CHARACTERIZATION OF A MICROBIAL CULTURE CAPABLE OF REMOVING TASTE- AND ODOR-CAUSING 2-METHYLISOBORNEOL FROM WATER

By

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This thesis is dedicated to Marley Lauderdale.

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A common blue-green algae metabolite, 2-methylisoborneol (MIB), is responsible for unpalatable drinking water in Asia, Australia, North American and Europe. Current water treatment technologies, are ineffective in removing MIB from potable water. The focus of this project was to examine the potential for microbial transformation of MIB by a culture isolated from a drinking water reservoir, Lake Manatee, in Manatee County, Florida. This culture was characterized using phenotypic and genotypic methods as well as by assessing its growth and MIB-depletion potentials using growth and oxygen uptake experiments and microcosms coupled with solid-phase microextraction (SPME) and gas chromatography/mass spectrophotometry (GC/MS). The predominant strain in the isolated culture was bacillus in shape and possessed spore and flagella, and 16S rRNA analysis determined that this isolated culture is most similar to *Bacillus sphaericus* (99% match). The ability of this culture to transform MIB was examined by running oxygen uptake measurements and by conducting depletion studies using SPME coupled with

GC/MS for MIB analysis. The results obtained from these studies demonstrated the isolated culture was capable of using MIB as its sole source of carbon and depleting MIB to below its odor threshold concentration (OTC) of 10 ng/l. Implications of these results are that microbial populations can be isolated from natural water sources for the removal of MIB and potentially other taste- and odor-causing compounds to concentrations that render aesthetically acceptable drinking water.

CHAPTER 1
INTRODUCTION

2-Methylisoborneol, a common blue-green algae metabolite released in surface waters typically from late spring to early fall, has been a cause of unpalatable drinking water in Asia, Australia, North American and Europe. Conventional water treatment technologies, consisting of breakpoint pre-chlorination, coagulation, sedimentation, and post chlorination, are not effective in removing MIB from potable water to below its odor threshold of 10 ng/l (Lalezary et al., 1986; Ashitani et al., 1988). As a supplement o water treatment, powdered activated carbon (PAC) is often added to water to remove MIB. However, PAC addition is not cost effective at higher MIB concentrations (Herzing et al. 1977). Seeking a possible solution, recent studies have begun to examine biological treatment as an alternative treatment method for MIB removal. To date, several strains of bacteria have been isolated from natural waters that are capable of using MIB as a growth substrate (Ishida and Miyaji, 1992; Egashira et al., 1992). Although these studies have shown MIB removal by these cultures, the potential for microbial communities to deplete MIB to below the odor threshold concentration (OTC) has not been shown. Also, the effect of MIB concentration on microbial growth and metabolic activity has not been fully explored.

The focus of this research was to examine the potential for microbial transformation of MIB by a culture isolated from water collected from Lake Manatee in Manatee County, Florida. The hypothesis of this project was that, because of the MIB

outbreaks experienced in this reservoir on a seasonal basis, bacterial populations capable of using MIB as a growth substrate could be isolated. The scope of this work was composed of 4 major objectives. The first included a literature review covering the current knowledge of the origins and properties of MIB as well as current technologies involving biological treatment. The next objective was to isolate a bacterial culture capable of growth on MIB from a water sample obtained from Lake Manatee. Subsequently, the isolated culture was to be characterized by using growth kinetics to determine specific growth rates, light and TEM microscopy to examine fine cell structures, and 16S rRNA phylogenetic analysis to assess the closest match of the unknown isolate(s) to known bacterial strains. The final objective was to assess the MIB transformation potential of the microbial community by using oxygen uptake methods and batch microcosm experiments combined with solid phase microextraction analysis to determine the ability of the culture to oxidize and degrade MIB at different initial concentrations and to deplete MIB to below the OTC.

The results from this project promise to benefit water treatment facilities and their customers by providing applied results directed towards the remediation of MIB contamination. Additionally, this research will add to the continuously growing body of knowledge addressing the use of biological treatment for improved potable water quality.

CHAPTER 2
LITERATURE REVIEW

Introduction to Taste and Odors in Drinking Water: Description, Causes, and Brief History

Tastes and odors are considered by most consumers to be significant factors when determining potable water quality. In 1973, a Gallup Poll indicated that most consumer complaints concerning drinking water involved tastes and odors. As far back as 1957, surveys given to both water utilities and consumers have consistently reported similar problems (Suffet et al., 1996). The occurrence of taste and odor problems is widespread and has been reported in Argentina, Australia, Canada, Denmark, England, Finland, Germany, Israel, Japan, The Netherlands, Norway, Poland, Sweden, U.S.A and U.S.S.R. (Ashitani et al., 1988; Juttner, 1995; Persson, 1983; Suffet et al., 1996; Zimmerman et al., 1995).

Some of the more prominent taste- and odor-causing substances are naturally occurring organic compounds that produce an earthy-musty odor in drinking water (Rashash et al., 1997). Examples of these compounds include 2-isopropyl-3-methoxypyrazine (IPMP), 2,3,6-trichloranisole (TCA), 2-isobutyl-3-methoxypyrazine (IBMP), *trans*-1,10-dimethyl-*trans*-9-decalol (geosmin) and 1,2,7,7-tetramethylbicyclo[2.2.1]heptan-2-ol, also known as 2-methylisoborneol (MIB). Although these compounds are not deleterious to human health, they can cause malodorous drinking water at extremely low concentrations (≤10 ng/l) (Persson et al., 1980; Lalezary et al., 1986; Rashash et al., 1997). The odor threshold concentrations (OTC) of these compounds, defined as the concentration at or above which odor can be detected, are provided in Table 2-1 (Lalezary et al., 1986; Persson, 1980).

Table 2-1: Odor threshold concentrations for 5 earthy-musty odor compounds (Lalezary et al., 1986; Persson, 1980)

Common Name	Chemical Formula	OTC (ng/l)
Geosmin	$C_{12}H_{22}O$	4
IPMP	$C_8H_{12}ON_2$	2
IBMP	$C_9H_{14}ON_2$	2
TCA	$C_7H_5OCl_3$	7
MIB	$C_{11}H_{20}O$	10

*Odor threshold concentrations determined by an odor panel

Water utilities typically rely on conventional treatment methods, supplemented with powder activated carbon (PAC), to remove taste- and odor-causing compounds. These methods can effectively decrease IPMP, TCA, IBMP and geosmin concentrations to below their odor thresholds; however, these processes have been shown to be unsuccessful in removing MIB to below its OTC (Ashitani et al., 1988). Today, some alternative methods for MIB treatment, including biological transformation, granular activated carbon, and ozonation, are being examined. This literature review summarizes previous studies reporting the origins and properties of MIB as well as current technologies involving biological treatment.

Methylisoborneol: Isolation, Formation, Properties, Conventional Treatment and Analysis

Initial Isolation of Methylisoborneol

In 1969, Medsker et al. first reported successful isolation and identification of MIB from actinomycetes, a group of terrestrial gram-positive bacteria that form branching filaments. They described the compound as “camphor-smelling” and the major odorous constituent in 3 out of 28 species of actinomycetes surveyed. The empirical formula (Table 2-1) and structure (Fig. 2-1) were identified by mass spectrometry.

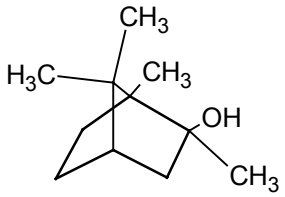

Figure 2-1: 2-Methylisoborneol, structure identified by Medskar et al. (1969)

Natural Formation of Methylisoborneol

Cyanobacteria are prokaryotic oxygenic phototrophs that are found in a variety of ecological settings, including terrestrial, freshwater and marine habitats (Madigan et al. 2000). In freshwater lakes, especially those that are eutrophic, cyanobacteria can develop massive accumulations, known as blooms. Cyanobacteria and their resulting blooms are responsible for the production of many odor-causing compounds, including MIB in natural waters.

Numerous species of MIB-producing cyanobacteria have been isolated and classified, and these include *Oscillatoria sp.*, *Anabaena sp.* and *Phormidium sp.* (Hosaka et al., 1995; Juttner et al., 1995; Zimmerman et al., 1995). These microorganisms synthesize MIB during normal growth. MIB is believed to be a methylation product of an unknown monoterpene formed from acetate and mevalonate (Juttner et al., 1995). The function of MIB in the cell has yet to be understood completely, and the compound may simply be a by-product of the photosynthetic pathway. After MIB is synthesized, it is found either bound to thylakoid membranes and cytoplasmic proteins or excreted by the cell.

The production of MIB varies by cyanobacteria strain. Some species, such as *Oscillatoria* sp. 3 and *Phormidium tenue*, demonstrate a proportionate relationship between the number of filaments, or chains of cells, and MIB concentration. A single filament of *Oscillatoria* sp. 3 produces an average of 19.5 picograms of MIB (Hosaka et al., 1995). The graph shown in Figure 2-2, compiled from data collected from the Ooba River in Japan,

further illustrates the effect of filament/ml (*Oscillatoria* sp. 3) on MIB concentration (Hosaka et al., 1995).

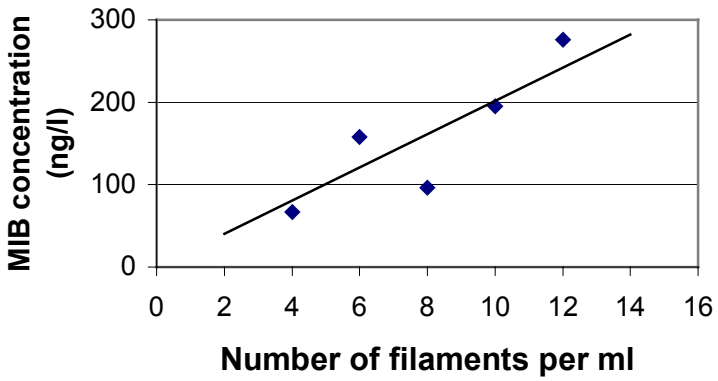

Figure 2-2: Plot depicting the linear relationship between MIB concentration and the filament concentration of *Oscillatoria* sp.3 (adapted from Hosaka et al., 1995)

These data imply that during a period of algal bloom, typically occurring from late spring to early fall, the MIB concentration in surface water supplies will increase. If eutrophication increases in the freshwater bodies used for drinking water, the potential for increased algal concentrations and, thus, MIB releases is suggested by these findings.

Physical and Chemical Characteristics of Methylisoborneol

The physical and chemical characteristics of MIB dictate its behavior in the environment and in water treatment plants. The fate of MIB in a treatment process may be predicted and explained, at least in part, by examining its following properties: density, aqueous solubility, octanol-water partition coefficient, and Henry's constant (Ney, 1990).

Density is a valuable characteristic when assessing the physical separation potential of a compound in the aqueous phase. The density of MIB is approximately 0.9288 g/cm^3 (Pirbazari et al., 1992). This value is similar to that of water, indicating MIB is unlikely to pool at either the surface or bottom of the water column.

A compound's aqueous solubility is a measurement of its affinity for water. Compounds with high aqueous solubility (>1000 mg/l) are hydrophilic, whereas compounds with low aqueous solubility (<10 mg/l) are hydrophobic. Compounds with a high aqueous solubility are typically more mobile and bioavailable, thus have a higher potential to be biodegraded (Ney, 1990). MIB has an aqueous solubility of 194.5 mg/l (Pirbazari et al., 1992), which is close to the low range, and would, therefore, be expected to be less mobile in the aqueous environment, less biodegradable, and more tending to sorb to sediments.

The octanol-water partition coefficient (K_{ow}) indicates a chemical's tendency to sorb to soils and sediments, bioconcentrate in aquatic organisms and accumulate in the soil. A K_{ow} value greater than 1000 suggests that a chemical has an affinity for bioaccumulation in the food chain, has a low aqueous solubility and has a low mobility in the soil and aqueous phases, whereas a chemical with K_{ow} value of less than 500 would be more bioavailable, soluble, and mobile (Ney, 1990). MIB has an octanol-water partition coefficient of approximately 1349 (Pirbazari et al., 1992), indicating it is lipophilic and thus has a tendency to partition out of the aqueous phase.

Henry's Constant (K_H) demonstrates the ability of a chemical to partition between the aqueous phase and the atmosphere and it can be estimated by ratioing the vapor pressure and the aqueous solubility. A chemical with a high K_H value (>0.4 l-atm/mol) is more likely to escape the from aqueous phase to the vapor phase, whereas a chemical low K_H value (<0.004 l-atm/mol) would most likely remain in the aqueous phase (Ney, 1990). The K_H value for MIB is 5×10^{-8} l-atm/mol (Pirbazari et al., 1992), indicating that MIB will not readily escape from the aqueous phase. Table 2-2 summarizes the physical and chemical characteristics of MIB.

Table 2-2: Physical-chemical characteristics of MIB (Pirbazari et al., 1992) compared to high and low values as described by Ney (1990)

Characteristic	Observed Value for MIB	Low Value	High Value
Density	0.9288 g/cm^3	-	-
Aqueous Solubility	194.5 mg/l	<10 mg/l	>1000 mg/l
Octanol/Water Coefficient	1349	<500	>1000
Henry's Law Constant	5.76×10^{-8} l -atm/mol	<0.004 l-atm/mol	>0.4 l–atm/mol

In summary, MIB is expected to have moderately low motility in the aqueous environment, with a large portion partitioned out of the aqueous phase via bioaccumulation and/or sorbtion onto sediments. The low Henry's constant indicates MIB is unlikely to escape into the atmosphere.

MIB Detection and Analysis Methods

The detection and quantitative analysis of MIB requires the use of technically complex and time-consuming analytical methods, as it is commonly found in natural waters at ultra-trace concentrations (Watson et al., 2000). Because MIB is found at such low natural concentrations, traditional analysis of MIB typically relies on concentrating large sample volumes (100-1000 ml). The preparation and analysis methods used, including liquid-liquid extraction, purge and trap, closed-loop stripping analysis and simultaneous distillation-extraction, are time-intensive and/or require high-resolution mass spectrophotometers (Lloyd et al., 1998).

As a means of avoiding the constraints of time and cost involved in using the large sample volumes required in these concentration methods, solid phase microextraction (SPME) methods coupled with a gas chromatograph-mass spectrophotometer (GC/MS) system have been recently developed By combining SPME and GC/MS, detection of MIB at ng/l levels is possible without the large sample volumes. Typically, a sample volume of 25

ml is sufficient. SPME-GC/MS is an inexpensive and rapid method for the analysis of volatile and semi-volatile compounds occurring in the headspace of water matrices (Eisert and Levsen ,1996). Techniques using SPME-GC/MS were originally applied by the Des Moines Water Works for taste and odor analysis and have recently been employed by the City of Tampa for the analysis of MIB and geosmin (Brand, 1995). Currently, a SPME-GC/MS method is undergoing balloting for consideration as Standard Method 6040D (APHA, 2001). This SPME method is based on the adsorption of MIB on a fiber coated with divinylbenzene-carboxen-polydimethyloxane cross-link. This fiber is placed in the headspace of a sealed vial and allowed to equilibrate with an aqueous sample. After equilibrium is reached (typically 30-35 minutes), the fiber is removed and injected into the port of a GC/MS system, where it is heated allowing the analytes to be desorbed for analysis. The minimum detectable concentration of MIB analyzed by these methods < 5 ng/l and the recovery of the laboratory control standard of 20 ng/l is 95±10% depending on the laboratory (APHA, 2001). Although, this standard method appears to be a good alternative to other forms of MIB analysis, it has not been validated in for many matrices, therefore, prior to sample analysis, recoveries using these matrices with known additions of MIB should be examined (APHA, 2001).

Conventional Water Treatment for MIB Removal

As stated previously, municipal water utilities have confronted problems with MIB for many years. Typical water treatment consists of breakpoint pre-chlorination, coagulation, sedimentation, rapid sand filtration and post-chlorination. Many studies have reported that current conventional treatment methods do not sufficiently remove MIB (Ashitani et al., 1988; Rashash et al., 1997; Suffet et al., 1996). Powder activated carbon (PAC) is often added as a supplement to a treatment line to decrease the MIB concentration; however, this

practice is cost-effective only for low dosing (Herzing et al., 1977). A study conducted by Ashitani et al. (1988) examined several possible water treatment scenarios, including coagulation alone, pre-chlorination followed by coagulation/sedimentation, pre-chlorination followed by coagulation/sedimentation then PAC, and full conventional treatment (including PAC) (Ashitani et al., 1988). These results (Table 2-3) show MIB removal by coagulation alone was greater than that of prechlorination with coagulation. The authors' explanation is that a significant part of the MIB in the raw water was present in the responsible microorganism. These microorganisms are typically removed during coagulation and sedimentation; however, when chlorination was applied prior to coagulation, the cells lysed, releasing intracellular material that contained MIB. Although the PAC (10 ppm)/pre-chlorination/coagulation method was able to remove an average of 42% of the influent MIB, none of the treatment methods studied were capable of decreasing MIB concentrations to below the OTC; thus, alternative technologies must be found. Recently, such water treatment methods have been devised and are showing preliminary success in the removal of MIB. As the focus of this work is microbial transformation of MIB, the remainder of this discussion examines the literature reporting success with biological treatment of MIB.

Table 2-3: Conventional treatment methods and powdered activated carbon (PAC) average MIB removal efficiencies (Ashitani et al., 1988)

Treatment Method	**Raw Water MIB (ng/l)**	**Treated Water MIB (ng/l)**	**Removal (%)**
Coagulation	53	34	36
Pre-Chlorination/Coagulation	53	39	26
PAC (10ppm)/ Pre-Chlorination/Coagulation	53	31	42

Bacterial Transformation of MIB

Isolation and Identification of Potential MIB-Degrading Bacteria

Most work reporting the identification of microorganisms that are capable of degrading MIB involved the use of a MIB-contaminated raw water as the inoculum source for a liquid culture. The bacterial strains were isolated from the resulting liquid cultures by colony morphology using spread plate methods. Each study then identified isolated strains by using biochemical test kits. These kits are commonly used for many groups of bacteria to determine phenotypic activity (oxidase, catalase, nitrate reduction, amino acid-degrading enzymes, fermentation or utilization of carbohydrates). The tests are typically conducted by adding a small amount of culture to a multi-welled plate containing various biochemical reagents. The reaction in each well is then observed and compared to a database containing phenotypic characteristics of known microorganisms (Chester and Cleary, 1980). Biochemical test kits are commonly used in the identification of gram type negative, nonfermentative bacteria, such as *Pseudomonas*, *Acinetobacter*, *Flavobacterium*, *Moraxella* and fermentative bacteria not belonging to the *Enterobacteriaceae*, such as *Vibrio* and *Aeromonas.* There are limitations with these kits; however, such as previously unknown, rare, or newly described strains that are not in the database (Chester and Cleary, 1980). This can often lead to strains being unidentified or misidentified when their results are compared to only known species. The two testing kits employed by the studies discussed below for characterization of isolated MIB-degrading microorganism are the Rapid Nonfermentor Test (NFT) and the Minitek Test Kit.

Izaguire et al. (1998) reported use of a common technique for the isolation of a pure MIB-degrading culture. In this method, water and sediment samples were collected from contaminated lakes and used as inoculum for a MIB-spiked mineral salts medium. The MIB-

degrading bacteria were identified as soon as the cultures showed a decrease in MIB concentration, typically after incubation periods of 3-20 days. Purified isolates were identified from the cultures using the NFT kit, described above. Examples of some of the isolated MIB-degrading bacteria included *Pseudomonas aeruginosa*, *Pseudomonas pseudoalcaligenes*, *Pseudomonas paucimobilis*, and *Pseudomonas mendocina*. Other publications claiming successful isolation of MIB degraders from natural waters used similar techniques; however, different strains were identified, including *Bacillus subtilis* and *Flavobacterium multivorum* (Ishida and Miyaji, 1992; Egashira et al., 1992).

Another means of identifying these bacteria would be to compare each gene sequence in a given strain with the gene sequences other known species. The ribosomal ribonucleic acid (rRNA) of one organism can be compared with that of any other organism by a method called 16s rRNA gene sequencing. The results of gene sequencing provides an estimate of the percentage of divergence within sequences that are related but not identical and provides a higher level of accuracy compared to phenotypically based methods (Chester and Cleary, 1980). This information could then be used to construct a phylogenetic tree comparing the isolated culture to known species.

Characteristics of MIB-Degrading Bacteria

Biological treatment systems are most effective when the conditions for microbial growth are met, including adequate pH and temperature (Egashira et al.,1992). Unfortunately, while some of the microorganisms found capable of MIB transformation have been partially characterized (Ishida and Miyaji, 1992; Egashira et al., 1992), the growth characteristics and environmental requirements of many MIB degraders have not been clearly defined.

Ishida and Miyaji (1992) investigated the kinetics of MIB degradation for a strain these authors isolated, named *Bacillus sp.* HI-5. This pure strain was isolated by inoculating a minimal salts medium spiked with 100 μg/l of MIB with backwash water obtained from a rapid sand filter. The culture was incubated for 18 days while MIB removal was continuously observed. This experiment showed that 30 μg/l MIB was removed after the first 70 hours, and, after 7 days all of the MIB had been removed below the minimum analytical detection limit, or MDL, of 20 ng/l. Batch experiments, conducted with *Bacillus sp.* HI-5, yielded maximum specific growth rates (μ_{max}) of 0.10 hr^{-1} and 0.03 hr^{-1} in the presence of 8 and 0.1 mg MIB/l, respectively. A saturation constant (the concentration of substrate where the growth rate μ is equal to 1/2 μ_{max}) was found to be 205 μg/l for the culture grown in 8 mg MIB/l. This study also reported corresponding observed MIB depletion rates (Figure 2-3) of 7.7 μg/l/hr for the culture grown at an initial concentration of 8 mg/l MIB and 0.5 μg/l/hr for the culture grown at the initial concentration of 0.1 mg/l MIB.

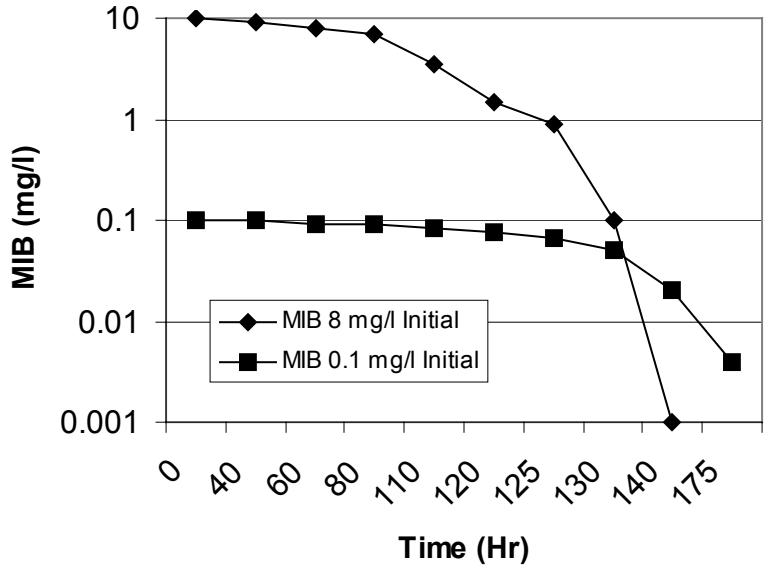

Figure 2-3: Depletion by *Bacillus* HI-5 at 2 different initial concentrations of MIB (adapted from Ishida and Miyaji, 1992)

The results of the growth study, as shown in Figure 2-4, show the lag time for each culture to be approximately 40 hours. The stationary growth phase for the 0.1 mg/l and 8 mg/l cultures was reached at approximately 130 hours, and the maximum number of microorganisms grown at each MIB concentration were 5.0 x 10^5 CFU/ml and 3.5 x 10^7 CFU/ml, respectively. However, the authors do not report use of a chemical control containing MIB and no cells or a killed control with autoclaved cells. Thus, it is implied that these investigators did not account for volatilization or cell sorption losses of MIB, causing an overestimation of MIB depletion.

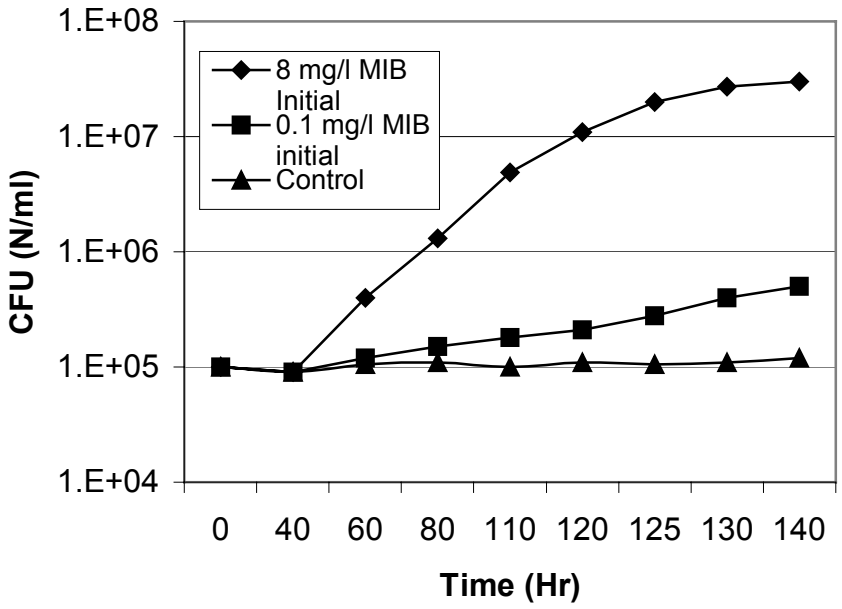

Figure 2-4: Growth curves for *Bacillus* HI-5 at 2 different initial MIB concentrations (adapted from Ishida and Miyaji, 1992)

Egashira et al. (1992) reported the effects of water temperature and pH on the MIB depletion potential in a pilot plant study with a biological filtration system (packed-column). The microorganisms that were responsible for the depletion in the column were isolated and identified, and their individual depletion potentials were studied. In the biological filter study, a constant concentration of MIB (0.2 μg/l) was added to the natural water fed into the

column. The pH and temperature were then systematically adjusted, and the changes in MIB depletion were then measured by GC/MS and recorded. The results for these experiments are shown in Figures 2-5 and 2-6.

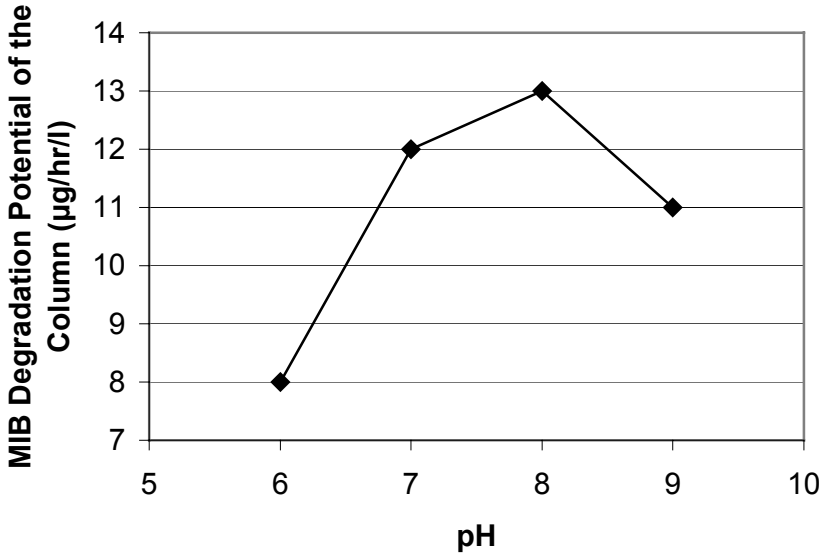

Figure 2-5: Effect of pH variation on the MIB depletion potential of a biologically active column (adapted from Egashira et al., 1992)

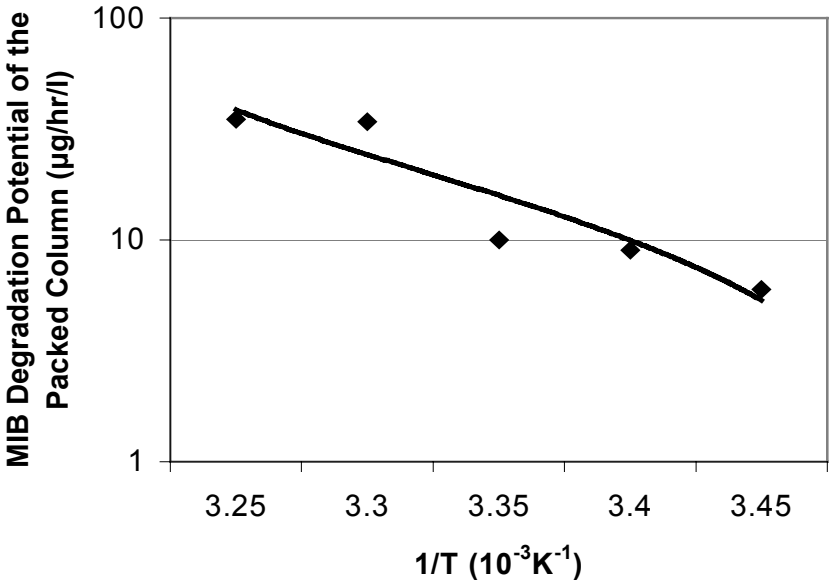

Figure 2-6: The logarithmic relationship between temperature and the MIB depletion potential of a biologically active column (adapted from Egashira et al., 1992)

Although no error is reported, the trends in Figures 2-5 and 2-6 suggest that changes in temperature and pH can have a significant influence on the MIB depletion potential of the microbial populations residing in the biological filter used in this study. The maximum MIB depletion potential of the column was observed at a temperature of 30 °C and a pH of 8. This correlation may be useful in determining appropriate operating conditions for biological treatment systems designed to remove MIB.

After the completion of the biological filtration study, the granular ceramic medium used in the column was treated by ultrasonication. The resulting biological sludge suspension was used as an inoculum in a tripticase soy culture agar that was then incubated for 3 days at 25 °C. Microorganisms were then isolated from the agar based on colony morphology. The MIB depletion potential of each isolated bacterium was determined using batch experiments. The isolate bacteria were used to inoculate a minimal mineral medium that was incubated for 7 days with an initial MIB concentration of 20 μg/l, under carefully controlled temperature and pH, ranging from 26 to 29 °C and 7.3 to 7.6 respectively. MIB depletion potential was determined by the difference between the initial and final MIB concentrations, measured by purge and trap and GC/MS. The isolated species were then characterized using a Minitek identification kit. The results of the MIB depletion potential study for cultures isolated from the biological filter are presented in Table 2-4.

Table 2-4: MIB depletion potential for the identified species isolated from a biological treatment filter (Egashira et al., 1992)

Strain	*Pseudomonas* sp.	*P. aeruginosa*	*Flavobacterium* sp.	*F. multivorum*
Initial MIB Concentration	20 ug/l	20 ug/l	20 ug/l	20 ug/l
Final MIB Concentration	15.7 ug/l	14.82 ug/l	14.0 u/l	16.7 ug/l
Incubation Time	24 hr	24 hr	24 hr	24 hr
MIB Depletion Rate	0.18 ug/l/hr	0.21 ug/l/hr	0.25 ug/l/hr	0.14 ug/l/hr
Total MIB Depletion %	21.6	25.9	29.7	16.7
MIB Depletion %/hr	0.9	1.08	1.24	0.70

As shown in Table 2-4, several species of bacteria that can potentially transform MIB were isolated in this study. This study did not provide; however, the cumulative depletion rate of the original mixed culture composed of these isolated strains. It is unknown whether the depletion rate of the mixed culture differs significantly from those reported. The inclusion of controls to measure the effects of volatilization or cell sorption on MIB depletion was not reported in this study.

Applications of Technology Using Biological Transformation for MIB Removal in Water Treatment

Introduction to the Use of Bioremediation in Water Treatment

Bioremediation is the use of microorganisms to eliminate or detoxify toxic or unwanted chemicals, and can be employed in water treatment to eliminate natural and anthropogenic chemicals from raw water (Characklis and Marshall, 1990). One of the most common bioremediation applications in water treatment is biological filtration. Microorganisms that are present in filtration media oxidize biodegradable organic matter and nitrify ammonium compounds. Recently, laboratory- and pilot- scale experiments have shown that some microorganisms have the potential to transform MIB (Izaguirre et al., 1988; Oikawa et al., 1995; Namkung and Rittmann, 1987; Tanaka et al., 1996). Important factors that may dictate the effectiveness of a biological treatment method for

MIB removal include microbial diversity, transformation pathways (intermediate formation), growth characteristics and biofilm development.

Biological Filtration

Microbial cells often attach firmly to submerged surfaces in aquatic environments. These immobilized cells grow, reproduce, and produce extracellular polymers that can form a tangled matrix of fibers. A biofilm is the assemblage of these fibers (Characklis and Marshall, 1990). Biofilm-based technologies are currently implemented in the treatment of air, water and wastewater. Packed-bed reactors, in which biofilms accumulate on solid substrata or granular media packed within a tower or bed, are often used in water treatment. One example of a packed-bed reactor is a trickling filter where the influent liquid is spread over the top of a granular media by a sprinkler system and allowed to flow through the bed in a thin water layer over the biofilm (Viessman and Hammer, 1998). Oxygen, a required gas for aerobic processes, is drawn up the bed by natural convection. Since biofilm development depends on the adsorbable surface area of the media, a high surface-to-volume ratio, such as small and/or porous particles, is optimal. As mentioned earlier, sand is a typical example of medium used in conventional trickling filters. These trickling filters have shown little success for the removal of MIB from drinking water; thus, alternative media types must be explored (Ashitani et al, 1988).

Yagi et al. (1988) examined one example of a biologically active filter for MIB reduction. The filter media for this study included activated carbon (coconut shell, 10x32 mesh), zeolite, and sand, and *Bacillus subtilis* IAM 12118 was used as the inoculum. The dimensions of the filter are provided in Table 2-5. Each filter was fed 0.9 liters of raw water, which was supplemented with additional MIB to maintain a constant concentration

for each run (1.5 – 1.6 mg/l). The microbial transformation was estimated by subtracting the total MIB influent by the amount of MIB adsorbed and MIB extracted from the filtrate of a control, consisting of autoclaved medium to eliminating biological activity. The study also examined the amount of MIB adsorbed by the filter medium and the biological cells. The concentration of MIB was determined in these tests by GC/MS, with a minimum detection level of 0.1 mg/l. The results from this study can be found in Table 2-6.

Table 2-5: Column parameters for the biological filtration study conducted by Yagi et al. (1988)

Internal Diameter	1.5cm
Length	20 cm
Media volume	35 cm^3
Empty Bed Contact Time	2.4 min
Hydraulic Loading	8.3 cm/min

Table 2-6: Results for the biological filtration study conducted by Yagi et al. (1988)

Parameter	**Carbon Medium**		**Sand Medium**		**Zeolite Medium**	
Biological Activity	Bio-Active	Control	Bio-Active	Control	Bio-Active	Control
MIB in raw water (mg/l)	1.6	1.5	1.5	1.5	1.5	1.6
Volume of filtrate (l)	0.9	0.9	0.9	0.9	0.9	0.9
Loading of MIB (mg/l)	1.4	1.4	1.4	1.4	1.4	1.4
MIB in Filtrate (mg/l)	0.2	<0.01	1.7	1.5	1.7	1.5
MIB in filtrate (mg)	0.18	<0.009	1.5	1.4	1.5	1.4
Estimated Adsorbed (mg)	1.2	1.4	-0.1	0	-0.1	0
Amount Extracted (mg)	0.53	1.33	0.02	0.01	0.06	0.2
Estimated Transformation (mg)	0.7	0.07	0	0	0	0
Estimated Depletion %	58	5	0	0	0	0

*These values, determined by GC/MS, represent the MIB removal characteristics of each column type.

These data show that the activated carbon control removed MIB to the lowest effluent concentration. The effectiveness the activated carbon filter to remove MIB was diminished by biological activity; however, it appears that the bacteria present in the in

this filter were responsible for the removal of some MIB via transformation. The data provided also shows that the sand and zeolite filters were not capable of removing MIB regardless of biological activity. It is important to note; however, that no information is provided quantifying the biomass concentration on the filter, allowing the possibility that there was insufficient inoculum present for significant MIB depletion. Also, the raw water fed into the column may have lacked the necessary nutrient concentrations for MIB transformation.

The Effect of Ozonation the Biological Transformation of MIB

Ozonation is becoming a popular drinking water disinfection method in the United States (Bitton, 1999). This is primarily because of its ability to kill microorganisms without producing trihalomethanes or other halogenated disinfection byproducts. Another consequence of ozonation is the oxidation of large organic compounds (i.e., humics and fulvics) into smaller compounds, such as ketones, ketoaldehydes, alkanes, and alcohols, that may be more easily biodegraded. The reduction of large organics in the influent may also increase the life of a carbon adsorber by saving pore space. Thus, the application of ozonation before a biological system may increase its effectiveness (Muramoto et al., 1995).

Muramoto et al. (1995) reported the results of a full-scale application of ozonating water immediately before biological treatment. These researchers examined part of the treatment line in the Kanamachi Purification Plant in Tokyo, Japan (Fig 2-7). The MIB concentrations were analyzed by GC/MS after each stage to determine the removal efficiencies of each process. Figure 2-8 presents the average MIB concentrations that Muramoto et al. (1995) observed at each collection point over 3 months. Regardless of the month of sampling, approximately 20 percent of the influent MIB was removed by

coagulation-sedimentation, and the remainder was removed by the combined ozonation biological treatment system.

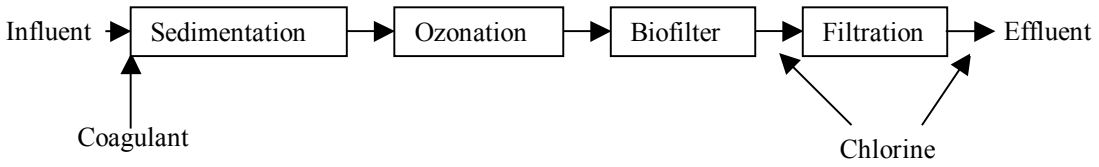

Figure 2-7: Kanamachi treatment line (adapted from Muramoto et al., 1995)

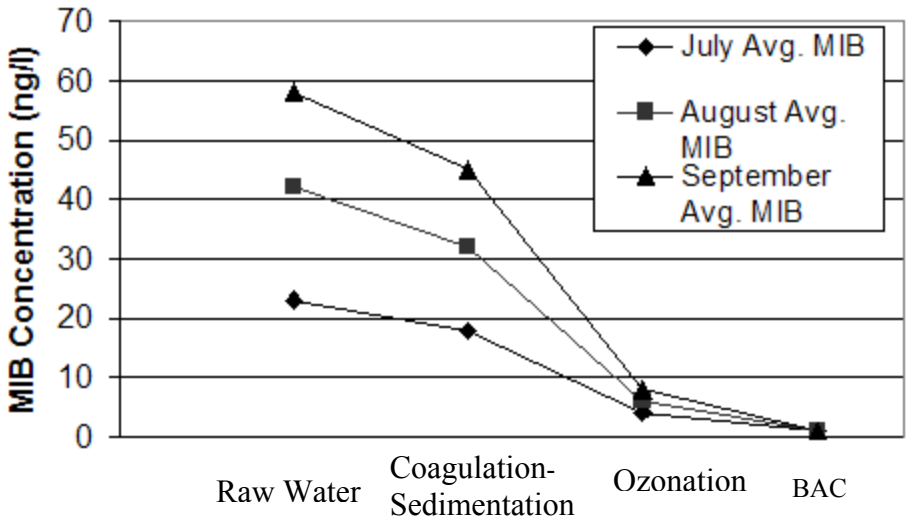

Figure 2-8: MIB concentrations in raw and processed waters (adapted from Muramoto et al., 1995)

As shown in Figure 2-8, the Kanamachi treatment plant was capable of removing MIB to below the OTC, regardless of the MIB loading. Although coagulation and sedimentation processes removed a portion of the MIB, ozonation and BAC treatment effectively removed all of remaining MIB. Although the BAC system shows promise for the removal of MIB, it remains unclear whether the MIB was depleted through bacterial degradation or by adsorption onto the activated carbon.

Conclusions

Most consumer complaints regarding drinking water quality are due to malodors and tastes. Often, the odors are described as earthy-musty. Once an earthy-musty odor event occurs, water utilities can typically remove the responsible compounds using conventional water treatment with PAC. However, one chemical responsible for the earthy-musty odor, MIB (a cyanobacteria metabolite), is not effectively removed by these methods. This literature review examined previous studies of isolation and identification of microorganisms and microbial systems capable of removing MIB. Included in this review are papers covering the characteristics and origins of MIB, the isolation of microbial species capable of depleting MIB, the characterization and transformation kinetics of MIB-degrading bacteria, and selected water treatment technologies currently incorporating microbial degradation for MIB removal. Many of the studies mentioned reported MIB removal to below analytical detection limits; however, these studies did not clearly report the final concentration of MIB at the ng/l levels commonly found in natural waters or to a concentration that did not confer odor. To improve upon these methods, additional studies are needed to further assess the MIB transformation potential of microbial communities, particularly, the ability of cultures to deplete MIB at different initial concentrations, including the ng/l levels commonly found in natural waters. Also, improved methods of identification of MIB-degraders that involve genetics-based techniques are desirable because of their increased accuracy. By better understanding MIB concentration effects on the activity of MIB-degrading bacteria and the phylogeny of these microorganisms, drinking water facilities will be better able to design more effective biologically based systems that cater to these specific requirements.

CHAPTER 3
CHARACTERIZATION OF A MICROBIAL CULTURE CAPABLE OF REMOVING TASTE- AND ODOR-CAUSING 2-METHYLISOBORNEOL FROM WATER: A MANUSCRIPT TO BE SUBMITTED TO *WATER RESEARCH*

Introduction

Tastes and odors are considered by most consumers to be significant factors when determining potable water quality. The occurrence of taste and odor problems is widespread and has been reported in Asia, Austrailia, North America and Europe (Ashitani et al., 1988; Persson, 1983; Suffet et al., 1996; Zimmerman et al., 1995). Some of the more prominent taste- and odor-causing substances are naturally occurring organic compounds that produce an earthy-musty odor in drinking water (Rashash et al., 1997). Many of these organics have been identified and include 2-isopropyl-3-methyoxy pyrazine (IPMP), 2,3,6-trichloranisole (TCA), 2-isobutyl-3-methyoxy pyrazine (IBMP), trans-1, 10-dimethyl-trans-9-decahol (geosmin) and 2-methylisoborneol (MIB). These compounds are released by blue-green algae, typically from late spring through early fall, in concentrations reported to be as high as 100 ng/l (Tenauchi et al., 1995); however concentrations ranging from 10 ng/l can cause malodorous drinking water (Persson, 1980). While not a concern in terms of health impacts, the offensive odor and taste of MIB may lead to psychosomatic effects, such as headaches, stress, or stomach upsets (Young et al., 1996).

Water utilities typically rely on conventional treatment methods to remove taste- and odor-causing compounds. These methods can effectively decrease IPMP, TCA, IBMP and geosmin concentrations to below their odor thresholds; however, they are

typically unsuccessful in removing MIB to below its commonly accepted odor threshold concentration (OTC) of 10 ng/l (Ashitani et al., 1988). Recent studies have begun to examine biological treatment as an alternative method for MIB removal. Ishida et al. (1992) and Egashira et al. (1992) isolated several strains of bacteria from natural waters that are capable of using MIB as a growth substrate, including *Pseudomonas aeruginosa*, *Pseudomonas pseudoalcaligenes*, *Bacillus* sp., and *Flavobacterium multivorum*. Ishida et al. (1992) conducted batch and continuous feed experiments studying the removal of MIB by a bacterium isolated from Lake Kasumigaura in Japan. The results for the batch experiments showed the ability of the bacterium to deplete MIB at the mg/l and μg/l levels, while the continuous feed study showed the isolated strain was capable of reducing influent MIB concentrations of 600 ng/l to approximately 60 ng/l. The researchers identified the isolated strain as a *Bacillus* sp. using phenotypic identification tests. Egashira et al. (1992) examined the removal of μg/l concentrations of MIB in drinking water by a biological filter inoculated with surface water from Lake Biwa, Japan. This study also reported the effects of temperature and pH on MIB depletion in a packed column and identified isolated cultures from the filter media using a biochemical test kit. No error was reported in the studies performed by either Egashira et al. (1992) or Ishida et al. (1992); however, the trends of their results support the potential of MIB depletion by bacteria.

Although many reports show positive findings on microbial depletion of MIB, the potential for microbial communities to deplete MIB to below the OTC has not been shown. Also, the effect of MIB concentration on microbial growth and metabolic activity has not been fully explored. The purpose of this study was to further assess the MIB

transformation potential of microbial communities, particularly, the ability of cultures to deplete MIB at different initial concentrations including the ng/l levels commonly found in natural waters. The effect of varying MIB concentration on microbial growth and oxidation potential was also examined. The characterization and identification of the isolated culture was conducted by using transmission electron and light microscopy to examine fine cell structures and 16S rRNA gene sequencing to construct alignment profiles and a neighbor joining phylogenetic tree.

Materials and Methods

Enrichment and Isolation of MIB Transforming Bacteria

Source water used as initial inoculum

The source of inoculum used in this study was Lake Manatee in Manatee County, FL. Lake Manatee currently feeds the Manatee County Water Treatment Facility to the supply of potable water for Manatee County. This reservoir experiences periods of extensive algal blooms from late spring through early fall, resulting in average MIB concentrations in the raw water of approximately 25 ng/l. Water samples were collected in July 2000 in sterile glass bottles from the raw water source, which were then stored at 4 ºC until their use in the batch enrichment experiments.

Enrichment procedures

The enrichment for potential MIB-degrading bacteria was conducted by pumping 1 l of feed solution of buffered mineral salts medium (MSM) (Table 3-1) (Izaguirre et al., 1988) inoculated with 200 ml lake water and spiked with 6 mg/l MIB (Wako Pure Chemicals, LTD., Osaka, Japan) through anthracite-packed glass columns (Figure 3-1). The feed solution was circulated through the columns at a flow rate of 0.5 ml/min by a peristaltic pump for 5 days, at which time biological growth was visible on the anthracite.

Table 3-1: Components of mineral salts media (Izaguirre et al., 1988)

Species	Concentration (mg/l)
NH_4Cl	50
K_2HPO_4	100
$MgSO_4$	50
$CaCl_2$	20
$FeCl_3$	1

A control column was also run without added MIB. Further enrichment was then achieved in batch enrichments with flasks containing 10 ml MSM, 6 mg/l MIB, and 0.5 grams of anthracite that was removed from the biologically active columns. These flasks were then incubated in an incubator shaker at 30 ºC and 300 rpm until turbidity was observed after approximately 3 days. Repeated transfers to fresh MIB-enriched medium were performed before characterization was conducted.

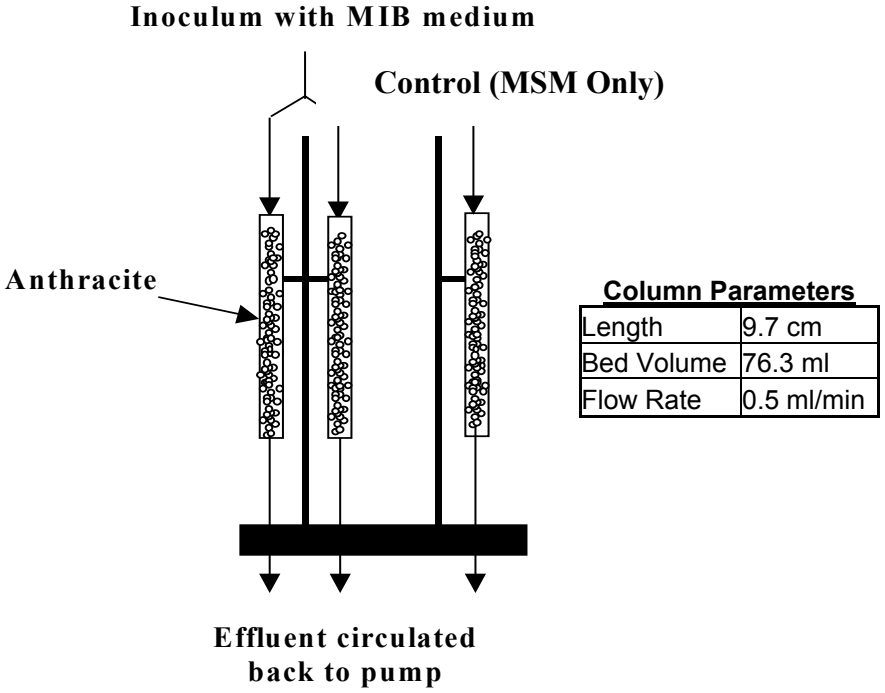

Figure 3-1: A schematic of the anthracite column apparatus used to isolate a culture capable of depleting MIB.

Solid culturing of the MIB-degrading culture was performed by streaking from the liquid cultures onto agar plates composed of MSM medium and 1.5% (w:v) agar . Plates were subsequently placed in a sealed 4-liter dessicator equipped with an uncovered beaker containing 10 ml of 100 mg MIB/l of deionized water and incubated at 30 °C.

Characterization of the Isolated Mixed Culture

Cellular morphology

The cellular morphologies of the strains present in the isolated mixed culture were identified using light microscopy and transmission electron microscopy (TEM). The liquid cultures were sampled during the stationary phase in order to check for the presence of spores. For light microscopy observations, a pipet tip was touched to a colony and then inserted into a drop of deionized water on a glass slide. The glass slide was then examined and photographed using a Nikon Optiphot-2 microscope (Nikon, Tokyo, Japan) with either differential interference contrast or phase contrast optics.

Liquid and solid cultures examined using TEM methods were prepared by first placing samples on a formvar-coated 300 mesh copper grid and treating with a negative stain. The liquid culture was prepared by placing 1 drop of the culture on the grid with an equal amount of 1% aqueous uranyl acetate. The liquid was wicked off the grid with filter paper after 2 minutes and rinsed once with deionized water. For the solid culture, a pipet tip was touched to a colony, inserted into a drop of deionized water mixed with an equal amount of 1% uranyl acetate on the grid, and wicked off the grid after 2 minutes as previously described. The negatively stained sample grids were then observed and photographed at 100 kv on a Zeiss EM10 electron microscope (Zeiss, Oberkochen, Germany).

Genotypic characterization

16S rRNA genetic analysis of the dominant species in the isolated culture was performed by MIDI Labs Inc., (Newark, DE, USA) by using MicroSeq 500 16S ribosomal RNA (rRNA)-based bacterial identification system (Applied Biosystems, Foster City, CA). The 16S rRNA gene was PCR amplified from genomic DNA isolated from the isolated culture. The PCR primers used for the amplification corresponded to *E. coli* positions 005 and 1540 (full length packages) and 005 and 531 (500 base pair packages). Amplification products were purified from excess primers and deoxy nucleotide triphosphates (dNTPs) using Microcon 100 (Millipore, Billerica, MA, USA) molecular weight cut-off membranes and checked for quality and quantity by running a portion of the products on agarose gel.

PCR cycling parameters used were 95 ^{o}C and 10 min for initial incubation, 95 ^{o}C and 30 seconds for melting, 72 ^{o}C and 45 seconds for chain extension and 60 ^{o}C and 30 seconds for annealing. Cycle sequencing of the 16S rRNA amplification products was carried out using AmpliTaq FS DNA polymerase and dRhodamine dye terminators (Roche Molecular Systems, Inc., Pleasanton, CA, USA). Excess dye-labeled terminators were removed from the sequencing reactions using a Sephadex G-50 spin column (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The products were collected by centrifuge, dried under vacuum and frozen at –20º C until they were ready to load. Samples were then resuspended in a solution of formamide/blue dextran/EDTA and denatured prior to loading. The samples were electrophoresed on an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA). The results were analyzed using Applied Biosystems DNA editing and assembly software (Applied Biosystems, Foster City, CA, USA).

Sequences were compared with previously identified sequences in the Microseq database using Microseq software (Applied Biosystems, Foster City, CA, USA) and in the National Center for Biotechnology Information (NCBI) GenBank database using BLAST (Altschul et al. 1990). Isolated culture sequences were aligned with closely related sequences from the Microseq database based on the percent genetic difference of the unknown culture strains with those of known strains. This alignment match was then used to construct the neighbor joining phylogenetic tree.

Growth characterization of the isolated culture

Specific growth rates and generation times of the isolated culture were determined from turbidity measurements using a UV-VIS spectrophotometer (Milton Roy Company, Ivyland, PA, USA) at a 600 nm wavelength. To assess the effect of MIB concentration on growth, initial MIB concentrations of 5, 10, and 20 mg/l were prepared in triplicate in MSM medium with 20% inoculum (v:v). These concentrations were chosen based on previous publications reporting observed microbial growth on MIB in the given range (Ishida and Miyaji, 1992; Izaguirre et. al, 1988). Chemical (MIB only) and biological (cells only) controls were also included with each set of concentrations. The specific growth rates were calculated using linear fits to the slope of the exponential growth phase of each growth curve obtained.

MIB Depletion Potential of the Isolated Culture

Oxygen uptake studies

Oxidation has been reported as one significant microbial pathway for MIB transformation (Tanaka et. al, 1996; Oikawa et. al, 1995). In order to assess the MIB oxidation potential of the isolated culture, oxygen uptake analysis was first performed. Cell suspensions of the isolated culture were prepared for the oxygen uptake experiments

by harvesting cultures at ¾-log phase by centrifugation at 1.94 g for 25 minutes in a J2-HS Beckman centrifuge (Beckman Coulter, Fullerton, CA, USA). Cells were washed once in MSM to remove residual MIB and resuspended to a wet cell weight concentration of 0.2 g/ml. The final cell suspension was stored in an ice bath during the oxygen uptake experiments.

A 1.9-ml glass, water-jacketed reactor (Figure 3-2) was used at a constant temperature of 30 ^{o}C to measure the rates of oxygen consumption at various initial substrate concentrations as described by Lindner et al. (2000). An electrolyte and membrane-covered Clarke-type electrode (Instech Laboratories, Plymouth, MA, USA) was inserted into the reactor using a ground-glass port with two rubber o-rings and was connected to a biological oxygen monitor (Yellow Springs, Yellow Springs, OH, USA). Monitor output was sent to an A/D converter board (DAS08-PGL, Computer Boards, Mansfield, MA, USA) for data collection using Labtech Notebook software (Wilmington, MA, USA). In all assays, the reaction chamber was filled with MSM before the addition of cells or substrate. The electrode was calibrated daily (following manufacturer's instructions) after application of fresh electrolyte and membrane. Runs for each concentration were conducted in triplicate. Oxygen uptake rates were calculated following the methods presented by Hitchman (1978). To verify oxygen uptake by the isolated culture, controls included runs with MIB only and cells only, and all rates were corrected for endogenous metabolism.

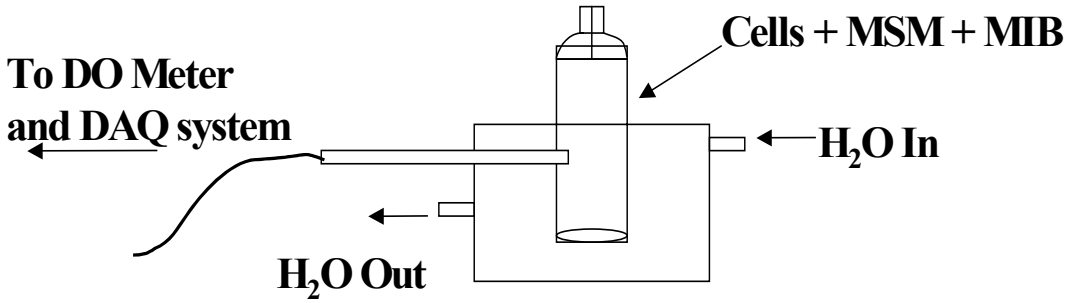

Figure 3-2: A schematic of the reactor used for the oxygen uptake experiments

MIB depletion studies

The protocol for the analysis of MIB depletion was developed using Solid Phase Micro Extraction (SPME) procedures described in Standard Method 6040D (APHA, 2001). The MIB depletion potential of the isolated culture was examined at two MIB concentrations, 5 mg/l and 25 ng/l. These concentrations were chosen because the highest rate of oxygen uptake was observed at 5 mg/l, and 25 ng/l represents average environmental concentrations observed in Lake Manatee during periods of high algal bloom. Microcosms were prepared in triplicate by addition of a stock solution of 100 mg/l MIB to flasks containing a mixture of 50 ml MSM and inoculum added to yield an optical density of 0.5 (λ = 600 nm). Controls included with each set of experiments were a substrate control (MIB only) and a killed control with autoclaved cells. All microcosms were incubated in an incubator shaker at 30 ºC and 300 rpm and sampled at regular time intervals for analysis. The 5 mg/l and 25 ng/l cultures were incubated until no change in MIB concentrations were observed. Samples were collected from the 25 ng/l cultures by transferring 10 ml of the culture to a 20 ml scintillation vial containing 3 g of NaCl, which was then immediately capped. Samples were taken from the 5 mg/l MIB cultures and then serially diluted in deionized water to ng/l MIB concentrations, and these diluted solutions were transferred to 20 ml scintillation vials as previously described. Sample

vials were stored at 4 ºC prior to analysis. All samples were analyzed by a Varian 3900 gas chromatograph (Varian, Inc., Palo Alto, CA, USA) with an ion trap ion detector, an Equity 5 fused silica capillary column (Supelco, Bellefonte, PA, USA), and a Saturn 2100 mass spectrophotometer (Varian, Inc., Palo Alto, CA, USA) with an auto-sampling system (CTC Analytics, Zweigen, Switzerland) fitted with a SPME fiber (Supelco, Bellefonte, PA, USA). The fiber, coated with divinyl-carboxen-polydimethylsiloxane cross-link, was injected into the headspace of each sample and allowed to equilibrate with the aqueous solution for 30 minutes. The fiber was then removed and inserted into the injection port of the GC/MS system where it was allowed to desorb for 5 minutes. Saturn Workstation version 5.52 data acquisition software (Varian, Inc., Palo Alto, CA, USA) was used for data analysis. Duplicates of each culture sample, a set of MIB standards, and a blank were run during the depletion studies to regularly calibrate the system.

Results and Discussion

Characterization of the Isolated Mixed Culture

Cellular and colony morphology

Homogenous, cream-colored, opaque colonies were observed on the agar 36 hours after inoculation, and full growth development was reached after an additional 60 hours of incubation. As summarized in Table 3-2, the colonies, approximately 2 mm in diameter, were round with a glassy surface, minimal elevation, and smooth edges.

Table 3-2. Colony and cellular characteristics of the dominant strain in the isolated culture

Characteristics	**Isolated Culture**
Colony Morphology	
Size	2 mm
Shape	Round
Color	Cream
Topography	Glassy
Full Development	96 hours
Elevation	Minimal
Edge	Smooth

Phase contrast light microscopy and transmission electron microscopy were used to elucidate cellular morphologies and fine structure of the strain(s) present in the isolated culture. Both techniques revealed that the isolated culture was composed of predominantly bacillus-shaped bacteria (Figure 3-3). The average cell size was approximately 5 μm in length and 1 μm in width. The presence of opaque structures, denoted by arrows in Figures 3-3A and 3-3B, indicates morphological evidence of sporulation. When observed under light microscopy the cells were highly motile, and flagella were observed in the TEM micrographs (Figure 3-3B). Flagellation and sporulation are cellular responses that are indicative of nutrient deprivation and allow the cells to move towards nutrient-rich regions or to remain dormant in periods of nutrient deprivation, respectively (Harwood, 1989). Both characteristics of these cells— flagellation and sporulation— provide insight into the modes of survival that these cells possess in their natural reservoir environment that undergoes seasonal changes in MIB concentrations.

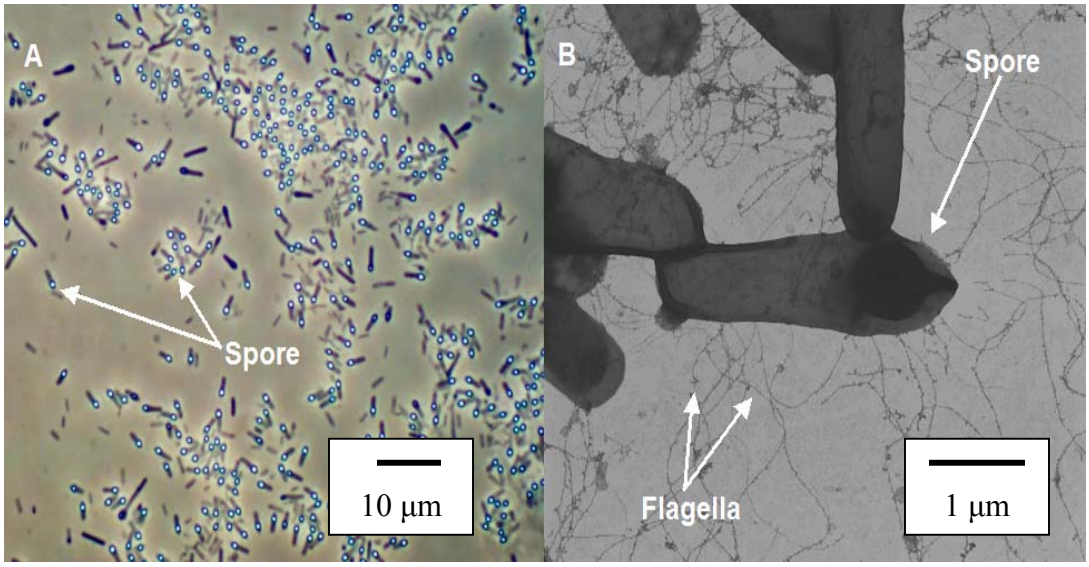

Figure 3-3: Phase contrast photograph (A) and transmission electron photograph (B) of the MIB-degrading isolated culture when grown on solid agar. Flagella and sporulation denoted with arrows.

Genotypic characterization

The 16S rRNA phylogeny results (Figure 3-4) from the MicroSeq alignment confirms placement of the isolated culture the *Bacillaceae* family of bacteria within a branch dominated by *Bacillus* strains. The isolated culture showed a 1.68% genetic difference (%GD) with its nearest relative *Bacillus fusiformis* (accession # AF2132169). Although this is not sufficient enough to place the unknown to the species level, it does match the isolated strain to the *Bacillus* genus level.

It is important to note; however, that results from the BLAST search of the GenBank database showed that the closet match of the MIB unknown was with *Bacillus Sphaericus*, at a 1% GD. *Bacillus sphaericus* and its sub-species *Bacillus fusiformis* are both strictly aerobic, mesophilic bacilli that are capable of forming flagella and spherical endospores in order to adapt to nutrient deprivation. *Bacillus sphaericus* is currently applied in the remediation of other contaminants, including: urea herbicides (Doi and McGloughlin, 1992).

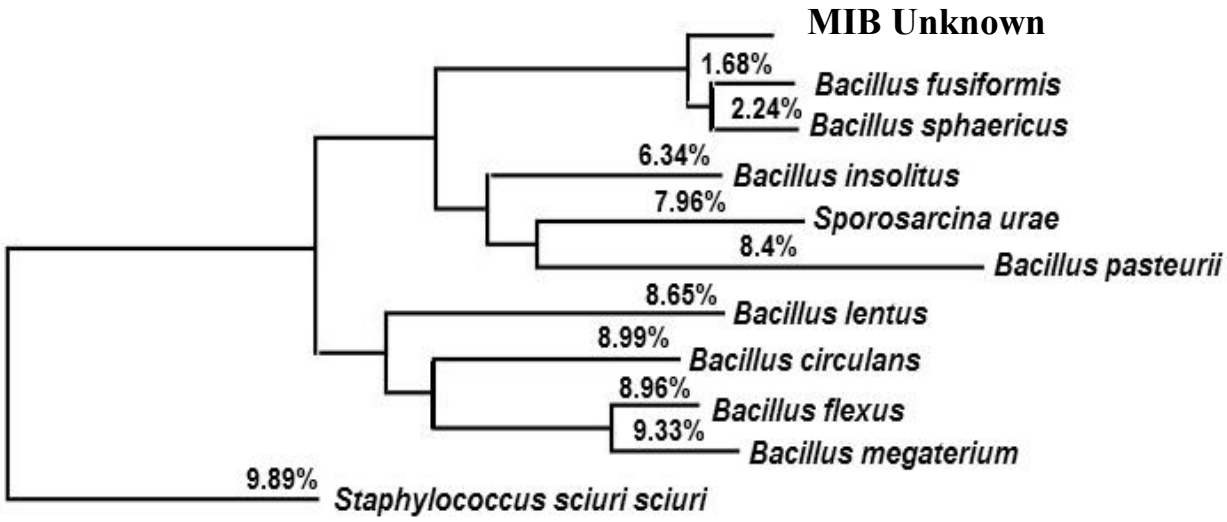

Figure 3-4: The neighbor joining phylogenetic tree of the isolated culture constructed from the MicroSeq alignment report. The top 10 closest genetic matches of the isolated culture are presented with their percent genetic difference. A lower % difference indicates a closer match.

Growth characterization

The effects of varying initial MIB concentration on specific growth rates were determined using the spectrophotometric methods as previously described. Figure 3-5 shows the growth curves of the cultures grown with initial MIB concentrations of 5, 10, and 20 mg/l. The highest specific growth rate calculated from each curve corresponded to an initial MIB concentration of 10 mg/l (0.067 ± 0.015 hr^{-1}). A summary of the specific growth rates and generation times calculated for each concentration is provided in Table 3-3.

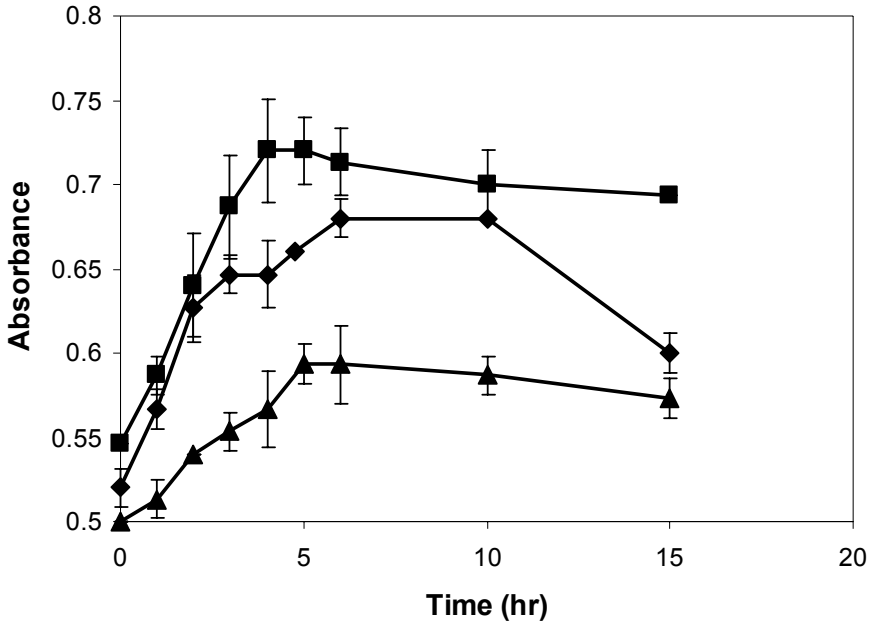

Figure 3-5: Growth curves of isolated culture at initial MIB concentrations of 5 mg/l (◆), 10 mg/l (■), and 20 mg/l (▲). All absorbance measurements were taken at a wavelength of 600 nm.

Table 3-3: A summary of the growth kinetics for the isolated culture grown at 5, 10, and 20 mg/l MIB.

MIB Concentration	Specific Growth Rate (μ)	Generation Time
5 mg/l	0.045 ± 0.005 hr^{-1}	15.64 + 1.73 hr
10 mg/l	0.067 ± 0.0015 hr^{-1}	10.74 ± 2.67 hr
20 mg/l	0.034 ± 0.004 hr^{-1}	20.56 ± 2.42 hr

MIB Depletion Potential of the Isolated Culture

Oxygen uptake studies

Figure 3-6 presents the oxygen uptake rates observed over a range of MIB concentrations from 2.5 mg/l to 20 mg/l. A maximum rate of 0.04 ± 0.004 μmoles O_2/s/ml was observed at 5 mg/l followed by a dramatic decrease in rates at higher MIB concentrations tested. As no effects on the probe were observed in controls with only MIB, this behavior, reported in other oxygen uptake studies (Lindner et al., 2000, 2003),

suggests a toxicity effect on the cells. Whether this is caused by excessively high MIB concentrations or formation of toxic intermediates is not known.

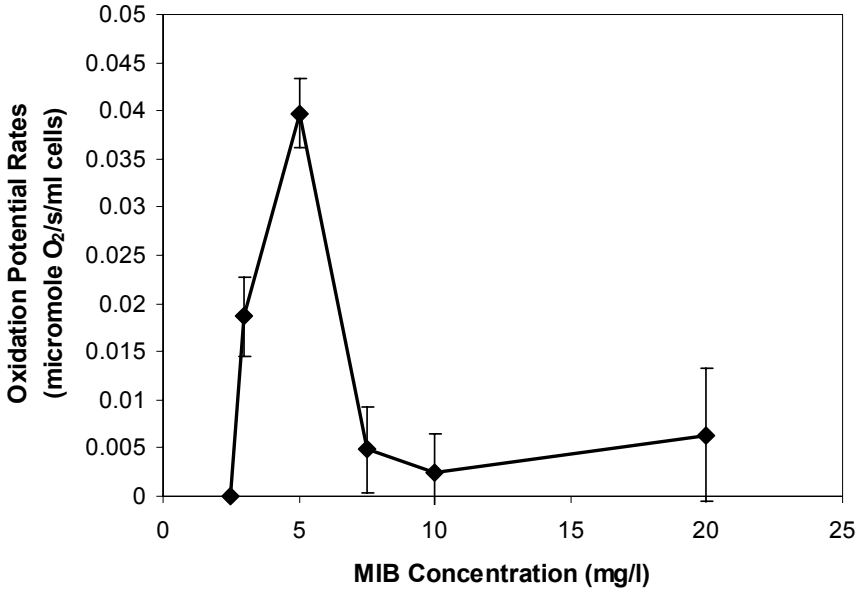

Figure 3-6: Oxygen uptake rates for the isolated culture over a range of MIB concentrations.

MIB depletion studies

The ability of the isolated culture to remove MIB at mg/l and ng/l concentrations was examined (Figure 3-7 and Figure 3-8). Five mg/l MIB, representing the high range of concentrations, was chosen because this was the concentration where the maximum rate of oxygen uptake was observed, as discussed previously. To represent the low and perhaps more environmentally relevant range of MIB concentrations, 25 ng/l was selected.

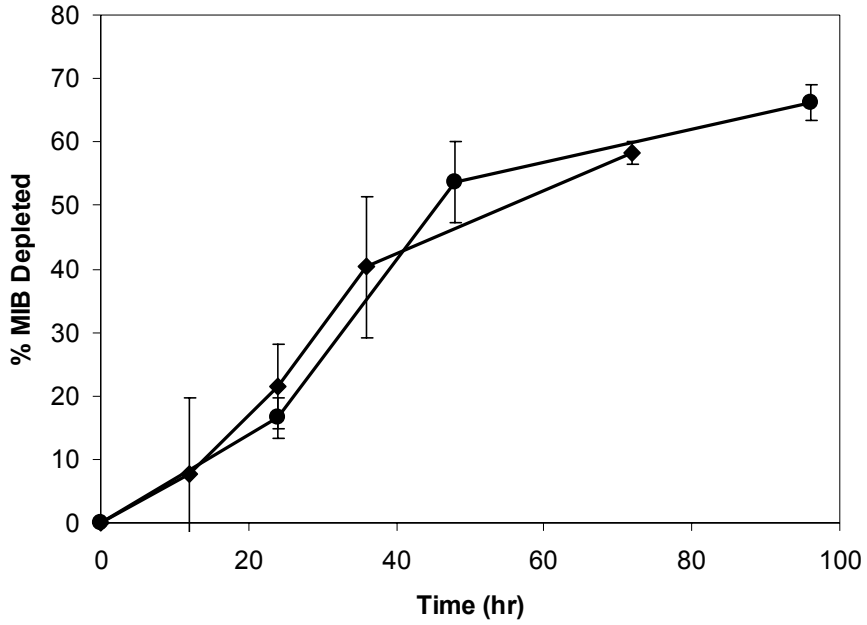

Figure 3-7: MIB depletion percent removal curves for isolated culture microcosms grown with initial MIB concentrations of 25 ng/l (●) and 5 mg/l (◆).

As shown in Figure 3-7, each culture was capable of decreasing the concentration of MIB by over 50% in less than 60 hours. There was also no noticeable lag time experienced by either culture before depletion occurred. This is most likely due to each culture's acclimation to the initial concentrations through routine transfers conducted prior to the depletion studies. The culture grown with an initial MIB concentration of 5 mg/l (4.2 mg/l measured) depleted MIB at an average rate of 0.35 $\pm$ 0.004 mg/l/hr, removing nearly 66% of the MIB to yield a final concentration of 1.8 $\pm$ 0.02 mg/l MIB after 72 hours of incubation. The culture grown with an initial concentration of 25 ng/l (28.5 ng/l measured) removed MIB at a rate of 0.20 $\pm$ 0.05 ng/l/hr and, after 96 hours of incubation, had reduced the concentration of MIB by approximately 58% to 9.6 $\pm$ 0.3 ng/l. While the isolated culture was capable of depleting MIB at both concentration levels, it is interesting to note that it was able to remove MIB to below the OTC of 10 ng/l in the 25 ng/l microcosms and no odor was observed in the culture at the end of the

study. The controls used in this study did not show any significant cell or substrate effects on MIB depletion and/or analytical recovery.

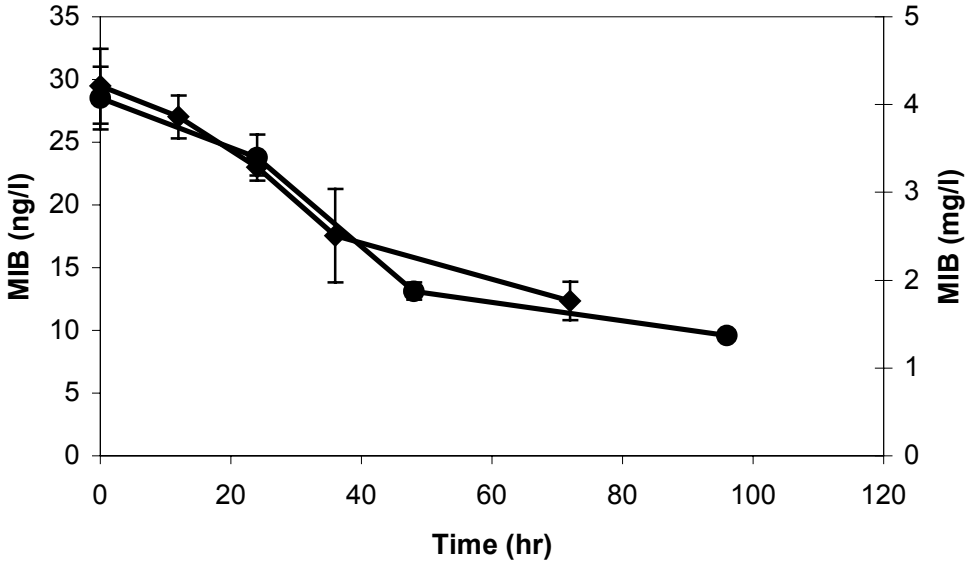

Figure 3-8: MIB depletion curves for isolated culture microcosms grown with initial MIB concentrations of 25 ng/l (●) and 5 mg/l (◆).

Conclusions

Microorganisms indigenous to aqueous surface-water environments that experience seasonal blue-green algae outbreaks may play an important role in cycling taste- and odor-causing compounds released by these microorganisms. Degradation of these compounds, such as MIB, may be a result of pure-culture or mixed-culture activity. Furthermore, opportunity may exist in using these bacteria for enhanced biodegradation for either *in situ* or *ex situ* applications. We obtained samples from a drinking water reservoir servicing Manatee County, FL, as it was our hypothesis that, because of the MIB outbreaks experienced in this reservoir on a seasonal basis, bacterial populations capable of using MIB as a growth substrate could be isolated. We report in this study successful isolation of a culture that is capable of degrading MIB to below odor threshold

levels. The predominant strain in this culture was matched to the *Bacillus* genus level and most closely related by 16S rRNA analysis to *Bacillus sphaericus*. While previous studies that focused on isolation of MIB-degrading bacteria have also identified *Bacillus* strains (Ishida and Miyaji, 1992), these results showed the influence of MIB concentration on microbial activity. The maximum growth rates were observed at 10 mg/l MIB, whereas observed oxygen uptake rates were the highest at 5 mg/l MIB. Depletion of MIB was shown at 5 mg/l and 25 ng/l initial concentrations, and final concentrations of MIB below the OTC of 10 ng/l were observed in the latter case.

The implications of these results are that microbial populations can be derived from natural water sources for removal of MIB and possibly other taste- and odor-causing compounds to concentrations that render drinking water as "wholesome," defined by Young et al. (1996) as both toxicologically and aesthetically acceptable. The culture isolated in this study was capable of using MIB as a growth substrate at relatively high and low concentrations of substrate, thus suggesting the ability of this culture to remove MIB throughout the phases of a seasonal outbreak of blue-green algae. Future work on this study should include further examination of the effectiveness of biological removal of MIB from natural surface waters under water treatment operational conditions that include continuous flow and variation in water characteristics.

CHAPTER 4
CONCLUSIONS

The focus of this project was to examine the potential for microbial transformation of MIB by a culture isolated from water collected from Lake Manatee in Manatee County, Florida. This study was part of a larger project that included an investigation on tailoring granular activated carbon specifically for MIB removal. This work included a literature review and laboratory studies focusing on the isolation and characterization of microbial systems capable of removing MIB. The hypothesis that drove the laboratory phase of this project was that, because of the MIB outbreaks experienced in this reservoir on a seasonal basis, bacterial populations capable of using MIB as a growth substrate could be isolated.

In this project, a MIB-degrading bacterial culture was isolated from a Lake Manatee water sample. The sample was used as an inoculum in a feed solution, composed of MSM and 6 mg/l MIB, which was passed through an anthracite column until growth was observed. Subsequently, the isolated culture was characterized by using growth kinetics to determine specific growth rates, light and TEM microscopy to examine fine cell structures, and 16S rRNA phylogenetic analysis to assess the closest match of the unknown isolate(s) to known bacterial strains. Finally, the MIB transformation potential of the microbial community was assessed by using oxygen uptake methods and batch microcosm experiments combined with SPME coupled with

GC/MS to determine the ability of the culture to oxidize and degrade MIB at different initial concentrations and to deplete MIB to below the OTC.

The results of this study supported the hypothesis by showing that a bacterial population was isolated from Lake Manatee that is capable of using MIB as a growth substrate. The predominant strain in this culture was matched to the *Bacillus* genus level and most closely related by 16S rRNA analysis to *Bacillus sphaericus*. While previous studies that focused on isolation of MIB-degrading bacteria have also identified *Bacillus* strains (Ishida et al., 1992), these results showed the influence of MIB concentration on microbial activity. Maximum growth rates were observed at 10 mg/l MIB, whereas observed oxygen uptake rates were the highest at 5 mg/l MIB. Depletion of MIB was shown at 5 mg/l and 25 ng/l initial concentrations, and final concentrations of MIB below the OTC of 10 ng/l were observed after 96 hr in the latter case.

These results imply that microbial populations can be derived from natural water sources for removal of MIB and possibly other taste- and odor-causing compounds to concentrations that render aesthetically acceptable drinking water. The culture isolated in this study was capable of using MIB as a growth substrate at over a relatively large range of concentrations of substrate, thus suggesting the ability of this culture to remove MIB throughout the phases of a seasonal outbreak of blue-green algae.

Currently, most drinking water facilities are equipped with powdered activated carbon and oxidation processes, such as chlorination, to remove taste- and odor-causing compounds. However, these technologies are insufficient in removing MIB. Alternatively, biological treatment systems should be considered for the removal of this

compound. One possible biological system application is a biologically active granular activated carbon filter to be used as a polishing step of the treatment process. This system would have several advantages over conventional treatment, including the removal of MIB through carbon adsorption and biodegradation, the production of biologically stable water, the removal of trihalomethane precursors, and the extension of activated carbon bedlife (Bitton, 1999).

Future work on this study should address questions concerning the effectiveness of biological removal of MIB from natural surface waters. A closer study of the biodegradation pathway(s) followed and toxicity mechanism(s) should be pursued to elucidate whether intermediates formed render odor and/or toxicity effects. Also, the effect of water treatment operational conditions, including continuous flow and variation in water characteristics, should be determined before a pilot-scale system is designed. Finally, filtration media of different physical and chemical characteristics should be assessed for optimal microbial growth. Answers to these questions will ensure that the ultimate design of a large-scale biological system is the most effective in terms of economics and performance.

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BIOGRAPHICAL SKETCH

I began studying at the University of Florida in August 1997. The multidisciplinary approach to solving problems for the health and well being of society drew me to the field of environmental engineering. As I progressed through my B.S. degree in environmental engineering, I found particular interest in classes covering the biological treatment of contaminants. When I completed my B.S. degree in 2001, I knew I wanted to further explore these subjects; therefore, I decided to attend graduate school for a master's degree in environmental engineering with a focus on biological remediation. The challenge of pursuing this degree has been unparalleled; however, the skills and experience I have obtained along the way, I know, will benefit me for the rest of my life. I look forward to continuing my education in environmental engineering when I begin work as a potable water engineer this June.