BIODEGRADATION OF METHYL TERT-BUTYL ETHER AND TERT-BUTYL ALCOHOL USING BIOAUGMENTATION WITH BIOWISH® AQUA

A Thesis

presented to

the Faculty of California Polytechnic State University,

San Luis Obispo

In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Civil and Environmental Engineering

By

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December 2022
COMMITTEE MEMBERSHIP

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ABSTRACT

Biodegradation of Methyl Tert-Butyl Ether and Tert-Butyl Alcohol Using Bioaugmentation with BiOWiSH® Aqua

Elizabeth Villanueva

Aqua, a commercial product manufactured by BiOWiSH® Technologies, was utilized in this research to study its effectiveness to biodegrade methyl tert-butyl ether (MTBE) and tert-butyl alcohol (TBA). Microcosms containing varying concentrations of MTBE and TBA as well as a growth media and mineral salt solution were examined. Analytical instrumentation used in this study included the use of a gas chromatograph-mass spectrometer (GC/MS) to determine concentrations of MTBE and TBA and a spectrophotometer to extrapolate approximate active biomass concentrations in each experiment. Four different environmental conditions were tested for both MTBE and TBA. The environmental conditions tested for each contaminant included: biodegradation under aerobic conditions, biodegradation under anaerobic conditions, biodegradation under denitrifying conditions, and biodegradation under aerobic conditions with glucose present.

This study concluded that there is potential for degradation of MTBE and TBA using Aqua under the conditions tested. Maximum MTBE biodegradation was observed under aerobic conditions which yielded a first order rate constant of 0.019/hour and a 99.8 percent decrease in MTBE over 14 days. Maximum TBA biodegradation was observed under aerobic conditions with glucose present which yielded a first rate order constant of 0.009/hour and a 95.03 percent decrease in TBA concentrations over 14 days. It is
presumed that under both conditions a monooxygenase enzymatic reaction involving Cytochrome P-450 aids in breaking down both MTBE and TBA. However, the results presented are indicative of biodegradation under lab conditions with little to no interference. Further research is needed to determine the effectiveness of Aqua utilizing groundwater or soil samples from MTBE or TBA contaminated sites in order to truly analyze Aqua’s potential to be used as a bioaugmentation product in real world applications.

Keywords: MTBE, TBA, bioaugmentation, biodegradation, BiOWiSH
ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Nirupam Pal, for his continued guidance throughout this project. Without his patience and support, this project would not have been possible. Thank you to Dr. Oulton and Dr. El Badawy for being helpful committee members.

A special thank you to Darin Son for being a great lab assistant. Thank you for staying by my side through all the long nights in lab and for helping develop a SPME extraction method.

Finally, I would like to thank my friends and family who have been my main support source along this journey. I would especially like to thank my parents for their eternal love and support. I am so grateful to have such wonderful parents who have endlessly helped me achieve all the goals I have set. None of my accomplishments would ever have been possible without their selfless support.
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1. INTRODUCTION

According to the California Office of Environmental Health Hazard Assessment, in the late 1990s, methyl tert-butyl ether (MTBE) was the second most produced chemical in the United States. At its peak in 1999, production of MTBE was estimated at 78.9 million barrels per year, equivalent to 12.5 billion liters per year (U.S. Environmental Protection Agency 2008). Its high production and use rate were attributed to the Clean Air Act Amendments of 1990 which required fuel oxygenates be added to gasoline in order to promote efficient combustion. Although other oxygenates were available to meet the requirement, MTBE was the oxygenate of choice for oil refiners due to its ability to be easily integrated into refinery operations and its low production cost (Franklin 2000). The high production and use rate of MTBE was diminished when its widespread contamination of groundwater was discovered. The extensive groundwater contamination of MTBE prompted the United States Environmental Protection Agency (EPA) to impose a Drinking Water Advisory and the focus of MTBE was quickly shifted to groundwater remediation.

Similar to MTBE, tert-butyl alcohol (TBA) can be utilized as an oxygenate according to the EPA. TBA has also been found to be a byproduct of MTBE degradation (Bradley 2002). Although the EPA has not set a drinking water maximum contaminant level (MCL) for TBA, groundwater remediation of TBA has been a major focus because it is a known toxin and possible carcinogen (Cirvello 1995).

Physical and chemical treatment technologies for MTBE include air stripping, granular activated carbon (GAC), hydrophobic hollow fiber membranes, and advanced oxidation
processes (AOP)-using ozone (Keller 2000). Bioremediation is a method of removing contaminants from soil or water using naturally occurring microorganisms or by introduction of microorganisms that break down contaminants. According to the EPA, bioremediation is a proven method of reducing contamination and can be more cost effective than physical or chemical treatment technologies. Though MTBE and TBA were initially thought to be recalcitrant contaminants, recent studies have shown that both contaminants can be biodegraded.

This thesis work focused on testing the efficacy of BiOWiSH® Aqua. Aqua is a commercially available bioaugmentation product created by BiOWiSH® Technologies, Inc. This proprietary biological product is intended to remove contaminants of concern in water. Aerobic and anaerobic microcosm experiments were set up at varying concentrations of both MTBE and TBA. Samples were taken over the course of the study period and analyzed using gas chromatography-mass spectrometry. Additionally, the effects of adding glucose and nitrate to the microcosms were analyzed. The specific objectives of this study were as follows:

1. Establish a lab method to analyze MTBE and TBA concentrations using gas chromatography-mass spectrometry and solid phase microextraction techniques.
2. Evaluate the effectiveness of using bioaugmentation product, BiOWiSH® Aqua, to biodegrade MTBE and/or TBA.
2. BACKGROUND

After the EPA’s inception in 1970, major focus was placed on the need to control and mitigate air pollution. In an effort to do so, the Clean Air Act of 1970 was enacted in December 1970. The act allowed for federal and state entities to impose regulations to limit harmful emissions from stationary and mobile sources. Automobiles, in particular, were targeted for their use of leaded gasoline. The EPA began to phase out leaded gasoline in 1973 due to lead’s known toxicity and harmful environmental impacts. The eventual ban of leaded gasoline gave way for the use of MTBE as an octane booster to replace lead and subsequent Clean Air Act requirements paved the way for widespread use of MTBE.

2.1 Clean Air Act Amendments of 1990

According to the EPA, the Clean Air Acts Amendments of 1990 were enacted in November 1990. The amendments were specifically designed to target four main air related issues, including acid rain, urban air pollution, toxic air emissions and stratospheric ozone depletion. Requirements of the amendments included the establishment of the Wintertime Oxygenated Fuel program which required gasoline to be oxygenated in forty areas of carbon monoxide (CO) nonattainment. In 1990, the EPA estimated that in the United States automobiles were responsible for approximately 29% of the total volatile organic compound (VOC) emissions, 33% of the total oxides of nitrogen (NOx) emissions, and 65% of all CO emissions (Kirchstetter 1996). Since CO
emissions tend to increase in cold weather, oxygenated fuel was required to contain a minimum oxygen content of 2.7 or greater weight percent during the winter.

The Clean Air Act Amendments also required the use of reformulated gasoline (RFG) in nine areas with the worst ozone problems. These areas included Los Angeles, San Diego, Houston, Baltimore, Philadelphia, New York, Hartford, Chicago and Milwaukee. During production of RFG, volatile compounds are removed from crude oil thereby reducing the emission of volatile organic compounds released into the atmosphere from RFGs. RFG was required to contain a minimum oxygen content of 2.0 or greater weight percent year round. The EPA states that the target oxygen content of 2.0 weight percent can be achieved by utilizing 11 percent MTBE by volume. The same oxygen content could be achieved by utilizing 5.4 percent ethanol by volume. Other oxygenated compounds that can be used include alcohols, like methanol and TBA as well as ethers, like ethyl tert-butyl ether (ETBE) and tert-amyl methyl ether (TAME). Due to MTBE’s low cost and ease of integration into production processes, it was overwhelmingly preferred when compared to the second most commonly used oxygenate, ethanol. MTBE became the primary oxygenate utilized to comply with the Clean Air Act Amendments of 1990 until its detrimental environmental impacts were discovered.

2.2 Methyl tert-Butyl Ether Overview

MTBE was first synthesized in the 1960s by the Atlantic Richfield Corporation, now known as ARCO. The compound is created by a chemical reaction involving 2-methylpropene (isobutene) and methanol using an acid catalyst (Figure 1).
MTBE is a manmade VOC and is chemically similar to other ethers, such as ETBE (Table 1). It is a clear and flammable liquid at room temperature. MTBE has a distinctive odor and taste likened to turpentine. MTBE’s taste and odor in drinking water are strong enough to be detected at concentrations as low as 2.5 ppb for odor and 2.0 ppb for taste (Fiorenza 2002).

Table 1. Physical and chemical properties of MTBE.

<table>
<thead>
<tr>
<th>Physical and Chemical Properties of Methyl tert-Butyl Ether</th>
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<tbody>
<tr>
<td>Abbreviation</td>
</tr>
<tr>
<td>Structural Formula</td>
</tr>
<tr>
<td>Molecular Weight (g/mol)</td>
</tr>
<tr>
<td>Density (g/cm³)</td>
</tr>
<tr>
<td>Boiling Point</td>
</tr>
<tr>
<td>Solubility (mg/L)</td>
</tr>
<tr>
<td>Henry’s Law Constant (at 25 °C)</td>
</tr>
<tr>
<td>Log K_OC</td>
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<tr>
<td>Log K_OW</td>
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¹ EPA 2000
² Fayolle 2001

MTBE first rose to prominence when the EPA began phasing out leaded gasoline. Beginning in the 1920s, gasoline contained tetraethyl lead (lead) which reduced engine
knocking and improved performance. The addition of lead was an important practice because knocking could lead to overheating, waste of gasoline, and, potential damage to the engine (Seyferth 2003). Following the ban of lead, the EPA approved MTBE as a substitute to reduce engine knocking in 1987. Gasoline blends were cleared by the EPA to contain 11 percent MTBE by volume (Franklin 2000).

As stricter regulatory limits were imposed by the EPA’s Clean Air Act Amendments of 1990 for the use of cleaner fuel, MTBE production rose dramatically in the 1990s. MTBE and ethanol have long been considered primary oxygenates. However, the widespread use of MTBE was eased by its relatively inexpensive production cost and its ability to be transported through existing pipelines. In contrast, ethanol separates from gasoline when it comes in contact with water resulting in the need for separate infrastructure.

Following the implementation of MTBE as the primary gasoline oxygenate, questions were raised about its health effects on humans when exposed to MTBE, such as when refueling. Health effects of MTBE through inhalation and ingestion have long been debated and no clear findings have been established. Exposure to MTBE as an oxygenate has been documented to cause headaches, nausea, and sensory irritation (Health Effects Institute 2016). In laboratory studies using rats and mice, MTBE has been proven to be carcinogenic at high exposure levels. However, these mechanisms are not well understood and the likelihood of MTBE being carcinogenic to humans is unknown (Franklin 2000). MTBE is currently classified as a possible carcinogen and was placed on the Drinking Water Contaminant Candidate List by the EPA. No national standards are in
effect for MTBE but the EPA advises that concentrations between 20 and 40 parts per billion (ppb) are not expected to cause harmful health effects. Many states began banning MTBE as a fuel oxygenate in the early 2000s. These bans lead to a nationwide phase out of MTBE by 2006. Some states have implemented drinking water regulatory limits for MTBE. California, for example, established a secondary MCL of 5 ppb in response to taste and odor concerns in 1999 and in 2000 established a primary MCL of 13 ppb in response to health concerns (CA SWRCB n.d.).

2.3 Tert-Butyl Alcohol Overview

TBA is a manmade chemical created by the catalytic hydration of isobutylene or by the reduction of tert-butyl hydroperoxide (Toumari 2021). It has also been found as a byproduct of MTBE biodegradation. TBA is a clear, flammable liquid with a camphor-like smell. Physical and chemical properties of TBA are shown in Table 2.

<table>
<thead>
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<th>Physical and Chemical Properties of tert-Butyl Ether</th>
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<tr>
<td>Abbreviation</td>
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<tr>
<td>Structural Formula</td>
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<tr>
<td>Molecular Weight (g/mol)</td>
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<tr>
<td>Density (g/cm&lt;sup&gt;3&lt;/sup&gt;)</td>
</tr>
<tr>
<td>Boiling Point</td>
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<tr>
<td>Solubility (mg/L)</td>
</tr>
<tr>
<td>Henry’s Law Constant (at 25 °C)</td>
</tr>
<tr>
<td>Log K&lt;sub&gt;OC&lt;/sub&gt;</td>
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<tr>
<td>Log K&lt;sub&gt;OW&lt;/sub&gt;</td>
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</table>

<sup>a</sup> Toumari, 2021

<sup>b</sup> National Library of Medicine (n.d.)

<sup>c</sup> TBA is fully miscible in water.
Similar to MTBE, TBA can be used as a fuel oxygenate though its use was less common after the Clear Act Amendments of 1990 were enacted. Other uses of TBA include its addition to lacquers, paint removers, and nail polishes as well as its use as a solvent for pharmaceuticals according to the EPA. TBA’s smell also makes it an attractive option for use in the production of perfumes and fruit essences.

Because the likelihood of detecting TBA in MTBE contaminated groundwater is relatively high, TBA’s health effects have been heavily studied. Following a toxicology review by the EPA, the EPA stated that TBA is a potential carcinogen. Exposure of TBA has been known to cause irritation of mucous membranes, nausea, defatting of the skin, and intoxication (Clark 2001). Animal studies conducted on mice and rats showed that exposure to TBA contaminated water via ingestion caused kidney tumors in male rats and thyroid tumors in female mice. Carcinogenic effects on humans have not been well documented but based on animal studies, the evidence suggests TBA is carcinogenic. A MCL has not been established by the EPA for TBA, however, some states have set regulatory limits. California, for example, has a Notification Limit (NL) of 12 ppb for TBA. It’s important to note that unlike MCLs, NLS serve as advisories and are not enforceable limits.

2.4 Leaking Underground Storage Tanks

The widespread contamination of groundwater by MTBE is primarily linked to leaking underground storage tanks (LUST). The EPA estimates there are approximately 760,000 regulated underground storage tanks and between three to four million smaller,
unregulated storage tanks across the United States. Following the implementation of
RFG, municipalities began detecting high levels of MTBE in public wells. In August
1995, the City of Santa Monica detected levels of up to 610 ppb in drinking water supply
wells. The discovery lead the City to shut down half of the public wells and forced to find
an alternate water source. According to the EPA, at least 24 states report finding MTBE
contamination at sixty percent of gasoline contaminated sites. Happel et al. (1998) found
that in California, MTBE is found at 78 percent of gasoline contaminated sites.

MTBE is known to be persistent in groundwater due to its physical and chemical
properties. MTBE is highly miscible in water and is known to migrate at speeds similar to
groundwater, resulting in large MTBE plumes. Biodegradation of MTBE involves a
series of oxidation-reduction reactions. Oxygen is the most common electron acceptor in
soil systems and biodegradation rates are typically more efficient under aerobic
conditions (Figure 2). Anaerobic microorganisms are capable of breaking down organic
contaminants once oxygen is depleted at a slower rate under different reactions such as
denitrification.
Aside from the influence of its physical and chemical characteristics, the persistence of MTBE can be attributed to several site specific conditions. Unfavorable site conditions may prevent native microorganisms from degrading MTBE. Conditions associated with unfavorable growth conditions can include lack of electron acceptors such as oxygen, lack of nutrients such as nitrogen and phosphorus, and unfavorable pH levels. Additional persistence of MTBE could be due to the lack of native microorganisms able to degrade MTBE because of the absence of required enzymes.

2.5 Pathways for Biological Degradation of MTBE

MTBE was initially thought to be a recalcitrant contaminant. Jensen et al. (1990) studied the solubility and biodegradability of MTBE, TAME and BTEX (benzene, toluene, ethylbenzene, xylenes) using different inoculums. The first inoculum, described as sandy aquifer material, was added to an experiment containing 10 mg/L of MTBE and a separate experiment containing 3.5 mg/L of BTEX. It was observed that inoculation with sandy aquifer material degraded 100 percent of the BTEX concentration within thirteen days, but after sixty days, zero percent of MTBE had been degraded. Similar results were
identified when experiments were inoculated with aquifer top soil and activated sludge.
Utilizing the top soil inoculation yielded 100 percent degradation of BTEX in nine days
and zero percent degradation of MTBE after forty days. The study also tested the effects
of MTBE inhibition in the presence of BTEX. It was shown that at concentrations of up
to 40 mg/L, the presence of MTBE had no effect on the degradation rates of BTEX. At
concentrations of 200 mg/L, MTBE presented low inhibitory effects on BTEX
degradation by increasing the degradation rates of BTEX compound o/m-xylene by one
day. The study concluded that, like other ether compounds, MTBE is presumably
recalcitrant due to its tertiary carbon structure.

Similarly, Yeh et al. (1995) studied the biodegradation of MTBE, TBA and ETBE
utilizing aquifer material derived from an area near Virginia Tech. The study found that
of the three contaminants tested, TBA was the most easily degraded compound while
MTBE was the most recalcitrant compound. Under unamended, denitrifying soil
conditions, TBA was shown to have a degradation rate of approximately 0.05 mg/L/g dry
soil. Under the same conditions, no MTBE degradation was observed after 250 days.

Initial field studies concluded that natural attenuation via biodegradation is slow and
resulted in half-lives of approximately two years for MTBE. In contrast, BTEX,
considered the most water-soluble hydrocarbon of gasoline, has a natural attenuation half
live of approximately two to three months (Fayolle 2001). The recalcitrance of MTBE is
inherent to its structure, specifically, the presence of an ether bond and a tertiary carbon
structure. Degradation of MTBE was initially only observed under aerobic conditions.
However, subsequent microcosm studies have shown that biodegradation of MTBE under anaerobic conditions is possible though degradation rates are typically slower.

Biodegradation of MTBE using pure cultures and mixed cultures has been proven in microcosm studies. Degradation of MTBE has been observed when MTBE is the sole carbon source and also under cometabolic conditions. Though many studies have been conducted regarding MTBE biodegradation, the degradation pathway(s) of MTBE have yet to be fully understood. Multiple studies appear to suggest a monooxygenase enzyme, specifically cytochrome P-450, is involved in the biodegradation process of MTBE.

Steffan et al. (1997) studied the cometabolic degradation pathway of MTBE and confirmed the involvement of the monooxygenase enzyme, cytochrome P-450. Propane oxidizing bacteria were isolated from various water and soil samples, including pond water and soils contaminated with motor oil. ENV 425 was among the selected bacteria strains to degrade MTBE because of its high growth rate. ENV 425 is described as a gram-variable, filamentous, rod-shaped bacteria linked to the genus Nocardia (Steffan 1997). The study showed ENV 425 cells grown on propane and incubated with 200 mg/L of MTBE completely degraded MTBE to tertiary alcohol products such as TBA within 8 hours. Complete degradation of TBA using ENV 425 required approximately 35 hours.

According to Steffan et al. (1997) cell extracts of ENV 425 were obtained and a spectral scan showed peaks at 420 nm and 450 nm which are expected results consistent with the presence of cytochrome P-450 enzyme. The study further confirmed the presence of
cytochrome P-450 by incubating MTBE with known cytochrome P-450 inhibitors. Carbon monoxide (CO) and 1-aminobenzotriazole (ABT) were added to a microcosm studies containing 100 mg/L of MTBE or TBA. MTBE degradation was reduced by approximately 85 percent in the presence of ABT and approximately 70 percent in the presence of CO. TBA degradation was reduced by approximately 64 percent in the presence of ABT and approximately 67 percent in the presence of CO. In contrast, when CO and ABT were added to microcosms containing 2-propanol, degradation of 2-propanol was not affected since cytochrome P-450 was presumably not involved in this process.

Hardison et al. (1997) also studied the degradation of MTBE and diethyl ether (DEE) using Graphium sp. grown on n-butane. Graphium sp. is one of the few known organisms able to grow on n-alkanes. n-butane was chosen as a substrate in this study because it is an alkane known to be oxidized by cytochrome P-450. The study further confirmed the presence of cytochrome P-450 by adding inhibitors of Graphium sp. to the microcosms. Graphium sp. is known to be inhibited by alkenes and alkynes, such as acetylene and ethylene. Currently, the only monooxygenase known to be inactivated by acetylene and ethylene is cytochrome P-450 (Hardison 1997). Therefore, it is suggested that n-alkanes and MTBE are oxidized by the same enzyme, cytochrome P-450. The study also showed that though n-butane and MTBE can be degraded cometabolically, in the presence of n-butane, MTBE degradation rates decreased. Graphim sp. grown on n-butane was introduced to 18 mg/L of MTBE and was observed to have a maximum degradation rate of 0.92 mg MTBE/g cells/h. Degradation rates of MTBE were presumably lower in the
presence of n-butane due to competitive inhibition where n-butane is the preferred substrate.

Figure 3 illustrates possible degradation pathways of MTBE as documented by various studies. The monooxygenase enzyme cytochrome P-450 is believed to be the first step in the degradation process of MTBE. Monooxygenases are oxidoreductase enzymes that incorporate a hydroxyl group (-OH) into substrates. The product of introducing a hydroxyl group into MTBE is tert-Butoxy methanol. tert-butoxy methanol is oxidized in terms of hydrogen and the resulting product is tert-Butyl formate (TBF). The interaction of a hydroxyl radical and TBF result in the production of TBA. Studies conducted by Church et al. (1997) suggest that TBF is readily hydrolyzed and converted to TBA therefore no detectable levels of TBF are accumulated. Further degradation of TBA has been shown to produce two known intermediates, 2-methyl-2-hydroxy-1-propanol (MHP) and 2-hydroxyisobutyric acid (HIBA), and further metabolism concludes with CO₂ production. Studies suggest the formation of formaldehyde from MTBE degradation may be possible as a result of a non-enzymatic interaction (Fayolle 2001).

To further characterize the degradation of MTBE utilizing cytochrome P-450, studies were conducted using Pseudomonas Putida CAM. According to studies conducted by Steffan et al. (1997), Pseudomonas Putida CAM is known to produce the well characterized P-450 cytochrome. The pure culture was grown on camphor and yielded stoichiometric conversion of MTBE to TBA at a rate of 0.4 nmol/min/mg of protein (Steffan 1997).
Figure 3. Possible degradation pathway(s) of MTBE. Adapted from Fayolle 2001.
Maubert et al. (2017) studied the biodegradation of MTBE using the strain of bacteria *Bacillus amyloliquefaciens* which was isolated from agar contaminated with levels of MTBE ranging from 1,000 ppm to 5,000 ppm. Experiments using MTBE or TBA as the sole carbon source showed successful growth of *Bacillus amyloliquefaciens* and degradation of MTBE and TBA over a matter of hours. Agarose containing MTBE at a concentration of 1,000 ppm showed growth of *Bacillus amyloliquefaciens* after only 48 hours while agarose containing TBA at a concentration of 500 ppm showed growth of *Bacillus amyloliquefaciens* after 72 hours. Both cultures were incubated at a temperature of 37°C. Viable counts of *Bacillus amyloliquefaciens* were performed on experiments containing nutrient broth and varying concentrations of MTBE. After 8 hours of incubation, the growth stabilized for all different concentrations tested indicating the cell stationary phase had been reached. Degradation rates of MTBE were also analyzed using GC/MS. MTBE at a concentration of 100 ppm was added to a growth medium with *Bacillus amyloliquefaciens*. After 2 hours of incubation at 37°C, MTBE concentrations were 40 ppm and after 4 hours of incubation, MTBE concentration was 9 ppm indicating a 91% decrease over the course of 4 hours. The presence of TBA was not detected in this experiment. This study showed *Bacillus amyloliquefaciens*’ capability to degrade MTBE and TBA as the sole carbon source. Given that organisms of the genus *Bacilli* are capable of tolerating growth in various ecosystems, successful growth on MTBE and TBA is promising.

Recent studies have shown that degradation rates of MTBE under anaerobic conditions with varying electron acceptors is possible. Nitrate, iron, sulfate and methanogenesis are
among the possible electron acceptors for anaerobic biodegradation of MTBE.

Denitrification is the most energetically efficient process amongst the previously mentioned anerobic terminal electron accepting processes (Bradley 2001). Denitrification is a process in which denitrifying bacteria reduce nitrate into nitrogen gas in the presence of a carbon source. The process involves the reduction of nitrate to nitrite, further reduction involving the formation intermediaries nitric oxide and nitrous oxide, and the final reduction to nitrogen gas (Equation 1).

\[
\text{Equation 1: } \text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2
\]

MTBE is degraded under denitrifying conditions according to Equation 2 (Finneran 2001).

\[
\text{Equation 2: } \text{C}_5\text{H}_{12}\text{O} + 6\text{NO}_3^- + \text{H}^+ \rightarrow 5\text{HCO}_3^- + 3\text{N}_2 + 4\text{H}_2\text{O}
\]

According to the American Petroleum institute, biodegradation of MTBE under anaerobic conditions is not fully elucidated because no pure culture able to degrade MTBE under these conditions have been isolated. Microbiologists utilize pure cultures to establish the biochemical reactions that occur in the biodegradation process. The lack of identified pure cultures able to break down MTBE may indicate that under anaerobic conditions, degradation of MTBE may occur as a result of combined activity from mixed cultures. Kohatkar et al. (2002) indicated that biodegradation of MTBE under anaerobic
conditions may be a result of enzyme-catalyzed hydrolysis. Still, this potential pathway is not well understood because the enzymes involved are not known.

Bradley et al. (2001) studied the biodegrading of MTBE under denitrifying conditions utilizing NO₃ amended microcosms. Results of MTBE biodegradation were compared to degradation rates of unamended conditions. Radiometric detection gas chromatography (GC/RD) was utilized to determine the amount of MTBE degraded under denitrifying conditions. Microcosms containing surface water sediments collected from South Carolina were analyzed after 77 days. The recovery of ¹⁴C radioactivity was quantified to determine the percentage of MTBE that was degraded. Under denitrifying conditions, ¹⁴C total recovery was approximately 96 percent. Of the 96 percent, MTBE constituted a recovery rate of 70 percent as ¹⁴C, while TBA, CO₂, and CH₄ constituted 0, 26, and 0 percent, respectively. Because of the presence of CO₂, these results indicate that MTBE was degraded. The absence of TBA recovery also indicates that microorganisms are able to biodegrade TBA under denitrifying conditions since TBA is a known intermediary of MTBE. Compared to aerobic biodegradation, MTBE degradation rates are generally lower under anaerobic, denitrifying conditions.

### 2.6 Pathways for Biological Degradation of TBA

Similar to MTBE, the biological degradation pathway of TBA is not fully elucidated. The intermediaries 2-methyl-2-hydroxy-1-propanol and 2-hydroxyisobutyric acid have been shown in previous TBA degradation studies. Complete biodegradation of TBA yields
CO₂ and H₂O as products under aerobic conditions (Figure 4). Under denitrifying conditions, complete biodegradation of TBA yields CO₂ and N₂ as products.

Under aerobic conditions, a monooxygenase enzyme is presumably involved in the biodegradation of TBA, resulting in the formation of 2-methyl-2-hydroxy-1-propanol. MTBE degradation studies using pure and mixed cultures have shown complete degradation of MTBE in the absence of TBA indicating that microorganisms capable of degrading MTBE can degrade TBA using the same mechanisms.
Figure 4. Possible biological degradation pathway of TBA. Adapted from Shamsipur et al. 2012.
Like MTBE, the American Petroleum Institute, states that no pure cultures able to biodegrade TBA under anaerobic conditions have been isolated. Because isolation a pure culture able to degrade TBA under anaerobic conditions has not been identified, the biodegradation process under these conditions is not well understood. Mixed cultures have been documented to degrade TBA under anaerobic conditions.

TBA degradation under denitrifying conditions is presumed to be in accordance with Equation 3 based on the MTBE reaction documented by Finneran et al. (2001).

Equation 3: \[ C_4H_{10}O + 4.8NO_3^- + 0.8H^+ \rightarrow 4HCO_3^- + 2.42N_2 + 3.4H_2O \]

Bradley et al. (2002) studied the biodegradation of TBA under aerobic and anaerobic conditions using microcosms containing native microorganisms from surface water sediments collected in South Carolina. The effect of varying terminal electron acceptors was also analyzed. Degradation under oxic, denitrifying, and Mn(IV) reducing conditions were found to suitably degrade TBA. However, degradation under SO\(_4\) reducing conditions showed minimal mineralization of TBA to CO\(_2\) and methanogenic and Fe(III) reducing conditions indicated no degradation of TBA. To quantify the amount of TBA degraded, GC/RD was utilized. After incubating for 198 days, the recovery of \(^{14}\)C radioactivity was quantified to determine the percentage of TBA that was degraded. Under unamended conditions 100 percent of the \(^{14}\)C-total was recovered as TBA and no percentage of CO\(_2\) \(^{14}\)C was detected indicating no biodegrading of TBA occurred. Under oxic conditions, the study showed no detection of TBA and 99 percent of the \(^{14}\)C-total was recovered as CO\(_2\) indicating near complete degradation of TBA to CO\(_2\). Under
denitrifying conditions amended with NO₃, 90 percent of total ¹⁴C was recovered. Recovery of TBA as ¹⁴C was evaluated at 20 percent while 70 percent of ¹⁴C was recovered as CO₂ indicating successful biodegradation of TBA under denitrifying conditions. In contrast, the study found that under Fe(III) reducing conditions, 97 percent of ¹⁴C was recovered as TBA and no percentage of ¹⁴C was covered indicating anaerobic degradation rates vary based on environmental conditions.

Successful degradation of TBA under denitrifying conditions has been shown to break down TBA to yield CO₂ and N₂ as products. Yeh (1995) studied the degradation rate of MTBE, TBA and ETBE under denitrifying conditions utilizing nitrate as an electron acceptor. The addition of nitrate resulted in increased degradation rates of TBA and a reduction in lag time. Under unamended conditions, the observed TBA degradation rate was 0.05 mg/L/day/g dry soil while denitrifying conditions amended with nitrate and nutrients showed a degradation rate of 0.2 mg/L/day/g dry soil, an increase in degradation rates of approximately 300 percent. The concentration of TBA was degraded to 80 percent of the original concentration after nearly 600 days under unamended conditions in comparison to less than 200 days under nitrate and nutrient amended conditions. The presence of ethanol showed a decrease in TBA biodegradation rates indicating that ethanol may have stimulated the growth of microorganisms that compete with TBA degrading microorganisms or the presence of ethanol may inhibit TBA degrading enzymes. To date, studies suggest that TBA degradation under anaerobic conditions is possible but the process is sensitive to available electron acceptors.
2.7 BiOWiSH Overview

Biowish® Aqua (Aqua) is commercially available product produced by Biowish Technologies. It consists of a unique consortium of bacteria, nutrients and enzymes that aid in accelerating the biological removal of contaminants. According to Biowish Technologies, Aqua is capable of degrading complex organic compounds into simpler end products by enhancing biological hydrolysis and oxidation. The bacterial content of the proprietary blend is known to include primarily *Lactobacillus* and *Bacillus*. Active microbial cultures make up less than one percent of the composition of Aqua while dextrose makes up between 95-99.5% of the composition and salt makes up the remaining one percent (BiOWiSH n.d.).

Gorsuch et al. (2012) isolated various distinct bacterial isolates from Aqua. The bacterial isolates identified are shown in Table 3.

*Table 3. Bacterial Isolates of BiOWiSH Aqua identified by Gorsuch et al. (2012).*

<table>
<thead>
<tr>
<th>Bacterial Isolates from BiOWiSH Aqua</th>
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<tbody>
<tr>
<td><em>Bacillus licheniformis</em></td>
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<tr>
<td><em>Bacillus amyloliquefaciens</em></td>
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<tr>
<td><em>Bacillus mojavensis</em></td>
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<tr>
<td><em>Bacillus subtilis</em></td>
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<tr>
<td><em>Bacillus pumilus</em></td>
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<tr>
<td><em>Pediococcus pentosaceus</em></td>
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<tr>
<td><em>Pediococcus acidilactici</em></td>
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<tr>
<td><em>Lactobacillus plantarum</em></td>
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</table>
The genus *Bacillus* is among the species that has been shown to successfully biodegrade MTBE and TBA under aerobic conditions and has also shown the ability to break down contaminants under denitrifying conditions.
3. MATERIALS AND METHODS

MTBE and TBA biodegradation rates were analyzed using solid phase microextraction techniques and Gas Chromatography – Mass Spectrometry (GC/MS). In conjunction with GC/MS, optical density measurements were taken to estimate microbial cell growth.

3.1 Gas Chromatography – Mass Spectrometry

Gas chromatography – Mass Spectrometry analysis consists of volatilization of a sample that is carried by an inert gas through a capillary column. The sample is then separated into its various components by the gas chromatograph. The time it takes for each compound in the sample to elude is referred to as retention time. Retention time is based on the compounds boiling point and polarity. The retention time is used to identify the compound by comparing it to commercial libraries. Compounds leaving the gas chromatograph are then ionized and fragmented by mass spectrometry. A mass-to-charge (m/z) ratio is identified allowing the compounds of the sample to be identified and quantified by comparing the ratio to commercial libraries. The m/z ratio is 73 and 59 for MTBE and TBA, respectively.

Extracted MTBE and TBA samples were analyzed using Agilent Technologies 6890N Gas Chromatograph (splitless inlet) with an Agilent 5975B inert Mass Selective Detector (Figure 5). The gas chromatograph used a 30-m fused silica column with a film thickness of 0.25 micrometers and an inner diameter of 0.25 millimeters (Agilent Catalog #19091S-
Helium was utilized as the carrier gas. The temperature of the gas chromatograph was maintained at 50°C for 4 minutes, then ramped to 90°C at a rate of 20°C per minute and held for 3 minutes, then ramped to 200°C at a rate of 40°C for the remainder of the run time.

3.2 Solid Phase Microextraction

Solid phase microextraction (SPME) is a widely used technique for extracting and quantifying VOCs. It consists of a solvent free process that involves the use of an adsorptive fiber exposed to the headspace above a sample. During extraction time, the analytes in the sample partition from the sample matrix and are adsorbed by the exposed fiber. The fiber can then be removed and inserted into a gas chromatograph for analysis.
Samples were collected using a Supelco SPME fiber (Sigma Aldrich #57334-U) and SPME fiber holder (Sigma Aldrich #57330-U) intended for manual sampling. The selected fiber was an 85 micrometer carboxen-polydimethylsiloxane (CAR/PDMS) fiber. According to Sigma Aldrich, this fiber is recommended for gases and low molecular weight compounds with molecular weights between 30 and 225. The fiber was conditioned per manufacturer’s recommendations. The fiber was inserted into vials fitted with PTFE-lined silicone septum to collect samples.

*Figure 6. Supelco 85 micrometer CAR/PDMS SPME fiber was utilized for MTBE and TBA extraction and GC analysis.*
The SPME technique is a very sensitive process. It is critical to keep the process as consistent and precise as possible. Factors that influence the extraction method, and should thus remain constant, are:

- Extraction Time
- Temperature
- Agitation
- Salt content
- Amount of Headspace
- SPME fiber purging and Sampling Vials (cleanliness)

Extraction time for each sample was set at 20 minutes. Cassada et al. (2000) reported the effect of varying extraction times versus MTBE response resulted in minimal response differences for extraction times over 10 minutes. After 25 minutes, responses began decreasing. This study tested extraction times at 10 minutes and 20 minutes and found area responses were more accurate at 20 minute extraction times.

Optimum temperature for headspace extraction range from 20 to 65°C. This study used an extraction temperature of 35°C. The hot plate was set to an agitation rate of 100 rotations per minute.

Adjusting salt concentration can improve the extraction efficiency by changing the solubility of the analytes in the sample. Salt increases the ionic strength and promotes volatilization of the analytes to the headspace. Acten et al. (2001) reported salt
concentrations less than 10 percent (w/w) yielded lower MTBE responses while concentrations higher than 25 percent w/w yielded fluctuating results. A salt concentration of 10 percent w/w was utilized in this study after testing varying concentrations that resulted in discrepancies.

Optimal headspace volume used was 0.5 volume ratio (ml of solution/ml of vial) as determined by Arambarri et al (2004).

The SPME fiber was purged between samples at a temperature of 250°C using the GC. Blanks were also run between samples to verify that all adsorbed analytes from previous samples had been removed.

3.3 Optical Density Measurements

Optical density measurements utilize spectrophotometers to determine biomass present. Light passes through a cuvette containing microorganisms in suspension and the amount of scattered light in turn indicates the biological growth in the system. Measurements were taken with a wavelength set at 460 nm using a Thermo Scientific GENESYS 10S UV-Visible Spectrophotometer (Figure 6). Using a pipette, 1 mL of sample was pipetted into sterilized cuvettes and optical measurements were recorded.
Figure 7. Thermo Scientific GENESYS 10S UV-Visible Spectrophotometer was utilized for optical density measurements.

3.4 pH Measurements

pH measurements were taken at the start and end of each experiment to ensure pH levels were not inhibiting biological activity. Measurements were taken using an Oakton Acorn series pH/temperature meter. The meter was calibrated per the manufacturer’s recommendations.

3.5 Chemicals

MTBE (assay percentage rate 99.97%) and TBA (assay percentage rate 99%) were obtained from Fisher Scientific. Stock solutions of MTBE and TBA (20,000 ppm) were prepared and stored in a refrigerator. Fresh working solutions were prepared by diluting
the stock solution in deionized water whenever required. Sodium nitrate was obtained from Fisher Scientific to introduce nitrate and simulate denitrifying conditions.

3.6 BiOWiSH Aqua Dosing

To increase microbial populations prior to inoculation, Aqua was activated 24 hours prior (Figure 8). A 10,000 ppm stock solution of activated Aqua was prepared by adding 3 g of dry Aqua product to 300 mL of growth media (Appendix A). The stock solution was placed in an incubator shaker at 25°C and 100 rpm for 24 hours to allow microbial growth. To further promote microbial growth, a solution containing micronutrients was added to the growth media as recommended by BiOWiSH (Appendix B).
Prior to activation and inoculation, all materials including glassware, stoppers, growth media, and micronutrient solution were sterilized using an autoclave. Sterilized growth media and micronutrient solution were inoculated according to the prescribed MTBE or TBA concentration and placed into an incubator shaker at 25°C and 100 rpm until ready to be used.

3.7 Aerobic Degradation of MTBE and TBA
The goal of this experiment was to test the effectiveness of Aqua to biodegrade MTBE and TBA at various concentrations under aerobic conditions. The concentrations tested were 5 ppm, 10 ppm and 25 ppm. Each system consisted of 200 mL of sterile growth media, 2 mL of mineral salt solution, activated Aqua, and MTBE or TBA at the respective concentrations previously mentioned (Figure 9 and Figure 10).

*Figure 9. Duplicate experiments at MTBE concentrations of 5, 10 and 25 ppm under aerobic conditions.*
Sterilized 250 mL flasks were utilized for each system and were capped using silicone sponge closures in order to maintain aerobic conditions but minimize evaporation. Samples were stored in an incubator shaker and taken over the course of two weeks. 25 mL samples were collected using a transfer pipette and stored in a 50 mL glass container for analysis. Table 4 summarizes the experimental design.

Table 4. Experimental design for aerobic biodegradation of MTBE and TBA.

<table>
<thead>
<tr>
<th>Aerobic MTBE and TBA Experiment</th>
<th>Media</th>
<th>Sterilized Growth Media, Sterilized Mineral Salt Solution</th>
</tr>
</thead>
</table>
**MTBE Concentrations**  5 ppm, 10 ppm, 25 ppm  
**TBA Concentrations**  5 ppm, 10 ppm, 25 ppm  
**Electron Acceptor**  Oxygen  
**Aqua**  1,000 ppm  
**Duration**  14 days  
**Temperature**  25°C  
**Speed**  100 rpm  
**Analysis and Frequency**  
- pH @ initial and final  
- Optical Density @ Day 0, 3, 7, 10, 14  
- GC/MS @ Day 0, 3, 7, 10, 14

### 3.8 Anaerobic Degradation of MTBE and TBA

The goal of this experiment was to test the effectiveness of Aqua to biodegrade MTBE and TBA at various concentrations under anaerobic conditions. The concentrations tested were 5 ppm, 10 ppm and 25 ppm. Each system consisted of 200 mL of sterile growth media, 2 mL of mineral salt solution, activated Aqua, and MTBE or TBA at the respective concentrations previously mentioned (Figure 11 and Figure 12).
Figure 11. Duplicate experiments at MTBE concentrations of 5, 10 and 25 ppm under anaerobic conditions.
Sterilized 250 mL flasks were utilized for each system and were capped using rubber stoppers in order to simulate anaerobic conditions. Samples were stored in an incubator shaker and taken over the course of two weeks. The rubber stopper was removed prior to sample collection and replaced immediately after to minimize introduction of air and thus a strict anaerobic conditions were not fully employed. While an attempt to simulate anaerobic conditions was made, the conditions most likely represented anoxic conditions. 25 mL samples were collected using a transfer pipette and stored in a 50 mL glass container for analysis. Table 5 summarizes the experimental design.
### Table 5. Experimental design for anaerobic biodegradation of MTBE and TBA.

<table>
<thead>
<tr>
<th>Anaerobic MTBE and TBA Experiment</th>
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<tbody>
<tr>
<td><strong>Media</strong></td>
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<tr>
<td><strong>MTBE Concentrations</strong></td>
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<td><strong>TBA Concentrations</strong></td>
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<tr>
<td><strong>Electron Acceptor</strong></td>
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<tr>
<td><strong>Aqua</strong></td>
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<td><strong>Duration</strong></td>
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<td><strong>Temperature</strong></td>
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<td><strong>Speed</strong></td>
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<td><strong>Analysis and Frequency</strong></td>
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### 3.9 Anaerobic Degradation of MTBE and TBA Under Denitrifying Conditions

The goal of this experiment was to test the effectiveness of Aqua to biodegrade MTBE and TBA at various concentrations under denitrifying conditions. The concentrations tested were 5 ppm, 10 ppm and 25 ppm. Each system consisted of 200 mL of sterile growth media, 2 mL of mineral salt solution, activated Aqua, and MTBE or TBA at the respective concentrations previously mentioned (Figure 13 and Figure 14). Crystalline sodium nitrate was added to each system to simulate denitrifying conditions in which
nitrate performs as the terminal electron acceptor. Optimal sodium nitrate amounts were calculated and added to each system based on Equation 2 and 3 for MTBE and TBA, respectively.

Figure 13. Duplicate experiments at MTBE concentrations of 5, 10 and 25 ppm under denitrifying conditions.
Sterilized 250 mL flasks were utilized for each system and were capped using rubber stoppers in order to simulate anaerobic conditions. Samples were stored in an incubator shaker and taken over the course of two weeks. Similar to the anaerobic experimental design, rubber stoppers were removed prior to sample collection and replaced immediately after resulting in unstrict anaerobic conditions. 25 mL samples were collected using a transfer pipette and stored in a 50 mL glass container for analysis. Table 6 summarizes the experimental design.
Table 6. Experimental design for biodegradation of MTBE and TBA under denitrifying conditions.

<table>
<thead>
<tr>
<th>Denitrification MTBE and TBA Experiment</th>
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<tbody>
<tr>
<td>Media</td>
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<tr>
<td>MTBE Concentrations</td>
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<tr>
<td>TBA Concentrations</td>
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<tr>
<td>Electron Acceptor</td>
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<td>Aqua</td>
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<td>Duration</td>
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<td>Analysis and Frequency</td>
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3.10 Aerobic Degradation of MTBE and TBA with Glucose Present

The goal of this experiment was to test the effectiveness of Aqua to biodegrade MTBE and TBA at various concentrations under aerobic conditions in the presence of glucose. Previous studies have indicated the presence of other carbon sources can enhance the biodegradation rate of MTBE and TBA. The concentrations of MTBE and TBA tested were 5 ppm, 10 ppm and 25 ppm. Each system consisted of 200 mL of sterile growth
media, 2 mL of mineral salt solution, activated Aqua, glucose, and MTBE or TBA at the respective concentrations previously mentioned (Figure 15 and Figure 16).

Figure 15. Duplicate experiments with glucose added at MTBE concentrations of 5, 10 and 25 ppm under aerobic conditions.
Sterilized 250 mL flasks were utilized for each system and were capped using silicone sponge closures in order to maintain aerobic conditions but minimize evaporation. Samples were stored in an incubator shaker and taken over the course of two weeks. 25 mL samples were collected using a transfer pipette and stored in a 50 mL glass container for analysis. Table 7 summarizes the experimental design.
Table 7. Experimental design for biodegradation of MTBE and TBA under aerobic conditions with glucose added.

<table>
<thead>
<tr>
<th>Denitrification MTBE and TBA Experiment</th>
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<tbody>
<tr>
<td>Media</td>
</tr>
<tr>
<td>MTBE Concentrations</td>
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<tr>
<td>TBA Concentrations</td>
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<tr>
<td>Additional Carbon Source</td>
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<tr>
<td>Electron Acceptor</td>
</tr>
<tr>
<td>Aqua</td>
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<tr>
<td>Duration</td>
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<td>Analysis and Frequency</td>
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3.11 Quality Assurance/Quality Control (QA/QC)

QA/QC methods were employed to ensure precise and accurate results. Methods used included sample replicates, spikes, and splits. Sample replicates were utilized to verify accuracy of extraction and analysis methods. Split samples needed to be within ±15% of the expected value. Results of spike samples also needed to be within ±15%.
4. RESULTS AND DISCUSSION

To understand the effectiveness of Aqua to degrade MTBE and TBA, it was crucial to set up good extraction and analysis methods. The success of biodegradation depends on a number of environmental factors and this study aimed to determine which conditions could prove optimal in the degradation process of MTBE and TBA utilizing Aqua.

GC/MS calibration curves helped determine the estimated concentration of MTBE and TBA during each experiment. Previously mentioned stock solutions and methods were used to create a curve for MTBE and TBA. Calibration curves for MTBE and TBA are shown below as Figure 17 and Figure 18, respectively.

Calibration curves were created for varying concentrations between 0 and 25 ppm and yielded $R^2$ values of 0.987 and 0.955 for MTBE and TBA, respectively. It’s important to note that standard concentrations higher than 25 ppm, including 50 ppm, 75 ppm and 100 ppm, were created and tested; however, at higher concentrations results were skewed and resulted in lower $R^2$ values. This indicates that while the method used is suitable for concentrations less than 25 ppm, there is still room for improvement and it is especially crucial to continue developing the method to analyze the effect of Aqua at higher concentrations of MTBE and TBA.
Figure 17. MTBE calibration curve.

Figure 18. TBA calibration curve.
pH is considered one of the most important environmental factors that affect biodegradation. Optimal pH levels for microbial degradation is between 6 and 8 (ICSS 2006). pH was monitored at the start of each experiment and at the end to confirm pH levels were suitable for the bioremediation process.

Optical density measurements at 460 nm were utilized to estimate microbial growth. Dilutions of concentrated MTBE and TBA were created and the optical density of each dilution was recorded. 200 mL dilution samples were then filtered using Fisherbrand G4 Glass Fiber Filters and put in an oven at 90°C for 12 hours. After removing from the oven, the dry weights were recorded and graphed against the samples respective recorded optical density. Figure 19 shows the relationship between biomass concentrations of MTBE and TBA and optical density. Given the optical density of a sample, the calibration curve shown in Figure 19 could then be used to estimate biomass concentration in the sample.
Figure 19. Relationship between absorbance at 460 nm and estimated biomass concentration.

For each of the following aerobic conditions tested for MTBE and TBA, it is presumed a monooxygenase enzymatic reaction is involved. Specifically, the monooxygenase cytochrome P-450 is assumed to be present in the Aqua mixed culture used and therefore involved in breaking down MTBE and TBA. De Mot et al. (2002) confirmed cytochrome P-450 is present in the genus Bacilli. The biological reaction involved for MTBE and TBA is suspected to be in accordance with Figures 3 and 4. In all MTBE degradation experiments, trace amounts of TBA were present indicating that degradation of MTBE and TBA was occurring in these experimental set ups.

Under anaerobic conditions, the degradation pathway for MTBE and TBA has yet to be fully elucidated. Because of this, this study cannot indicate which enzymatic reactions are presumably occurring in the conducted experiments. Previous studies have indicated a pure culture able to anaerobically biodegrade MTBE and TBA has not been identified but
mixed cultures have been found to breakdown both contaminants under anaerobic conditions. It is presumed that due to the combined activity of the cultures present in Aqua, MTBE and TBA were biodegraded under anaerobic conditions. However, as previously mentioned, strict anaerobic conditions were not held throughout the studies. Introduction of oxygen was minimized every time samples were taken, however, the presence of trace amounts of oxygen could skew results.

Under denitrifying conditions, Aqua has been shown to perform well by other studies testing its performance in reducing Total Nitrogen concentrations. Under denitrifying conditions with nitrate conditions, Aqua was able to degrade MTBE and TBA.

Each experiment showed degradation of MTBE and TBA to varying degrees. The following sections describe the results of each set up.

**4.1 Results of Aerobic Degradation of MTBE**

Under aerobic conditions near complete degradation of MTBE occurred in the presence of Aqua. Average initial pH conditions of each experiment ranged from 6.68 to 6.77 while average final pH conditions ranged from 6.66 to 6.74 (Figure 20). The results indicated a slight drop in pH values, however the conditions remained within the previously mentioned optimal pH range of 6 to 8. Therefore it is assumed pH had no inhibitory effects on the biodegradation of MTBE.
Optical density measurements for all aerobic MTBE experiments showed a rapid increase in absorbance between Day 0 and Day 3 indicating microbial growth was rapid and this time frame was presumably the exponential growth phase (Figure 21). It is assumed a lag phase is not apparent due to the activation of Aqua. The rapid growth between Day 0 and Day 3 can be attributed to Aqua’s composition of 99% dextrose where microbial growth significantly increased as a result of dextrose being consumed by the bacteria. Following the peak growth, a slight decrease in absorbance was observed and followed by a slight increase and thereafter a steady growth of bacteria, presumed to be a stationary phase.
The relationship between recorded absorbance measurements and biomass concentration was used to calculate approximate biomass concentration throughout the 14 days as compared to MTBE concentration (Figure 22, 23 and 24). In all cases the growth in biomass concentration correlates with the decrease in MTBE concentration indicating that as Aqua microorganisms matured, they consumed MTBE.
Figure 23. MTBE concentration vs. biomass concentration for starting MTBE concentration of 5 ppm.

Figure 22. MTBE concentration vs. biomass concentration for starting MTBE concentration of 10 ppm.
In every case tested under aerobic biodegradation conditions, trace amounts of MTBE were detected after 14 days. On average, aerobic degradation of MTBE was estimated at 99.80 percent (Table 8). As previously mentioned, trace amounts of TBA were detected indicating that both MTBE and TBA degradation was occurring under the conditions presented.

Table 8. Percent of MTBE biodegradation under aerobic conditions.

<table>
<thead>
<tr>
<th>MTBE Percent Biodegradation</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>5 ppm</td>
<td>99.84</td>
</tr>
<tr>
<td>10 ppm</td>
<td>99.90</td>
</tr>
<tr>
<td>25 ppm</td>
<td>99.64</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>99.80</strong></td>
</tr>
</tbody>
</table>

*Figure 24. MTBE concentration vs. biomass concentration starting MTBE concentration of 25 ppm.*
4.2 Results of Aerobic Degradation of TBA

Biodegradation of TBA under aerobic conditions was observed in this experiment. During this experimental set up, pH was maintained between the optimal range of 6 to 8. Initial average pH values ranged from 6.69 to 6.76 while final average pH values ranged from 6.64 to 6.8 (Figure 25). This again indicated a slight decrease in pH over the course of the experiment but not enough to bring the pH below a level that would have detrimental effects to the biodegradation process.

![Initial and final pH measurements for aerobic MTBE biodegradation.](image)

Optical density measurements for aerobic TBA biodegradation showed similar trends to MTBE microbial growth. A lag phase was not observed in this experiment also presumably due to the activation period of Aqua. An exponential growth phase was observed between Day 0 and Day 3 where it is presumed bacteria are consuming the dextrose present in Aqua, followed by a slight decrease in absorbance but an increase in
measured absorbance for the 10 ppm set, a decrease for the 5 ppm set up, and a stable, stationary phase for the 25 ppm set up (Figure 26).

![Graph](image)

*Figure 26. Optical density results for aerobic TBA biodegradation.*

In all cases the growth in biomass concentration correlates with the decrease in TBA concentration indicating that TBA was utilized as a substrate by Aqua (Figure 27, 28 and 29). While biomass concentrations followed the same trends as MTBE wherein biomass increased as concentration decreased, it is interesting to note that in this experiment there was a higher concentration of active biomass at all times compared to the aerobic MTBE experiment, but degradation rates were lower in this experiment. This indicates that under aerobic conditions, MTBE is the preferred substrate when compared to TBA.
Figure 27. TBA concentration vs. biomass concentration for starting TBA concentration of 5 ppm.

Figure 28. TBA concentration vs. biomass concentration for starting TBA concentration of 10 ppm.
Under aerobic conditions, TBA degradation was less than MTBE but still yielded high degradation rates. On average, an 81.26 percent decrease in TBA concentration was found utilizing Aqua under aerobic conditions (Table 9).

4.3 Results of Anaerobic Degradation of MTBE

Under anaerobic conditions degradation of MTBE in the presence of Aqua was observed but at a slower rate. Average initial pH conditions of each experiment ranged from 6.68 to 6.87 while average final pH conditions ranged from 6.71 to 6.90 (Figure 30). The
results indicated a slight drop in pH value for the 25 ppm experiment but pH levels were for the other experiments remained near the initial pH values or slightly increased. These changes, however remained within the previously mentioned optimal pH range of 6 to 8. pH is not assumed to have had an inhibitory effect on the biodegradation of MTBE under anaerobic conditions.

![Figure 30. Initial and final pH measurements for anaerobic MTBE biodegradation.](image)

Optical density measurements for anaerobic MTBE biodegradation showed an increase in measured absorbance between Day 0 and Day 3 presumably due to the composition of Aqua which includes 99% dextrose, followed by a slight decrease in absorbance levels and ending with another increase that did not level off by Day 14 indicating that by Day 14 the microbes had not yet reached a stationary phase (Figure 31).
Under anaerobic conditions, biomass concentration had a semi-direct correlation with concentration decrease (Figure 32, 33 and 34). Between Day 0 and Day 7 biomass concentration varied as the concentration of MTBE kept decreasing. At Day 7, calculated biomass concentration dropped to near starting rates and was followed by a steep increase in biomass concentration thereafter. One theory about the fluctuations in biomass concentrations is that the introduction of oxygen could have skewed the results.

Anaerobic experiments were capped with rubber stoppers to prevent oxygen from entering, however, at the time of sampling the rubber stoppers were removed, a sample was pulled, and the rubber stopper was quickly returned. During the short sampling times, the introduction of oxygen could have interfered with the experiment as oxygen will always be the preferred electron acceptor. The figures indicate an upwards trend in biomass concentration indicating that microbes had not yet reached a stationary phase and given more time, degradation would have continued.
Figure 32. MTBE concentration vs. biomass concentration for starting MTBE concentration of 5 ppm.

Figure 33. MTBE concentration vs. biomass concentration for starting MTBE concentration of 10 ppm.
Under anaerobic conditions presented in this study, MTBE degradation did occur but anaerobic conditions were not strict. On average, a 55.4 percent decrease in MTBE concentration was found utilizing Aqua under anaerobic conditions (Table 10).

<table>
<thead>
<tr>
<th>MTBE Percent Biodegradation</th>
<th>5 ppm</th>
<th>10 ppm</th>
<th>25 ppm</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 ppm</td>
<td>69.99</td>
<td>27.43</td>
<td>62.76</td>
<td>53.40</td>
</tr>
</tbody>
</table>

4.4 Results of Anaerobic Degradation of TBA

Biodegradation of TBA under anaerobic conditions was observed in this study at rates slower than aerobic biodegradation. During this experimental set up, pH was maintained between the optimal range of 6 to 8. Initial average pH values ranged from 6.67 to 6.8...
while final average pH values ranged from 6.64 to 6.8 (Figure 35). Although a slight decrease in pH was observed, pH is not assumed to have any effects on the results.

![Figure 35. Initial and final pH measurements for anaerobic TBA biodegradation.](image)

Optical density measurements for anaerobic TBA biodegradation showed an increase in measured absorbance between Day 0 and Day 3 like previous experiments due to the presence of dextrose, followed by a decrease in absorbance and a repeated increase. Similar to MTBE anaerobic conditions, absorbance did not level off by Day 14 indicating that by Day 14 the microbes had also not yet reached a stationary phase (Figure 36).
Under anaerobic conditions for TBA biodegradation, a similar phenomenon to MTBE anaerobic biodegradation was observed wherein biomass concentration had a semi-direct correlation with concentration decrease (Figure 37, 38 and 39). Between Day 0 and Day 7 biomass concentration varied as the concentration of TBA kept decreasing. Yet again at Day 7, calculated biomass concentration dropped to near starting rates and was followed by a steep increase in biomass concentration thereafter. Following the theory presented in the previous section about non-strict anaerobic conditions possibly explaining the initial drop of biomass due to the microbes affinity to use oxygen as an electron acceptor before using nitrate could potentially explain the biomass trends observed. The figures for TBA also indicate an upwards trend in biomass concentration after Day 7 indicating that microbes had not yet reached a stationary phase and given more time, degradation would have continued.
Figure 37. TBA concentration vs. biomass concentration for starting TBA concentration of 5 ppm.

Figure 38. TBA concentration vs. biomass concentration for starting TBA concentration of 10 ppm.
Under anaerobic conditions presented in this study, TBA was observed at rates similar to MTBE. On average, a 52.08 percent decrease in MTBE concentration was found utilizing Aqua under anaerobic conditions (Table 11). TBA biodegradation under anaerobic conditions is presumed to be a result of the combined activity of cultures present in Aqua.

Figure 39. TBA concentration vs. biomass concentration for starting TBA concentration of 25 ppm.

<table>
<thead>
<tr>
<th>Concentration (ppm)</th>
<th>Control</th>
<th>Biomass (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>5.00</td>
<td>0.00</td>
</tr>
<tr>
<td>5.00</td>
<td>10.00</td>
<td>5.00</td>
</tr>
<tr>
<td>10.00</td>
<td>15.00</td>
<td>10.00</td>
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<td>15.00</td>
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<td>15.00</td>
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<td>20.00</td>
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<td>60.00</td>
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<tr>
<td>60.00</td>
<td>65.00</td>
<td>60.00</td>
</tr>
<tr>
<td>65.00</td>
<td>70.00</td>
<td>65.00</td>
</tr>
</tbody>
</table>

Table 11. Percent of TBA biodegradation under anaerobic conditions.

<table>
<thead>
<tr>
<th>TBA Percent</th>
<th>Biodegradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 ppm</td>
<td>53.63</td>
</tr>
<tr>
<td>10 ppm</td>
<td>50.59</td>
</tr>
<tr>
<td>25 ppm</td>
<td>52.02</td>
</tr>
<tr>
<td>Average</td>
<td>52.08</td>
</tr>
</tbody>
</table>
4.5 Results of Anaerobic Degradation of MTBE Under Denitrifying Conditions

Biodegradation of MTBE under denitrifying conditions was observed at rates similar to MTBE degradation under anaerobic conditions. Like all other experiments performed, the pH of these microcosms was maintained between the optimal range of 6 to 8. Initial average pH values ranged from 6.84 to 6.86 while final average pH values ranged from 6.84 to 6.9 (Figure 40). Under these conditions, final pH increased in the 5 and 10 ppm microcosm and remained nearly constant in the 25 ppm microcosm. pH levels are assumed to have had no inhibitory effects on the results.

![Figure 40. Initial and final pH measurements for MTBE biodegradation under denitrifying conditions.](image)

Optical density measurements under denitrifying conditions were observed to show an exponential growth phase between Day 0 and Day 3 (Figure 41). This growth can be attributed to the presence of dextrose in Aqua and the microbes affinity to consume dextrose. Following Day 3, the 5 ppm microcosm showed a decrease in microbial growth, reaching a near stationary phase but still indicating a slow growth until Day 14. The 10
ppm microcosm presumably entered a stationary phase after Day 3 as absorbance levels remained near constant for the remainder of the experiment while the 25 ppm microcosm showed a sharp decrease in absorbance for the remainder of the experiment.

![Figure 41: Optical density results for MTBE biodegradation under denitrifying conditions.](image)

In all cases the growth in biomass concentration directly correlated with the decrease in MTBE concentration indicating that MTBE was consumed by Aqua under denitrifying conditions (Figure 42, 43 and 44). The 5 ppm microcosm showed a continuous increase in biomass and a continuous decrease in MTBE concentration. Whereas the 10 ppm microcosm showed a constant biomass concentration between Day 3 and Day, this trend can also be observed in MTBE concentration where during this time concentration remained mostly unchanged. After Day 10, the slight increase in biomass concentration correlated with a drop in MTBE concentration. For the 25 ppm microcosm, after Day 3 biomass concentrations continued dropping indicating MTBE was not readily being
broken down as the concentration was shown to decrease at very low levels throughout the remainder of the experiment.

Figure 42. MTBE concentration vs. biomass concentration for starting MTBE concentration of 5 ppm.
Figure 43. MTBE concentration vs. biomass concentration for starting MTBE concentration of 10 ppm.

Figure 44. MTBE concentration vs. biomass concentration for starting MTBE concentration of 25 ppm.
Under denitrifying conditions presented in this study, MTBE concentrations were observed to decrease at levels less than aerobic conditions. On average, a 54.37 percent decrease in MTBE concentration was found utilizing Aqua under denitrifying conditions (Table 12). Degradations under denitrifying conditions proved to be only slightly higher compared to anaerobic conditions which yielded an average percent decrease of 53.4 percent.

<table>
<thead>
<tr>
<th>MTBE Percent Biodegradation</th>
</tr>
</thead>
</table>
| 5 ppm                       | 64.45         
| 10 ppm                      | 44.47         
| 25 ppm                      | 54.20         
| **Average**                 | **54.37**     |

**4.6 Results of Anaerobic Degradation of TBA Under Denitrifying Conditions**

Under denitrifying conditions degradation of TBA in the presence of Aqua was observed at a rate equivalent to biodegradation under anaerobic conditions. Average initial pH conditions of each experiment ranged from 6.92 to 7.02 while average final pH conditions ranged from 6.93 to 7.06 (Figure 45). pH values were observed to fluctuate over the course of the study period, but remained within the previously mentioned optimal pH range of 6 to 8. pH is not assumed to have had an inhibitory effect on the biodegradation of TBA under denitrifying conditions.
Optical density measurements, like all previous experiments, indicated an increase in microbial growth between Day 0 and Day 3 due to the presence of dextrose in Aqua (Figure 46). After Day 3, absorbance dropped for the 5 ppm microcosm showing a presumed decrease in microbial growth and reaching a near stationary phase by Day 14. The 10 ppm microcosm showed decreases in absorbance levels after Day 7 and reached absorbance levels similar to initial conditions by Day 14 indicating new microbial growth was no longer occurring. In contrast, the 25 ppm microcosm showed fluctuating absorbance measurements and no trend or explanation could be identified for these results.
Results of biomass concentration versus TBA concentration are shown in Figure 47, 48 and 49 for 5 ppm, 10 ppm and 25 ppm microcosms, respectively. The 5 ppm microcosm showed an increase in biomass concentration between Day 0 and Day 3 but thereafter showed a decrease in biomass concentration until reaching a stationary phase. TBA concentrations correlate with this observation as TBA concentrations remained nearly constant between Day 3 and Day 7 and ultimately showed a slight decrease by Day 14. The 10 ppm microcosm indicated an increase in biomass between Day 0 and Day 10 and reached a stationary phase between Day 10 and Day 14. Observed TBA degradation rates were not observed to directly correlate with these biomass results as TBA concentrations remained unchanged during times of presumed microbial growth. The 25 ppm microcosm showed a continuous increase in biomass and a continuous decrease in MTBE concentration. Given more time, TBA concentrations are expected to have continued decreasing under denitrifying conditions particularly in the 25 ppm microcosm.
Figure 47. TBA concentration vs. biomass concentration for starting TBA concentration of 10 ppm.

Figure 48. TBA concentration vs. biomass concentration for starting TBA concentration of 5 ppm.
Under denitrifying conditions presented in this study, TBA concentrations were observed to decrease at levels less than aerobic conditions. On average, a 41.02 percent decrease in MTBE concentration was found utilizing Aqua under denitrifying conditions (Table 13).

<table>
<thead>
<tr>
<th>TBA Percent Biodegradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 ppm 26.31</td>
</tr>
<tr>
<td>10 ppm 41.77</td>
</tr>
<tr>
<td>25 ppm 54.98</td>
</tr>
<tr>
<td><strong>Average</strong> 41.02</td>
</tr>
</tbody>
</table>

4.7 Results of Aerobic Degradation of MTBE with Glucose Present

The final experiment conducted tested degradation under aerobic conditions with glucose present. In theory, in the presence of glucose, Aqua would consume this readily
degradable substrate and increased microbial rates would subsequently result in increased biodegradation of MTBE given that glucose would allow for the microbes to grow at a faster rate and adapt to the given environment.

pH conditions were tested at the beginning of each experiment and also at the end. Average initial pH values ranged from 6.72 to 6.82 while final pH values ranged from 6.75 to 6.81 (Figure 50). pH is not expected to have had any detrimental effects on the biodegradation process as the initial and final pH values recorded are within optimal biodegradation pH values.

![Figure 50. Initial and final pH measurements for MTBE biodegradation with glucose present.](image)

Optical density measurements for aerobic MTBE biodegradation with glucose present showed microbial growth between Day 0 and Day 3 due to the known inclusion of
dextrose in Aqua (Figure 51). After Day 3, absorbance levels dropped but once again increased after Day 7 and continued increasing until the end of the study period.

Figure 51. Optical density results for MTBE biodegradation with glucose.
Results of biomass concentration versus MTBE concentration are shown in Figure 52, 53 and 54 for 5 ppm, 10 ppm and 25 ppm microcosms, respectively. For each condition, between Day 0 and Day 3, concentrations of above 10 mg/L were observed for active biomass. However, during this same period, MTBE degradation was very slow. It is believed that during this time, the high microbial growth was due to the presence of glucose. Microbes consumed the glucose present and rapidly grew. Glucose was the preferential substrate and microbes consumed glucose until it was fully consumed before moving on to MTBE. By Day 10, active biomass concentrations began to increase and MTBE concentrations simultaneously decreased indicating the substrate being utilized during this time period was MTBE. By Day 14, biomass concentration appeared to be in an upwards trend for all microcosms indicating continued degradation would have occurred past Day 14.

![Figure 52. MTBE concentration vs. biomass concentration for starting MTBE concentration of 5 ppm.](image-url)
Under aerobic conditions with glucose, MTBE concentrations were observed to decrease.

On average, a 52.02 percent decrease in MTBE concentration was found utilizing Aqua
with glucose present (Table 14). However, it is important to consider that the bulk of the degradation rate occurred between Day 10 and Day 14—a 4 day window compared to a 14 day window observed for near complete degradation of MTBE under aerobic conditions—and that continued degradation is expected to have occurred by mature microbes that first grew on glucose and were acclimated to the test conditions.

<table>
<thead>
<tr>
<th>MTBE Percent Biodegradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 ppm</td>
</tr>
<tr>
<td>10 ppm</td>
</tr>
<tr>
<td>25 ppm</td>
</tr>
<tr>
<td><strong>Average</strong></td>
</tr>
</tbody>
</table>

**Table 14. Percent of MTBE biodegradation with glucose present.**

### 4.8 Results of Aerobic Degradation of TBA with Glucose Present

Average initial pH conditions of each experiment conducted under aerobic conditions with glucose present ranged from 6.66 to 6.79 while average final pH conditions ranged from 6.7 to 6.82 (Figure 55). It is assumed pH had no inhibitory effects on the biodegradation of MTBE due to the fact that pH fluctuated but remained with the optimal biodegradation pH range of 6 to 8.
Optical density measurements indicate that a steep increase in microbial growth was observed between Day 0 and Day 3 based on recorded absorbance measurements. This growth is expected as with other experiments due to the presence of dextrose in Aqua. Except for a slight decrease in absorbance on Day 7, the absorbance measurements indicate a general upwards trend in microbial growth during the duration of the experiment (Figure 56).
In all cases the growth in biomass concentration directly correlated with the decrease in TBA concentration indicating that TBA was consumed by Aqua under aerobic conditions with glucose present (Figure 57, 58 and 59). Each microcosm showed a general increase in biomass concentration throughout the study period and a simultaneous decrease in TBA concentrations. Unlike MTBE, where it is believed that glucose was the preferential substrate and MTBE consumption did not occur until all glucose was consumed, it is presumed glucose and TBA were cometabolized in these experiments. Under these cometabolic conditions, an exponential growth in microbes occurred that were able to consume both glucose and TBA yielding high levels of TBA biodegradation.

*Figure 56. Optical density results for MTBE biodegradation with glucose.*
Figure 57. TBA concentration vs. biomass concentration for starting TBA concentration of 5 ppm.

Figure 58. TBA concentration vs. biomass concentration for starting TBA concentration of 10 ppm.
Under aerobic conditions with glucose present, TBA concentrations were observed to decrease at the highest levels compared to all other experimental set ups involving TBA. On average, a 95.03 percent decrease in MTBE concentration was found utilizing Aqua under aerobic conditions with glucose present (Table 13).

Table 15. Percent of TBA biodegradation with glucose present.

<table>
<thead>
<tr>
<th>TBA Percent Biodegradation</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>5 ppm</td>
<td>95.90</td>
</tr>
<tr>
<td>10 ppm</td>
<td>94.50</td>
</tr>
<tr>
<td>25 ppm</td>
<td>94.68</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>95.03</strong></td>
</tr>
</tbody>
</table>
4.9 First Order Rate Constants

First order rate constants were calculated for each experiment and are shown in Table 16 below. Aerobic conditions were shown to be the most effective conditions in which degradation of MTBE and TBA utilizing Aqua occurred. For MTBE a first order rate constant of 0.019/hour and a 99.8 percent decrease in MTBE concentrations was observed under aerobic conditions. For TBA a first rate order constant of 0.009/hour and a 95.03 percent decrease in TBA concentrations was observed under aerobic conditions with glucose present. It is presumed that under both conditions a monooxygenase enzymatic reaction involving Cytochrome P-450 aids in breaking down both MTBE and TBA using Aqua.
Table 16. Calculated first order rate constants for each experimental set up.

<table>
<thead>
<tr>
<th>Experimental Set Up</th>
<th>5 ppm</th>
<th>10 ppm</th>
<th>25 ppm</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic MTBE + Biowish</td>
<td>0.019</td>
<td>0.021</td>
<td>0.017</td>
<td>0.019/hr</td>
</tr>
<tr>
<td>Aerobic TBA + Biowish</td>
<td>0.003</td>
<td>0.005</td>
<td>0.008</td>
<td>0.006/hr</td>
</tr>
<tr>
<td>Anaerobic MTBE + Biowish</td>
<td>0.004</td>
<td>0.001</td>
<td>0.003</td>
<td>0.002/hr</td>
</tr>
<tr>
<td>Anaerobic TBA + Biowish</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002/hr</td>
</tr>
<tr>
<td>MTBE + Nitrate</td>
<td>0.003</td>
<td>0.002</td>
<td>0.003</td>
<td>0.003/hr</td>
</tr>
<tr>
<td>TBA + Nitrate</td>
<td>0.001</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002/hr</td>
</tr>
<tr>
<td>Aerobic MTBE + Glucose</td>
<td>0.002</td>
<td>0.003</td>
<td>0.002</td>
<td>0.002/hr</td>
</tr>
<tr>
<td>Aerobic TBA + Glucose</td>
<td>0.010</td>
<td>0.009</td>
<td>0.009</td>
<td>0.009/hr</td>
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</tbody>
</table>
5. CONCLUSIONS AND FUTURE IMPROVEMENTS

The study of MTBE biodegradation has progressed over the years. While initial studies suggested MTBE was a recalcitrant compound, subsequent studies, including this thesis, have shown successful biodegradation of MTBE and TBA using bacteria of varying genera.

Under the varying conditions tested in this study, biodegradation of MTBE using Aqua was most effective under aerobic conditions yielding a first order rate constant of approximately 0.019/hour over the course of a 14 day study period. TBA biodegradation was found to be most effective under aerobic conditions with glucose present as an additional substrate yielding a first order rate constant of approximately 0.009/hour over the course of a 14 day study period. In comparison, the first order rate constant of TBA under aerobic conditions and the same study period was estimated at 0.006/hour indicating biodegradation under these conditions is only slightly less favorable than conditions with glucose present. Under all conditions, except denitrifying, TBA degradation rates were less than MTBE degradation rates but degradation of TBA was always observed and only found in trace amounts in MTBE experiments. Overall, this study showed that under the conditions presented, biodegradation of MTBE and TBA utilizing Aqua was observed and further large scale testing should be conducted.

However, because this study could not develop a successful method for MTBE and TBA concentrations above 25 ppm, before moving on to larger scale testing, it is crucial that
the testing method be further refined to be able to test concentrations of MTBE and TBA higher than 25 ppm.

In developing an improved method, it is also recommended that automated analyzing equipment be used. The method used in this study had an approximate sampling time for each sample of about 50 minutes between sample preparation, extraction time, GC/MS analyzation time, and purging. The long sample time limited the amount of samples that could be tested over the course of the study. Automated SPME sampling equipment would greatly increase the amount of samples that could be analyzed.

Further, it is important to note that while microcosm studies using bioaugmentation may show successful biodegradation of certain contaminants, bioaugmentation of contaminated subsurface environments may not yield the same results. Microcosm studies are performed under controlled environments with limited interferences that may not truly simulate subsurface conditions, thereby providing limited information. Groundwater systems vary from location to location resulting in the need for site specific conditions and ultimately varying biodegradation rates. This study aimed to evaluate the effectiveness of Aqua to biodegrade lab grade MTBE and TBA in nutrient broth known to stimulate Aqua growth. It is recommended that future studies test the effectiveness of Aqua utilizing soils or groundwater from MTBE and TBA contaminated sites. Studies have suggested that the presence of other gasoline constituents typically found at LUST sites, like BTEX, may or may not affect the biodegradation rate of MTBE. The effectiveness of Aqua should be further examined utilizing samples from contaminated
sites in order to study Aqua’s performance in the presence of potential substrates other than MTBE to provide more accurate results.

Bioaugmentation is known to be a cost effective, sustainable, and effective process. Given the promising results that this thesis found in utilizing Aqua to biodegrade MTBE and TBA under lab conditions, the possibility of using Aqua in real world applications could lead to an additional effective treatment for MTBE and TBA contaminated sites. The addition of Aqua as a bioaugmentation could be appropriate for sites where native bacteria are unable to biodegrade MTBE and TBA or where expedited biodegradation is necessary.
REFERENCES


Appendix A. Growth Media Recipe

Recipe for Growth Media:

1. 2g/L K2HPO4
2. 1g/L KH2PO4
3. 0.75g/L NH4Cl
4. 0.5g/L MgSO4
5. 0.018g/L CaCl2
Appendix B. Micronutrient Solution Recipe

Recipe for Micronutrient Solution:

1. 3.0g/L MgSO₄-7H₂O

2. 0.5g/L MnSO₄-H₂

3. 1.0g NaCl

4. 0.1g/L FeSO₄-7H₂O

5. 0.1g/L CoCl₂-6H₂O

6. 0.1g/L CaCl₂

7. 0.1g/L ZnSO₄-7H₂O

8. 0.01g/L CuSO₄-5H₂O

9. 0.01g/L AlK(SO)₄-12H₂O

10. 0.01g/L H₃BO₃

11. 0.01g/L Na₂MoO₄-2H₂O