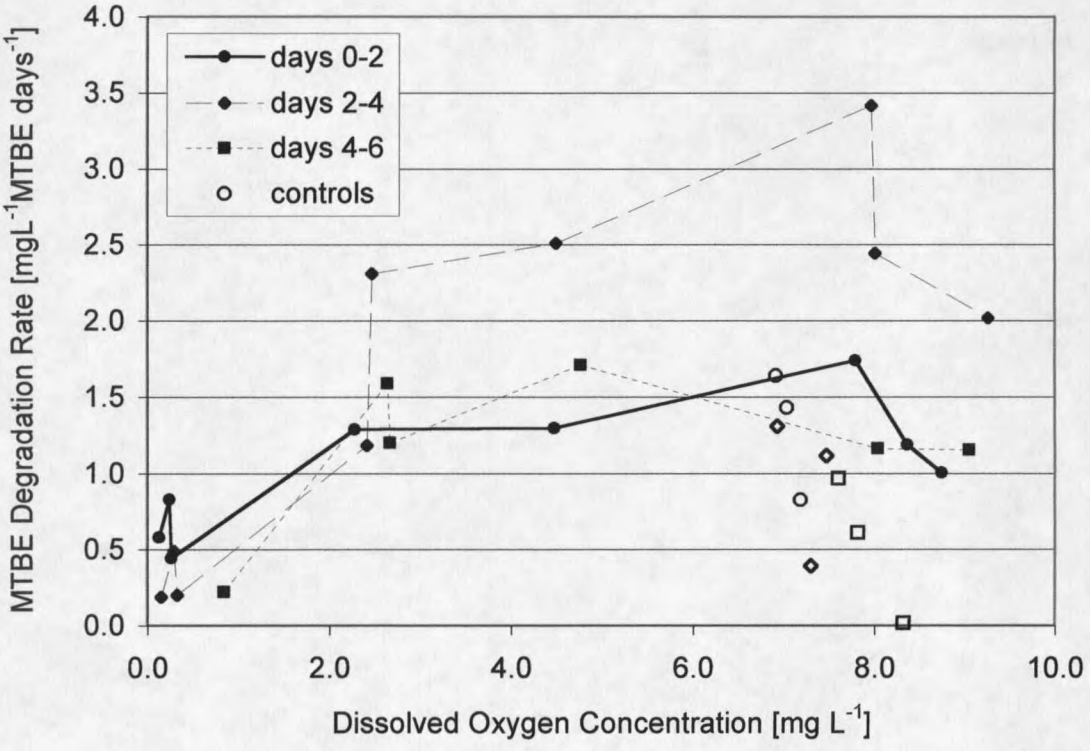

Figure 4.5 Experiment Two: Unadjusted Data. MTBE Degradation Rate Versus Dissolved Oxygen Concentration.

Experiment Two: Adjusted Data

As in Experiment One, data for each microcosm in Experiment Two were also analyzed after being normalized to MTBE concentration at the beginning of each interval. The data for all three intervals is characterized by an initial region of accelerating $\mu_{adjusted}$, followed by a region in which $\mu_{adjusted}$ levels off (Figure 4.6).

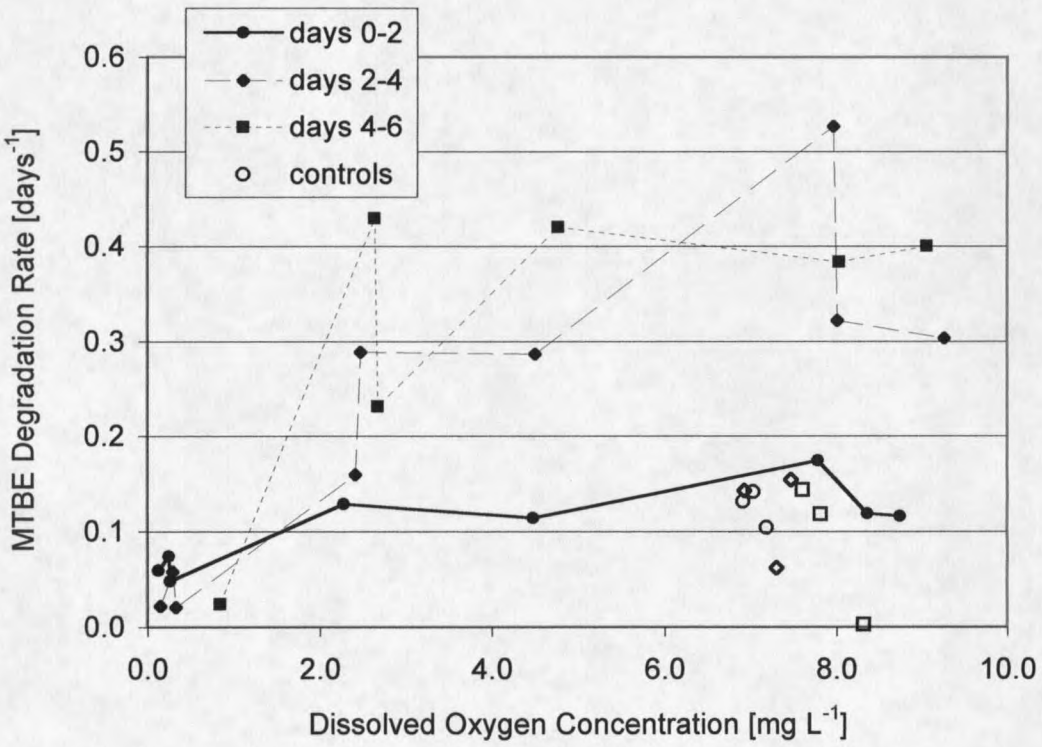

Figure 4.6 Experiment Two: Adjusted Data. MTBE Degradation Rate Versus Dissolved Oxygen Concentration.

Adjusted MTBE degradation rates varied at the low DO treatment between 0.05 and 0.07 $days^{-1}$ for the first interval, between 0.02 and 0.06 $days^{-1}$ during the second interval, and was 0.02 $days^{-1}$ for the third interval. At the medium treatment, the adjusted MTBE degradation rates ranged from 0.11 to 0.13 $days^{-1}$ during the first

interval, from 0.16 to 0.29 $days^{-1}$ during the second interval, and from 0.23 to 0.43 $days^{-1}$ over the third interval. The adjusted MTBE degradation rates for the high treatment varied from 0.12 to 0.17 $days^{-1}$ during the first interval, from 0.30 to 0.53 $days^{-1}$ over the second interval, and from 0.38 to 0.40 $days^{-1}$ during the third interval.

Comparison of Experiments One and Two

Experiment Two was conducted to verify the findings of Experiment One. Figure 4.7 is a plot of the composite data from all three intervals of Experiments One and Two. The trend of the data is similar for all data series. The data show the characteristics of a Michaelis-Menton curve. The data are characterized by an initial region in which MTBE degradation rate increases with increasing dissolved oxygen concentration. This is followed by a region in which the MTBE degradation rate does not accelerate as quickly with increases in dissolved oxygen concentration.

Small variations between the experiments may have caused the differences in the data between the two experiments. It is worth noting that the maximum MTBE degradation rates seen in Experiment Two are generally lower than those in Experiments One. This may be due to the fact that the culture densities in Experiment Two were lower than those in Experiment One. It was noted in experiments not presented in this thesis that higher cell densities corresponded to faster MTBE degradation rates.

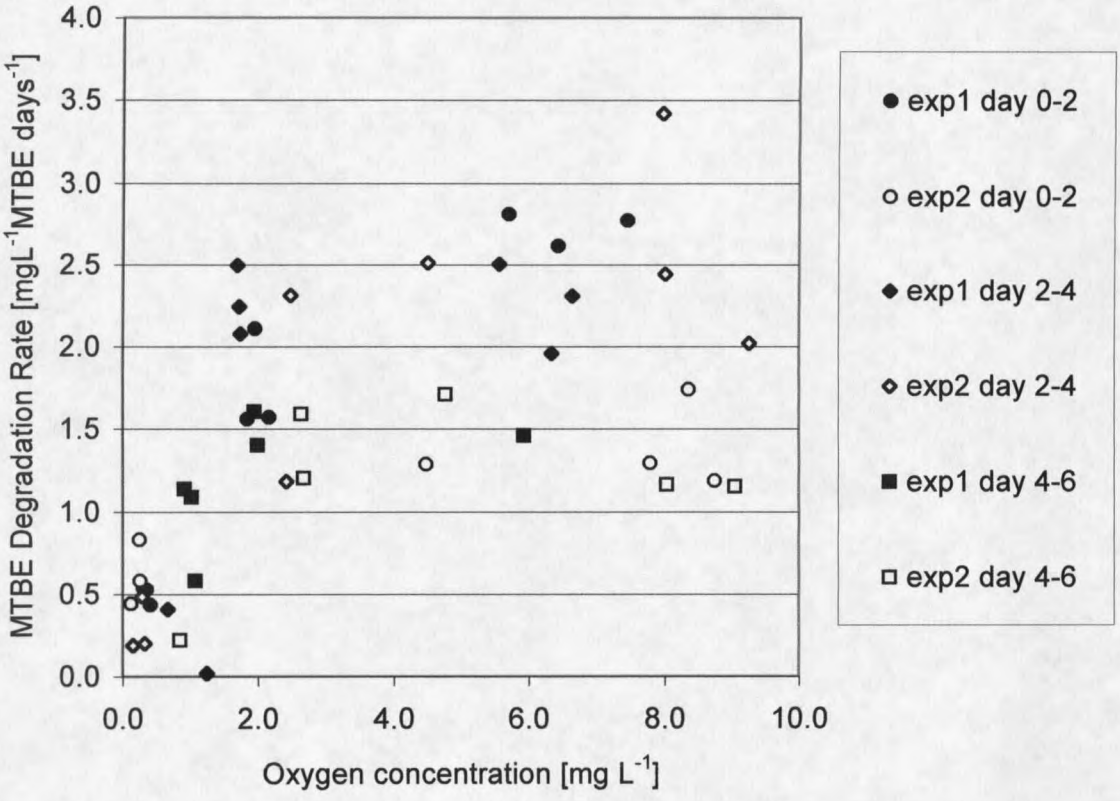

Figure 4.7 Comparison of Data from Experiments One and Two.

Experiment Three

The primary purpose of Experiment Three was to determine the effect of allowing a culture of PM1 to acclimate to MTBE before the culture was introduced into the microcosm environment. The total time that the culture was exposed to MTBE prior to the experiment was 10 days. A secondary purpose of Experiment Three was to more closely bracket the region of rapidly increasing MTBE degradation rates. This was accomplished by adjusting the three dissolved oxygen treatments to lower dissolved oxygen concentrations.

Figure 4.8 shows the dissolved oxygen concentration in each microcosm over the duration of Experiment Three. The dissolved oxygen concentration ranges for each treatment over all three intervals were as follows: low (0.07 to 0.41 mg L^{-1} O_2), medium (0.9 to 2.0 mg L^{-1} O_2), and high (2.8 to 4.8 mg L^{-1} O_2). These treatments represent lower values of dissolved oxygen concentration for all three treatments as compared to Experiments One and Two. Table 4.3 gives the cell densities for each microcosm at each sample event in Experiment Three.

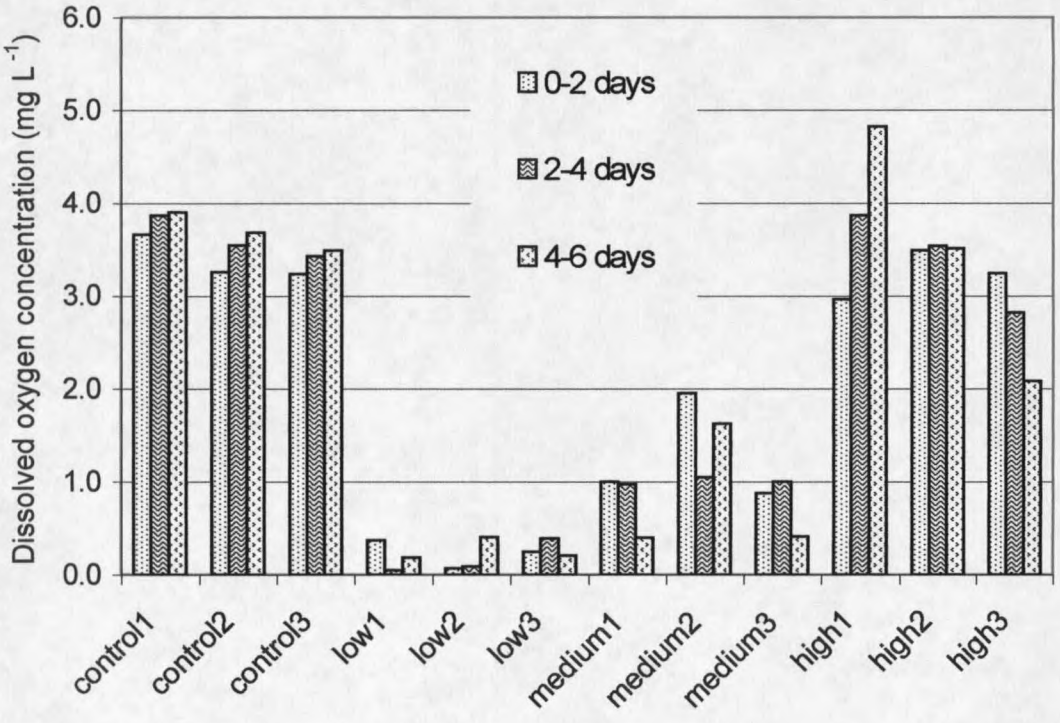

Figure 4.8 Experiment Three: Dissolved Oxygen Concentration in Each Microcosm at the End of Each Interval.

Table 4.3 Experiment Three: Cell Density in Each Microcosm at the Beginning of Each Interval.

sample ID	Cell Density CFU mL^{-1}		
	day 0-2	day 2-4	day 4-6
low1	1.40E+07	6.00E+06	4.00E+06
low2	1.40E+07	8.00E+06	1.00E+07
low3	1.80E+07	8.00E+06	1.40E+07
medium1	1.40E+07	8.00E+06	1.40E+07
medium2	1.20E+07	8.00E+06	1.40E+07
medium3	1.40E+07	1.20E+07	1.80E+07
high1	1.40E+07	8.00E+06	1.60E+07
high2	1.40E+07	1.00E+07	1.60E+07
high3	1.40E+07	1.00E+07	2.00E+07

Experiment Three: Unadjusted data

In Experiment Three, there is a wider variation in μ at all three dissolved oxygen concentrations for each interval than is seen in Experiments One and Two (Figure 4.9). During the first interval, the wide variation in μ at the low dissolved oxygen treatment levels off somewhat at the medium and high treatments. In the second interval, μ accelerates rapidly between the low and medium dissolved oxygen treatments, and then varies widely at the high dissolved oxygen treatment. During the third interval, μ varies widely at all three treatment levels. This wide variation in the data may be due to the fact that biodegradation rates by acclimated bacteria is dependent on both dissolved oxygen concentration as well as MTBE concentration.

The MTBE degradation rates for the low dissolved oxygen treatment varied from 0.04 to 3.14 mg L^{-1} MTBE $days^{-1}$ for the first interval, from 0.22 to 0.56 mg L^{-1} MTBE $days^{-1}$ for the second interval, and from 0.95 to 2.30 mg L^{-1} MTBE $days^{-1}$ for the third interval. For the medium treatment, MTBE degradation rates varied from 0.93 to 1.45 mg L^{-1} MTBE $days^{-1}$ during the first interval, from 1.71 to 1.75 mg L^{-1} MTBE $days^{-1}$ for the second interval, and 3.65 to 5.95 mg L^{-1} MTBE $days^{-1}$ during the third interval. For the high treatment, μ ranged between 0.83 and 1.91 mg L^{-1} MTBE $days^{-1}$ during the first interval, from 1.96 to 4.83 mg L^{-1} MTBE $days^{-1}$ during the second interval, and from 1.07 to 6.20 mg L^{-1} MTBE $days^{-1}$ during the third interval.

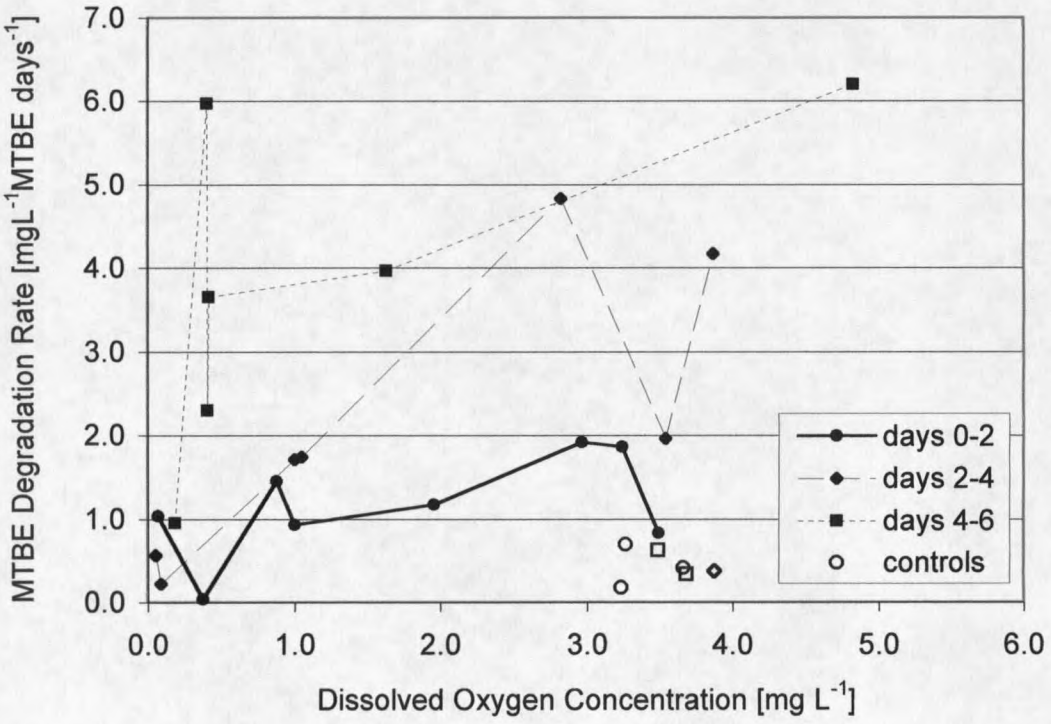

Figure 4.9 Experiment Three: Unadjusted Data. MTBE Degradation Rate Versus Dissolved Oxygen Concentration.

Experiment Three: Adjusted Data

Adjusting the data for initial MTBE concentration causes there to be less variation in the data (Figure 4.10). In intervals one and three, $\mu_{adjusted}$ accelerates between the low and medium treatments, and then remains fairly constant between the medium and high treatments. $\mu_{adjusted}$ in the second interval continues to increase with increasing dissolved oxygen concentration.

Taking the initial MTBE concentration into account appears to significantly improve the model. There is much less variation in the adjusted data than was seen in the unadjusted data. This suggests that in Experiment Three, the behavior of degradation rate is more dependent on MTBE concentration than in Experiments One and Two. It may be that as the bacteria become acclimated to MTBE degradation, biodegradation rates are not as affected by enzyme activity and instead are influenced by MTBE concentration.

The adjusted MTBE degradation rates for the low dissolved oxygen treatments varied from 0.004 to 0.49 $days^{-1}$ during the first interval, from 0.01 to 0.06 $days^{-1}$ during the second interval, and 0.11 to 0.13 $days^{-1}$ during the third interval. At the medium dissolved oxygen treatment, adjusted MTBE degradation rates ranged from 0.06 to 0.10 $days^{-1}$ for the first interval, is a constant 0.15 $days^{-1}$ for both data points measured in the second interval, and was 0.46 $days^{-1}$ in all three microcosms for the third interval. At the high treatment, adjusted MTBE degradation rates ranged from 0.08 to 0.11 $days^{-1}$ over the first interval, from 0.20 to 0.33 $days^{-1}$ for the second interval, and equaled 0.47 $days^{-1}$ for all three microcosms during the third interval.

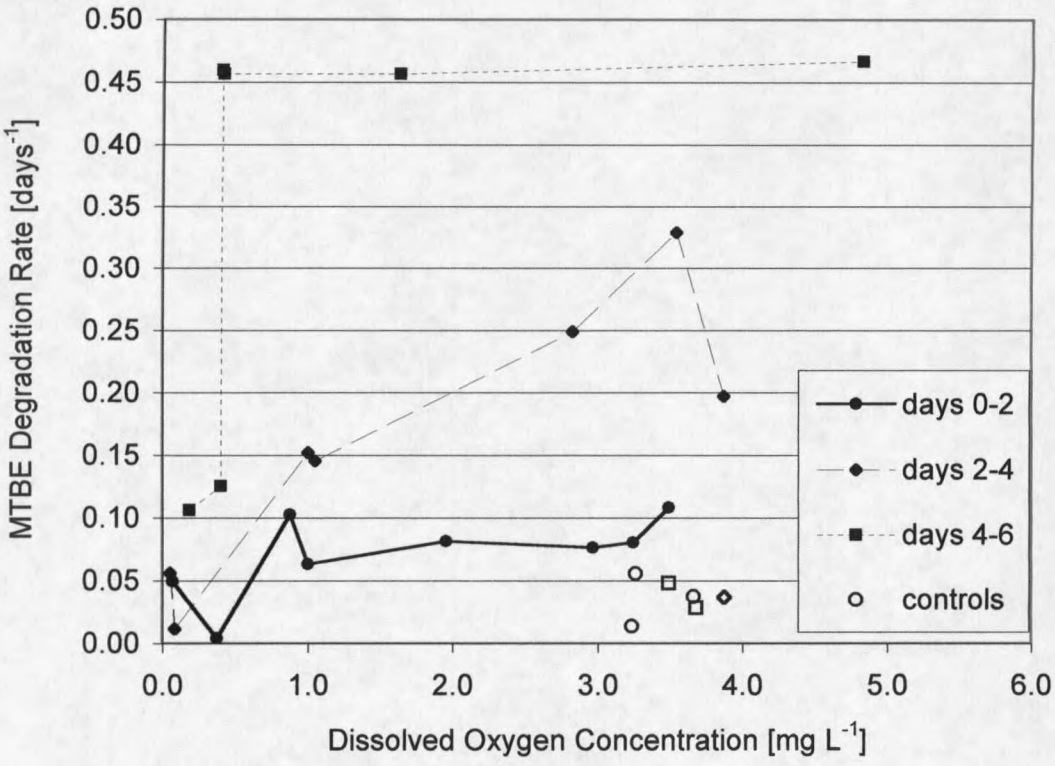

Figure 4.10 Experiment Three: Adjusted Data. MTBE Degradation Rate Versus Dissolved Oxygen Concentration.

Fitting the Data to the Michaelis-Menton and Hill Models

The data was fit to the Michaelis-Menton and Hill models using the *genfit* function in MathCad. The *genfit* function determines the parameters that make a function f(x) with n parameters best approximate the data given in a data set composed of x and y components. This form of nonlinear regression is based on minimizing the sum of the squares of the residuals. The algorithm uses a Taylor series expansion to express the original non-linear equation in an approximate, linear form. The Taylor series is

truncated after the first derivative, which is approximated by the partial first derivatives of the function with respect to each unknown parameter. The solution calculation proceeds iteratively, converging on a solution within a given tolerance. The tolerance for the MathCad solution is 0.001.

Figures 4.11 through 4.28 show the model fits for both the Michaelis-Menton and Hill models for all three intervals of each experiment for both the unadjusted and adjusted data. For ease of comparison, a plot of model fit to the adjusted data for each experimental interval follows a plot of the models fit to the unadjusted data.

Experimental intervals are modeled separately because of the fact that MTBE concentrations in the microcosms may be affecting the observed degradation rates. As the experiment proceeds, MTBE concentration within the microcosm decreases. If degradation rates are dependent not only on dissolved oxygen concentration but also on MTBE concentration, then the decrease in MTBE concentration would affect reaction rates. Based on this, different values of μ_{max} would be expected from the model. In the case of Experiments One and Two, the models are also fit to the composite data from the three intervals of each experiment. This is done to determine the general trend in the experimental data, as well as to determine a value of dissolved oxygen common to the composite data below which MTBE biodegradation rates are inhibited.

Michaelis-Menton and Hill Model Fits: Unadjusted Data.

Table 4.4 is a summary of the fitted parameters, μ_{max}, K_m and n, as well as calculated R^2 values for each model fit. R^2 is the fraction of total squared error that is explained by the model. R^2 alone is not satisfactory for determining which model most

accurately predicts the behavior of the data. The root mean squared error (RMSE) is a measure of the variance between the model and observed data. Low values of RMSE indicate that observed values do not deviate greatly from predicted values. Similarly, large values of RMSE occur when there is a lot of deviation of observed values from the model. The RMSE is of the same order of magnitude as the observed and predicted values. For this reason, it is not appropriate to compare values of RMSE between the adjusted data and the unadjusted data. Comparing values of RMSE within the adjusted data or within the unadjusted data along with comparisons of R^2 provides a means of determining the relative goodness of fit of the two models.

For this analysis, an R^2 of 0.7 is taken as an arbitrary point above which the model is found satisfactory. The calculated R^2 values for the Michaelis-Menton model are at or above 0.7 for 4 of the 9 data sets. The Hill model is at or above 0.7 for 6 of the 9 data sets. Table 4.5 gives a comparison of R^2 and RMSE values for the Michaelis-Menton and Hill models for the unadjusted data.

Table 4.5 Unadjusted data: Comparison of R^2 Values from Michaelis-Menton and Hill Models.

		R^2		RMSE	
Experiment #	Interval (days)	Michaelis-Menton	Hill	Michaelis-Menton	Hill
1	0-2	0.96	0.96	0.18	0.18
1	2-4	0.43	0.94	0.69	0.23
1	4-6	0.44	0.51	0.25	0.23
2	0-2	0.68	0.68	0.22	0.22
2	2-4	0.83	0.86	0.46	0.45
2	4-6	0.46	0.89	0.35	0.22
3	0-2	0.44	0.50	0.49	0.40
3	2-4	0.69	0.72	0.89	0.84
3	4-6	0.47	0.11	1.35	1.80

Table 4.4 Unadjusted Data: Michaelis-Menton and Hill Model Parameters.

Experiment #	Interval (days)		μ_{max} (mg L^{-1}MTBE $days^{-1}$)	K_m (mg L^{-1})	n	R^2
1	0-2	Michaelis-Menton	3.62	0.69	1.00	0.96
		Hill	3.50	1.97	1.05	0.96
1	2-4	Michaelis-Menton	2.93	0.46	1.00	0.43
		Hill	2.26	1.31	74.45	0.94
1	4-6	Michaelis-Menton	1.80	0.25	1.00	0.44
		Hill	1.55	0.81	2.48	0.51
2	0-2	Michaelis-Menton	1.36	0.08	1.00	0.68
		Hill	1.39	0.25	0.88	0.68
2	2-4	Michaelis-Menton	3.32	0.68	1.00	0.83
		Hill	2.96	1.66	1.28	0.86
2	4-6	Michaelis-Menton	1.63	0.37	1.00	0.46
		Hill	1.36	0.92	17.46	0.89
3	0-2	Michaelis-Menton	1.40	0.002	1.00	0.44
		Hill	2.80	2.86	0.41	0.50
3	2-4	Michaelis-Menton	5.43	0.58	1.00	0.69
		Hill	3.65	1.04	6.56	0.72
3	4-6	Michaelis-Menton	4.44	0.05	1.00	0.47
		Hill	5.06	0.28	3.40	0.11

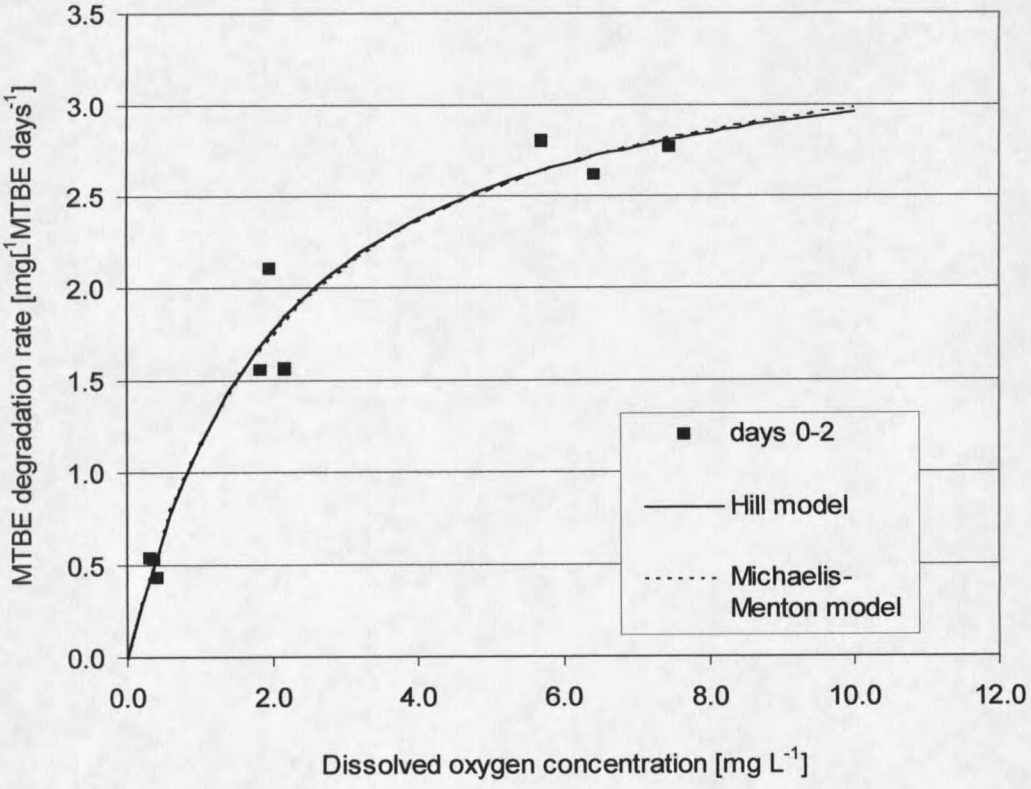

Figure 4.11 Experiment One: Unadjusted Data, Days 0-2. MTBE Degradation Rate as a Function of Dissolved Oxygen Concentration. Michaelis-Menton Model: $\mu_{max} = 3.6$, $K_m = 0.69$, $R^2 = 0.96$. Hill model: $\mu_{max} = 3.5$, $K_m = 1.97$, $n = 1.05$, $R^2 = 0.96$.

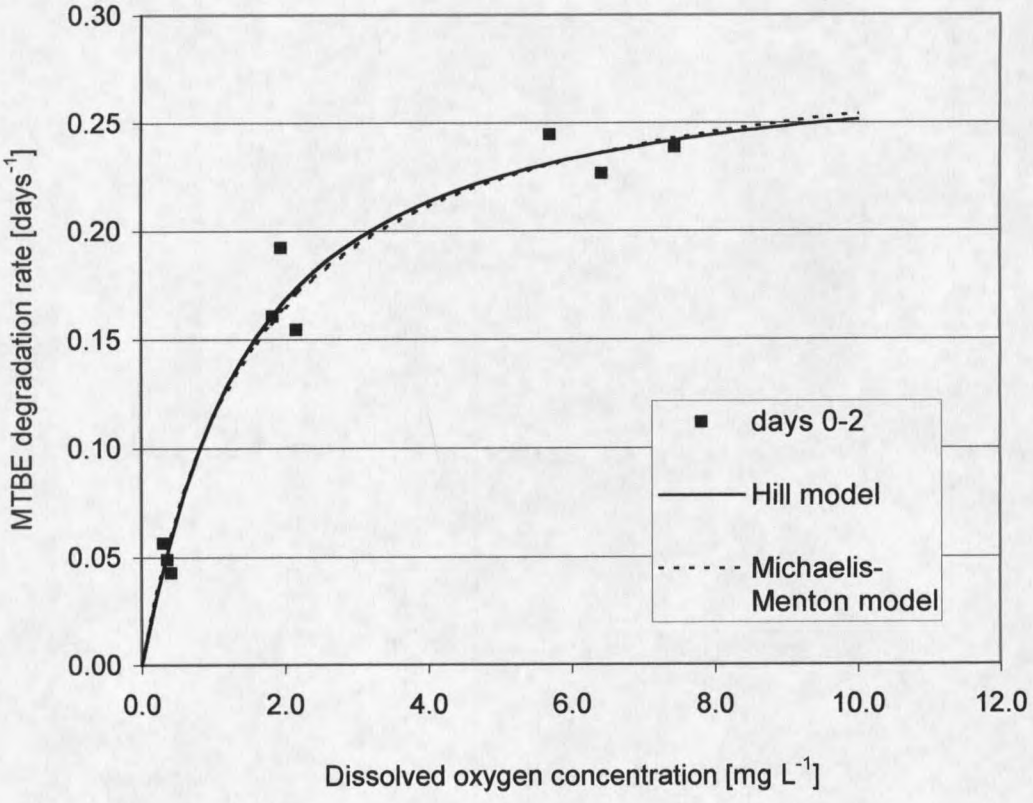

Figure 4.12 Experiment One: Adjusted Data, Days 0-2. MTBE Degradation Rate as a Function of Dissolved Oxygen Concentration. Michaelis-Menton model: $\mu_{max} = 0.29$, $K_m = 0.50$, $R^2 = 0.97$. Hill model: $\mu_{max} = 0.28$, $K_m = 0.45$, $n = 1.09$, $R^2 = 0.97$.

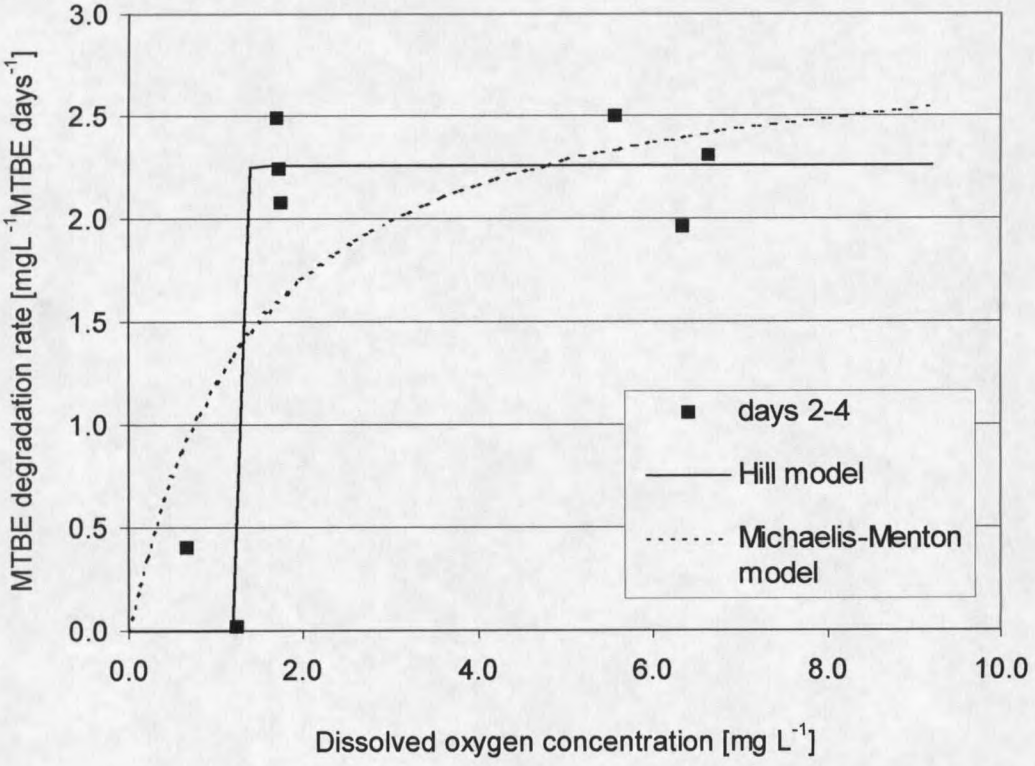

Figure 4.13 Experiment One: Unadjusted Data, Days 2-4. MTBE Degradation Rate as a Function of Dissolved Oxygen Concentration. Michaelis-Menton model: $\mu_{max} = 2.9$, $K_m = 0.46$, $R^2 = 0.43$. Hill model: $\mu_{max} = 2.3$, $K_m = 1.31$, $n = 74.45$, $R^2 = 0.94$.

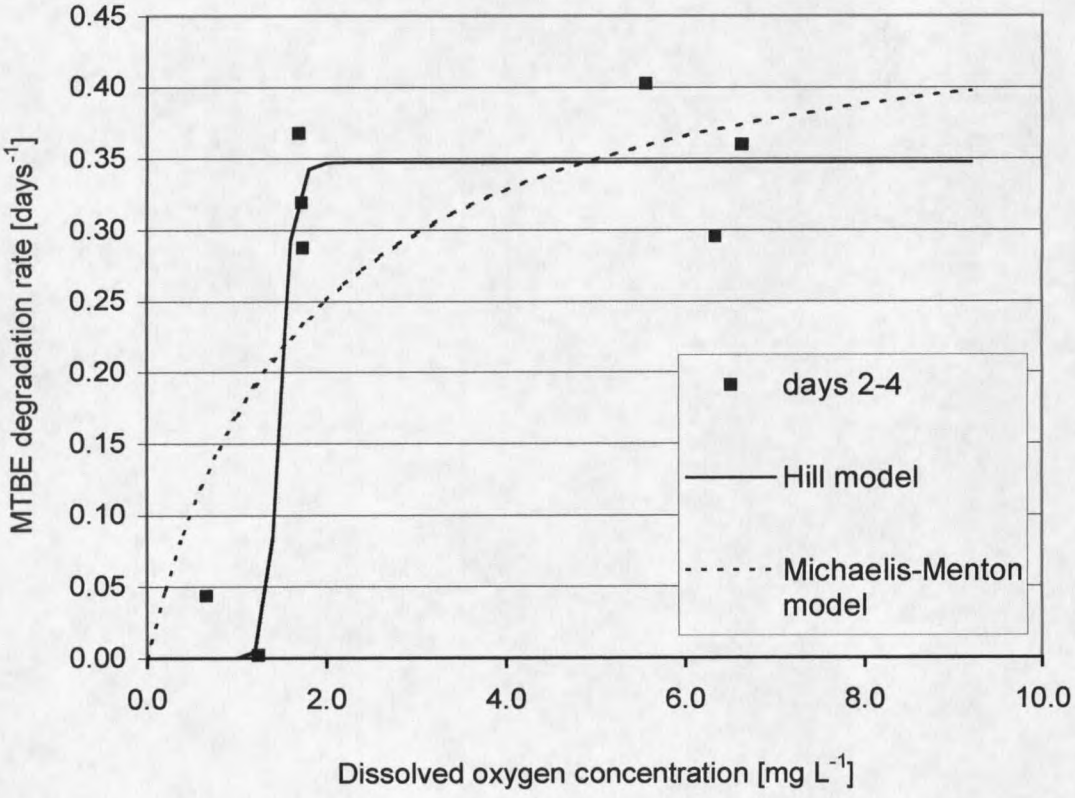

Figure 4.14 Experiment One: Adjusted Data, Days 2-4. MTBE Degradation Rate as a Function of Dissolved Oxygen Concentration. Michaelis-Menton model: $\mu_{max} = 0.48$, $K_m = 0.58$, $R^2 = 0.53$. Hill model: $\mu_{max} = 0.35$, $K_m = 0.48$, $n = 20.26$, $R^2 = 0.93$.

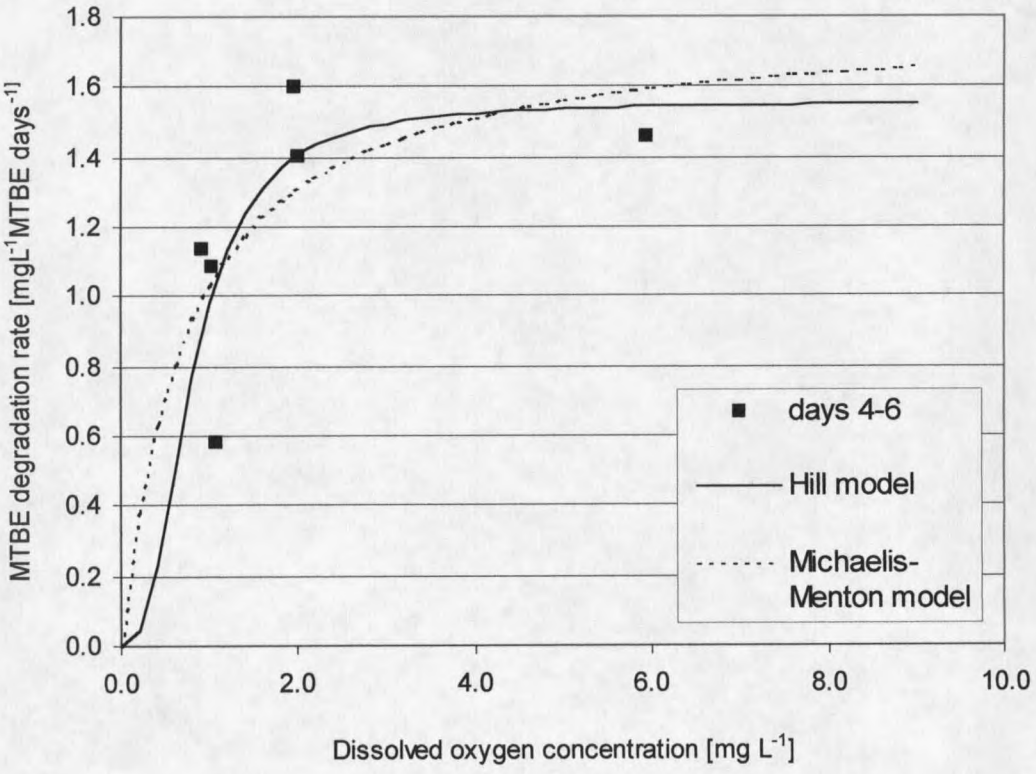

Figure 4.15 Experiment One: Unadjusted Data, Days 4-6. MTBE Degradation Rate as a Function of Dissolved Oxygen Concentration. Michaelis-Menton model: $\mu_{max} = 1.8$, $K_m = 0.25$, $R^2 = 0.44$. Hill model: $\mu_{max} = 1.6$, $K_m = 0.81$, $n = 2.48$, $R^2 = 0.51$.

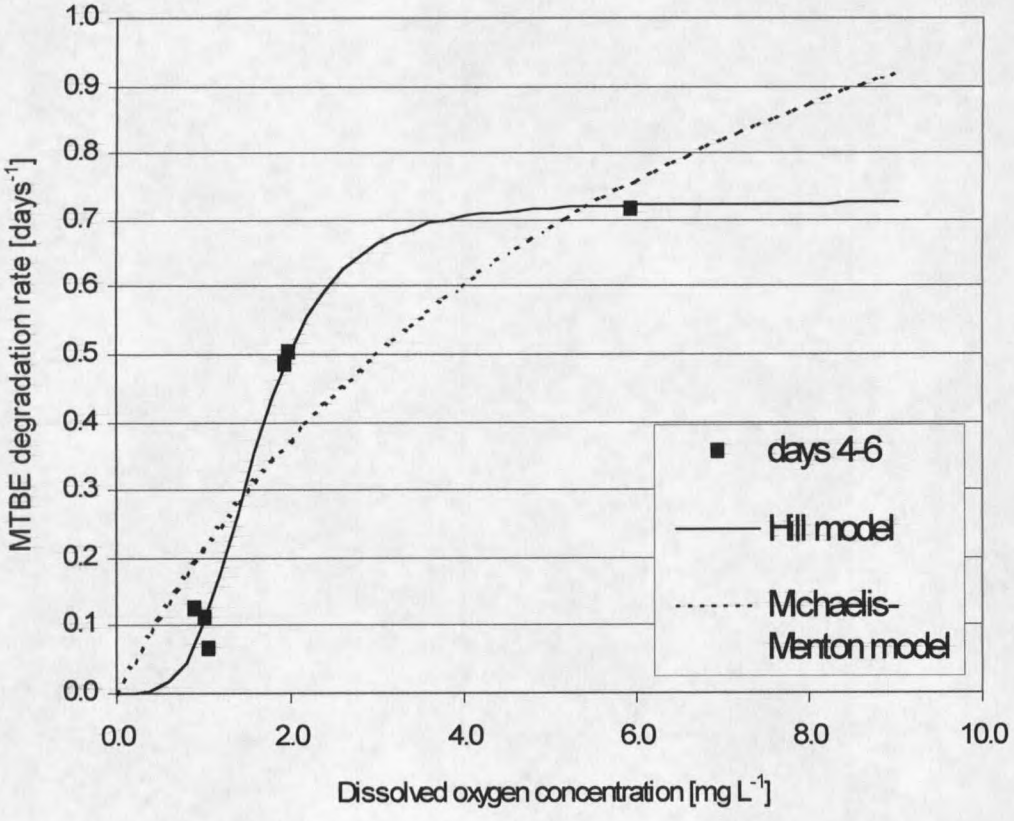

Figure 4.16 Experiment One: Adjusted Data, Days 4-6. MTBE Degradation Rate as a Function of Dissolved Oxygen Concentration. Michaelis-Menton model: $\mu_{max} = 1.6$, $K_m = 2.2$, $R^2 = 0.81$. Hill model: $\mu_{max} = 0.73$, $K_m = 0.54$, $n = 3.78$, $R^2 = 0.98$.

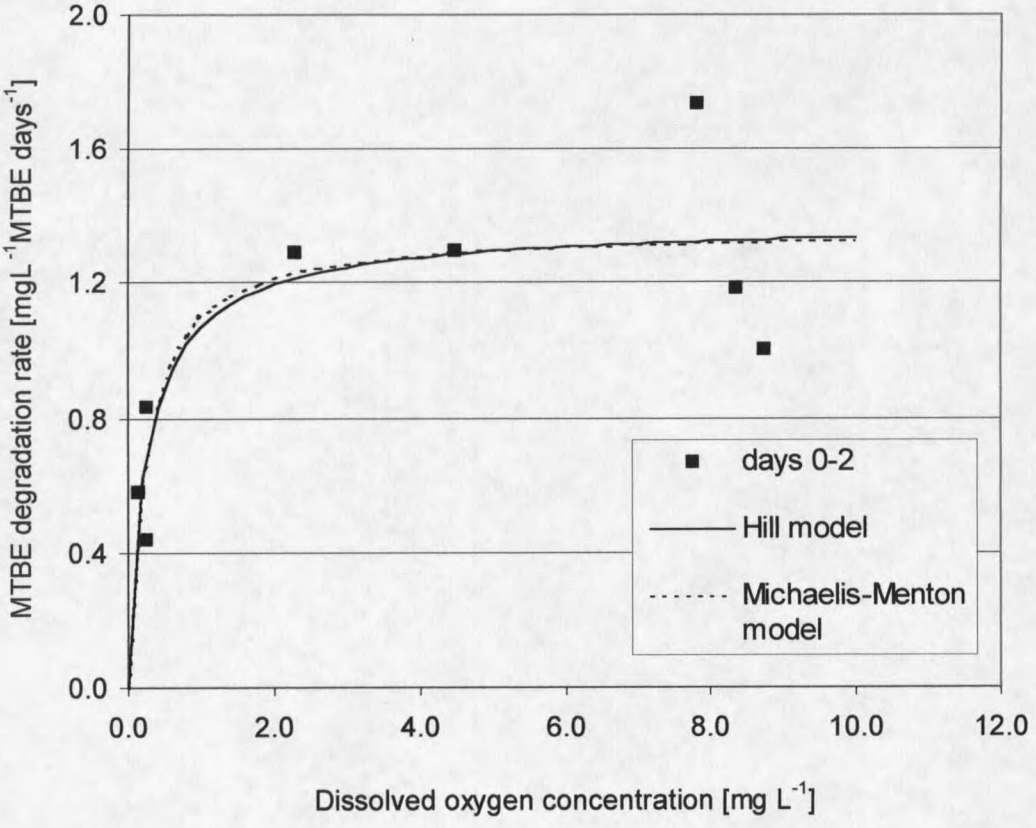

Figure 4.17 Experiment Two: Unadjusted Data, Days 0-2. MTBE Degradation Rate as a Function of Dissolved Oxygen Concentration. Michaelis-Menton model: $\mu_{max} = 1.4$, $K_m = 0.08$, $R^2 = 0.68$. Hill model: $\mu_{max} = 1.4$, $K_m = 0.25$, $n = .88$, $R^2 = 0.68$.

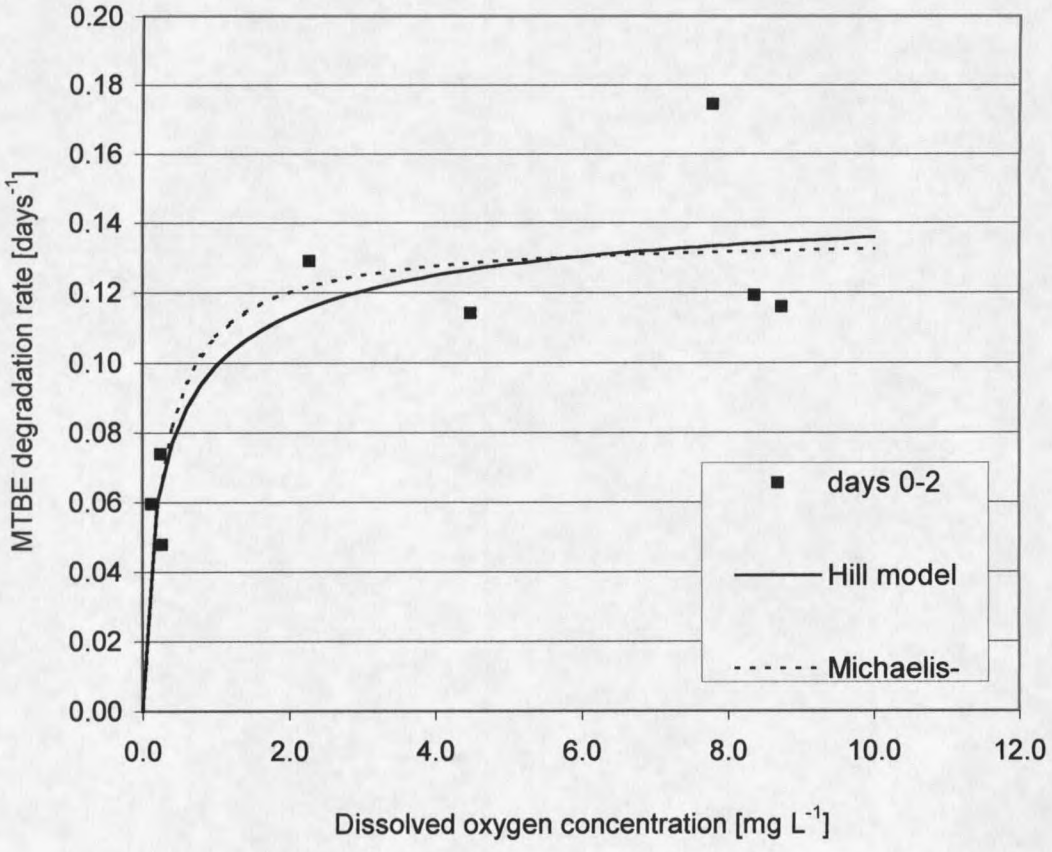

Figure 4.18 Experiment Two: Adjusted Data, Days 0-2. MTBE Degradation Rate as a Function of Dissolved Oxygen Concentration. Michaelis-Menton model: $\mu_{max} = 0.14$, $K_m = 0.08$, $R^2 = 0.67$. Hill model: $\mu_{max} = 0.15$, $K_m = 0.12$, $n = 0.65$, $R^2 = 0.68$.

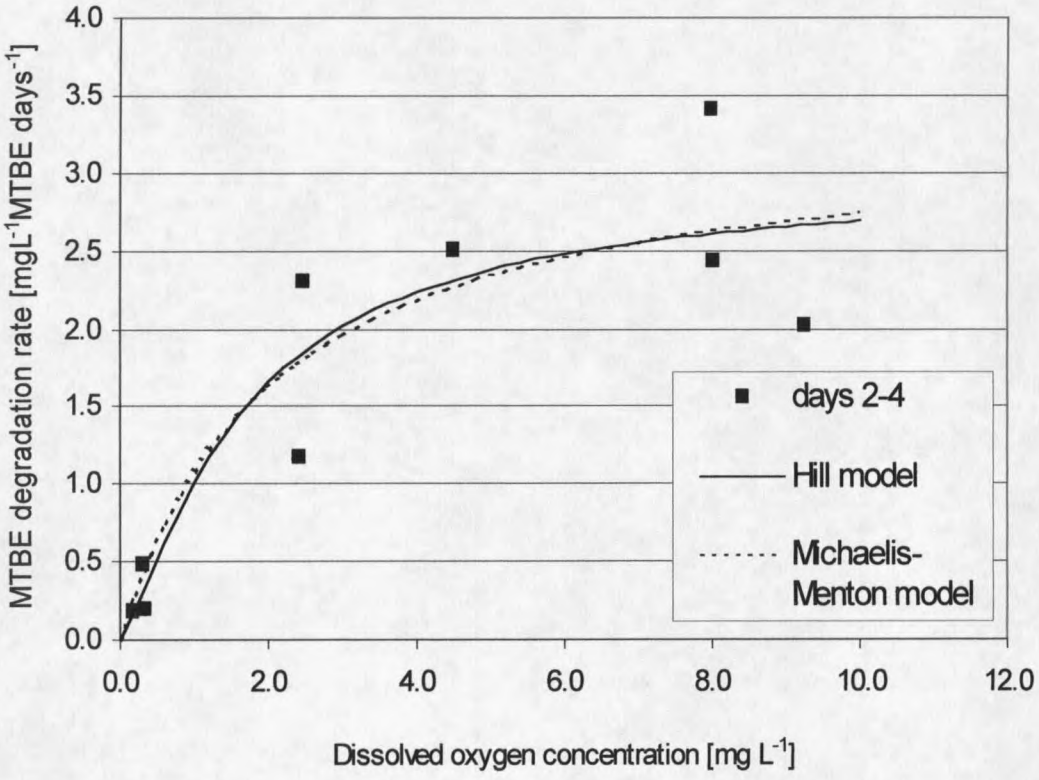

Figure 4.19 Experiment Two: Unadjusted Data, Days 2-4. MTBE Degradation Rate as a Function of Dissolved Oxygen Concentration. Michaelis-Menton model: $\mu_{max} = 3.3$, $K_m = 0.68$, $R^2 = 0.83$. Hill model: $\mu_{max} = 3.0$, $K_m = 1.66$, $n = 1.28$, $R^2 = 0.86$.

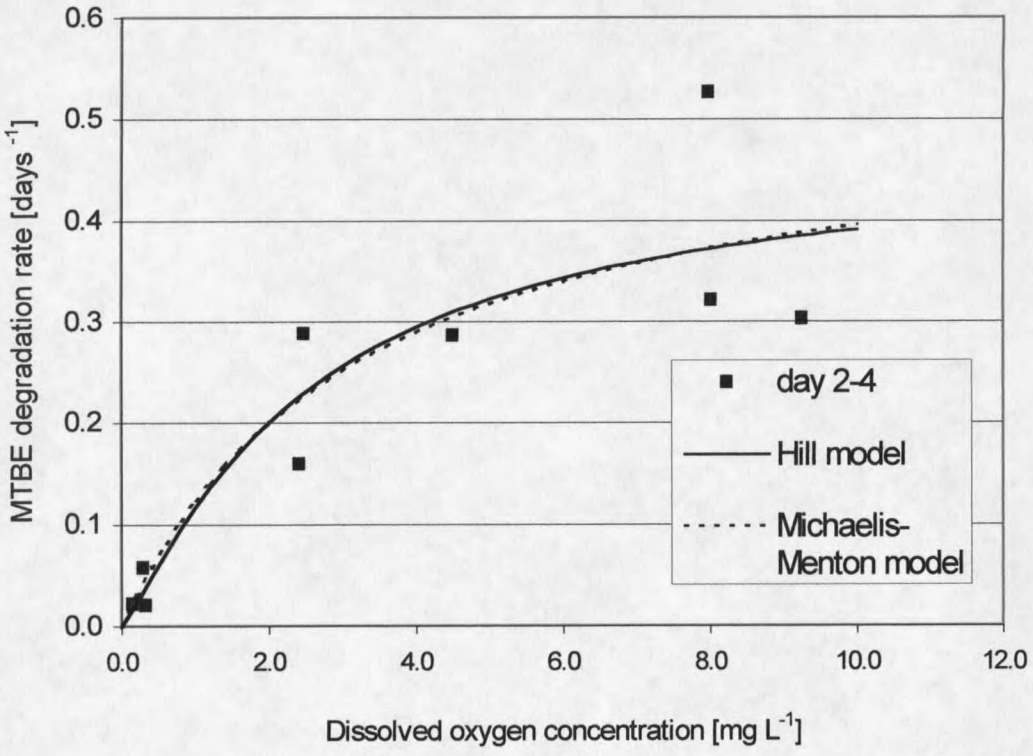

Figure 4.20 Experiment Two: Adjusted Data, Days 2-4. MTBE Degradation Rate as a Function of Dissolved Oxygen Concentration. Michaelis-Menton model: $\mu_{max} = 0.53$, $K_m = 1.06$, $R^2 = 0.81$. Hill model: $\mu_{max} = 0.47$, $K_m = 0.83$, $n = 1.19$, $R^2 = 0.81$.

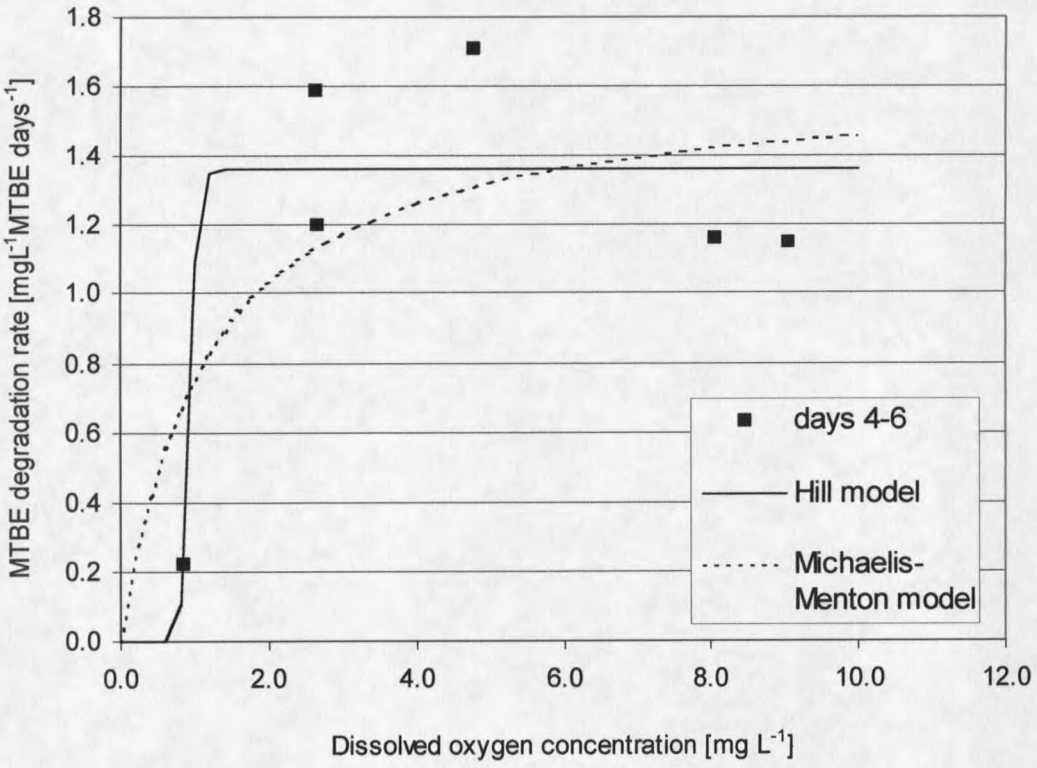

Figure 4.21 Experiment Two: Unadjusted Data, Days 4-6. MTBE Degradation Rate as a Function of Dissolved Oxygen Concentration. Michaelis-Menton model: $\mu_{max} = 1.6$, $K_m = 0.37$, $R^2 = 0.46$. Hill model: $\mu_{max} = 1.4$, $K_m = 0.92$, $n = 17.46$, $R^2 = 0.89$.

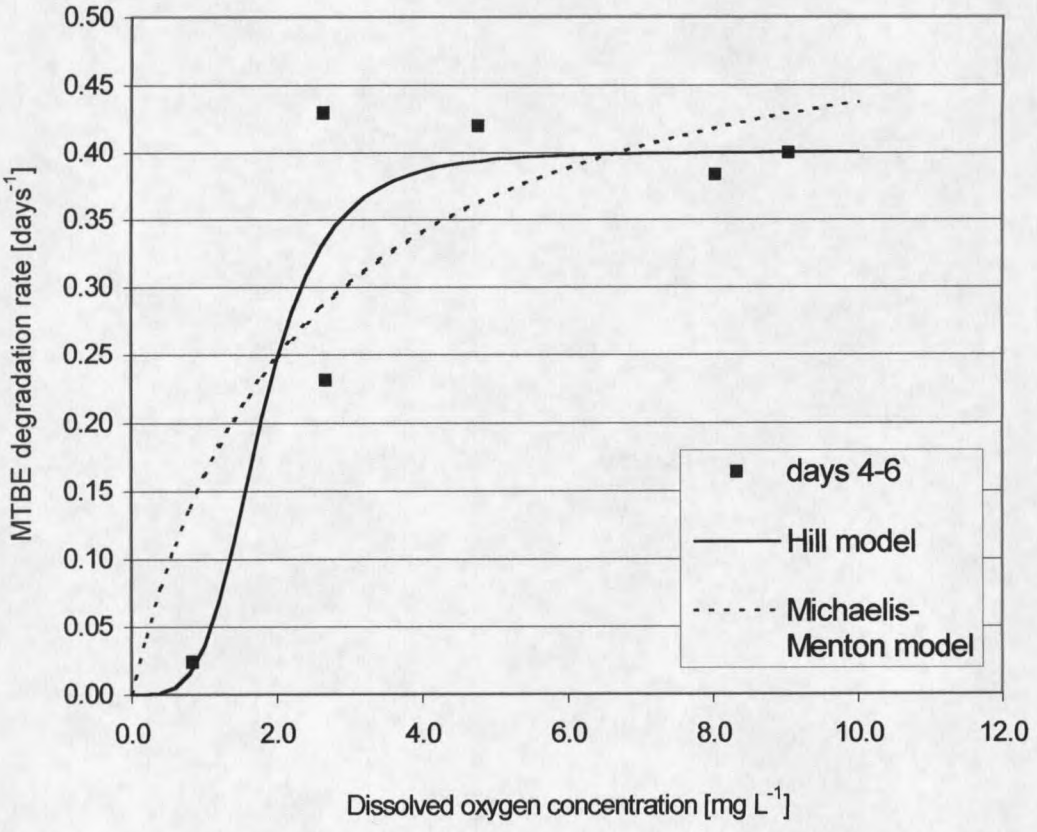

Figure 4.22 Experiment Two: Adjusted Data, Days 4-6. MTBE Degradation Rate as Function of Dissolved Oxygen Concentration. Michaelis-Menton model. $\mu_{max} = 0.54$, $K_m = 0.78$, $R^2 = 0.65$. Hill model: $\mu_{max} = 0.40$, $K_m = 0.57$, $n = 4.01$, $R^2 = 0.82$.

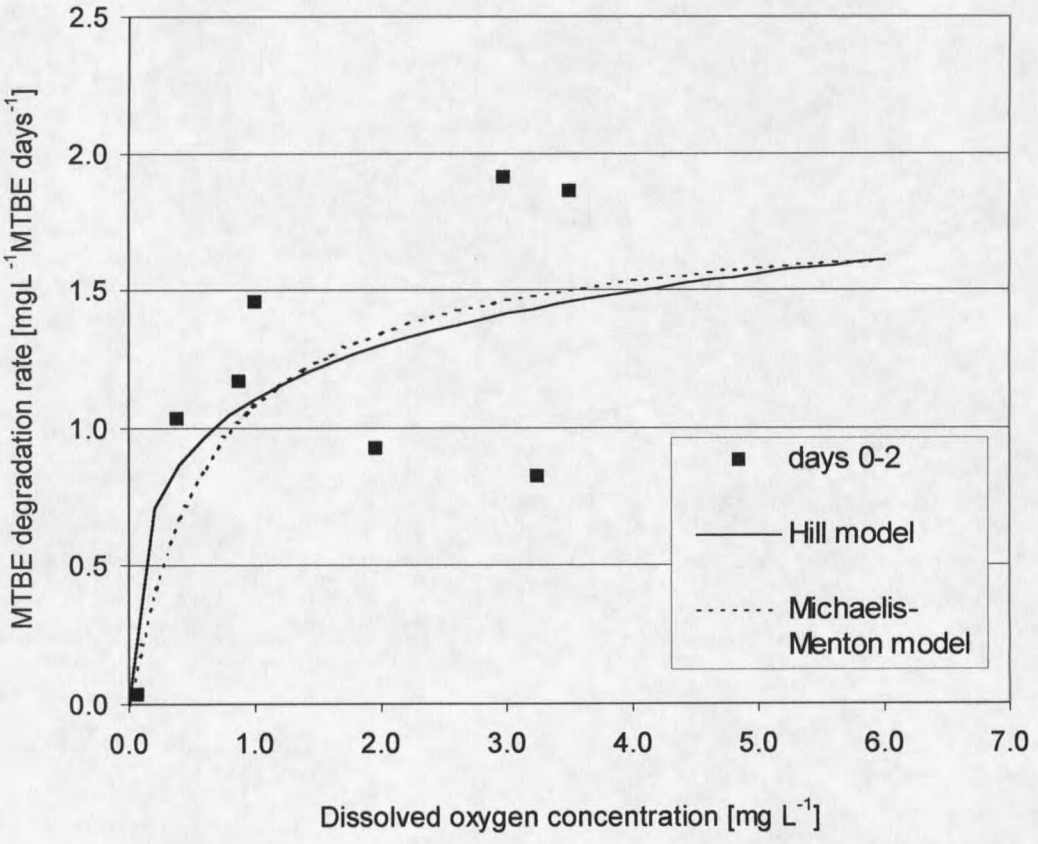

Figure 4.23 Experiment Three: Unadjusted Data, Days 0-2. MTBE Degradation Rate as a Function of Dissolved Oxygen Concentration. Michaelis-Menton model: $\mu_{max} = 1.4$, $K_m = 0.002$, $R^2 = 0.44$. Hill model: $\mu_{max} = 2.80$, $K_m = 2.86$, $n = 0.41$, $R^2 = 0.50$.

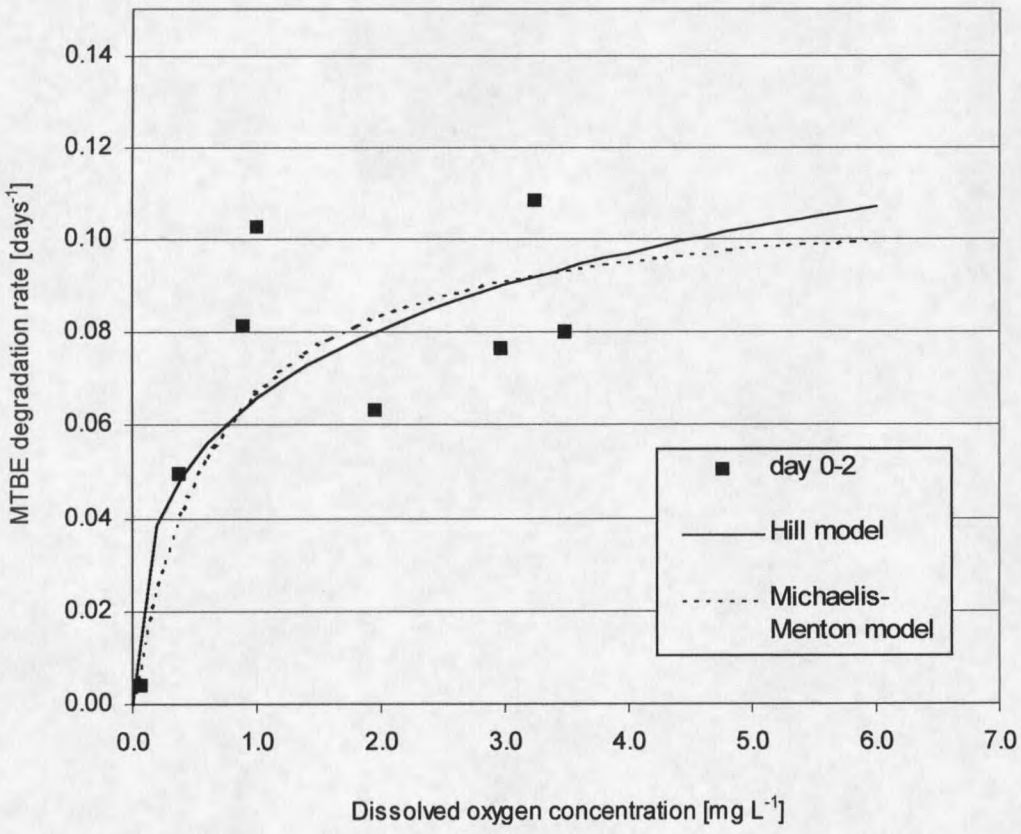

Figure 4.24 Experiment Three: Adjusted Data, Days 0-2. MTBE Degradation Rate as a Function of Dissolved Oxygen Concentration. Michaelis-Menton model: $\mu_{max} = 0.11$, $K_m = 0.20$, $R^2 = 0.41$. Hill model: $\mu_{max} = 0.09$, $K_m = 0.15$, $n = 12.65$, $R^2 = 0.54$.

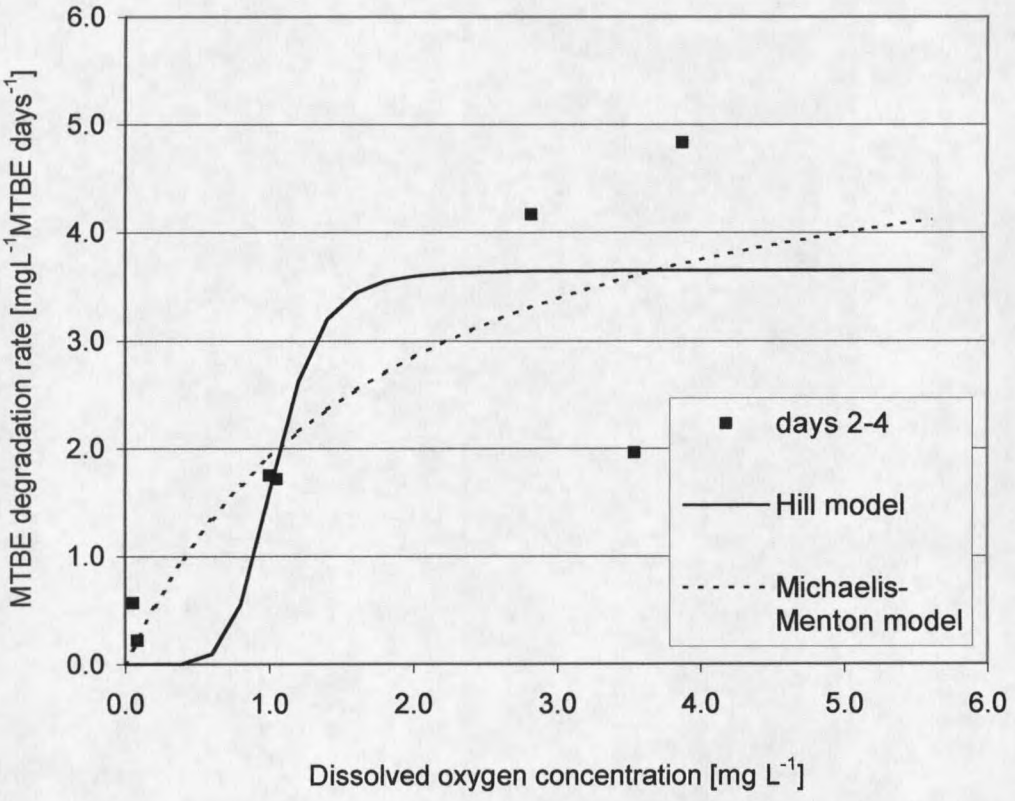

Figure 4.25 Experiment Three: Unadjusted Data, Days 2-4. MTBE Degradation Rate as a Function of Dissolved Oxygen. Michaelis-Menton model: $\mu_{max} = 5.4$, $K_m = 0.58$, $R^2 = 0.69$. Hill model: $\mu_{max} = 3.6$, $K_m = 1.04$, $n = 6.56$, $R^2 = 0.72$.

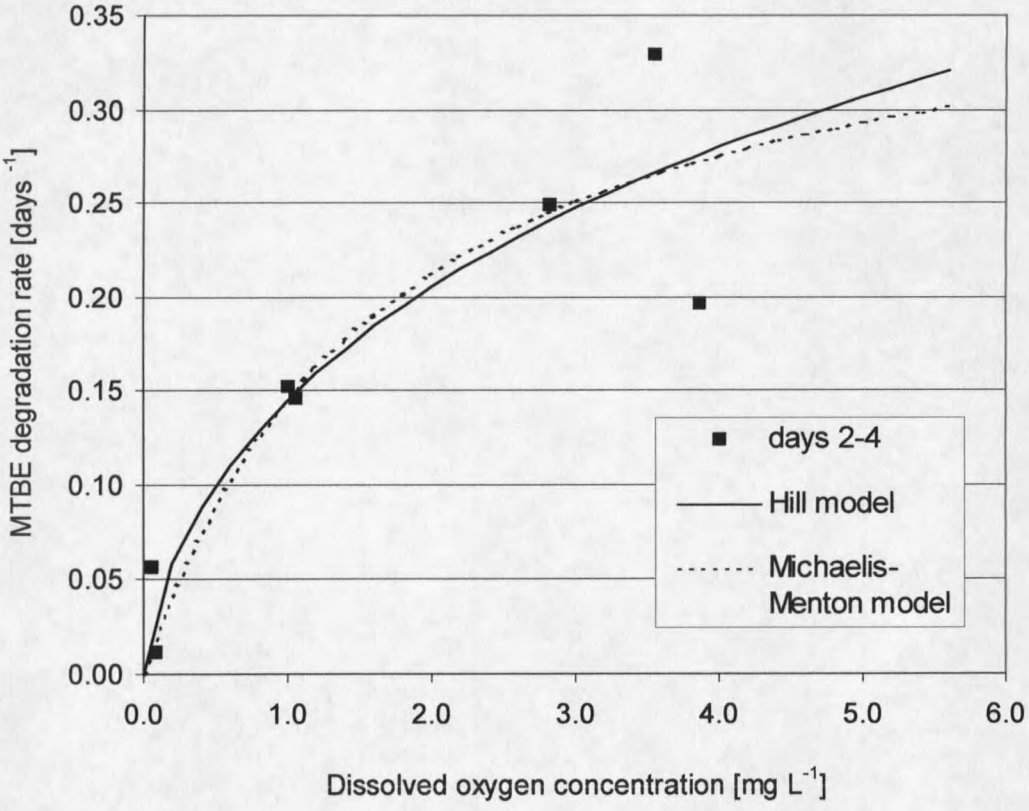

Figure 4.26 Experiment Three: Adjusted Data, Days 2-4. MTBE Degradation Rate as a Function of Dissolved Oxygen. Michaelis-Menton model: $\mu_{max} = 0.36$, $K_m = 0.42$, $R^2 = 0.84$. Hill model: $\mu_{max} = 0.44$, $K_m = 0.70$, $n = 0.79$, $R^2 = 0.84$.

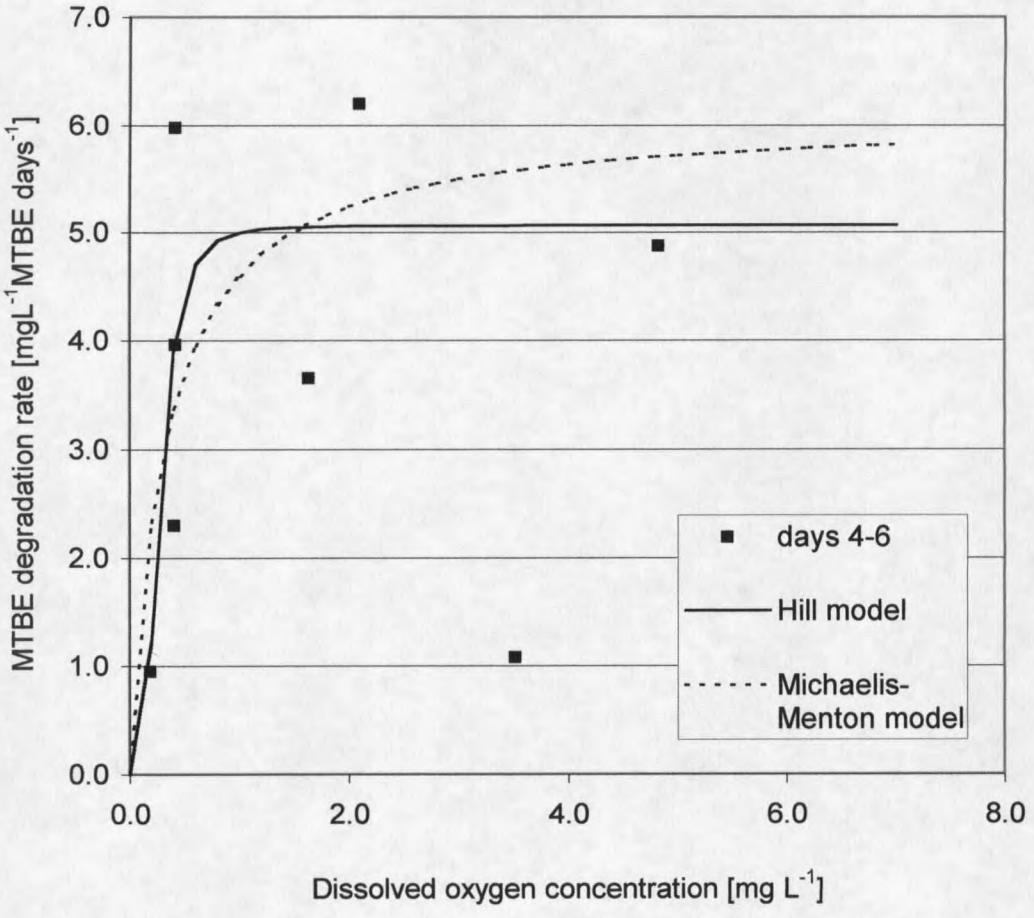

Figure 4.27 Experiment Three: Unadjusted Data, Days 4-6. MTBE Degradation Rate as a Function of Dissolved Oxygen. Michaelis-Menton model: $\mu_{max} = 4.4$, $K_m = 0.05$, $R^2 = 0.47$. Hill model: $\mu_{max} = 5.0$, $K_m = 0.28$, $n = 3.40$, $R^2 = 0.11$.

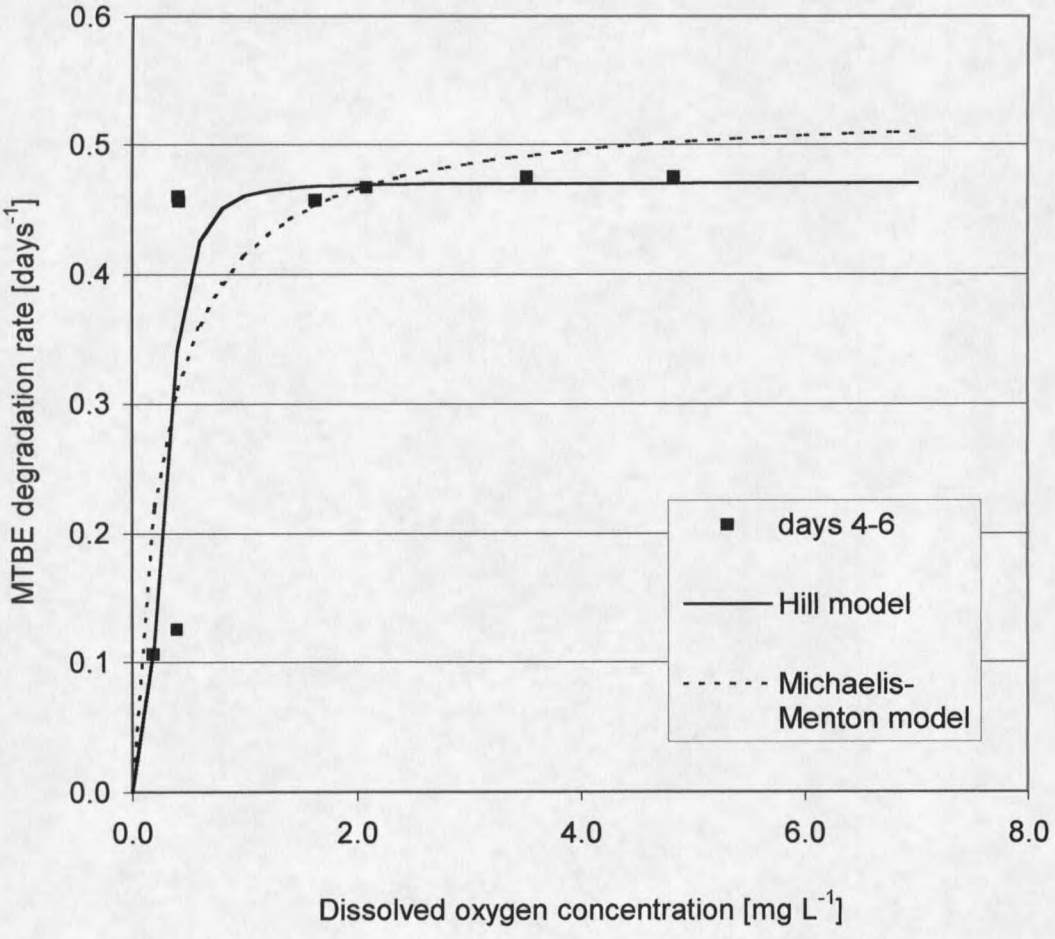

Figure 4.28 Experiment Three: Adjusted Data, Days 4-6. MTBE Degradation Rate as a Function of Dissolved Oxygen. Michaelis-Menton model: $\mu_{max} = 0.52$, $K_m = 0.09$, $R^2 = 0.48$. Hill model: $\mu_{max} = 0.47$, $K_m = 0.09$, $n = 2.92$, $R^2 = 0.57$.

A visual inspection shows that in many cases the Hill model and the Michaelis-Menton model are nearly identical. In these cases, the value of n in the Hill model is very close to 1.0. When the Hill model returns much better R^2 values than the Michaelis-Menton model, rapid increases in degradation rate characterize the model (Figures 4.13, 4.21, 4.25, 4.27). This is shown in these figures as nearly vertical lines. In these cases, it is unlikely that reaction rate is increasing in this way. It is more likely that the biodegradation rates are increasing with increasing dissolved oxygen concentration as shown by the Michaelis-Menton model. The model fit of the Michaelis-Menton model is likely a more accurate predictor of the actual behavior of the system.

In other cases, the Hill model fits all of the data points, but is sometimes passing through one data point at the high treatment level (Figure 4.15). The added polynomial in the Hill model increases its ability to fit the individual data points. However, this does not mean that the Hill model necessarily represents a better tool for predicting the behavior of the data. Without biochemical justification for using the Hill model, it may be more appropriate to model data of this type using the Michaelis-Menton model.

Michaelis-Menton and Hill Models: Adjusted Data

Table 4.6 gives a comparison of R^2 and RMSE values for the Michaelis-Menton and Hill models for the adjusted data. Table 4.7 is a summary of the fitted parameters, μ_{max}, K_m and n, as well as calculated R^2 values for each model fit. The R^2 values are at or above 0.7 for 6 of the 9 data sets for the Michaelis-Menton model. The Hill model gives R^2 values at or above 0.7 for 7 of the 9 data sets. The R^2 values for the Hill model are significantly better in some instances, such as the second interval of Experiment One. In

the cases when the R^2 value is significantly better, the RMSE values are better for the Hill model as well. However, again a visual inspection of the data suggests that the Michaelis-Menton model may be more accurately predicting the behavior of the data.

Table 4.6 Adjusted data: Comparison of R^2 Values from the Michaelis-Menton and Hill Models.

		R^2		RMSE	
Experiment #	Interval (days)	Michaelis-Menton	Hill	Michaelis-Menton	Hill
1	0-2	0.95	0.97	0.01	0.01
1	2-4	0.50	0.93	0.10	0.04
1	4-6	0.80	0.98	0.11	0.03
2	0-2	0.73	0.74	0.02	0.02
2	2-4	0.81	0.81	0.07	0.08
2	4-6	0.66	0.83	0.08	0.06
3	0-2	0.35	0.59	0.02	0.02
3	2-4	0.83	0.83	0.04	0.04
3	4-6	0.44	0.61	0.12	0.09

Table 4.8 gives a comparison of R^2 values for the Hill model fitted to both the unadjusted and the adjusted data. The adjusted data provides better model fits for both the Michaelis-Menton and Hill models than the unadjusted data. This suggests that the behavior of the MTBE degradation rate is dependent on both the concentration of MTBE as well as dissolved oxygen.

Table 4.7 Adjusted Data: Michaelis-Menton and Hill Model Parameters.

Experiment #	Interval (days)		μ_{max} ($days^{-1}$)	K_m ($mg\ L^{-1}$)	n	R^2
1	0-2	Michaelis-Menton	0.294	0.501	1	0.97
		Hill	0.280	0.449	1.09	0.97
1	2-4	Michaelis-Menton	0.475	0.578	1	0.53
		Hill	0.349	0.482	20.26	0.93
1	4-6	Michaelis-Menton	1.594	2.188	1	0.81
		Hill	0.727	0.539	3.78	0.98
2	0-2	Michaelis-Menton	0.136	0.084	1	0.67
		Hill	0.152	0.122	0.65	0.68
2	2-4	Michaelis-Menton	0.525	1.056	1	0.81
		Hill	0.469	0.832	1.19	0.81
2	4-6	Michaelis-Menton	0.544	0.775	1	0.65
		Hill	0.401	0.574	4.01	0.82
3	0-2	Michaelis-Menton	0.109	0.199	1	0.41
		Hill	0.085	0.151	12.65	0.54
3	2-4	Michaelis-Menton	0.356	0.419	1	0.84
		Hill	0.439	0.704	0.79	0.84
3	4-6	Michaelis-Menton	0.521	0.085	1	0.48
		Hill	0.469	0.090	2.92	0.57

Table 4.8 Hill Model: Comparison of R^2 Values from Unadjusted and Adjusted Data.

Experiment #	Interval (days)	R^2	
		Unadjusted	Adjusted
1	0-2	0.96	0.97
1	2-4	0.94	0.93
1	4-6	0.51	0.98
2	0-2	0.68	0.74
2	2-4	0.86	0.81
2	4-6	0.89	0.83
3	0-2	0.50	0.59
3	2-4	0.72	0.83
3	4-6	0.11	0.61

Michaelis-Menton and Hill Models Fit to Composite Data from Experiments One and Two

The Michaelis-Menton and Hill Models were fit to the composite data from Experiments One and Two. The regression is shown in Figure 4.29. The models are very similar. The value of n in the Hill model is very near 1.0. Model parameters are shown in Table 4.9.

Table 4.9 Composite Data: Experiments One and Two. Michaelis-Menton and Hill Model Parameters.

Experiment #		μ_{max} (mg L^{-1}MTBE/day)	K_m (mg L^{-1})	n	R^2
Composite 1&2	Michaelis-Menton	2.364	1.146	1	0.51
	Hill	2.133	0.933	1.456	0.52

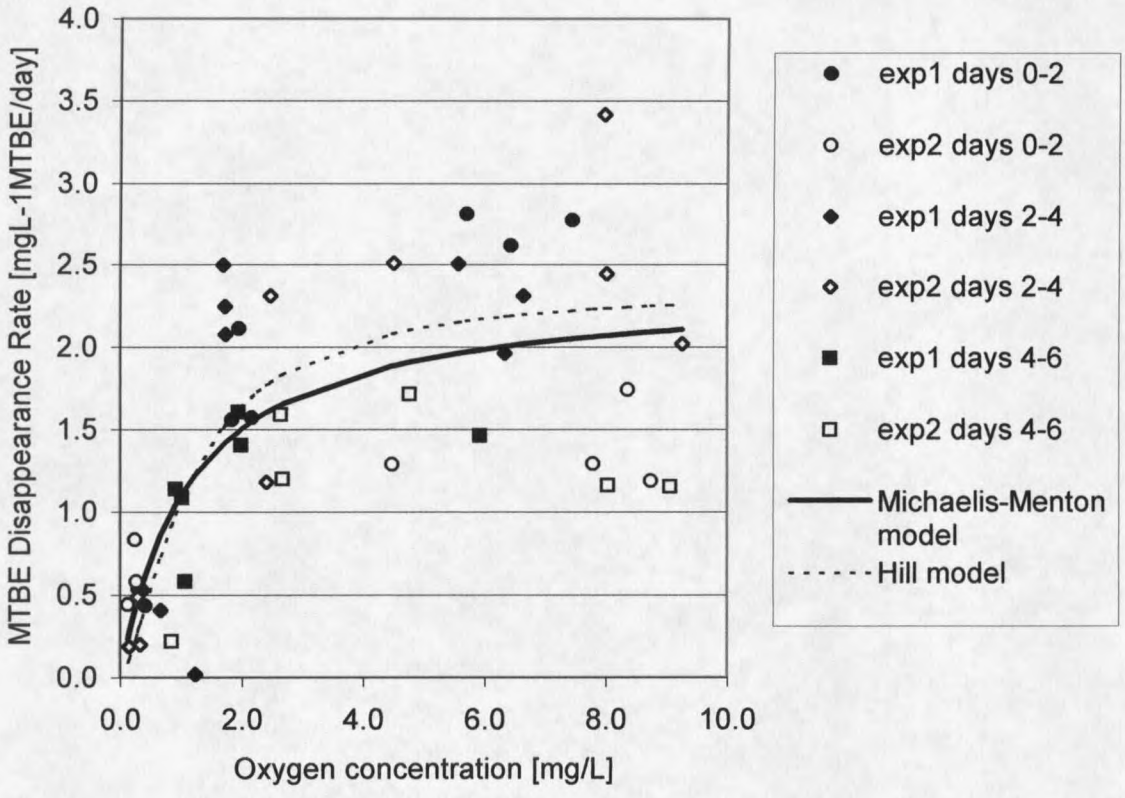

Figure 4.29 Composite Data: Experiments One and Two. MTBE Degradation Rate as a Function of Dissolved Oxygen. Michaelis-Menton model: $\mu_{max} = 2.4$, $K_m = 1.15$, $R^2 = 0.51$. Hill model: $\mu_{max} = 2.1$, $K_m = 0.93$, $n = 1.46$, $R^2 = 0.52$.

Dissolved Oxygen at 75% μmax

The Michaelis-Menton model fit to the composite data of Experiments One and Two was used to calculate the dissolved oxygen concentration at 75% μ_{max}, or $[DO]_{75}$. Because the model asymptotes to μmax, it is not possible to determine the dissolved oxygen concentration associated with 100% μ_{max}. However, at 75% μ_{max}, large increases in dissolved oxygen will produce only small changes in degradation rate.

The value of $[DO]_{75}$ is useful because it represents the minimum level of oxygen necessary to reach a nearly maximum reaction rate. This is significant in aquifer environments that are characterized by low levels of dissolved oxygen. By raising the dissolved oxygen a small amount, a large return in reaction rate can be induced. It is also useful to note that increasing the dissolved oxygen concentration to saturation would not correspondingly increase the reaction rate, and thus may not be an economically sound practice.

The calculated $[DO]_{75}$ from the composite data of Experiments One and Two is 3.4 mg DO L^{-1}. This is well below the saturation concentration of oxygen in water. Furthermore, this is similar to the findings by Park and Cowan (1997) in which it was found that below 2 mg DO L^{-1}, biodegradation rate was strongly dependent on dissolved oxygen concentration. A visual inspection of the composite data shows that below 2 mg DO L^{-1}, biodegradation rate shows a linear dependence on dissolved oxygen concentration, indicative of first order kinetics.

Regression of μ Versus Initial MTBE Concentration

To determine if MTBE concentration in the microcosms may be influencing MTBE biodegradation rates, regressions of MTBE degradation rate, μ, versus MTBE concentration at the start of each interval were performed. Figures 4.30 through 4.32 show plots of MTBE concentration versus MTBE degradation rate for each interval of each experiment. A linear regression was performed. Table 4.10 gives the regression parameters and associated R^2 values.

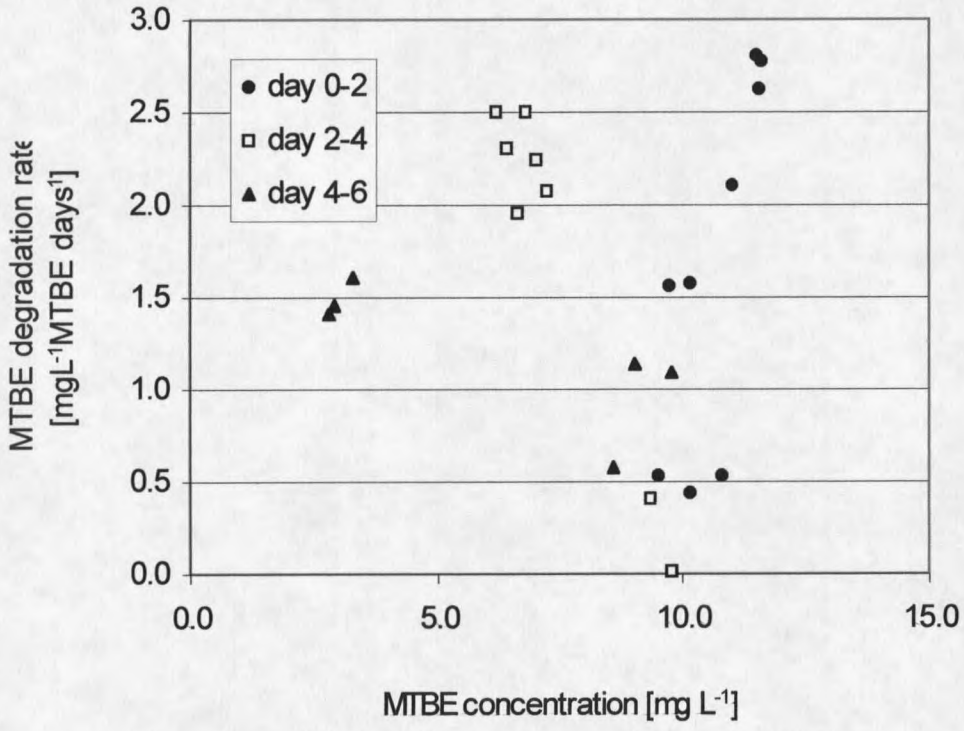

Figure 4.30 Experiment One: Plot of MTBE Degradation Rate Versus MTBE Concentration.

Table 4.10 Regression Parameters and R^2 Values for MTBE Degradation Rate Versus Initial MTBE Concentration.

Experiment #	interval (days)	slope	intercept	R^2
1	0-2	0.9416	-8.4	0.60
1	2-4	-0.781	7.4	0.84
1	4-6	-0.0007	1.7	0.59
2	0-2	0.1123	-0.1	0.06
2	2-4	-0.7531	7.6	0.42
2	4-6	-0.1774	2	0.65
3	0-2	0.0084	1.2	0.00
3	2-4	0.1692	-0.4	0.26
3	4-6	0.1739	1.8	0.16

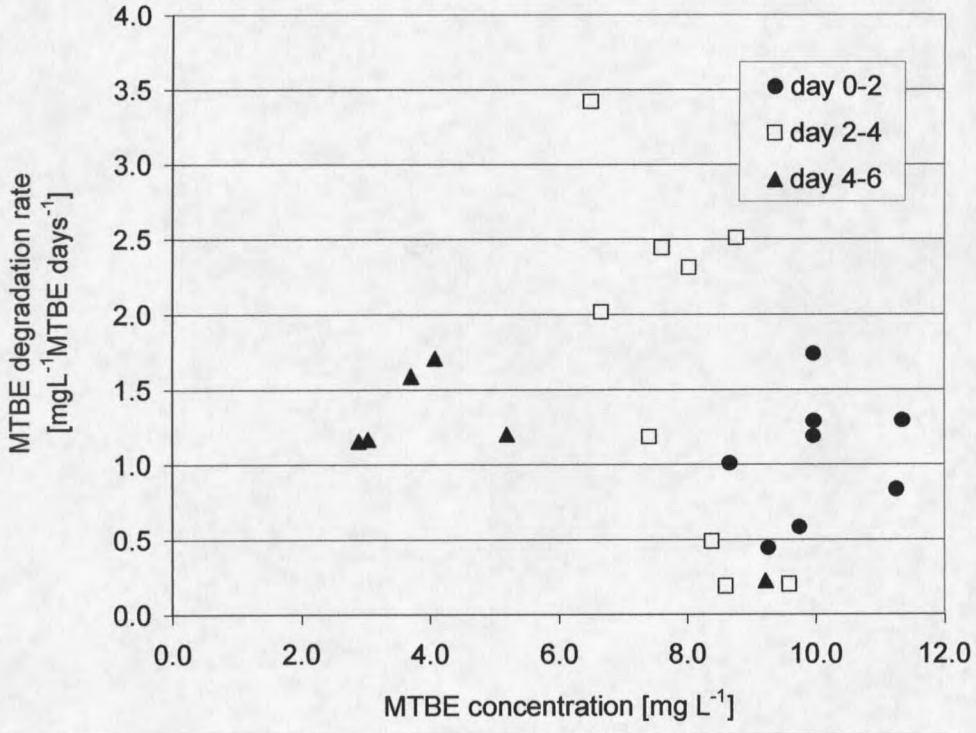

Figure 4.31 Experiment Two: Plot of MTBE Degradation Rate Versus MTBE Concentration.

It is difficult to determine from this data whether or not MTBE disappearance rate is linearly related to MTBE concentration. A more complex model may be warranted, or there may be no correlation. An exception to this assumption is the data for the medium and high oxygen treatments of the third interval of Experiment Three (Figure 4.32). These data show a strong linear correlation between MTBE degradation rate and MTBE concentration. By the third interval of Experiment Three, biodegradation rate appears to be strongly linearly correlated to MTBE concentration. This suggests first order kinetics in the region in which the bacterial population is the most acclimated to MTBE degradation.

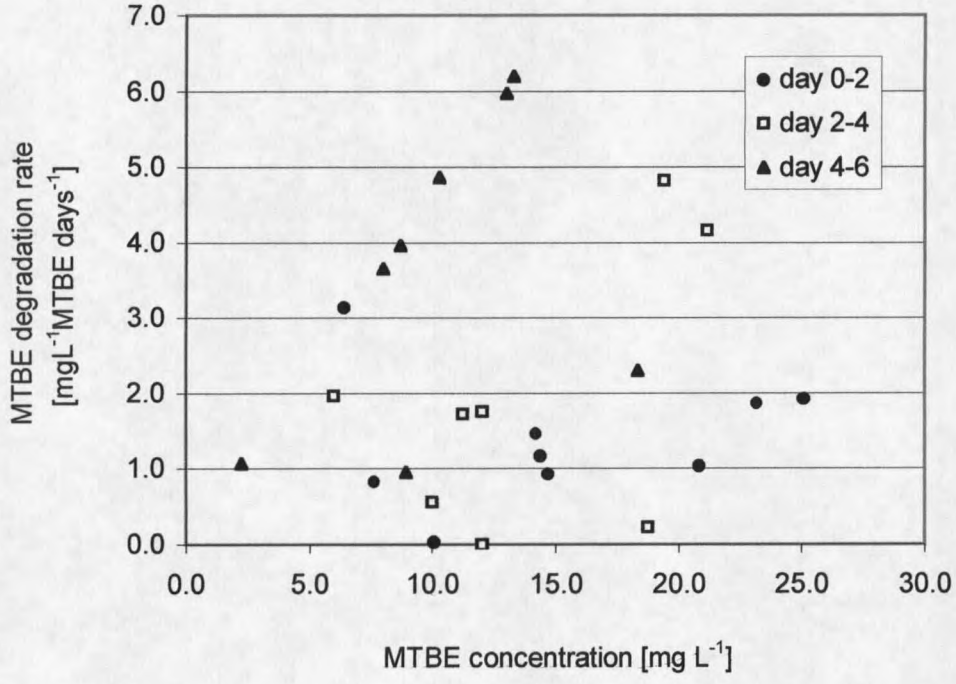

Figure 4.32 Experiment Three: Plot of MTBE Degradation Rate Versus MTBE Concentration.

Statistical Analysis

A statistical analysis was performed comparing the MTBE degradation rates in the high treatments with those from the controls from each data set. Recall that the controls were sterile controls originally given a high treatment of dissolved oxygen. A two-sample t-test assuming unequal variances was performed with the Data Analysis package in Excel. Alpha was set equal to 0.05. Table 11 gives a summary of the statistical parameters from the individual tests. A more detailed table of statistical parameters can be found in Appendix C.

Table 4.11 Two-Sample t-Test Analysis of High Treatments Versus Controls for Each Interval of Each Experiment.

Experiment	Interval	Control		High treatment		
#	#	Mean	Variance	Mean	Variance	P-value
1	1	0.58	0.05	2.73	0.01	0.0007
	2	-0.18	0.07	2.26	0.08	0.0004
	3	0.38	0.02	1.46	*	*
2	1	1.30	0.02	1.31	0.15	0.9750
	2	0.94	0.18	2.63	0.52	0.0279
	3	0.53	0.23	1.16	0.00	0.0548
3	1	0.42	0.07	1.53	0.38	0.0628
	2	-0.14	0.19	3.65	2.26	0.0525
	3	0.22	0.22	6.20	*	*

* Could not calculated due to single observation.

It should be pointed out that the sampling technique used was a destructive method. Samples were permanently removed, causing small amounts of MTBE to be lost from the microcosms. It was not possible to quantify the exact amount of loss due to sampling. In some instances the controls would show large amounts of loss; in other instances there were no losses observed. The losses were not consistent between individual samples. These losses have been attributed to variation in GC sensitivity rather that actual loss in MTBE in the controls. For this reason, the data were not adjusted to the losses seen in the controls. Instead, the data are reported as they were calculated keeping in mind that the rates reported are very nearly actual biodegradation rates. These rates can be compared to what was observed in the controls, and conclusions made accordingly.

An α of 0.05 represents a statistical analysis at the 95% confidence interval. P-values of 0.05 represent the threshold level at which to accept or reject the hypothesis that

the means of the treatments versus the means of the controls are statistically different. P-values smaller than 0.05 indicate that the means are statistically different with a 95% degree of confidence. P-values larger than this value indicate that the means are not statistically different at the 95% confidence interval. Accordingly, a P-value of 0.06 indicates that the means are statistically different at the 94% confidence level.

The P-values show that the means of the high treatments and the controls are statistically different in all intervals of all experiments with the exception of interval one of Experiment Two. During the first interval of Experiment Two, the calculated P-value is nearly one. This suggests that the MTBE degradation rates determined in the high treatments were not statistically different from those seen in the controls. This may be due to abnormally large losses in the controls on this date, or it may be that the microcosms did not behave differently than the controls on this date.

For each interval, the statistical analysis was performed on a small number of observations (usually 3, but sometimes only 1 or 2 measurements). The strength of the statistical analysis is affected by the sample size. A more accurate statistical analysis could be performed with a larger set of observations. For this reason, a statistical analysis was performed on the composite data from Experiments One and Two. Table 12 gives a summary of the calculated parameters from this analysis. Again, a two-sample t-test assuming unequal variances was performed with an alpha of 0.05. Note that the test proves that the MTBE degradation rates seen in the high treatments are strongly statistically different from those observed in the controls.

Table 12 Two-Sample t-Test Analysis of High Treatments Versus Controls for Composite Data of Experiments One and Two.

Experiment #	Interval #	Control		High treatment		P-value
		Mean	Variance	Mean	Variance	
composite 1&2	All	0.5919	0.3149	2.0367	0.5427	0.0000013

CHAPTER 5

DISCUSSION AND CONCLUSIONS

Dissolved oxygen is often limited in aquifer environments. Yet the aerobic degradation of MTBE is heavily dependent on oxygen. These investigations show that at low concentrations of dissolved oxygen, the degradation rate of MTBE increases rapidly with small increases in dissolved oxygen concentration. Furthermore, at higher levels of dissolved oxygen even large changes in oxygen concentration produce very small changes in reaction rate. These findings are significant to bioremediation treatments in which dissolved oxygen is limiting.

The calculated $[DO]_{75}$ provides a means of estimating a target dissolved oxygen concentration necessary to reach a nearly maximum reaction rate. This parameter represents a minimum level of dissolved oxygen that would provide an acceptably high rate of degradation. This parameter could be used to maximize the design of oxygen application systems by avoiding unnecessary waste. For example, it may not be economically warranted to increase dissolved oxygen concentrations to nearly saturation values, since MTBE degradation rate would not increase by a corresponding amount.

For the composite data of Experiments One and Two, the $[DO]_{75}$ was calculated to be 3.4 mg L^{-1}. Park and Cowan (1997) found that below 2 mg L^{-1} of dissolved oxygen, MTBE biodegradation rate was heavily dependent on dissolved oxygen. The findings from this experiment support that at low dissolved oxygen concentrations MTBE degradation rate is inhibited. The fact that the data from this experiment support the findings of Park and Cowan is significant in that a different MTBE degrading

microorganism was investigated. Park and Cowan looked at an MTBE degrading consortium. These experiments investigated a bacterial isolate capable of MTBE degradation. It may be that the phenomenon in which MTBE degradation rate is strongly dependent on dissolved oxygen at low oxygen concentrations is characteristic of MTBE degradation, regardless of the specific bacteria.

The Hill model contains a higher order polynomial than the Michaelis-Menton model. Because of this, the Hill model returns better R^2 values for the given data series. However, there is as of yet no biochemical basis for accepting this model over the Michaelis-Menton model. Furthermore, in some cases the parameter n in the Hill equation is one or nearly one. In these cases, this model is identical to the Michaelis-Menton model. In the context of these experiments, these models are used as predictive tools to determine a threshold dissolved oxygen concentration below which MTBE degradation is inhibited. For this purpose, the Michaelis-Menton model represents a satisfactory predictive tool.

The data also suggest that when the microorganism PM1 is initially introduced to MTBE as a sole carbon and energy source, enzyme activity that is dependent on dissolved oxygen concentration dominates the behavior of MTBE degradation rate. As time progresses and the microorganism becomes acclimated to MTBE as a substrate, the influence of MTBE concentration on degradation rate apparently increases. This is corroborated by the fact that the data from the medium and high treatment of the third interval of Experiment Three show a strong linear correlation between MTBE degradation rate and MTBE concentration. MTBE concentration most likely plays a role

in determining the behavior of MTBE degradation rate. The specific means by which MTBE should be mathematically incorporated into the model warrants further study.

The specific conclusions from this study are:

1. The microorganism PM1 is capable of complete degradation of MTBE at concentrations of up to 20 mg L^{-1} in 7 days when sufficient oxygen is present.
2. At low levels of dissolved oxygen, MTBE degradation rate is strongly dependent on dissolved oxygen concentration. This phenomenon is not evidenced at higher concentrations of dissolved oxygen.
3. The data support that aerobic MTBE degradation is an enzyme-mediated process as evidenced by agreement with the Michaelis-Menton model.
4. The Michaelis-Menton model is a satisfactory model for predicting the behavior of the data.
5. MTBE degradation rates are dependent upon both the MTBE concentration as well as dissolved oxygen concentration. Dependence on MTBE concentration becomes more pronounced as the microorganism PM1 acclimates to MTBE.

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APPENDICES

APPENDIX A

DATA

Experiment One

Sample ID	Sample Event day	MTBE concentration (mg/L)	Dissolved Oxygen Concentration (mg/L)
control1	0	11.5	13.1
control2	0	9.9	16.1
control3	0	13.0	18.9
low1	0	10.2	0.3
low2	0	9.5	0.3
low3	0	10.8	1.8
medium1	0	11.0	4.8
medium2	0	10.2	4.2
medium3	0	9.7	4.3
high1	0	11.6	18.4
high2	0	11.5	16.0
high3	0	11.5	14.5
control1	2	10.4	17.2
control2	2	9.2	17.0
control3	2	11.5	18.6
low1	2	9.4	1.0
low2	2	8.5	0.7
low3	2	9.8	0.9
medium1	2	7.0	4.7
medium2	2	7.2	5.3
medium3	2	6.8	4.5
high1	2	6.4	18.1
high2	2	6.6	15.6
high3	2	6.2	13.9
control1	4	11.1	11.5
control2	4	9.0	11.7
control3	4	12.1	12.1
low1	4	8.6	1.6
low2	4	9.0	2.1
low3	4	9.8	2.9
medium1	4	2.8	4.1
medium2	4	3.3	4.1
medium3	4	2.1	4.0
high1	4	2.0	15.7
high2	4	2.9	15.0
high3	4	1.5	13.1
control1	6	10.1	20.8
control2	6	8.1	16.2
control3	6	11.6	17.2

Sample ID	Sample Event day	MTBE concentration (mg/L)	Dissolved Oxygen Concentration (mg/L)
low1	6	7.4	2.5
low2	6	6.8	2.2
low3	6	7.6	2.4
medium1	6	0.0	4.7
medium2	6	0.2	4.6
medium3	6	ND	4.7
high1	6	ND	15.5
high2	6	0.1	14.0
high3	6	ND	13.7

Experiment Two

Sample ID	Sample Event #	MTBE concentration (mg/L)	Dissolved Oxygen Concentration (mg/L)
control1	0	12.4	18.0
control2	0	7.9	18.0
control3	0	10.1	18.3
low1	0	9.3	0.6
low2	0	11.3	0.7
low3	0	9.8	0.7
medium1	0	ND	5.8
medium2	0	10.0	6.1
medium3	0	11.3	6.3
high1	0	10.0	19.6
high2	0	10.0	19.2
high3	0	8.7	20.0
control1	2	9.2	16.8
control2	2	6.3	17.5
control3	2	7.2	17.1
low1	2	8.4	0.6
low2	2	9.6	0.6
low3	2	8.6	0.3
medium1	2	8.0	5.7
medium2	2	7.4	5.5
medium3	2	8.8	10.9
high1	2	6.5	19.0

Sample ID	Sample Event #	MTBE concentration (mg/L)	Dissolved Oxygen Concentration (mg/L)
high2	2	7.6	20.4
high3	2	6.7	21.3
control1	4	6.7	16.4
control2	4	5.6	17.3
control3	4	5.1	17.7
low1	4	7.5	0.7
low2	4	9.2	0.8
low3	4	8.2	0.4
medium1	4	3.7	5.8
medium2	4	5.2	5.7
medium3	4	4.1	10.6
high1	4	0.1	18.8
high2	4	3.0	18.9
high3	4	2.9	21.9
control1	6	4.7	17.5
control2	6	5.5	19.1
control3	6	3.9	18.0
low1	6	7.6	1.8
low2	6	8.8	1.9
low3	6	8.4	0.7
medium1	6	0.4	6.1
medium2	6	2.7	6.1
medium3	6	0.5	10.9
high1	6	ND	18.9
high2	6	0.6	18.5
high3	6	0.5	20.8

Experiment Three

Sample ID	Sample Event #	MTBE concentration (mg/L)	Dissolved Oxygen Concentration (mg/L)
control1	0	11.0	6.5
control2	0	12.4	6.7
control3	0	12.5	7.5
low1	0	10.1	0.4
low2	0	20.8	0.5
low3	0	6.4	0.4
medium1	0	14.7	1.7

Sample ID	Sample Event #	MTBE concentration (mg/L)	Dissolved Oxygen Concentration (mg/L)
medium2	0	14.4	1.9
medium3	0	14.2	2.1
high1	0	25.0	6.7
high2	0	7.6	10.5
high3	0	23.2	ND
control1	2	10.1	8.9
control2	2	11.0	7.9
control3	2	12.2	7.9
low1	2	10.0	0.9
low2	2	18.7	0.2
low3	2	ND	0.6
medium1	2	12.8	2.4
medium2	2	12.0	4.8
medium3	2	11.2	2.1
high1	2	21.2	7.2
high2	2	6.0	8.5
high3	2	19.4	7.9
control1	4	9.4	9.43
control2	4	11.8	8.63
control3	4	12.9	8.36
low1	4	8.9	0.13
low2	4	18.3	0.21
low3	4	ND	0.96
medium1	4	13.0	2.38
medium2	4	8.7	2.55
medium3	4	8.0	2.44
high1	4	13.3	9.42
high2	4	2.3	8.61
high3	4	10.3	6.87
control1	6	10.0	9.5
control2	6	11.1	9.0
control3	6	11.6	8.5
low1	6	6.9	0.5
low2	6	13.5	1.0
low3	6	ND	0.5
medium1	6	0.4	1.0
medium2	6	0.3	4.0
medium3	6	0.3	1.0
high1	6	0.2	11.7
high2	6	ND	8.6
high3	6	ND	5.1

APPENDIX B

SAMPLE CALCULATIONS

Sample Calculations

Experiment #3

Interval 3

Sample medium3

Calculation of MTBE Degradation Rate, μ

$$\mu = \frac{[MTBE]_{final} - [MTBE]_{initial}}{t_{final} - t_{initial}}$$

$$\mu = \frac{\left(8\frac{mg}{L}\right) - \left(0.3\frac{mg}{L}\right)}{6.19days - 4.08days}$$

$$\mu = 3.65\frac{mgL^{-1}MTBE}{day}$$

Calculation of dissolved oxygen concentration, [DO]:

Oxygen as % of total gaseous headspace = 1.01%

Volume of oxygen in headspace:

$$\forall_{O2gaseous} = \frac{1.01}{100} \circ 50mL$$

$$\forall_{O2gaseous} = 0.505mLO_2$$

Molar volume of oxygen in headspace:

$$n = \frac{PV}{RT}$$

$$n = \frac{\left(101325 \frac{N}{m^2}\right) \cdot \left(0.505 mL \cdot 10^{-6} \frac{m^3}{mL}\right)}{\left(8.314 \frac{N \cdot m}{K \cdot mol}\right) \cdot (293.15 K)}$$

$$n = 2.1 \cdot 10^{-5} mol O_2$$

Concentration of oxygen in headspace:

$$[O_2]_{gaseous} = \left(2.1 \cdot 10^{-5} mol\right) \cdot \left(32 \frac{g}{mol}\right) \cdot \left(\frac{1000 mg}{g}\right) \cdot \left(\frac{1}{50 mL}\right) \cdot \left(\frac{1000 mL}{L}\right)$$

$$[O_2]_{gaseous} = 13.43 \frac{mg}{L}$$

Concentration of dissolved oxygen:

$$[DO] = \frac{[O_2]_{gaseous}}{32.4}$$

$$[DO] = \frac{13.43 \frac{mg}{L}}{32.4}$$

$$[DO] = 0.415 \frac{mg}{L}$$

Calculation of $\mathbb{R}^2$:

$$y_i = \mu_i$$

$$x_i = [DO]_i$$

$$\bar{y} = \frac{\sum y_i}{n}$$

$$S_t = \sum (y_i - \bar{y})^2$$

$$y_p = \mu_{predicted}$$

$$S_r = \sum (y_i - y_p)^2$$

$$R^2 = \frac{S_t - S_r}{S_t}$$

APPENDIX C

STATISTICAL ANALYSES

Experiment 1
Interval 1
High treatments

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	0.5816	2.7323
Variance	0.0529	0.0105
Observations	3	3
Hypothesized Mean Difference	0	
df	3	
t Stat	-14.7969	
P(T<=t) one-tail	0.0003	
t Critical one-tail	2.3534	
P(T<=t) two-tail	0.0007	
t Critical two-tail	3.1824	

Experiment 1
Interval 2
High treatments

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	-0.1819	2.2564
Variance	0.0670	0.0762
Observations	3	3
Hypothesized Mean Difference	0	
df	4	
t Stat	-11.1595	
P(T<=t) one-tail	0.0002	
t Critical one-tail	2.1318	
P(T<=t) two-tail	0.0004	
t Critical two-tail	2.7765	

Experiment 1
Interval 3
High treatments

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	0.3847	1.4593
Variance	0.0175	#DIV/0!
Observations	3	1
Hypothesized Mean Difference	0	
df	0	
t Stat	-0.8878	
P(T<=t) one-tail	NA	
t Critical one-tail	NA	
P(T<=t) two-tail	NA	
t Critical two-tail	NA	

Experiment 2
Interval 1
High treatments

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	1.2988	1.3097
Variance	0.1776	0.1456
Observations	3	3
Hypothesized Mean Difference	0	
df	4	
t Stat	-0.0334	
P(T<=t) one-tail	0.4875	
t Critical one-tail	2.1318	
P(T<=t) two-tail	0.9750	
t Critical two-tail	2.7765	

Experiment 2
Interval 2
High treatments

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	0.9388	2.6262
Variance	0.2330	0.5160
Observations	3	3
Hypothesized Mean Difference	0	
df	4	
t Stat	-3.3770	
P(T<=t) one-tail	0.0139	
t Critical one-tail	2.1318	
P(T<=t) two-tail	0.0279	
t Critical two-tail	2.7765	

Experiment 2
Interval 3
High treatment

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	0.5293	1.1584
Variance	0.2301	0.0001
Observations	3	2
Hypothesized Mean Difference	0	
df	2	
t Stat	-2.2709	
P(T<=t) one-tail	0.0756	
t Critical one-tail	2.9200	
P(T<=t) two-tail	0.1511	
t Critical two-tail	4.3027	

Experiment 3
Interval 1
High treatment

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	0.4240	1.5346
Variance	0.0667	0.3753
Observations	3	3
Hypothesized Mean Difference	0	
df	3	
t Stat	-2.8934	
P(T<=t) one-tail	0.0314	
t Critical one-tail	2.3534	
P(T<=t) two-tail	0.0628	
t Critical two-tail	3.1824	

Experiment 3
Interval 2
High treatment

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	-0.1373	3.6527
Variance	0.1944	2.2605
Observations	3	3
Hypothesized Mean Difference	0	
df	2	
t Stat	-4.1897	
P(T<=t) one-tail	0.0263	
t Critical one-tail	2.9200	
P(T<=t) two-tail	0.0525	
t Critical two-tail	4.3027	