Evaluating the Use of Manganese-Oxidizing Bacteria in Surface Water Treatment Plants

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ABSTRACT

Soluble manganese (Mn) presents a significant treatment challenge to many water utilities, causing aesthetic and operational concerns. Surface water treatment plants using ozonation followed by biofiltration are unable to apply free chlorine across the filter, a method used by many utilities for soluble Mn control. These facilities are vulnerable to periodic problems of elevated Mn in the finished water. Manganese-oxidizing bacteria (MOB) readily oxidize Mn in groundwater treatment applications, which normally involve pH values above 7.0. However, the ability of MOB to facilitate Mn oxidation under lower pH conditions (6.2–6.3) often employed to optimize organics removal has not been demonstrated. Laboratory-scale biofilters were operated to evaluate the ability of MOB to accomplish soluble Mn control at a range of pH (6.3–7.3). The biofilters were able to oxidize Mn at a pH as low as 6.3 at greater than 98% removal. Ozonation by-product removal was also greater than 90% in all filter columns. Stress studies indicated that well-acclimated MOB can withstand variations in Mn concentration, hydraulic loading rate, and temperature typically found at surface water treatment plants, at least for relatively short (1-2 days) periods of time. Pilot studies demonstrated that there are unknown factors that affect MOB acclimation, and MOB may be present in full-scale biofilters already. MOB are difficult to identify with current biological analysis techniques and comprise small percentages of the total microbial population. MOB have demonstrated potential for use in surface water treatment plants, but further research is needed before this application is fully feasible.

Dedicated to my father, Richard Wheaton,
who would have been proud and delighted to see me graduate as a Hokie engineer

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LIST OF ABBREVIATIONS

BDOC	Biodegradable Dissolved Organic Carbon
BOM	Biodegradable Organic Matter
C	Carbon
CFU	Colony Forming Unit
DBP	Disinfection By-product
DGGE	Denaturing Gradient Gel Electrophoresis
DOC	Dissolved Organic Carbon
EPA	Environmental Protection Agency
EPS	Extracellular Polymeric Substance
Fl-AAS	Flame Atomic Absorption Spectroscopy
GAC	Granular Activated Carbon
gpm	gallons per minute
HPC	Heterotrophic Plate Count
ICP-MS	Inductively Coupled Plasma Mass Spectroscopy
ID	Inner Diameter
kb	kilobases
MCL	Maximum Contaminant Level
MCO	Multi-Copper Oxidase
MDL	Method Detection Limit
MIB	2-methylisoborneol
Mn	Manganese
MOB	Manganese-Oxidizing Bacteria
N	Nitrogen

NGE	Natural Greensand Effect
NOM	Natural Organic Matter
OTU	Operational Taxonomic Unit
P	Phosphorus
PCR	Polymerase Chain Reaction
ppm	parts per million
PTFE	Polytetrafluoroethylene
PVC	Polyvinyl Chloride
rep-PCR	Repetitive-Sequence-Based Polymerase Chain Reaction
rpm	rotations per minute
scfm	standard cubic feet per minute
SMCL	Secondary Maximum Contaminant Level
sp.	species
UV	Ultraviolet
V	Volts
v/v	volume to volume

INTRODUCTION

Manganese (Mn) is often present in drinking water sources in the reduced Mn(II) or oxidized Mn(IV) form. The soluble, reduced species can be oxidized in the presence of an oxidant and under appropriate pH conditions to form a dark brown MnO_2 precipitant, causing 'black water'. Elevated Mn levels can present a significant treatment challenge to many water utilities. Mn in drinking water can cause aesthetic and operational concerns even below the EPA Secondary Maximum Contaminant Level (SMCL) of 0.05 mg/L (EPA 1979) and has recently been linked to neurotoxic effects in children (Wasserman *et al.* 2006; Bouchard *et al.* 2010). One method of soluble Mn removal is to apply free chlorine to the influent directly upstream of a granular filter bed. The result is a natural greensand effect (NGE) where Mn(II) is initially adsorbed onto a MnO_x(s) surface on the media and then subsequently oxidized by free chlorine present in the filter-applied water (Knocke *et al.* 1988).

The enhanced usage of ozonation followed by biofiltration to increase finished water biostability and reduce the formation of chlorinated disinfection by-products (DBPs) has prevented the use of chlorine across filter media in some plants, thereby preventing soluble Mn removal by the NGE effect. Free chlorine application would reduce or eliminate biological activity by killing the microorganisms in the biofilter. As such, these utilities are seeking treatment options to help ensure effective soluble Mn removal and protection of their treated water from the associated aesthetic concerns. Mn oxidation by manganese-oxidizing bacteria (MOB) has been demonstrated in many studies; however, pH conditions associated with biological Mn removal have traditionally been above pH 7.0 (as high as 7.5) (Mouchet 1992). In comparison, water utilities using enhanced coagulation coupled with ozonation and biofiltration usually operate at a much lower pH (6.0 – 6.3) to optimize organics removal. While certain research studies have indicated that biological Mn removal may be possible at lower pH conditions (pH 6.5) (Burger *et al.* 2008a; Burger *et al.* 2008b), the evidence for Mn removal by MOB below pH 6.5 is lacking.

The goal of this research was to investigate the ability of MOB to oxidize and remove soluble Mn in water filter columns under a range of pH and changing influent conditions that may exist in surface water treatment facilities, especially those utilizing enhanced coagulation coupled with ozonation and biofiltration. The effect that a change in pH may have on organics removal via biofiltration (with focus on the removal of specific ozonation by-product organic compounds) was also evaluated. The objectives for this project were as follows:

1. To determine to what extent MOB can remove Mn in a water filter setup at low pH conditions while simultaneously monitoring any decrease in organics removal at higher pH conditions.

2. To determine how MOB respond to changes in influent Mn concentration, hydraulic loading rate, and temperature that simulate conditions commonly present at surface water treatment plants, and what effect these changes have on effluent Mn concentration.
3. To determine the feasibility of scale-up through a pilot-scale study.

Laboratory-scale biofilter columns, pilot-scale biofilter columns, and batch studies were used in various configurations to accomplish these objectives.

CHAPTER 1: LITERATURE REVIEW

1.1 MANGANESE IN DRINKING WATER

Manganese (Mn) is a transition metal that is commonly present in both groundwater and surface water sources. Mn can be found in the natural environment at oxidation states ranging from 0 to +7. Groundwater sources typically have high soluble Mn(II) concentrations year round; however, surface water sources (such as reservoirs and lakes) tend to have seasonal soluble Mn increases as thermal stratification and anaerobic reduction of oxidized Mn(IV) in sediments occurs. Insoluble Mn presents little treatment challenge to water utilities as it can be removed using conventional filtration techniques; however, soluble Mn must first be oxidized to be removed through standard filtration. Mn is not readily oxidized by oxygen at the neutral pH conditions at which treatment plants operate, so additional treatment is required. Conventional oxidation techniques involve the use of strong chemical oxidants (such as permanganate) or applying free chlorine across granular filter beds to stimulate a natural greensand effect (NGE) which causes soluble Mn to become incorporated in the filter media surface and effectively removed (Knocke *et al.* 1988).

Reduced Mn that is not removed at the treatment facility can be oxidized in the distribution system or in consumers' homes by residual disinfectants, bacteria, or household oxidants (such as bleach), causing black discoloration of water and scaling of pipes and fixtures (Sly *et al.* 1990). The EPA has set a non-enforceable Secondary Maximum Contaminant Level (SMCL) of 0.05 mg/L (EPA 1979) to address the aesthetic issues surrounding Mn; however, a target of 0.02 mg/L is more appropriate to ensure consumer confidence and minimize the potential for water discoloration (Sly *et al.* 1990). While Mn has not historically been perceived as a drinking water health risk, studies have shown elevated drinking water Mn concentrations to be associated with increased neurological symptoms in adults (Kondakis *et al.* 1989) and neurotoxic effects in children (Wasserman *et al.* 2006; Bouchard *et al.* 2010). As such, the removal of Mn from drinking water is potentially both an aesthetic and health concern.

1.2 BIOLOGICAL MANGANESE OXIDATION

Manganese-oxidizing bacteria (MOB) are able to oxidize Mn(II) to Mn(IV) through biological mechanisms. The purpose of this biological Mn oxidation is largely unknown. It has been hypothesized to be a source of cellular energy, a method for breaking down complex organic substrate, a means of protection from extracellular threats, or a fortuitous effect of unrelated cellular interactions (Tebo *et al.* 2004; Tebo *et al.* 2005). Biological Mn oxidation follows the equation below, although the process is most likely much more complex (Devrind *et al.* 1986; Boogerd and Devrind 1987):

$$Mn^{2+} + 0.5O_2 + H_2O \rightarrow MnO_2(s) + 2H^+.$$

Consistent with this equation, Vandenabeele *et al.* (1992) noted a pH decrease of 0.7 units within MOB flocs when Mn was present in the growth medium, refuting the hypothesis that biological Mn oxidation is caused by high pH microenvironments created by the bacteria.

The ability of MOB to oxidize Mn may be dependent on nutrient concentration, as organic-nutrient-limited growth media has been shown to better promote the growth of MOB over nutrient-rich media (Tani *et al.* 2003). Additionally, growth media enriched with Mn has been found to enhance the growth of MOB more than other bacteria species (Francis *et al.* 2001; Tani *et al.* 2003), suggesting that MOB are receiving some competitive benefit of Mn oxidation. Mn oxidation factors (the proteins presumably responsible for Mn oxidation) have highest Mn oxidation rates at pH 7.5 (Boogerd and Devrind 1987), which is consistent with the traditional theory based on empirical observations in groundwater treatment plants that MOB oxidize Mn best at pH conditions greater than 7.4 (Mouchet 1992). Mn oxidation has been linked to multi-copper oxidase (MCO) enzymes, which couple oxidation of metal and organic substrates to reduction of O_2 to H_2O (Tebo *et al.* 2005; Dick *et al.* 2008). Yet even with this discovery, the fundamental cellular benefit of biological Mn oxidation is still a mystery. Utilizing MOB in novel engineering applications may prove difficult without understanding the benefit MOB derive from Mn oxidation or the mechanism they use.

MOB are phylogenetically diverse and include various species (Tebo *et al.* 2004). Many species appear to have evolved the ability to oxidize Mn independently, so several phylogenetically-distinct model organisms have been studied to elucidate more information about the purpose and mechanism behind biological Mn oxidation. These model organisms include *Pseudomonas putida* strain GB-1 (Okazaki *et al.* 1997), *Leptothrix discophora* SS1 (Boogerd and Devrind 1987; Katsoyiannis and Zouboulis 2004) and SP-6 (Hope and Bott 2004; Burger *et al.* 2008b), and *Bacillus* sp. strain SG-1 (Devrind *et al.* 1986; Bargar *et al.* 2000). All of these bacteria deposit oxidized Mn on their external cellular structure (Tebo *et al.* 2004), which may provide a clue as to why they oxidize Mn.

In terms of bacterial growth phases, MOB have been shown to oxidize Mn during stationary phase, whereas spore-forming MOB oxidize Mn at the beginning of spore formation (Francis *et al.* 2001; Cerrato *et al.* 2010). *Leptothrix discophora* SS1 has been documented to have its highest Mn-oxidizing activity during stationary phase, but activity has also been observed during early exponential growth phase (Boogerd and Devrind 1987). According to another study of *Leptothrix discophora* SS1, biological Mn oxidation generally follows first-order reaction rates (Katsoyiannis and Zouboulis 2004). The ability to model biological Mn oxidation has great benefit in design applications.

Bacillus sp. strain SG-1 has been studied for its ability to oxidize Mn in the dormant spore form, where no metabolic activity occurs (Devrind *et al.* 1986; Bargar *et al.* 2000). Bacteria in spore form are known to be resistant to a host of environmental threats, including heat and oxidants (Nicholson *et al.* 2000), and many Mn-oxidizing *Bacillus* spores have been identified (Francis

and Tebo 2002; Cerrato *et al.* 2010). Targeting specific growth phases by correct timing of inoculation and control of environmental conditions may allow MOB to be more viable for engineering applications.

1.3 Biofiltration

Biofiltration occurs when a water utility stops applying residual disinfection to a filter bed which allows a community of microorganisms to become established on the filter media. Many utilities add ozonation for primary disinfection when upstream chlorination is removed. Ozonation causes natural organic matter (NOM) to become more biodegradable and therefore more easily removed by microorganisms present in a biofilter (Rittmann *et al.* 1989). The primary driver for allowing filters to become biologically active is to remove biodegradable dissolved organic carbon (BDOC). This increases the biostability of the finished water to reduce regrowth potential within the distribution system. Another benefit of biofiltration is a decrease in the formation of chlorinated disinfection by-products (DBPs). Chlorinated DBPs are formed when chlorine disinfectants interact with NOM found in the water, producing compounds that often have known or suspected adverse health effects. Chlorinated DBP formation can be decreased by removing much of the NOM through coagulation and filtration before adding chlorine and/or reducing the contact time of free chlorine in the water (Reckhow and Singer 2011), both of which occur when biofiltration is employed.

Biofilters have additional benefits aside from increasing the biostability of the finished water and reducing the formation of chlorinated DBPs. Biofilters have been demonstrated to be more effective than conventional filters at removing pathogens (Amburgey *et al.* 2005). They have also been shown to effectively remove other contaminants with aesthetic and health concerns, including the ozonation DBP bromate (Kirisits and Snoeyink 1999) and odor causing geosmin and MIB (Elhadi *et al.* 2006). Increased head loss and subsequent decreased filter run times are a common issue with biofilters as filter pore spaces become clogged from biological production of extracellular polymeric substances (EPS) (Mauclaire *et al.* 2004). Nutrient (nitrogen and phosphorus) addition and peroxide addition have been shown to decrease head loss (presumably due to decreasing EPS presence) and increase removal of NOM and other contaminants such as MIB (Lauderdale *et al.* 2012).

1.4 Manganese Removal Using Biofiltration

Traditional knowledge on the use of MOB in biofiltration applications was summarized by Mouchet (1992). According to this publication, MOB require specific conditions in order to oxidize Mn: pH > 7.4-7.5, dissolved oxygen > 5 mg/L, Eh > 300-400 mV, and rH > 25. These conditions can be readily met in groundwater treatment facilities which traditionally have alkaline pH (>7); however, surface water treatment plants traditionally operate at a much lower pH range (6.0-6.5) for enhanced coagulation. Biological removal of Mn has been used in groundwater treatment facilities in Europe since the 1980s and has been studied extensively in the treatment of groundwater (Mouchet 1992; Vandenabeele *et al.* 1992; Hope and Bott 2004; Katsoyiannis and Zouboulis 2004; Li *et al.* 2006; Burger *et al.* 2008a). Recent research has

started to expand the known operating range for MOB, particularly with regards to pH. MOB presence and biological removal of Mn have been observed in full-scale biofilters as low as pH 6.5 (Burger *et al.* 2008a). Additionally, biological Mn oxidation in lab-scale biofilters was greater at pH 6.5 than pH 7.5 (Burger *et al.* 2008b).

Mn removal in groundwater biofilters has been shown to be very effective, even when influent Mn concentrations are over 1 mg/L (Li *et al.* 2005; Pacini *et al.* 2005; Stembal *et al.* 2005). The presence of MOB in biofilters can increase Mn removal from 25% (based solely on chemical oxidation) to 98%, with combined chemical and biological oxidation (Gouzinis *et al.* 1998). Ammonia and iron have been shown to be inhibitory to biological Mn removal (Gouzinis *et al.* 1998); however, simultaneous removal of ammonia, iron, and Mn has also been demonstrated (Stembal *et al.* 2005; Tekerlekopoulou *et al.* 2010). Mn removal has been enhanced through supplementation of nitrogen and phosphorus as well as addition of hydrogen peroxide (Lauderdale *et al.* 2012).

Leptothrix discophora SP-6 has been the focus of several MOB biofiltration studies as a model organism (Hope and Bott 2004; Burger *et al.* 2008b). However, in a study of full-scale treatment plants with active biological Mn removal, *Leptothrix* was only detected in one out of the four plants analyzed, indicating that *Leptothrix* may not necessarily be a dominant Mn-oxidizing genus in full-scale operations (Burger *et al.* 2008a). MOB can be found in concentrations of 10^3 to 10^4 colony forming units (CFU)/g filter media in mature, full-scale groundwater treatment plants (Burger *et al.* 2008a). MOB are present in the natural environment and have been located in various stages of chlorinated drinking water systems, where they make up between 10^{-5}% and 10^{-2}% of the total CFU (Cerrato *et al.* 2010). Vandenabeele *et al.* (1992) found that MOB can make up 2-3% of the bacteria present in influent groundwater, but they can make up 25-33% of the bacteria present in the biofilms of a mature filter. Culturing MOB can be challenging, as Burger *et al.* (2008a) observed when no MOB were detected using Mn-specific plating methods from a biofilter with demonstrated biological Mn removal.

Startup time for filters designed to sustain biological removal of Mn is known to take from two weeks to two months (Mouchet 1992; Burger *et al.* 2008b) and may be a barrier to full-scale implementation. Time to reach filter maturation (when effluent Mn is stable and not impacted by changes in influent Mn concentration) has been shown to take up to nine months in some plants (Li *et al.* 2006). Media type has been found to have some impact on startup time, with uniformly-graded sand media reducing maturation time by one or two months over well-graded sand media, presumably due to increased filtration space for biofilm development (Li *et al.* 2005).

Seeding with backwash water from a filter with an active MOB community has been shown to substantially reduce startup time (Mouchet 1992). Similarly, Vandenabeele *et al.* (1992) successfully started biological Mn removal in filters by replacing 50% of the media with that from a mature filter; however, the authors did not discuss if there was any lag time between replacement of the media and stable Mn removal. They did note that increased influent iron

concentration could negatively affect establishment of an active MOB population as iron-oxidizing biofilms are much faster at maturing in filters and can lead to clogging and more frequent backwashing, further exacerbating the difficulty of MOB establishment.

Different hydraulic setups have been explored for their potential in reducing startup time. Through modeling of biological Mn removal in lab- and pilot-scale biofilters, Hope and Bott (2004) suggested that startup time can be reduced by using recirculating systems with Mn concentrations around 2.5 mg/L where the effluent is recirculated to the influent of the filter. Gouzinis *et al.* (1998) found that before biological Mn oxidation is established in a filter, fill-and-draw operation produces better Mn removal efficiencies than continuous flow operation. However, the continuous flow operation becomes much more efficient once MOB become established on the filter. They also recommend fill-and-draw reactors in series during startup to achieve effluent Mn requirements before the filters are mature.

1.5 SUMMARY

Soluble Mn control is an issue for drinking water treatment plants because of its associated aesthetic, operational, and health concerns. Many surface water treatment plants use biofiltration to achieve their finished water quality objectives; however, they are subject to chronic Mn control problems due to the inability to apply free chlorine to the filter beds. Biological removal of Mn has been proven to be a competitive treatment method for groundwater, but its use in surface water treatment is limited. Recent research has demonstrated that MOB can function under broader pH conditions than conventionally thought, potentially overlapping with pH conditions commonly found at surface water treatment plants. While simultaneous removal of various groundwater contaminants and Mn has been explored, there remains little research on the impact of varying surface water conditions on Mn removal. More research into the effects of pH and varying influent conditions needs to be completed in order for biological Mn removal to become a viable option for surface water treatment plants.

CHAPTER 2: MATERIALS AND METHODS

Laboratory-scale and pilot-scale biofilter experiments were designed to evaluate the use of manganese-oxidizing bacteria (MOB) for soluble manganese (Mn) removal in surface water treatment plants. The effects of pH, temperature, hydraulic loading rate, influent Mn concentration, and influent biodegradable organic matter (BOM) concentration on biological Mn removal were investigated. The methods and materials utilized in this research are outlined below.

2.1 MANGANESE-OXIDIZING BACTERIA

The MOB isolates used in the following experiments were chosen from 34 Mn-oxidizing isolates obtained from various locations within four water treatment facilities and distribution systems and identified using 16S rRNA sequences during a previous study at Virginia Tech (Cerrato *et al.* 2010). Of the original 34 isolates, 28 were chosen for further evaluation. The cultures were stored as 20% glycerol v/v suspensions at -70°C before use. Isolates capable of both reduction and oxidation of Mn were avoided. The isolates were streaked onto Mn-oxidation agar media at pH 6.0, 6.5, and 7.4 (see section 2.7.3) and incubated at 30°C to determine which isolates were still viable and able to oxidize Mn in that pH range. The plates were examined every three days for evidence of Mn oxidation and deposition of insoluble MnO_2, signified by colonies with brown centers or edges (Figure 2-1). The rate at which Mn oxidation and deposition occurred was categorized based on the number of days before brown colonies were observed. The isolates were labeled as fast (<10 days), average (10-20 days), or slow (>20 days). Table 2-1 summarizes the Mn oxidation rate for each of the isolates evaluated.

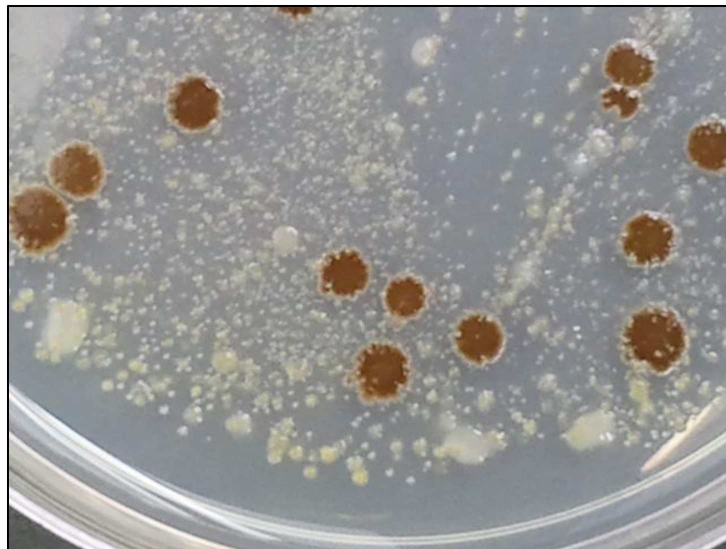

Figure 2-1. Dark Mn-oxidizing bacteria colonies on Mn-oxidation agar

Table 2-1. Mn-oxidizing isolate evaluation summary

Isolate #	Species Classification	Mn oxidation*	Growth Rate**
MB-1	*Bacillus cereus*	Average ~~	Unknown
MB-2	*Bacillus pumilus*	Fast	Fast
MB-3	*Bacillus pumilus*	Fast	Fast
MB-4	*Bacillus pumilus*	Slow ~~	Slow
MB-10	*Bacillus pumilus*	Fast	Fast
MB-11	*Bacillus pumilus*	Fast ~	Unknown
MB-12	*Pseudomonas saccharophilia*	Fast ~	Unknown
MB-14	*Brevundimonas nasdae*	No oxidation	Slow
MB-15	*Bacillus pumilus*	No growth	Unknown
MB-16	*Lysinbacillus sphaericus*	Fast	Average
MB-17	*Lysinbacillus sphaericus*	Fast	Average
MB-18	*Bacillus pumilus*	Slow	Slow
MB-19	*Bacillus pumilus*	Average	Average
MB-20	*Bacillus pumilus*	Fast ~	Fast
MB-21	*Lysinbacillus fusiformis*	Fast	Average
MB-22	*Lysinbacillus fusiformis*	Fast	Fast
MB-23	*Lysinbacillus fusiformis*	Fast	Average
MB-26	*Lysinbacillus sphaericus*	Fast	Fast
MB-27	*Lysinbacillus fusiformis*	Slow ~~	Slow
MB-28	*Bacillus pumilus*	No growth	Unknown
MB-29	*Bacillus cereus*	Fast	Slow
MB-30	*Bacillus cereus*	Average	Average
MB-31	*Brevibacillus brevis*	Slow	Unknown
MB-32	*Bacillus simplex*	Slow	Unknown
MB-33	*Pseudomonas aeruginosa*	Average ~	Average
MB-34	*Bacillus simplex*	Fast	Unknown
MB-37	*Bacillus pumilus*	Fast ~~	Average
MB-38	*Brevundimonas nasdae*	Fast	Fast

* Fast: MnO_2 deposition observed within 10 days
Average: MnO_2 deposition observed in 10-20 days
Slow: MnO_2 deposition first observed after >20 days

** Fast: Stationary phase reached within 2 days
Average: Stationary phase reached within 5 days
Slow: Stationary phase reached after >5 days
Unknown: Growth rate was not measured for this isolate

~ Mn oxidation much slower at lower pH

~~ Mn oxidation not observed at lower pH

Only 25 of the 28 isolates selected for evaluation were viable and were inoculated into tubes containing 5 mL pH 7.4 Mn-oxidation broth (see section 2.7.3) and incubated at 30°C until turbid (approximately one week) which indicated that enough cells had replicated for further use and analysis. The cell cultures were then streaked onto pH 7.4 Mn-oxidation agar media to verify purity of the cultures. Growth rates were measured for the isolates at pH 6.0, 6.5, and 7.4 by inoculating 0.1 mL of turbid cell culture suspension into 4.9 mL pH-adjusted Mn-oxidation broth. Turbidity was determined using a spectrophotometer (Milton Roy Company Spectronic 20) at a wavelength of 580 nm. The tubes were incubated on a shake table rotating at 120 rpm at 30°C, and turbidity was measured every three days for two weeks. Stationary phase was reached when the increase in absorbance began approaching zero asymptotically. Growth rates were categorized by how quickly stationary phase was reached: fast (<2 days), average (<5 days), and slow (>5 days). Table 2-1 summarizes the observed growth rates.

A consortium of six isolates was chosen based on the evaluations presented in Table 2-1. Rapid MnO_2 deposition and rapid growth rate were preferable to reduce acclimation time; further, Mn oxidation at all pHs was a requirement. Additionally, a diversity of species was desired to provide a robust community. Table 2-2 outlines the basic information about the chosen isolates. Detailed growth curves for these isolates were determined at pH 6.0, 6.5, and 7.4 as previously described, measuring turbidity every two hours to better characterize the growth curves. Two growth curves were developed and combined together to eliminate the need to sample every two hours for 54 hours. The separate growth curves are denoted by different marker shapes in Figures A-1 to A-6 in the Appendix.

Table 2-2. Summary of selected MOB isolates

Reference Number	Species Classification	Original Sample Site
MB-2	*Bacillus pumilus*	Blacksburg, Christiansburg, VPI Water Authority Radford, VA Sedimentation Basin – Bottom Sludge
MB-3	*Bacillus pumilus*	Newport News Waterworks (Lee Hall) Newport News, VA Sedimentation Basin – Top Sludge
MB-17	*Lysinbacillus sphaericus*	Newport News Waterworks (Lee Hall) Newport News, VA Sedimentation Basin – Top Sludge
MB-22	*Lysinbacillus fusiformis*	Newport News Waterworks (Lee Hall) Newport News, VA Sedimentation Basin – Top Sludge
MB-33	*Pseudomonas aeruginosa*	Honduras Distribution System – PVC Pipe
MB-38	*Brevundimonas nasdae*	Newport News Waterworks (Lee Hall) Newport News, VA Sedimentation Basin – Water

2.2 LABORATORY-SCALE BIOFILTERS

Lab-scale glass columns (Figure 2-2) were used to simulate the anthracite coal layer of a biologically active filter at a full-scale surface water treatment plant. MOB were inoculated into anthracite coal media in the columns, and Mn removal was monitored. When Mn removal reached steady-state, various stress studies were conducted to simulate the effects of changing influent characteristics commonly found at surface water treatment plants on biological Mn removal. The physical setup, feed water, MOB inoculation, sampling, and maintenance are described in the following subsections.

2.2.1 Biofilter Columns

Glass columns with 5 ft length and 1.5 inch inner diameter (ID) were constructed at the Virginia Tech Department of Chemistry Glass Shop by fitting one end with a threaded glass connection and compression cap and attaching threaded glass sampling ports along the length of the column at regular intervals (as shown in Figure 2-3). A smaller threaded glass connection with compression cap (which would serve as a manometer) was attached to the column across from the bottom sample port. The 1.5 inch ID was chosen to provide a compromise between minimizing wall effects and reducing influent flow requirements. The sampling ports along the length of the column were capped with open-top plastic screw caps with PTFE/silicone septa. This allowed for sampling throughout the two-foot media depth to determine profile behaviors of various parameters. Glass and PTFE-lined septa were the only materials in contact with the feed water between the influent (top) and effluent (bottom) sampling ports. These materials were chosen because they are inert would not interact with the carbon compounds in the feed water (see section 2.2.4.4). Therefore, any decrease in the concentration of the target carbon compounds across the media depth could be attributed to the biofilter microorganisms.

A six-inch gravel layer supported the anthracite coal media bed. A wire mesh square was placed above the compression cap to support the gravel layer. The columns were clamped to a pipe rack, and clear flexible tubing was compressed in the bottom compression cap of each glass column and connected to an effluent tube, which drained into a floor drain. A small diameter clear flexible tube was inserted into the manometer port and extended above the column. The effluent tubes were attached to the pipe rack with cable ties at a height approximately three inches above the media to ensure that the media was saturated at all times. Siphoning was prevented by attaching a small length of open-ended tubing to the effluent tube where it was tied to the pipe rack. Phototropic growth is uncharacteristic of surface water treatment plant biofilters, so the columns were covered in foil.

2.2.2 Biofilter Media

Anthracite coal media was obtained from a full-scale biofilter at the Newport News Waterworks Lee Hall Treatment Plant (Newport News, VA) by inserting a two-inch diameter pipe into the filter bed to a depth of approximately nine inches to collect media. The media had an effective size of 0.95-1.05 mm and a uniformity coefficient of <1.4. The media was shipped from Newport

Figure 2-2. Lab-scale biofilter columns

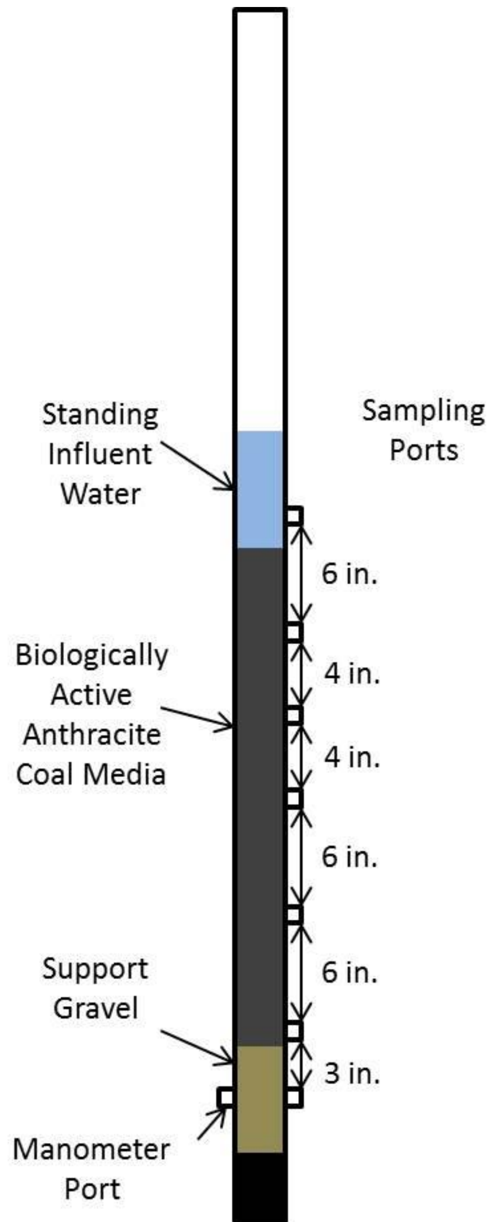

Figure 2-3. Lab-scale biofilter column design

News to Blacksburg, VA, in 1 L plastic Nalgene bottles, filled halfway with media and then filled with filter influent water. Upon arrival in Blacksburg, the media was stored at 4°C before filter startup. Carbon components in similar concentrations to the feed water described in section 2.2.4.4 were added. The media containers were opened and mixed every 2-3 days to prevent the biological community from becoming anaerobic. The media was backwashed to remove particulate still present from its use in the full-scale water treatment plant directly prior to inoculation with MOB.

2.2.3 Inoculation of Media with MOB

The media was inoculated with the six-isolate consortium previously mentioned. Late exponential growth phase of the pure cultures was targeted to provide cells that would be most likely to acclimate and grow in the less nutrient dense environment of the columns. The isolates were divided into three groups based on similar growth curves, which can be seen in Figures A-1 through A-6 in the Appendix. MB-33 was in Group 1, with a lag phase of approximately 30 hours, reaching stationary phase at around 50 hours. Group 2 consisted of MB-2, MB-17, and MB-38, which all had similar growth curves with a lag phase of 10-15 hours, reaching stationary phase at 30 hours. Group 3 contained MB-3 and MB-22, which had similar growth curves to Group 2 at pH 6.0 and 6.5, but at pH 7.4, the growth curve was more similar to Group 1.

A 250 mL baffled Erlenmeyer flask was filled with 100 mL pH 7.0 Mn-oxidation broth for each of the three groups. Each isolate in the group was inoculated into the broth by adding 2 mL of the turbid cell suspensions from the most recent growth curve study. The flasks were incubated at 30°C on a shake table at 120 rpm until turbid, approximately 48 hours. The groups were then combined in 1 L of pH 7.0 Mn-oxidation broth in a 2 L baffled Erlenmeyer flask by adding 7 mL turbid culture from each of the 250 mL flasks. MB-33 (Group 1) was added to the 1 L broth 12 hours before the other groups to account for the longer lag time. The flask was incubated at 30°C on a shake table at 120 rpm until turbid, approximately 20 hours after Groups 2 and 3 were added. The 1 L cell suspension was then centrifuged at 5000 x *g* for 30 minutes, and the cells were re-suspended in 300 mL of feed water (see section 2.2.4) and acclimated at room temperature for one week.

After acclimation, the 300 mL suspension was added to 3 L Lee Hall media in synthesized feed water (see section 2.2.4) and remained undisturbed for 24 hours to maximize attachment to the media surface. The inoculated media was then distributed into four columns to a depth of slightly less than two feet. The leftover media and enough liquid to ensure the media was fully saturated were kept at room temperature. The remaining liquid was distributed equally into the columns to retain as many cells as possible. The media was left in the columns for 24 hours without disturbance to allow for further cellular attachment. The columns were then lightly backwashed (see section 2.2.7) only to the point of bed fluidization to allow the media to expand and settle, and then additional media was added to the columns to even the depth to two feet. The media was undisturbed for 24 hours, and the light backwash and media addition, if required, were

repeated. After both backwashes, the backwash water was collected and poured equally into the columns over the course of eight hours to retain as many cells as possible. The columns were undisturbed for an additional 24 hours before the influent feed was started.

2.2.4 Feed Water System

Tap water from Blacksburg, Christiansburg, VPI Water Authority was dechloraminated and supplemented with biodegradable organic matter (BOM), Mn, and pH adjustment as described in the following subsections. The average characteristics of Blacksburg tap water can be found in Table 2-3. Figure 2-4 shows a schematic of the initial feed water design.

Table 2-3. Representative Blacksburg tap water characteristics

pH	7.7
Alkalinity	49 mg/L as $CaCO_3$
Hardness	46 mg/L as $CaCO_3$
DOC	1.2 mg/L C
Ammonia	0.73 mg/L as N
Mn	0.468 µg/L
Nitrate	0.45 mg/L as N
Phosphate	0.24 mg/L as P
Dissolved oxygen	9.25 mg/L

2.2.4.1 Chloramine Removal

A granular activated carbon (GAC) column was constructed from PVC pipe and filled with catalytic granular activated carbon (Calgon Centaur 12x40) to remove chloramine disinfectant from the Blacksburg tap water. The GAC unit had an empty bed contact time of ten minutes and was operated upflow. Total chlorine was measured daily to identify if and when breakthrough occurred. A faucet provided tap water at approximately 0.5 L/min and was outfitted with flexible tubing and connected to an influent port at the base of the GAC unit.

2.2.4.2 Hydraulic Setup

The dechloraminated water flowed from the top of the GAC column into a five-gallon bucket equipped with an overflow port to maintain constant head. This step provided a location for reaeration of the water after it had passed through the GAC unit where aeration would not strip out the BOM added in the main mixing bucket. The water was aerated with an air stone and pump, which provided a dissolved oxygen concentration of approximately 7.5-8.0 ppm. A tube at the base of the bucket allowed flow via gravity feed controlled by hose clamp into a ten-gallon bucket, which was the main mixing vessel.

Mn, BOM, and an initial pH adjustment were added to the ten-gallon bucket, which was mixed with a submersible pump. Each component was fed into the ten-gallon bucket from an individual 10 L glass container via peristaltic pump at a rate of approximately 0.8 mL/min. Individually

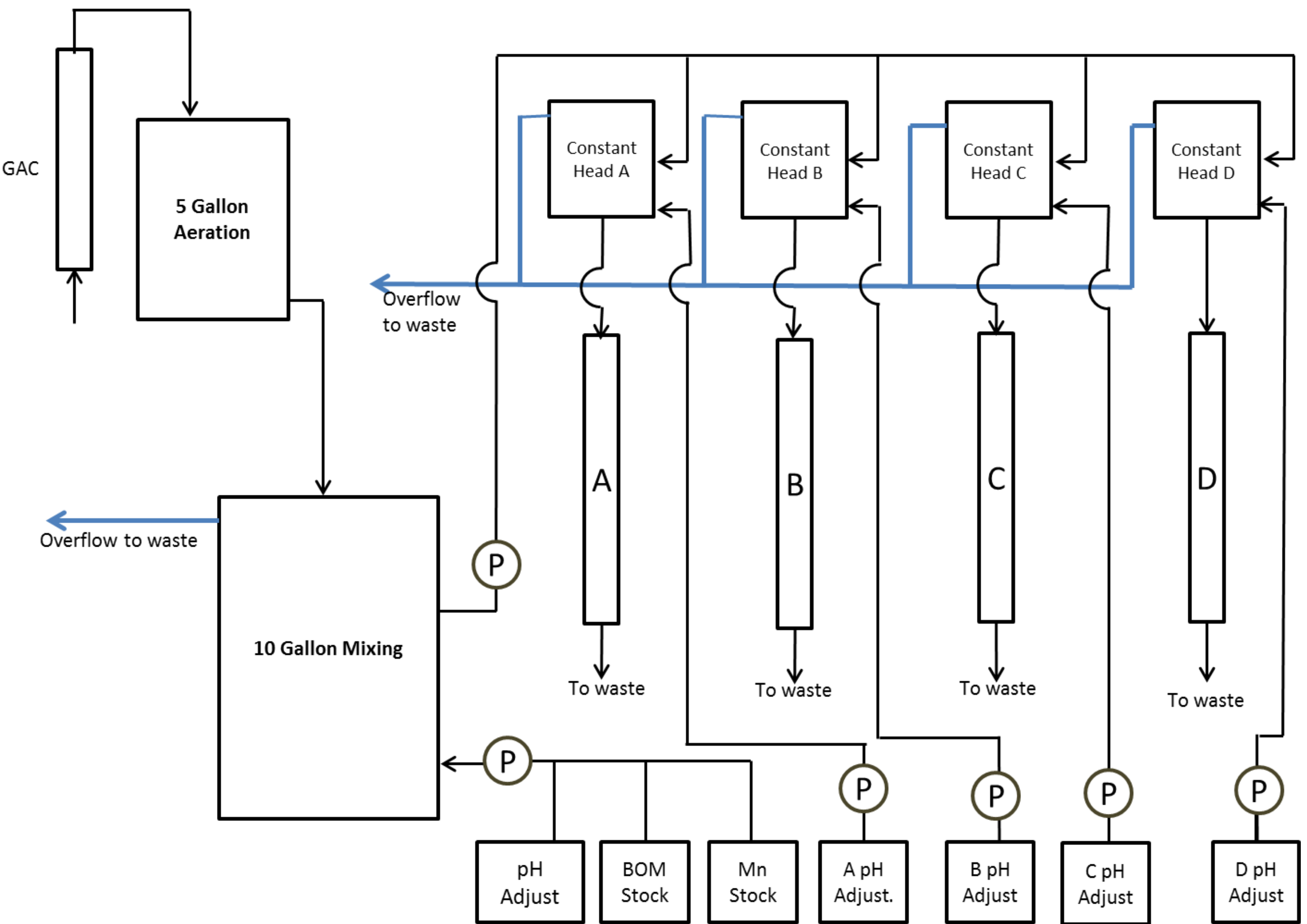

Figure 2-4. Lab-scale feed water Phase 1 schematic

adjusting the flow rates was not possible because the pump heads for the three components were attached to the same pump. There was some degree of flow variation among pump heads, so the concentrations in the 10 L containers were adjusted as needed to fine tune target influent concentrations. The chemical components used in these stock containers are discussed in subsequent sections.

Feed water from the ten-gallon mixing vessel was then pumped into four two-gallon constant head buckets, one for each of the four columns. Each constant head bucket was outfitted with an overflow port and a tube at the base of the bucket, to which a hose clamp was attached to control flow. An additional pH adjustment was added to the two-gallon containers via peristaltic pump operating at a flow rate of approximately 0.8 mL/min.

2.2.4.3 Mn and pH Adjustment

The columns were labeled A, B, C, and D. The target influent pH conditions are given in Table 2-4. Concentrated sulfuric acid diluted in distilled water was used for pH adjustment. As previously mentioned, the volume of acid added to the pH adjustment container for each column was adjusted as needed to maintain the target influent pH, since individually adjusting pump speed was not possible. The ten-gallon bucket target pH was 7.5. This initial decrease in pH made subsequent pH adjustment of the individual column influents easier as well as ensured that the pH was sufficiently low so as to eliminate any risk of physical-chemical Mn oxidation in the main mixing bucket.

Soluble Mn addition was achieved by adding manganous sulfate monohydrate ($MnSO_4 \cdot H_2O$) to 10 L distilled water. The concentration in the stock container was such that the final concentration in the ten-gallon mixing container was approximately 0.1 mg/L. The Mn concentration was increased 16 days after startup to 0.5 mg/L Mn in an attempt to select the MOB over other organisms present in the columns because Mn removal was not observed.

Table 2-4. Lab-scale biofilter influent pH targets for Phase 1

Column	Target pH
A	6.0 ± 0.1
B	6.3 ± 0.1
C	6.7 ± 0.1
D	7.0 ± 0.1

2.2.4.4 Biodegradable Organic Matter

The BOM added to the feed was intended to simulate post-ozonation by-products commonly found in water treatment plants utilizing ozone disinfection. The cocktail of formate, acetate, formaldehyde and glyoxal found in Elhadi *et al.* (2006) was slightly modified for use in this study by replacing acetate with oxalate. This was done because oxalate is the most prevalent component of ozonation by-products (Singer and Reckhow 1999) and the available carboxylic

acid determination method was better able to quantify oxalate than acetate (see section 2.7.2). The concentrations of oxalate, formate, formaldehyde, and glyoxal were selected to achieve a target biodegradable organic carbon (BDOC) concentration of 0.27 mg/L C, similar to the 0.28 mg/L C in Elhadi *et al.* (2006), while also following the percentage of post-ozonation by-products each component typically represents as found in Singer and Reckhow (1999). The target concentrations were 500 µg/L oxalate, 400 µg/L formate, 50 µg/L formaldehyde and 25 µg/L glyoxal. A target carbon/nitrogen/phosphorus ratio was maintained at 15:5:1 (C:N:P) to ensure that carbon was the limiting nutrient as described in Elhadi et al (2006). The nitrogen and phosphate levels in the influent tap water were sufficient without supplementation.

Carbon compounds were added to 10 L Nanopure water in the form of sodium formate, sodium oxalate, 40% glyoxal in water and 37% formaldehyde in water with preservatives. The BOM container was a 10 L glass carboy covered with foil to prevent organic compound degradation. The container was disinfected with chlorine in between refills and thoroughly rinsed with Nanopure before refilling. The degradation of the compounds between refills was determined to be negligible. On day 79 of the column startup, it was discovered that the formaldehyde addition was approximately 1/3 of the target value. The amount of formaldehyde added to the stock BOM container was increased over the course of three weeks to achieve the 50 µg/L target.

2.2.5 Startup Phase 2

No Mn removal was observed in the first 30 days of Phase 1 startup. When the media from the columns was plated on Mn-oxidation agar (see section 2.7.3), the biological community was primarily comprised of pseudomonads, the majority of which appeared very similar to MB-33. No MOB bacillus colonies were identified. Several issues with the Phase 1 hydraulic setup were also observed. The BOM was being removed by opportunistic microorganisms in the ten-gallon bucket and influent tubing lines, such that none of the added BOM cocktail was making it to the anthracite coal media in the columns. Additionally, the GAC and resulting microbial population within the GAC column were removing the chloramines and converting the ammonia to nitrite. A Phase 2 startup was implemented as outlined in the following subsections to rectify these issues and possibly stimulate Mn removal. Figure 2-5 shows the feed water setup for the Phase 2 startup.

2.2.5.1 Breakpoint Chlorination and GAC

A breakpoint chlorination system was added to address the ammonia issue. The influent tap water was rerouted into a 30-gallon bucket at a target flow of 750 mL/min. The flow was targeted higher than the required 400 mL/min needed for the column influent to account for occasional decreases in water pressure which would cause the flow rate out of the tap to decrease. The 30-gallon container was equipped with an overflow port and contained a submersible pump mixer and water heater that maintained the water temperature above 20°C.

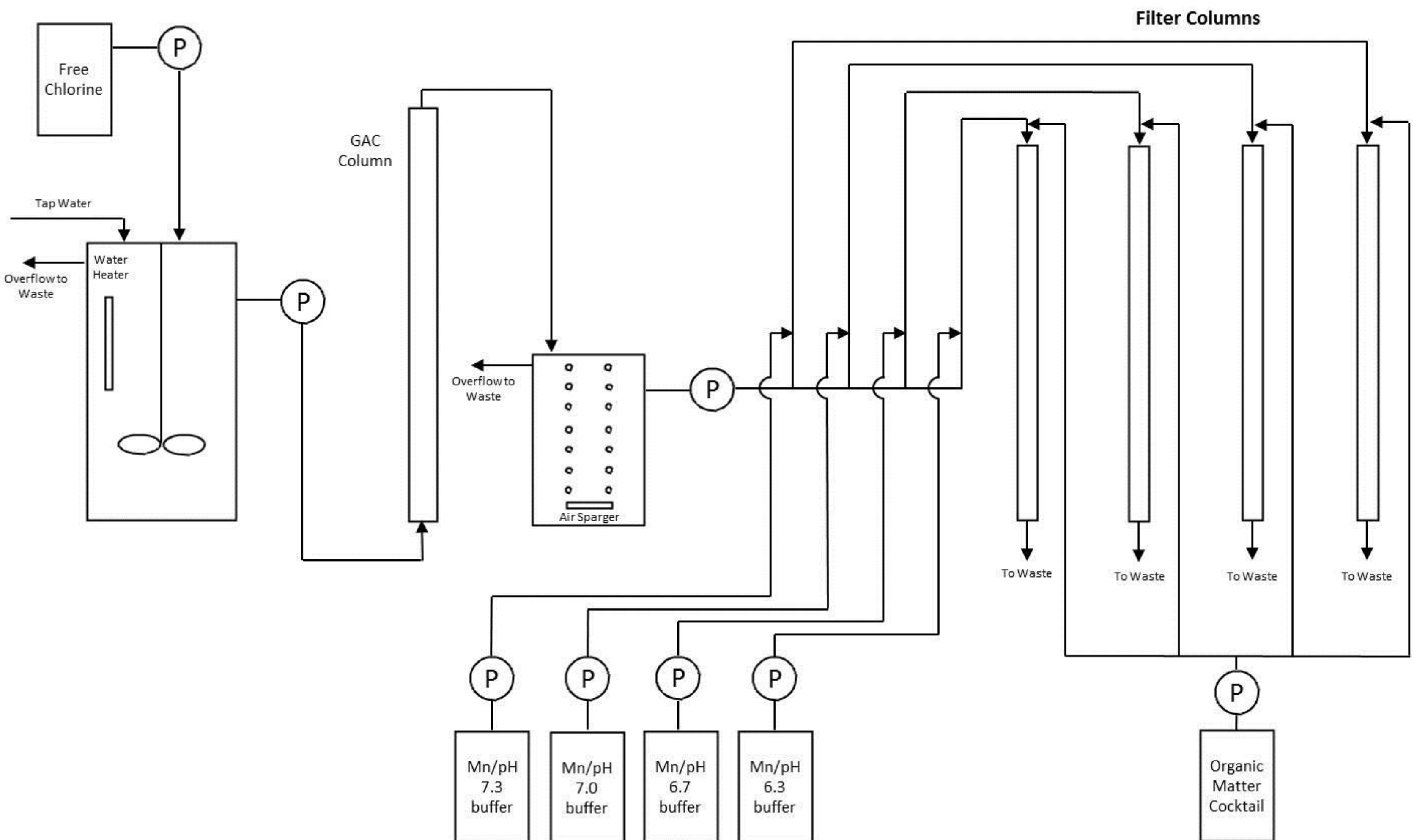

Figure 2-5. Lab-scale feed water Phase 2 schematic

A batch study was conducted to determine how much free chlorine needed to be supplemented to reach breakpoint. Increasing concentrations of free chlorine were added to 250 mL of tap water. The free chlorine was measured after a reaction time of 30 minutes. Figure 2-6 shows the results of this study. The initial target free chlorine concentration in the 30-gallon container was selected to be 3 mg/L as Cl_2, which corresponded to 7.5 mg/L as Cl_2 added free chlorine. A 10 L foil-covered glass container was filled with a solution of 10% bleach in distilled water, which was added to the 30-gallon container via a peristaltic pump at a flow rate of less than 1 mL/min. The flow rate of the tap water into the 30-gallon bucket varied to some degree with water pressure, and the concentration of free chlorine in the 10 L container decreased over time. The concentration of free chlorine in the 30-gallon bucket was measured daily, and the flow rate of the peristaltic pump was adjusted as needed to maintain the target free chlorine concentration.

Water was pumped via peristaltic pump from the 30-gallon bucket upflow through the GAC column to remove residual free chlorine. The free chlorine concentration in the 30-gallon bucket was decreased 2.5 weeks after the startup of Phase 2 to maximize the useful life of the GAC column. The ammonia concentration in the 30-gallon bucket was measured each day the free chlorine concentration was decreased, until a new optimum concentration of 1.5 mg/L as Cl_2 was determined to be the lowest free chlorine concentration needed to remove all ammonia. The ammonia, nitrite, and nitrate concentrations in the GAC effluent were measured before and after the addition of the breakpoint chlorination system, as shown in Figure 2-7. Note that the ammonia concentration was below the detection limit both before and after breakpoint chlorination. Before breakpoint chlorination was added, the ammonia was being oxidized to nitrite; after the installation of the breakpoint chlorination, the nitrite levels decreased substantially. Total and free chlorine in the GAC effluent was measured daily to identify if and when breakthrough of chlorine in some form had occurred.

2.2.5.2 Hydraulic Setup

The addition of the BOM cocktail into the ten-gallon mixing bucket was causing all of the added BOM to be consumed by opportunistic microorganisms before the feed water entered the columns. The hydraulic setup was substantially altered as seen in Figure 2-5 to remedy this. BOM was added directly before entry into the columns via peristaltic pump at approximately 0.8 mL/min. All columns drew out of the same BOM container. Additional changes to the feed water system were made to minimize maintenance issues. The two-gallon buckets were removed and replaced with a pump system as seen in Figure 2-5. The ten-gallon mixing container was converted into an air sparging vessel, from which water was pumped directly into the columns. Mn addition was combined with pH adjustment in 10 L glass carboys containing $MnSO_4 \cdot H_2O$ and sulfuric acid in distilled water in such concentrations to achieve the target influent pH and Mn concentration for each column. The influent Mn concentration for all four columns was maintained at 0.5 mg/L until significant Mn removal was observed, at which point it was decreased to 0.1 mg/L. The influent pH to each of the columns was increased as shown in Table 2-5

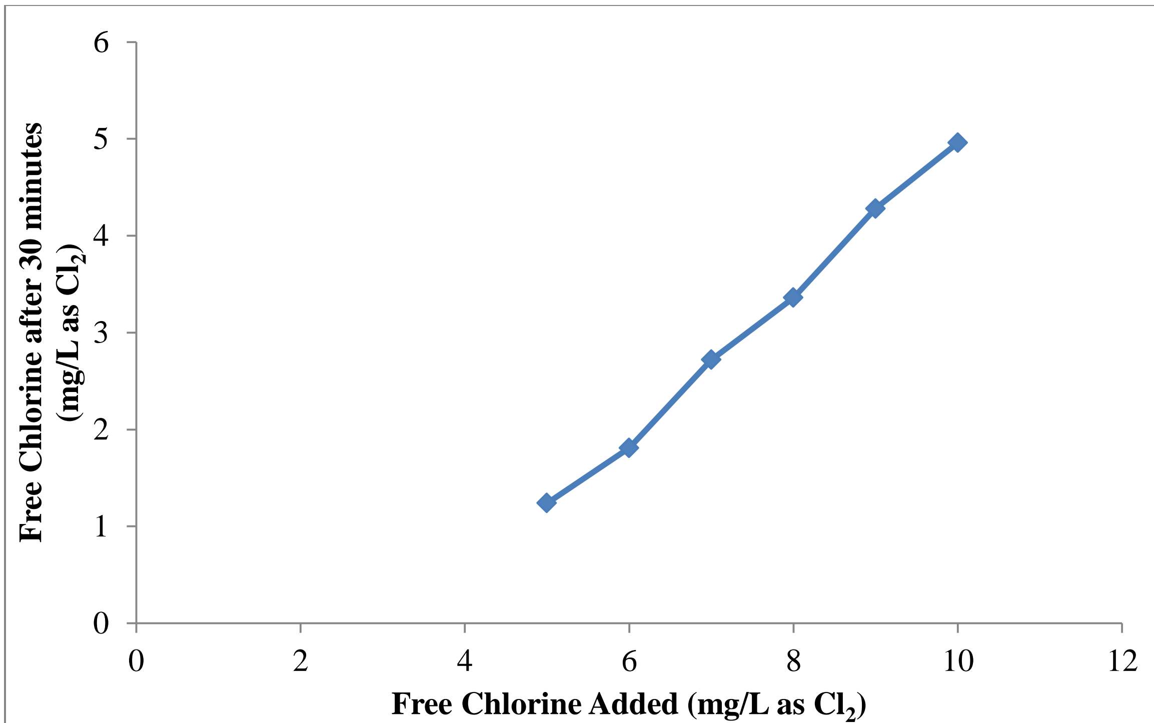

Figure 2-6. Breakpoint chlorination for Blacksburg tap water

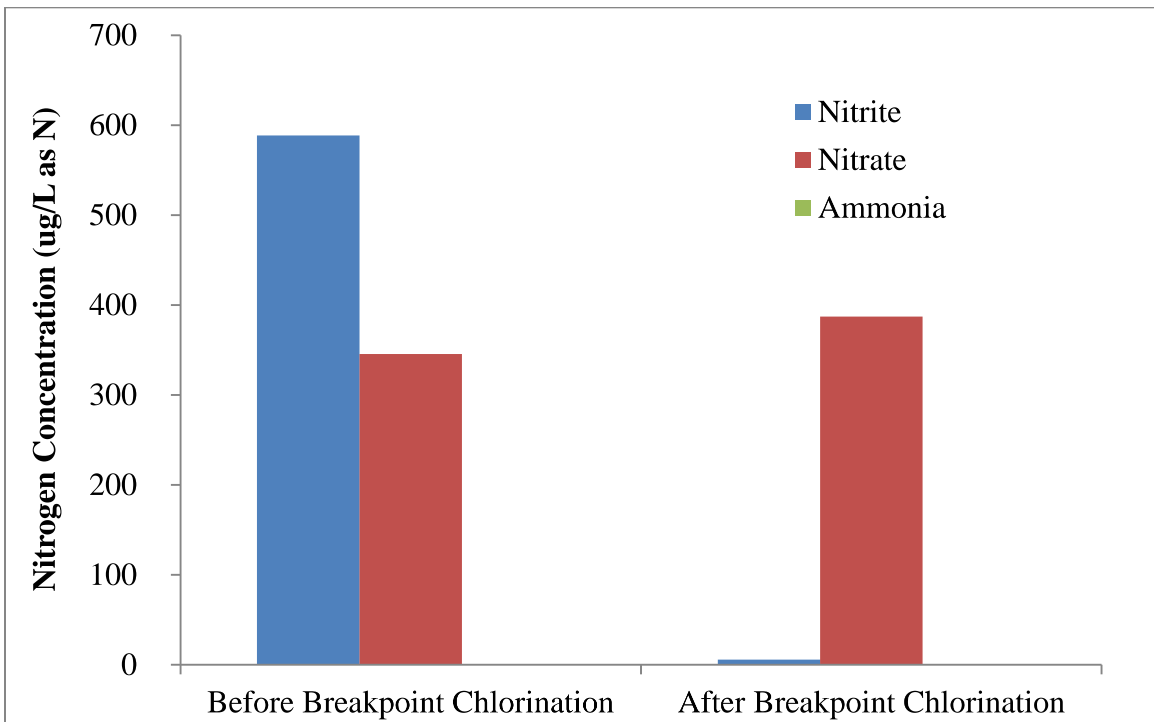

Figure 2-7. Nitrogen distribution in GAC effluent before and after breakpoint chlorination

Table 2-5. Change in lab-scale biofilter influent pH targets from Phase 1 to Phase 2

Column	Phase 1 Target pH	Phase 2 Target pH
A	6.0 ± 0.1	6.3 ± 0.1
B	6.3 ± 0.1	6.7 ± 0.1
C	6.7 ± 0.1	7.0 ± 0.1
D	7.0 ± 0.1	7.3 ± 0.1

2.2.5.3 Inoculation

Only the *Pseudomonas* isolate, MB-33 was observed when the column media biofilm was plated on Mn-oxidation agar. The columns were reinoculated as part of Phase 2 startup with the five other isolates: MB-2, MB-3, MB-17, MB-22, and MB-38. A 2 L baffled Erlenmeyer flask was filled with 1 L pH 7.0 Mn-oxidation broth, and 5 mL of each of the remaining 100 mL stocks for Group 2 and Group 3 were added. The flask was incubated at 30°C until turbid (approximately 20 hours) on a shake table at 120 rpm. The liquid was then centrifuged at 5000 x *g* for 30 minutes and the cells were re-suspended in 0.5 L feed water (see section 2.2.4) and acclimated at room temperature for one day. The columns were backwashed and then the MOB cell suspension was added to the columns by injecting 25 mL into each of the sampling ports down the column length (see Figure 2-3). The influent to the columns was stopped and a recirculation system was set up in which the effluent from each of the columns was captured in a five-gallon bucket and recirculated into the influent of the columns. BOM was added into the influent at normal concentrations, but the Mn concentration was increased to 2.5 mg/L. Hope and Bott (2004) determined that 2.5 mg/L Mn in a recirculation system may decrease MOB acclimation time. The columns were filled with this feed water and allowed to sit undisturbed for four days. Each day the liquid in the column was displaced with fresh feed water, and the effluent was captured. The columns were started on continuous-flow recirculation for an additional four days, after which normal influent operation resumed at a concentration of 0.5 mg/L Mn. BOM was added continuously to the recirculation flow as described in section 2.2.5.2 to replace the BOM that was consumed by the media biofilm. The recirculation flow and BOM addition were turned off overnight to prevent the buildup of some of the less biodegradable carbon compounds.

2.2.6 Sampling

Samples were collected using 30 mL syringes – glass for BOM samples, plastic for Mn samples – with stainless steel needle tips. Influent samples were collected from the top port above the media depth, and effluent samples were collected from the bottom sampling port in the gravel layer. Samples for Mn analysis were collected in 10 mL volumes, acidified to 2% nitric acid, and stored in 16 mL polystyrene tubes with snap caps. Particulate Mn in samples became an issue (especially during profile sampling), so Mn samples were filtered through 0.45 µm filters prior to acidification starting Day 93. Carbon samples were collected and analyzed the same day as described in section 2.7.2.

Media samples were collected by fluidizing the bed through backwashing to provide a representative sample and then opening the influent sample port and collecting the media and water mixture into a sterile bottle. The media was then transferred into sterile 2 mL vials for analysis and storage, and the remaining water/media mixture was returned to the column. Media samples for DGGE analysis were stored at -70°C.

2.2.7 Backwashing and Routine Maintenance

Backwashing of the media in each filter was performed on a weekly basis by connecting a peristaltic pump to the effluent tube of a column and pumping dechlorinated tap water upflow at approximately 25 gpm/ft^2, which corresponded to a 20% bed expansion. Air in the effluent tube provided an initial air scour before the backwash water reached the column media. The initial backwash time was five minutes; the time was increased to seven minutes starting Day 93 due to excessive particle buildup from biological growth.

In addition to backwashing, the tubing in the feed water system was periodically cleaned with a bleach rinse to remove unwanted biofilm. After the chlorine rinse, the tubing was rinsed with dechlorinated tap water until the free chlorine concentration in the tubing was less than 0.02 mg/L as Cl_2. The GAC column effluent tube was also replaced during chlorine rinses. Likewise, the column effluent and manometer tubes were replaced periodically when biofilm growth became apparent. The sampling port septa were replaced as needed when they lost their self-healing capabilities. Pump tubing was checked every two weeks for excessive wear and was replaced as needed.

2.3 STRESS EXPERIMENTS

Several stress studies were conducted to simulate the varying influent conditions present at surface water treatment plants, during which Mn removal performance of the columns was monitored. The effects of influent Mn concentration, hydraulic loading rate, temperature, and influent BOM concentration on Mn removal were evaluated as described in the following subsections.

2.3.1 Mn Concentration

Influent Mn concentration was doubled from 0.1 mg/L to 0.2 mg/L Mn in Columns A (pH 6.3) and C (pH 7.0) for a period of approximately 24 hours. The Mn concentration in the Mn/pH adjustment stock solution was incrementally increased over the course of two hours and maintained at the higher concentration. At the end of the 24 hour time period, the Mn concentration in the Mn/pH reservoir was incrementally decreased over the course of two hours to the original concentration. Influent and effluent Mn samples were collected at frequent intervals during and immediately following the transitions, and periodically throughout the experiment. Profile Mn samples were also collected from the ports along the depth of the column periodically during the study.

2.3.2 Hydraulic Loading Rate

Influent hydraulic loading rate was doubled from 2 gpm/ft^2 to 4 gpm/ft^2 in Column C (pH 7.0) for a period of approximately 24 hours. Batched feed water was made to maintain a consistent chemical composition while increasing the hydraulic loading rate. The batch feed water consisted of Blacksburg tap water (pH adjusted to 7.0) with $MnSO_4 \cdot H_2O$ added to achieve 0.1 mg/L Mn and BOM added to achieve the normal concentrations (see section 2.2.4.4). A peristaltic pump delivered the batch feed water to the column starting at 2 gpm/ft^2, and the flow rate was incrementally increased to 4 gpm/ft^2 over the course of two hours. The influent was then pumped at the higher rate using the traditional setup (see section 2.2.5.2), with doubled concentrations of Mn/pH adjustment and BOM in the respective stock containers. At the end of 24 hours, the setup was again placed on batched feed water and the hydraulic loading rate was decreased over two hours. Influent and effluent Mn samples were collected at frequent intervals during and immediately following the transitions, and periodically throughout the experiment. Mn profile samples were also collected periodically during the study.

2.3.3 Biodegradable Organic Matter

Influent BOM concentration was doubled from approximately 0.3 µg/L to 0.6 µg/L C in Column A (pH 6.3) for a period of approximately 24 hours. The BOM was increased by replacing the BOM stock solution for Column A with one that had twice as much of the four carbon compounds: oxalate, formate, formaldehyde, and glyoxal. There was no transition time as in the other stress studies. Only profile Mn samples were taken throughout and after the experiment. Frequent effluent Mn samples were deemed unnecessary as effluent Mn was not expected to be impacted. Column A was returned to normal BOM influent conditions by replacing the increased BOM stock solution with the original concentration. As before, there was no transition time.

2.3.4 Temperature Decrease

The influent water temperature of Columns A, B, and C was decreased to simulate low temperature conditions often found at surface water treatment plants. Column D was excluded from the study because it was undergoing a separate study as outlined in section 2.4.3. The feed water setup was altered slightly in that a five-gallon bucket equipped with an overflow port was added prior to the ten-gallon bucket in the setup shown in Figure 2-5. The dechloraminated tap water flowed from the GAC column into the five-gallon bucket, where the water was aerated with an air stone. Column D influent was pumped from this bucket at room temperature. Water was pumped at approximately 300 mL/min from the five-gallon bucket into the ten-gallon bucket, from which the influents for Columns A, B, and C were pumped. A chiller was used to cool the water in the ten-gallon bucket by circulating chilled antifreeze through a copper coil that was submerged in the bucket. The temperature of the water in the ten-gallon bucket was measured daily. Samples for Mn analysis were also collected daily. The columns were backwashed as described in section 2.2.7 with the chilled dechloraminated tap water when head

accumulation reached approximately one foot. The temperature was decreased approximately 0.5°C per day for 25 days.

The chiller was able to lower the ten-gallon temperature to approximately 11°C, at which point the chiller reached its cooling capacity. The flow through the ten-gallon bucket was decreased by removing Columns A and B from the study and the ten-gallon bucket was replaced with a cooler to further reduce the temperature. When the temperature in the cooler reached 7°C, it became clear that the temperature in the cooler and the temperature of the influent water at the column were at least 2-3°C different, and so the influent tubing connecting the cooler to the pump and the top of Column C was wrapped in pipe insulation to further reduce the influent temperature. When the influent temperature reached 7°C, the chiller again reached its cooling capacity, at which point the tubing insulation was removed and the influent temperature was raised over the course of 20 days. Mn removal across Column C was monitored for an additional 30 days after the column reached room temperature (22°C).

Once the discrepancy between the cooler temperature and influent temperature was discovered, both influent and cooler temperatures were measured daily. The temperatures after the insulation was removed were correlated as shown in Figure 2-8 and used to estimate the influent temperatures based on measured cooler temperatures prior to the addition of tubing insulation.

2.4 Studies Evaluating the Cause of Limited Mn Removal in Column D

Column D (pH 7.3) was unable to appreciably remove Mn for the first 230 days of the experiment, unlike the columns with lower influent pH. Several experiments were conducted to determine why Column D was not removing Mn. First a batch study was designed to determine whether there was direct pH inhibition. Inhibition plating was conducted to determine if there were any other microorganisms present in Column D that were inhibiting the growth and Mn oxidation of the MOB. Finally, the influent pH of Column D was lowered to see if Mn removal would be initiated.

2.4.1 Inhibition Batch Studies

Four flasks were filled with 250 mL feed water containing the normal BOM concentrations and 0.1 mg/L Mn. Two flasks were pH adjusted to pH 7.3, and two were adjusted to pH 6.5. MOB cell suspensions from Column B and D media (see section 2.7.3) were added to one flask at each pH in 100 µL volumes. The flasks were gently stirred with magnetic stir bars, and Mn samples were collected and filtered through 0.45 µm filters.

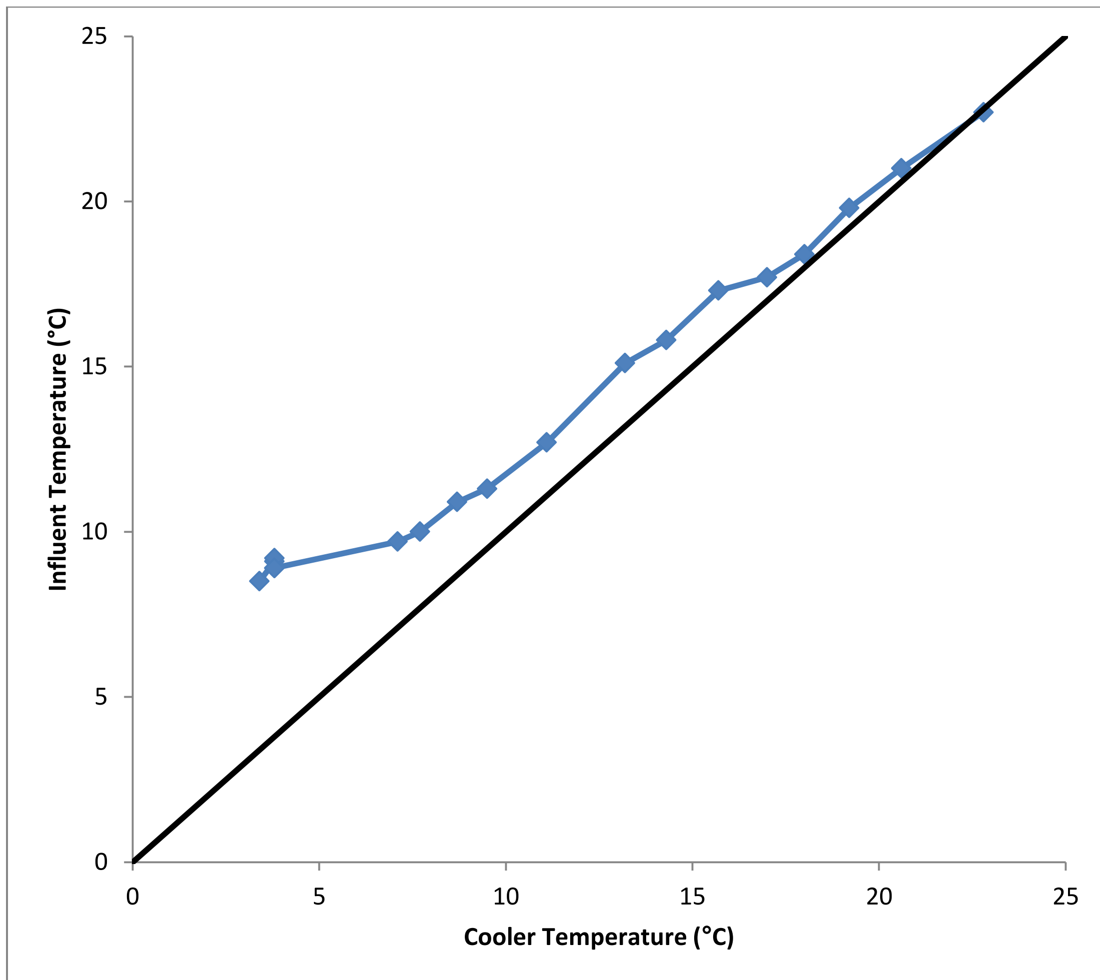

Figure 2-8. Temperature correlation between cooler and Column C influent. Black line marks 1:1 ratio.

2.4.2 Inhibition Plating

A modified standard inhibition method adapted from Bailey and Scott (1974) was used to determine if other microorganisms present in the Column D biofilm were inhibiting Mn oxidation by MOB. First, 100 µL of a 10^4 dilution of Column D cell suspension was spread onto Mn-oxidation agar media and incubated at 30°C for one week. Colonies with different morphology were selected and inoculated in tubes containing 5 mL Mn-oxidation broth. The tubes were then incubated at 30°C at 120 rpm until turbid (approximately three days). Inhibition was determined by spreading 100 µL of a turbid culture of the five reinoculated MOB strains onto Mn-oxidation agar media and spotting 10 µL of the turbid cell suspensions of the colonies obtained from Column D media. The plates were incubated at 30°C and observed daily for development of a zone of inhibition, usually indicated by decreased Mn oxidation or growth of MOB around the location of the Column D biofilm colonies.

2.4.3 Influent pH and BOM Changes

Because none of the above inhibition studies provided insight into the cause of the lack of Mn removal in Column D, the influent pH was decreased to pH 6.7 starting Day 233. The pH was maintained at 6.7 for approximately 60 days, when it was again increased to pH 7.3. Influent and effluent Mn samples were collected throughout the course of the experiment. pH was measured daily and any necessary adjustments to the pH/Mn stock were made. Column D was also used to further evaluate the effects of BOM on biological Mn removal. On Day 408, the BOM was removed from Column D. On Day 433, the BOM was resumed at five times the normal concentration. Influent and effluent samples were collected daily for Mn analysis throughout these changes.

2.5 Batch Studies

Batch studies were intended to provide information about optimum parameters for reducing acclimation time; however, repeated trials indicated that this may not be a predictive, useful method. Baffled Erlenmeyer flasks (1 L) were filled with 400 mL dechlorinated tap water (pH adjusted to either 6.3 or 6.7) and 100 mL anthracite coal media. The various trials included adding 1 mg/L or 1.5 mg/L Mn before or after autoclaving, adding 3 meq/L alkalinity or adding no alkalinity, adding the media before or after autoclaving, and using three different types of media: Lee Hall biofilter media, unused media from Blacksburg, Christiansburg, VPI Water Authority, and unused media from another source. Filter-sterilized BOM was always added to achieve normal feed water concentrations after autoclaving to prevent volatilization of the added organic matter. MOB were added in varying consortiums: the five reinoculated isolates, all six original isolates, isolate MB-3 or MOB from the biofilter column media. After adding water, media, Mn, BOM, and MOB, Mn samples were collected and filtered through 0.45 µm. The flasks were then placed on shake tables at 30°C at either 120 rpm or 60 rpm, depending on the trial. Mn samples were collected and BOM was added daily. In all trials, various issues arose, most notably decreases in solution pH and observed Mn removal in control flasks without MOB

being present. While it may have been possible to eventually determine an optimum procedure for using batch studies, laboratory-scale column experiments were developed and the batch studies were abandoned.

2.6 Pilot-Scale Biofilter Study

The pilot-scale biofilter setup at Lee Hall water treatment plant (Figure 2-9) was utilized to determine the feasibility of eventual implementation of MOB for Mn control in full-scale water treatment plants. The pilot-scale columns mirrored the design of the full-scale biofilters.

2.6.1 Pilot-Scale Setup

The setup consisted of four 4 in. ID plastic columns filled with 36 in. anthracite coal media (effective size 0.95-1.05 mm; uniformity coefficient <1.4), 12 in. silica sand (effective size 0.45-0.55 mm; uniformity coefficient <1.4), and 12 in. support gravel (Figure 2-10). Sampling ports were present at media depths of 4, 12, 18, 33, 39, and 49 inches below the media surface. The sampling ports at 33 and 49 inches below the media surface were converted to manometers to observe effects on head loss down the media depth. The media in the columns had been used in previous pilot-scale studies and was not replaced for this experiment; however, the pilot setup had not been in use for eight months prior to startup for this study. The media was dry and a biofilm had not been sustained during the eight months of non-use.

The pilot plant feed water was pumped from directly after the ozone contact basins in the full-scale plant (Figure 2-11) to each of the columns at a loading rate of 3.4 gpm/ft^2. Table 2-6 shows the average influent water quality characteristics for the pilot plant, and Figure 2-10 shows the feed water system. The columns were operated for approximately seven weeks prior to MOB inoculation to allow for the establishment of a biofilm similar to the full-scale biofilters. Three weeks prior to MOB inoculation, the hydraulic loading rate was decreased to approximately 1.15 gpm/ft^2 to promote attachment of MOB to the biofilm during acclimation. Pilot-scale Filter 1 effluent was collected for chemical batching and backwashing (see section 2.6.4). One week after reducing the flow rate, soluble Mn was supplemented into the main feed line going to all four columns to achieve a target of 0.1 mg/L soluble Mn. Mn feed stock was batched in 50 gallon volumes by adding manganese chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$) to Filter 1 effluent.

Table 2-6. Representative pilot plant influent characteristics

Parameter	Average	Range
pH	6.4	6.0 – 6.8
Total Mn (before Mn addition)	0.036 mg/L	0.021 – 0.052 mg/L
Total Mn (after Mn addition)	0.119 mg/L	0.081 – 0.166 mg/L
Soluble Mn (after Mn addition)	0.102 mg/L	0.062 – 0.143 mg/L
Dissolved Organic Carbon (DOC)	3.18 mg/L	2.54 – 3.66 mg/L

Figure 2-9. Pilot-scale biofilter columns

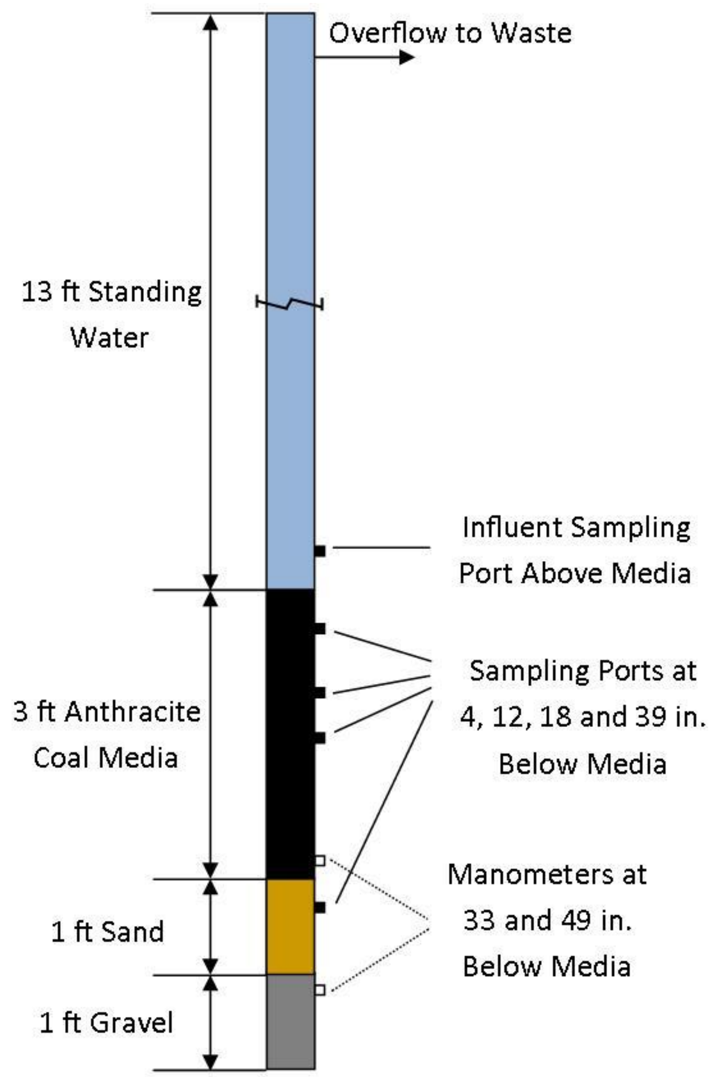

Figure 2-10. Pilot-scale biofilter column schematic

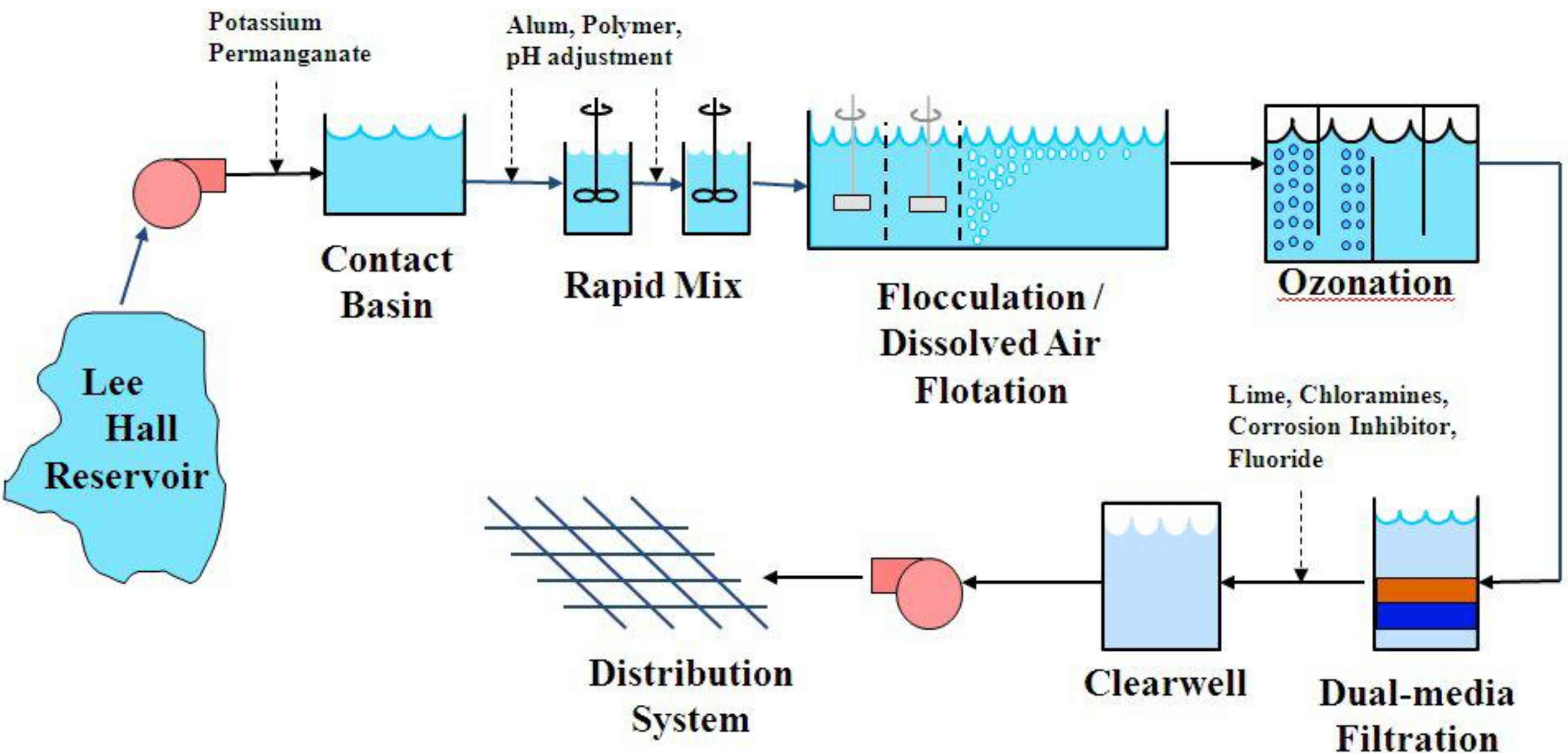

Figure 2-11. Lee Hall water treatment plant full-scale schematic (used with permission of Newport News Waterworks)

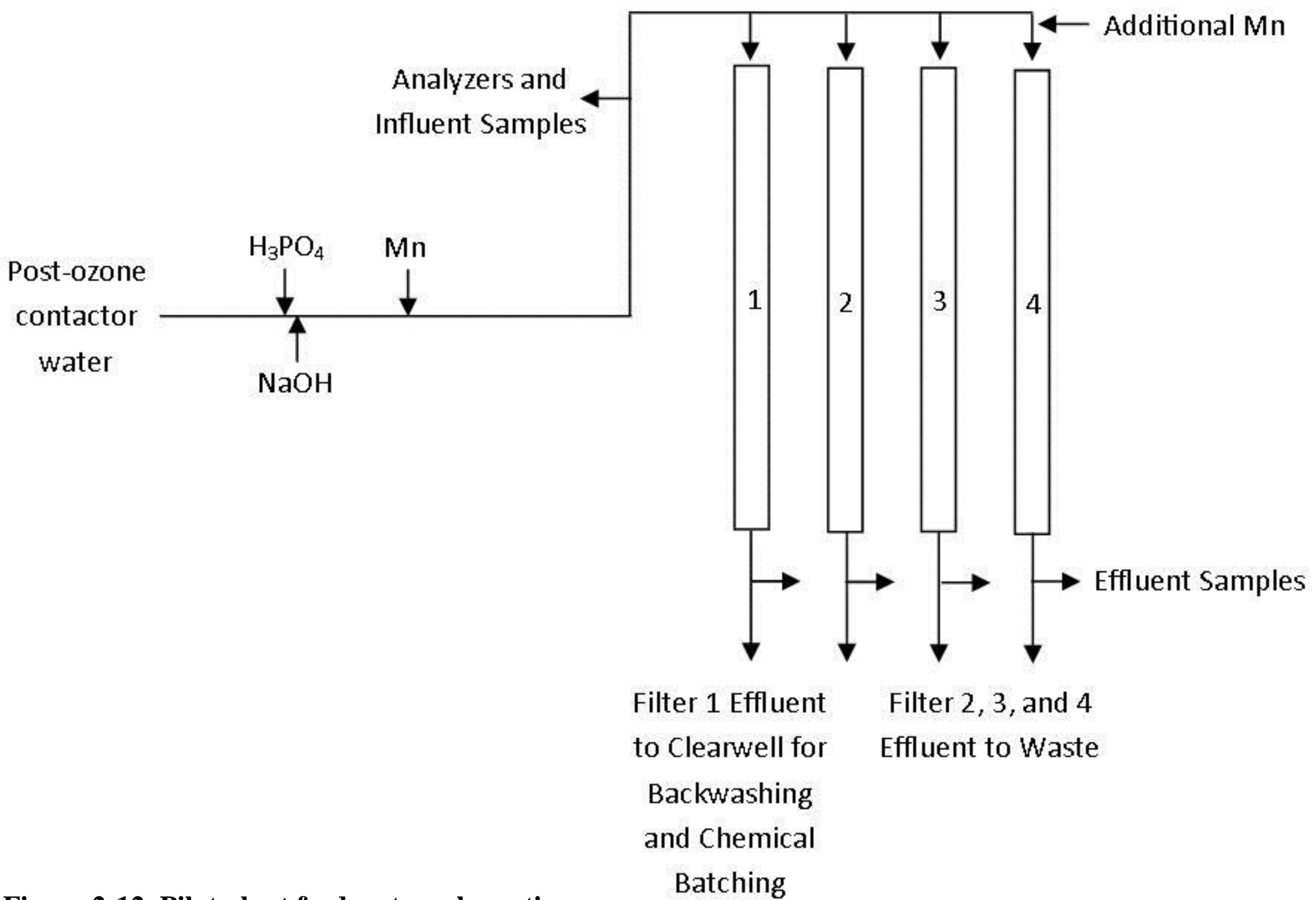

Figure 2-12. Pilot plant feed water schematic

2.6.2 MOB Inoculation

Dissolved organic carbon (DOC) removal across the filters was monitored to provide an indicator of biofilm activity during the initial pilot study period. The pilot-scale columns were inoculated with MOB when DOC removal was similar to that of the full-scale filters. Media from lab-scale Columns A (pH 6.3) and B (pH 6.7) were removed from the columns and stored in 1 L plastic bottles filled with lab-scale column influent water for transport to Newport News, VA. The media from Column A was added to the top of pilot-scale Filter 3, and the media from Column B added to the top of pilot-scale Filter 4. This media added approximately two inches to the total depth of anthracite coal media in Filters 3 and 4. The filters were allowed to sit undisturbed for one hour, at which point the flow to the filters was resumed at 1.15 gpm/ft^2.

2.6.3 Feed Water Additions

Mn removal in the MOB-inoculated columns began to decrease 22 days after MOB inoculation. Various changes to the influent feed water were implemented in an attempt to prevent further decreases in Mn removal. The pH in the full-scale plant decreased from an average of 6.5 to an average of 6.3 starting Day 38. Sodium hydroxide (NaOH) was added to the influent feed water to increase the pH back to 6.5 beginning on Day 50. The Mn feed to Filter 4 was increased on Day 51 from approximately 0.1 mg/L to 0.5 mg/L soluble Mn in an attempt to stimulate increased Mn removal. Phosphorus was added on Day 71 to the influent of all four pilot-scale columns in the form of phosphoric acid (H_3PO_4) at a concentration of 0.05 mg/L as P in a further attempt to increase Mn removal. All chemicals were batched using pilot-scale Filter 1 effluent in 50-gallon volumes. The hydraulic loading rate was increased from 1.15 gpm/ft^2 to 2 gpm/ft^2 on Day 105. The pilot study was divided into phases for analysis purposes based on these changes to feed water addition. The influent Mn increase in Filter 4 divides Phases 1 and 2. Phase 3 starts with the addition of phosphorus, and Phase 4 begins when the hydraulic loading rate was increased.

2.6.4 Sampling and Maintenance of Pilot-Scale Columns

Profile samples as a function of media depth were collected for Mn analysis from Filters 3 and 4 prior to MOB inoculation and twice a week following inoculation. Influent and effluent samples were collected daily from all four filters. Filtered (0.45 μm) and unfiltered samples from the influent and sample port 4 in. below the media depth were collected because significant particulate Mn was present at these two sampling points (most likely due to partial Mn oxidation occurring in the full-scale ozonation unit). Mn concentration was measured onsite using method 8149 on a Hach DR4000U spectrophotometer to ensure that Mn dosing was correct and that the filters did not require immediate attention (indicated by sharp increases in effluent Mn concentration). Samples were shipped on a weekly basis to Virginia Tech for preservation and ICP-MS analysis (see section 2.7.1). Turbidity and pH were monitored by an online Hach 1720D and bench top Thermo Orion 710A+ respectively. DOC samples were collected twice a week

and analyzed at Newport News Waterworks using a Shimadzu Total Organic Carbon Analyzer (model TOC-V cpn) following Standard Method 5310B (Clesceri *et al.* 1998).

The pilot-scale columns were operated as constant head filters with flow controlled at the effluent. The water level was maintained at 13 feet above the media level. The columns were backwashed when any one column reached head loss of 10-12 ft. Filter run times averaged between 70-90 hours. The pilot plant backwash procedure mimicked that of the full-scale plant and consisted of air scour for six minutes (4.5 scfm/ft^2), one minute of air scour (4.5 scfm/ft^2) with low water flow (4.0 gpm/ft^2), and seven minutes of high water flow (19 gpm/ft^2). Backwash water was pilot-scale Filter 1 effluent. The filters were operated at a lower hydraulic loading rate of 0.3 gpm/ft^2 Days 59 through 70 to allow time off for the pilot plant operator.

2.6.5 Statistical Analysis Methods

The Mn removal results were analyzed using JMP statistical software package. Ordinary least squares regressions were used to determine statistically significant factors that may be impacting Mn removal throughout the various pilot study phases.

2.7 Analytical Methods

The following analytical methods were used to measure various parameters in the water samples collected during the lab-scale and pilot-scale studies. Biological methods to determine the presence of Mn on the biofilter media were also used as outlined in the following subsections.

2.7.1 Manganese

Mn samples were preserved in 2% trace metal grade nitric acid. Samples above 0.05 mg/L Mn were measured using flame atomic absorption spectroscopy (Fl-AAS). Samples below 0.05 mg/L Mn were measured using inductively coupled plasma mass spectroscopy (ICP-MS) following Standard Method 3125b (Clesceri *et al.* 1998). The method detection limit (MDL) was 0.017 µg/L for ICP-MS and 13.4 µg/L for Fl-AAS. Phosphorus concentrations in the pilot study samples were also measured using ICP-MS to ensure that the dosing concentration was correct.

2.7.2 Biodegradable Organic Matter

Formaldehyde and glyoxal were measured following EPA Method 556 (Munch *et al.* 1998). Samples were derivatized immediately after sampling and were stored at 4°C. Samples were measured on a gas chromatograph with electron capture detection within two weeks of derivatization. The MDLs for formaldehyde and glyoxal were determined to be 0.64 and 4.5 µg/L respectively.

Formate and oxalate were measured using a modified version of the ion chromatography method outlined in Peldszus et al. (1996; 1998). An IonPac AS10 (4mm x 250mm) column was used with 1 mL sample loop. Utilizing an eluent gradient was not possible due to equipment limitations. An eluent concentration of 70 mM NaOH was determined to be the optimum

concentration to allow adequate separation between the fluoride and formate peaks while also minimizing total analysis time. Acetate was unable to be isolated from the fluoride peak; however, the oxalate peak was clearly defined. The total analysis time was 55 minutes. Nitrite, nitrate, and phosphate were also measured using this method to ensure that sufficient nitrogen and phosphorus were present in the lab-scale column influent. Initially, samples were analyzed immediately after sampling without preservation; however, it was observed on Day 184 that the oxalate was degrading in the samples as they were waiting on the autosampler to be measured. Starting Day 185, chloroform was added to the samples at 0.1% v/v to preserve the samples during analysis, as recommended in Peldszus et al. (1996; 1998). The MDLs for formate and oxalate were determined to be 36 and 6.2 μg/L, respectively. For all BOM components, half of the MDL was used for calculations involving concentrations below the detection limit.

2.7.3 Mn-oxidation Agar and Broth

Mn-oxidation broth was made using 10 mM HEPES buffer which was pH adjusted to pH 6.0, 6.5, or 7.4. The following was added per liter of pH-adjusted 10 mM HEPES solution: 0.001 g $FeSO_4 \cdot 7H_2O$, 0.15 g $MnSO_4 \cdot H_2O$, 2 g peptone, and 0.5 g yeast extract (Stein *et al.* 2001; Cerrato *et al.* 2010). Mn-oxidation agar plates were made by adding 15 g agar per liter solution. MOB isolates were stored for extended periods of time by inoculating Mn-oxidation broth with the desired strains, adding glycerol to 20% v/v, and storing the strains at -70°C.

Cell suspensions were collected from media samples by placing 0.5 mL media (which corresponded to approximately 0.35 g media) in a sterile 2 mL vial and filling the vial to the 1.5 mL line with the collection water, which corresponded to approximately 1.2 mL total water. The vials were then vortexed at the highest setting for one minute to shear the biofilm into suspension. Serial dilutions were made by adding a small volume of the cell suspension to sterile tap water. Plates were spread by placing 100 μL serial dilution suspension on either Mn-oxidation agar or R2A agar, depending on whether MOB or heterotrophic plate count was desired. The plates were incubated at 30°C. Spores were isolated by heating a cell suspension to 80°C in a water bath for ten minutes and then serially diluted and plating the suspension (Krieg 1981).

2.7.4 Repetitive-Sequence-Based Polymerase Chain Reaction (*rep*-PCR)

A repetitive-sequence-based polymerase chain reaction (*rep*-PCR) method was used to identify which of the five MOB isolates reinoculated during Phase 2 were present in the biofilm 200 days after reinoculation. The *rep*-PCR method required colonies to be isolated prior to DNA extraction. Cell suspensions from Columns A, B, C, and D media samples were spread onto Mn-oxidation agar as described in section 2.7.3. Spores were also isolated and spread-plated as described in section 2.7.3. The plates were incubated at 30°C. Colonies were picked and streaked for further isolation on Mn-oxidation agar. Selected colonies were either identified as MOB by the characteristic brown MnO_2 precipitate or had similar colony morphology as the MOB

isolates. DNA was extracted using a MO-BIO UltraClean® Microbial DNA Isolation Kit (Carlesbad, CA). Primers and temperature cycling followed that outlined in Cangelosi *et al.* (2004). Gels were made from low EEO agarose (Sigma, St. Louis, MO). Once solidified, gels were placed in 2x TAE buffer, and a 12 μL sample was placed in each well. A 1kb ladder was placed in the first well. Voltage across the gels was maintained at 70V until the orange indicator moved to the bottom of the gel, at which point the gel was stained in ethidium bromide solution for 30 minutes. Gels were then photographed using UV imaging. Gel images were analyzed with Quantity One 1-D software (BioRad). Comparisons between lanes within a gel were made based on shared number and migration of bands. Percent similarity was obtained by dividing the number of bands that were the same (±5%) by the total number of bands in the two lanes.

2.7.5 Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) was used to identify differences in biological community profiles and presence of the original MOB isolates in the lab-scale columns over time. The DGGE method followed the procedure outlined in Singh *et al.* (2012). Primers I-341f GC and I-533r were used to amplify the V3 variable region of bacterial 16S rRNA genes (Watanabe *et al.* 2001) and a 35% to 55% denaturant gradient was used. Table 2-7 and Table 2-8 present the amplification conditions and PCR reaction matrix. Band identification and intensity profiles were obtained using Quantity One 1-D analysis software (BioRad). Hierarchical clustering was based on Bray Curtis similarity of fourth-root transformed intensity of the bands. A p-value <0.05 was considered significant. Relative intensities of the bands compared to the intensity of the lane were used to estimate the relative abundances of bacteria represented by each band.

2.7.6 Other Parameters

pH was measured using an Accumet pH electrode probe and Oakton pH 110 series meter. Temperature was measured using a digital Fisher Scientific traceable thermometer. Free and total chlorine were measured using Hach Methods 8021 and 8167, respectively, on a DR 5000. Ammonia was measured using Hach Method 8155 on a DR 5000. Dissolved oxygen was measured using a YSI model 57 oxygen meter and probe.

Table 2-7. Primer sequences and annealing temperatures for PCR assay.

Primer[1]	Target Gene	Annealing Temp. (°C)	Primer (5'-3')
I-341 F GC[2]	V3 16S rRNA	47	CGCCCGCCGCGCGGCGGGC GGGGCGGGGGCACGGGGGGCC TACGGGAGGCAGCAG
I-533 R[2]	V3 16S rRNA	47	TIACCGIIICTICTGGCAC

[1] Integrated DNA Technologies Inc. (Coralville, IA)

[2] (Watanabe *et al.* 2001)

Table 2-8. PCR reaction matrix

Reagent	Amount per 25 µl master mix
10× PCR buffer[1]	2.5 µl
5× PCR buffer[1]	5 µl
dNTPs[2]	0.2 mM
Forward and reverse primer[3]	0.25 µM
Taq DNA polymerase[4]	1.75 U
Formamide[5]	0.25 µl
DNA template	1 µl
Mg^{2+}[1]	2 mM

[1] MasterTaq kit (Eppendorf, Westbury, NY)
[2] Promega Corporation (Madison, WI)
[3] Integrated DNA Technologies Inc. (Coralville, IA)
[4] TAQ DNA POL (5U/µl) (MP Biomedicals, Solon, OH)
[5] Formamide Mol Bio Grade (Fisher Scientific Company LLC, Suwanee, GA)

CHAPTER 3: RESULTS

3.1 Laboratory-Scale Biofilter Startup

Mn removal did not occur within the first 30 days following Phase 1 inoculation of the lab-scale biofilters, as seen in Figure 3-1. Startup Phase 2 was implemented to address hydraulic setup and inoculation issues (see section 2.2.5), and Mn removal was observed in Column A (pH 6.3) and B (pH 6.7) within one week of Phase 2 startup. Column C (pH 7.0) began removing Mn three weeks after Phase 2 startup. The Mn removal initiation cannot necessarily be attributed to Phase 2 startup because MOB are known to have extended acclimation times (Mouchet 1992). It is feasible that the MOB may have required 50 days from Phase 1 inoculation to become acclimated to the conditions present in the columns before beginning Mn removal, and the proximity of Mn removal initiation to Phase 2 startup may have been coincidence. The lab-scale column feed water did not contain particulate Mn, so Mn removal in the lab-scale columns only refers to soluble Mn removal. Biofilm shearing causing particulate Mn to be present in samples became an issue as the biofilm developed. Sheared biofilm would most likely be removed from the water in the sand layer common to most full-scale biofilters. Since a sand layer was not present in the lab-scale biofilters, samples were filtered through 0.45 μm.

After Phase 2 startup, essentially complete (>98%) Mn removal was reached within 52 days in Column B, 54 days in Column C, and 108 days in Column A. Column D (pH 7.3) did not remove an appreciable amount of Mn during the course of Phase 1 or Phase 2 startup. The lack of Mn removal in Column D warranted further study (see section 2.4) because Mn removal in groundwater systems has traditionally been thought to require alkaline pH (>7.4-7.5) (Mouchet 1992). The influent pH to Column D was closest to that of most groundwater systems that use MOB to effectively remove Mn. The results of these further studies can be found in section 3.3.

Profile water samples collected across the media depth demonstrated that most of the Mn removal occurred within the first four inches of the columns (Figure 3-2). Note that all profile figures are cumulative removal as a function of depth. Column B achieved essentially complete Mn removal within the first eight inches of media. Columns A and C achieved essentially complete Mn removal by a media depth of eighteen inches. Column D only achieved 16% Mn removal, and most of the removal (10%) occurred in the first four inches of media. This indicated that most of the Mn oxidation by MOB occurred at the top of the column, which is expected as this is the location of the greatest soluble Mn concentration.

All four columns were able to remove greater than 90% of the added BOM components, as shown in Figure 3-3. The exception was glyoxal, but glyoxal removal was in all cases greater than or just below 90%. The reason for glyoxal having lower removal than the other compounds can partly be attributed to its high method detection limit (MDL), relative to the influent concentration. In cases where the effluent concentrations were lower than the MDL, half of the

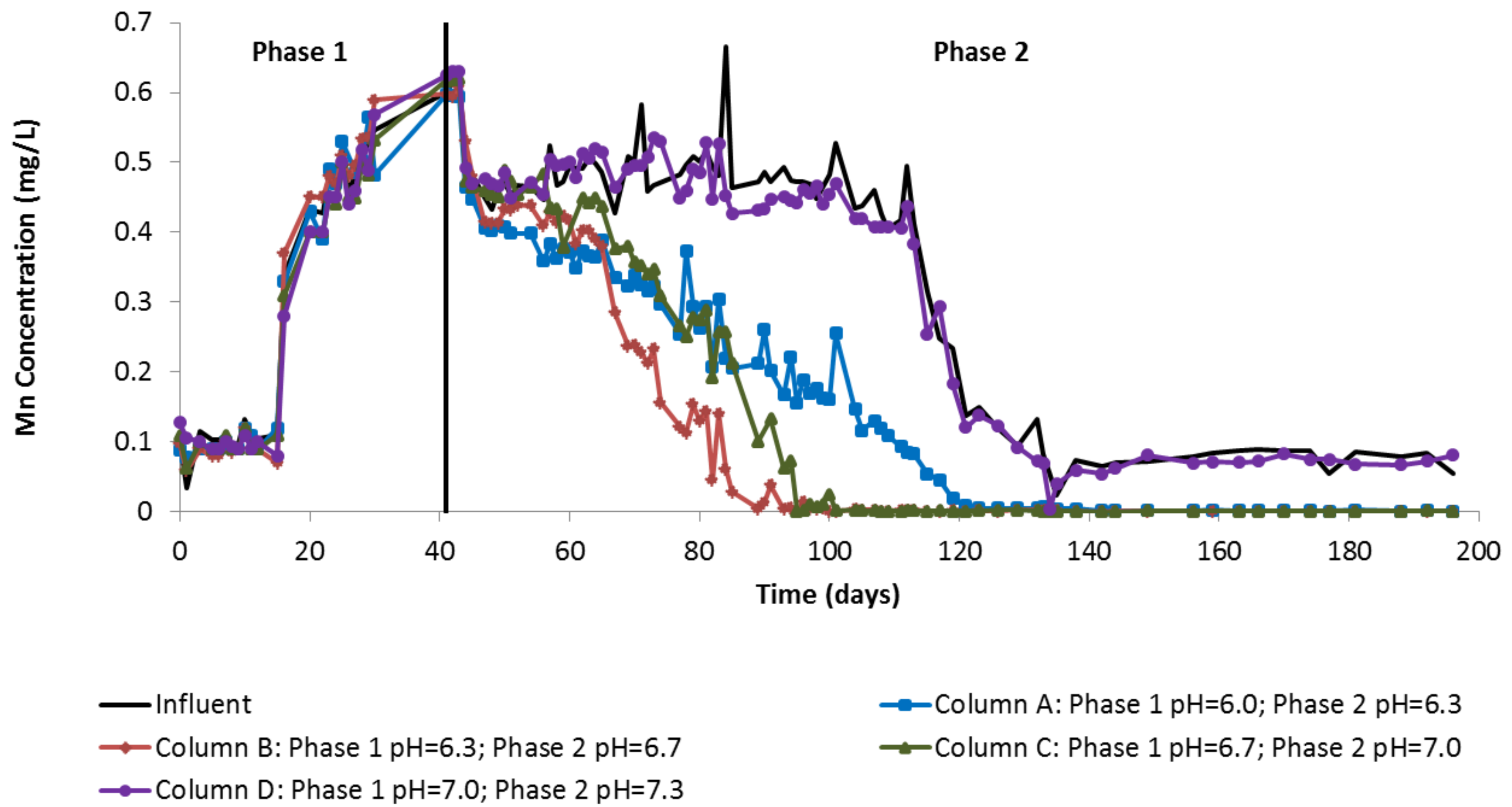

Figure 3-1. Influent and effluent Mn concentration for startup of the lab-scale columns

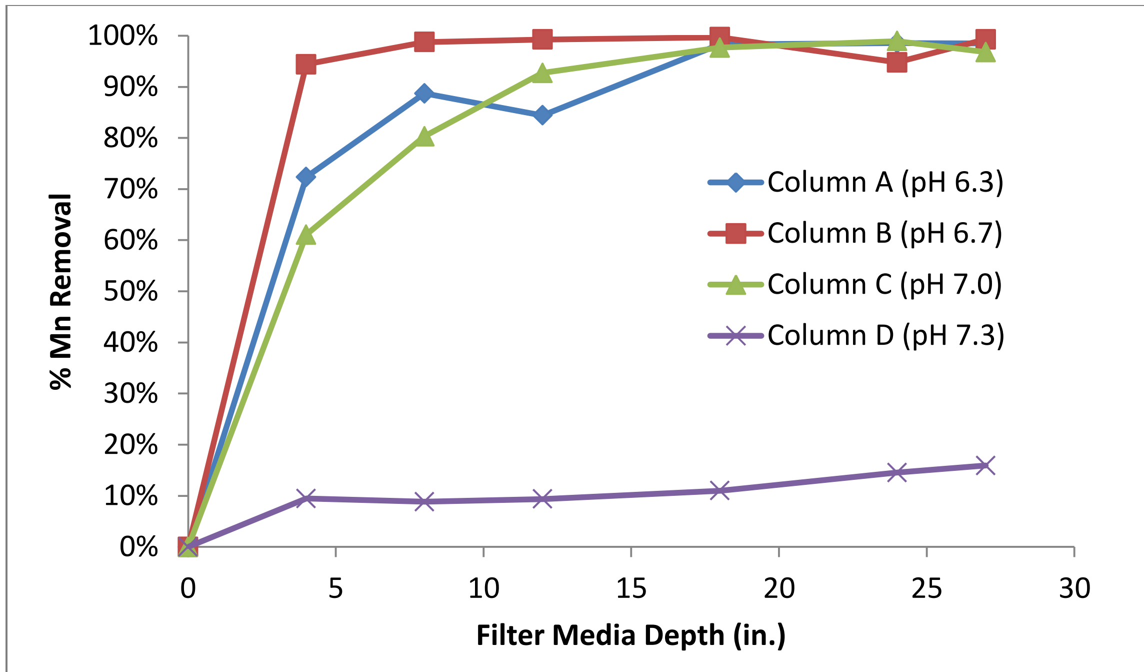

Figure 3-2. Mn removal profiles across the media depth of the lab-scale columns once essentially complete Mn removal was established in Columns A, B, and C

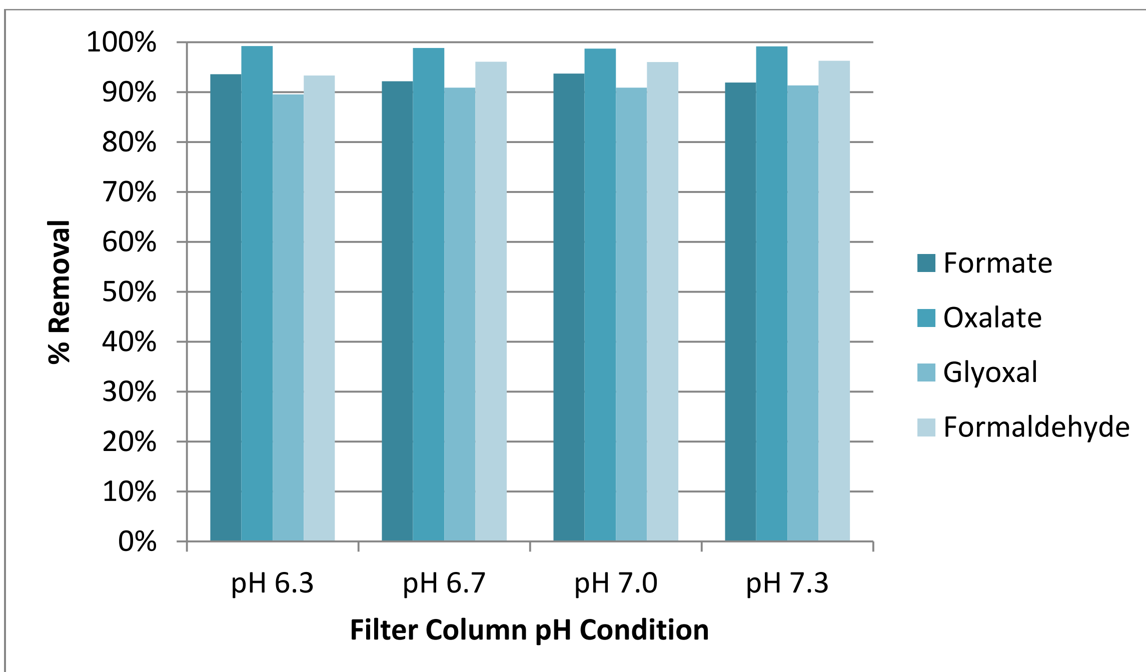

Figure 3-3. Average removal of supplemented biodegradable organic compounds in lab-scale columns during Phase 2 operation

MDL was used for calculating percent removal of a given compound. In most cases, 88-92% glyoxal removal was the highest achievable removal due to this calculation restriction. The high removal of BOM in all of the columns indicates that the presence of MOB at each of the pH conditions tested does not adversely affect the ability of a biofilter to increase the biostability of the finished water. Most of the BOM removal occurred in the first four inches of the column (Figure 3-4) similar to the Mn removal. This supports the claim that the majority of the biological activity is located at the top of the columns.

3.2 STRESS EXPERIMENTS

The following results summarize the outcomes of the various stress study experiments related to Mn removal in the laboratory-scale biofilters. These experiments were started once the columns had reached stable Mn removal at greater than 98% removal, indicating that a mature MOB biofilm had been established.

3.2.1 Mn Concentration

Column C (pH 7.0) and A (pH 6.3) were chosen for the influent Mn concentration increase stress studies. The pH in Column A during the experiment was measured to be 6.4 instead of the target 6.3. Effluent Mn concentration did not appreciably increase in either column when influent Mn concentration doubled (Figure 3-5 and Figure 3-7) indicating that the MOB in the mature biofilters were robust. Mn removal across the column depths shifted slightly as the time that the column remained at the higher Mn concentration increased (Figure 3-6 and Figure 3-8). This indicated that there was a minor effect of increasing the Mn concentration, but the biofilter media depth was sufficient for the MOB to adequately remove the Mn before it reached the effluent.

3.2.2 Hydraulic Loading Rate

Column C (pH 7.0) was selected for the hydraulic loading rate increase study. There was no noticeable effect on effluent Mn concentration when hydraulic loading rate doubled (Figure 3-9). Mn removal across the filter media depth was affected slightly by increased time at the higher hydraulic loading rate (Figure 3-10). The MOB were able to remove the Mn adequately, using more of the filter bed depth to do so. These results indicate that a mature MOB biofilter should be able to withstand the changes in hydraulic loading rate often experienced at surface water treatment plants, at least for relatively short (e.g. up to one day) time periods.

3.2.3 Biodegradable Organic Matter

The doubling of the influent BOM concentration in Column A (pH 6.3) caused a slight decrease in Mn removal down the column profile 3.5 hours after the BOM increase (Figure 3-11). The decrease was maintained during the approximately 24-hour experiment and did not return to the pre-BOM increase profile even after five days at normal influent BOM concentration (Figure 3-12). The effluent Mn concentration remained unchanged throughout the course of the experiment. This may indicate that MOB prefer conditions that are lower in biodegradable organic carbon.

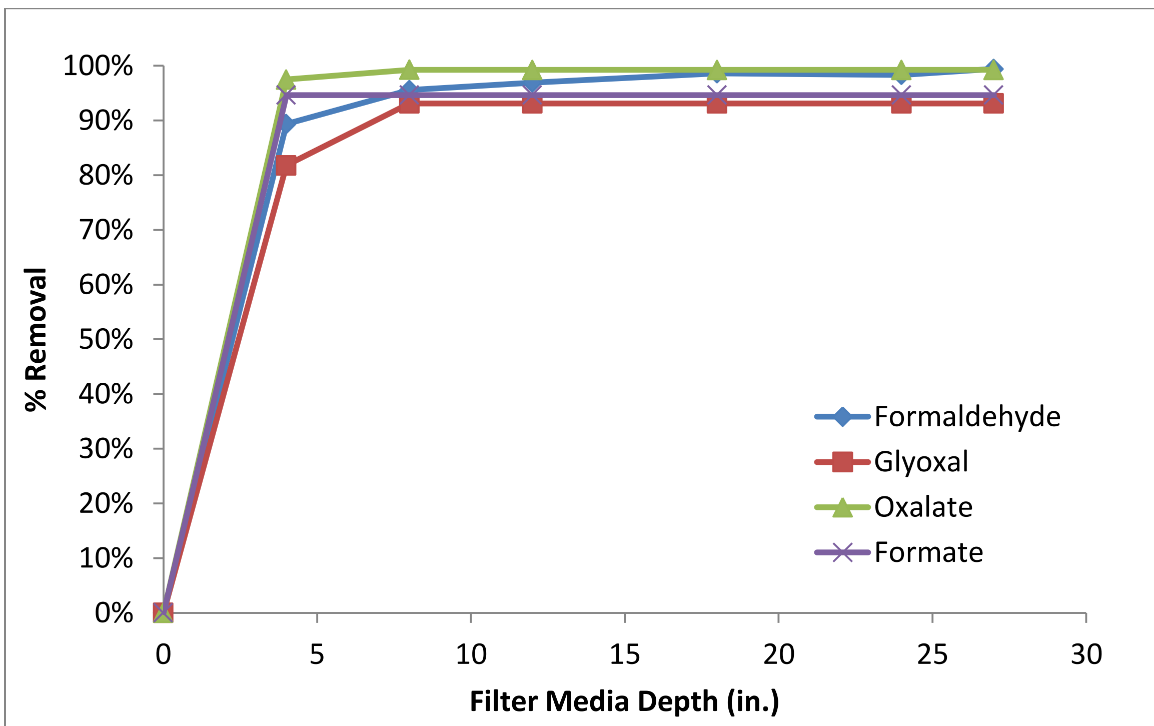

Figure 3-4. Removal of supplemented biodegradable organic compounds across Column C media depth (influent pH of 7.0)

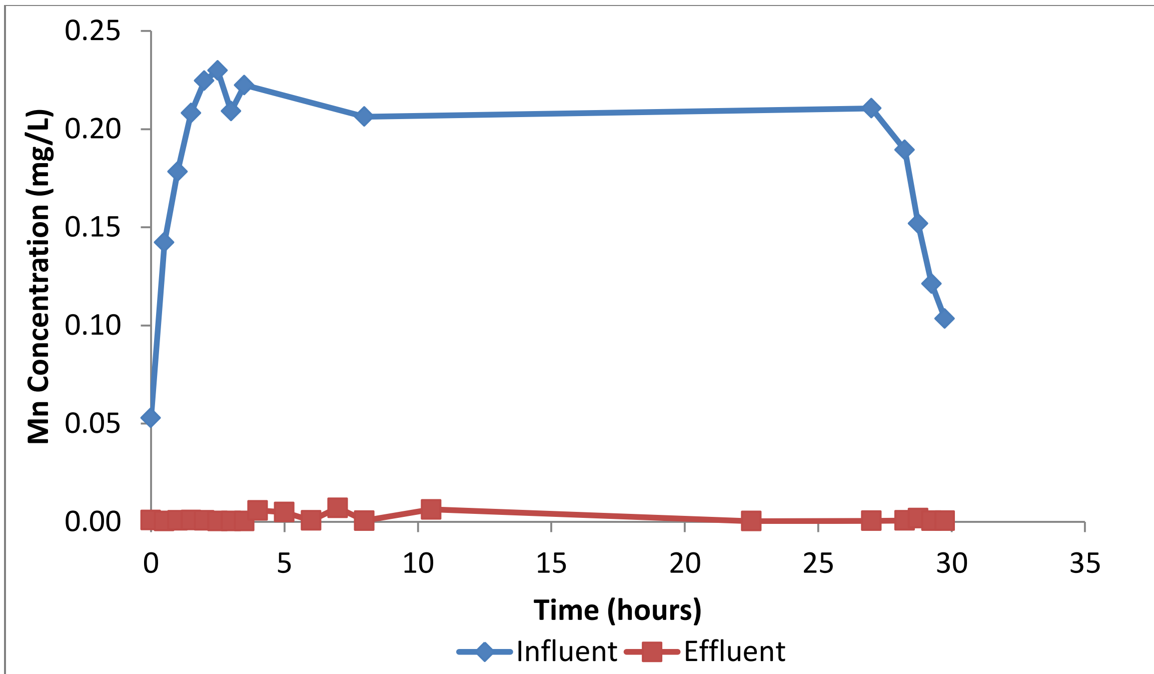

Figure 3-5. Mn removal in Column C during influent Mn concentration increase stress study (influent pH of 7.0)

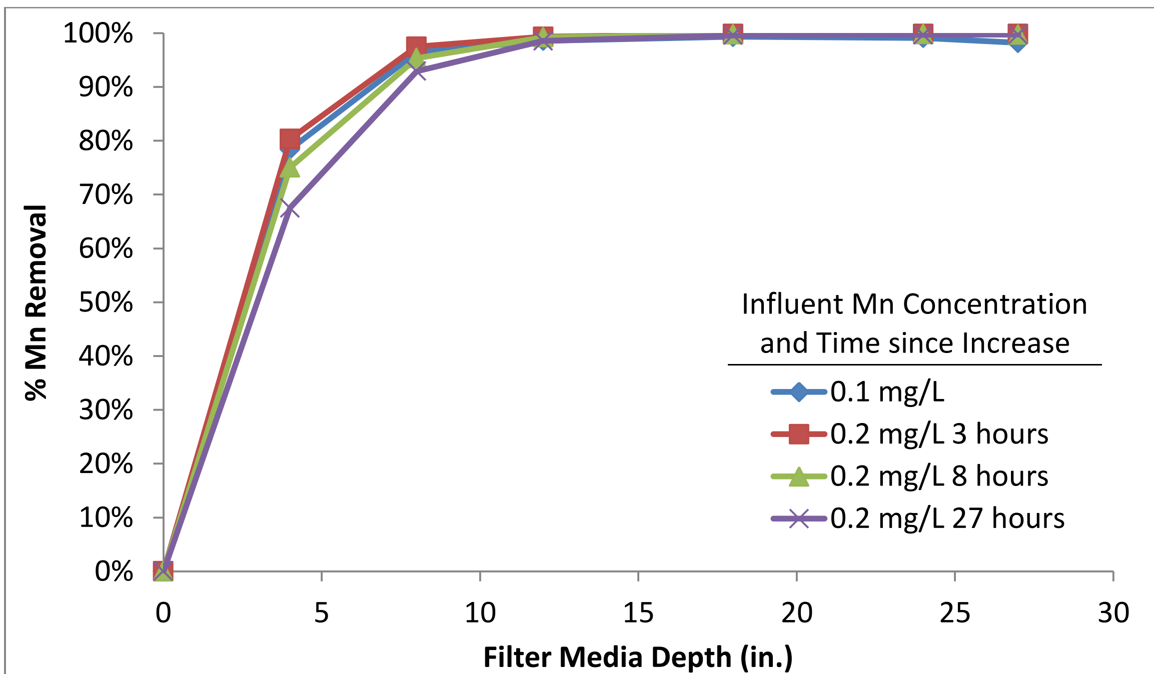

Figure 3-6. Mn removal profile across Column C filter media depth during influent Mn concentration increase stress study (influent pH of 7.0)

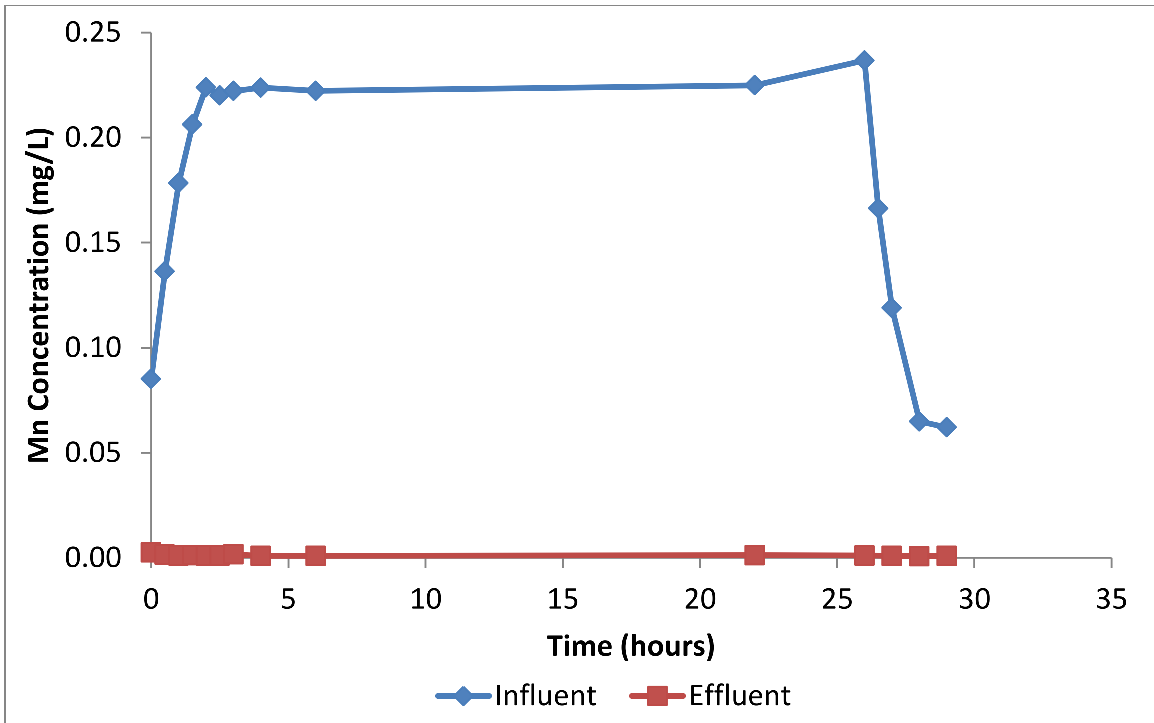

Figure 3-7. Mn removal in Column A during influent Mn concentration increase stress study (influent pH of 6.4)

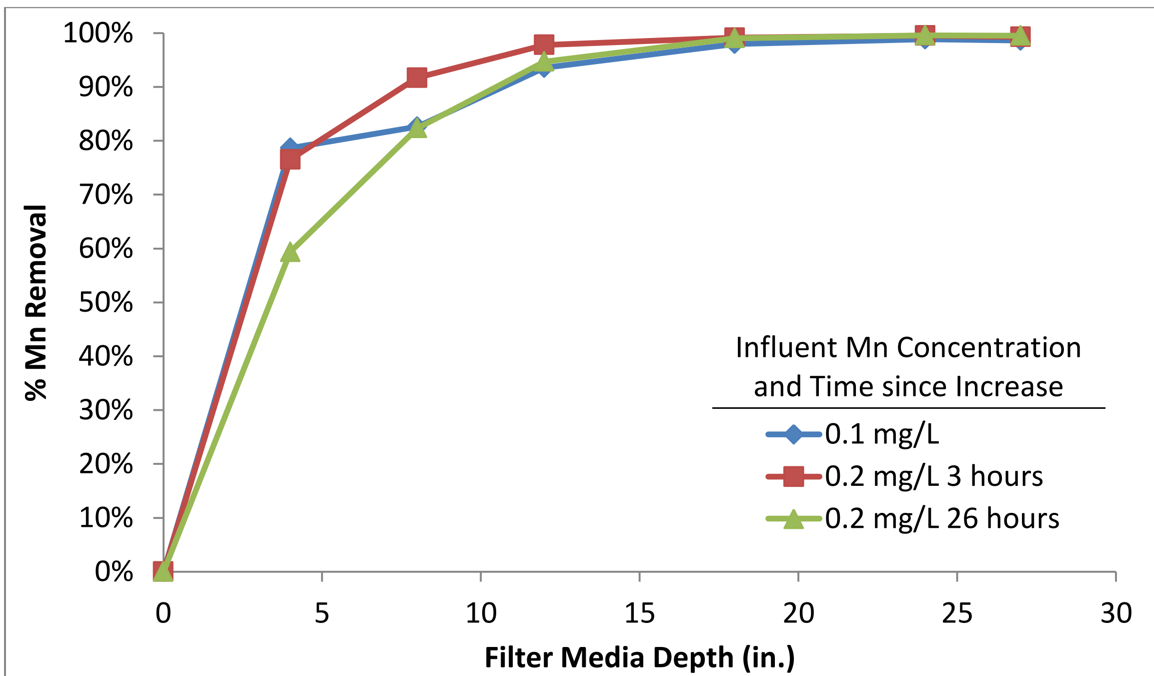

Figure 3-8. Mn removal profile across Column A filter media depth during influent Mn concentration increase stress study (influent pH of 6.4)

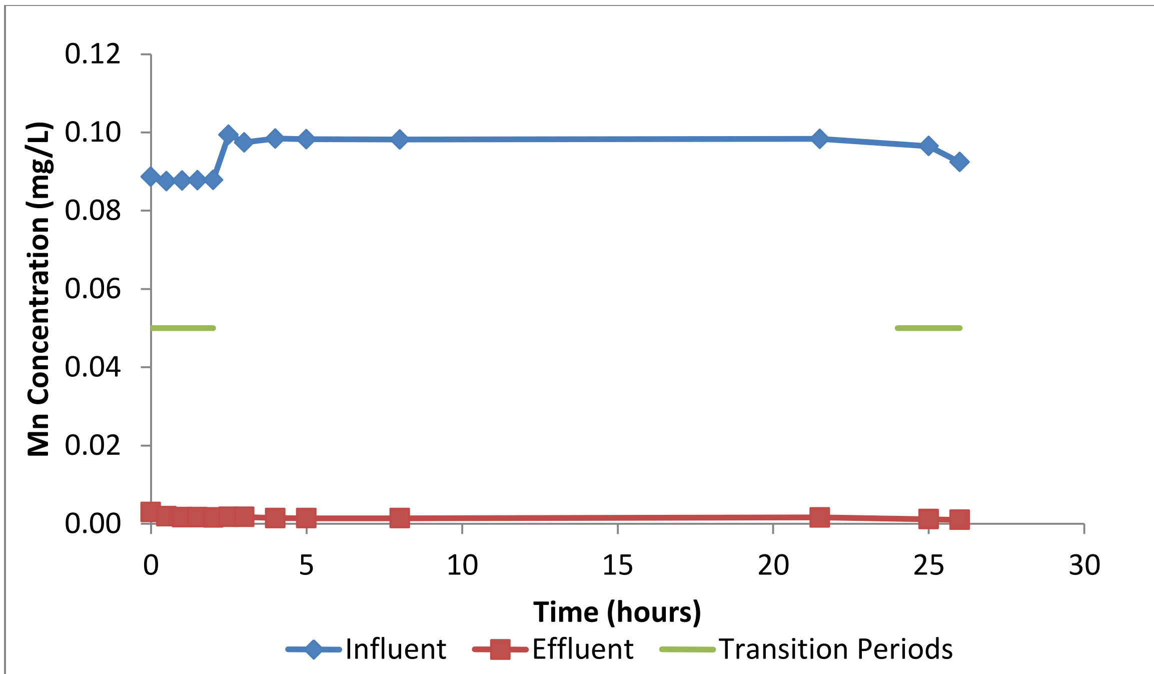

Figure 3-9. Mn removal in Column C during hydraulic loading rate increase from 2 gpm/ft^2 to 4 gpm/ft^2 (influent pH of 7.0)

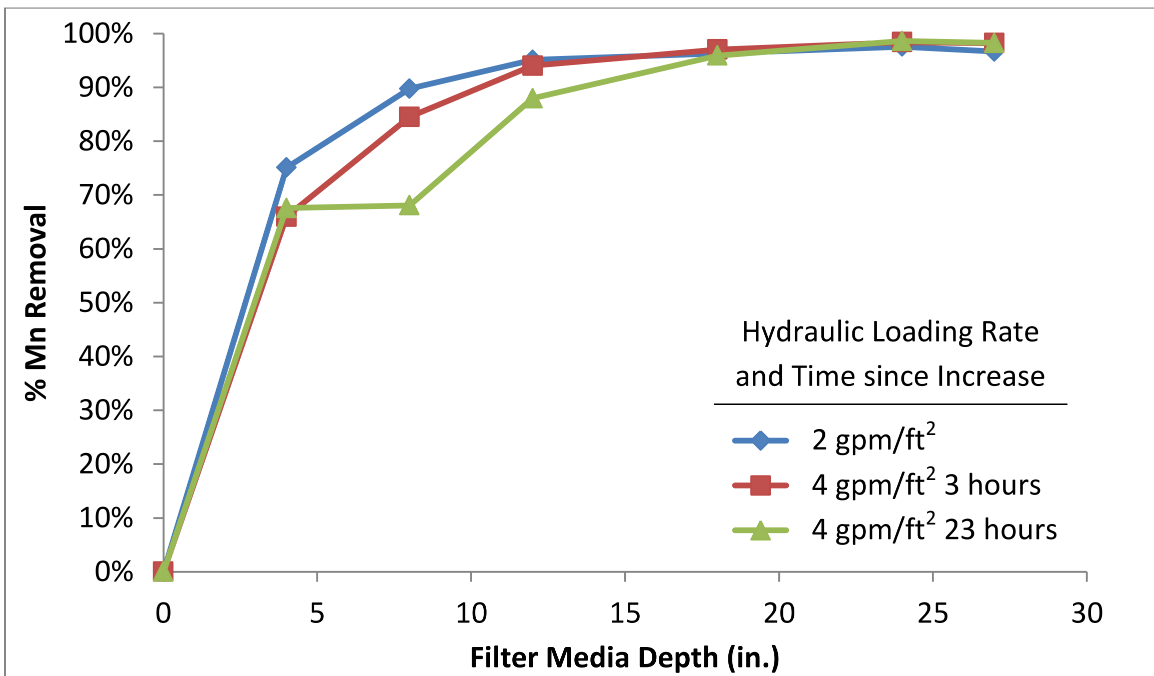

Figure 3-10. Mn removal profile across Column C filter media depth during hydraulic loading rate increase from 2 gpm/ft^2 and 4 gpm/ft^2 (influent pH of 7.0)

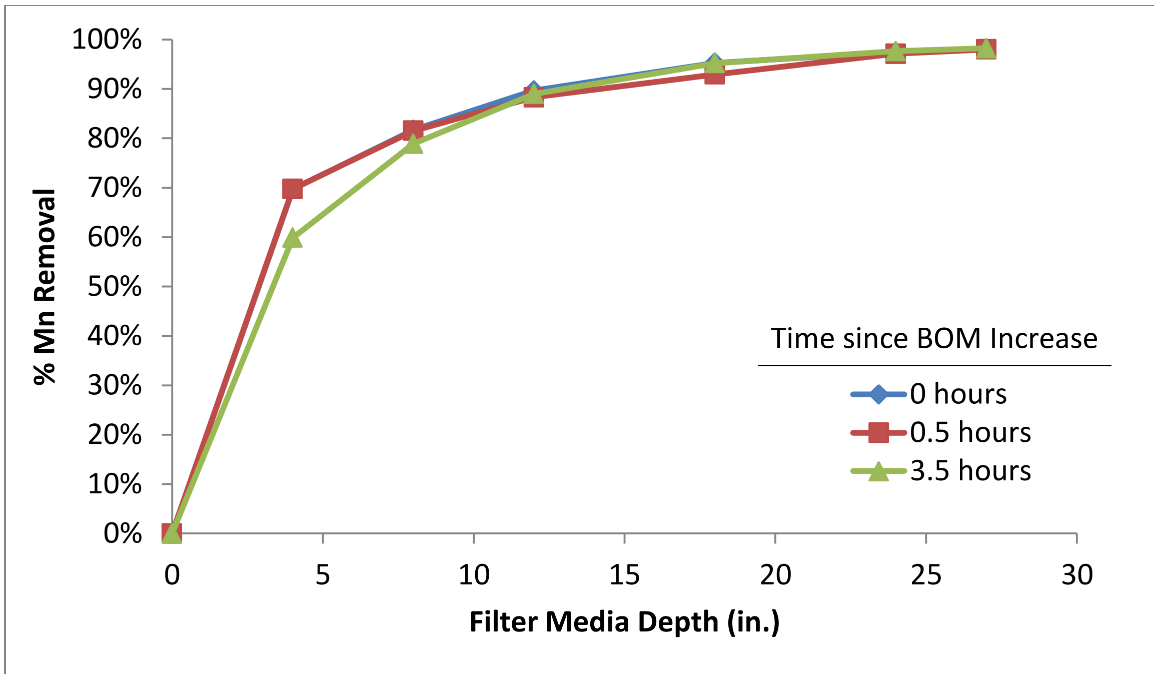

Figure 3-11. Short-term effect of influent biodegradable organic matter (BOM) increase from 0.3 to 0.6 mg/L as C on Mn removal across Column A media depth (influent pH 6.3)

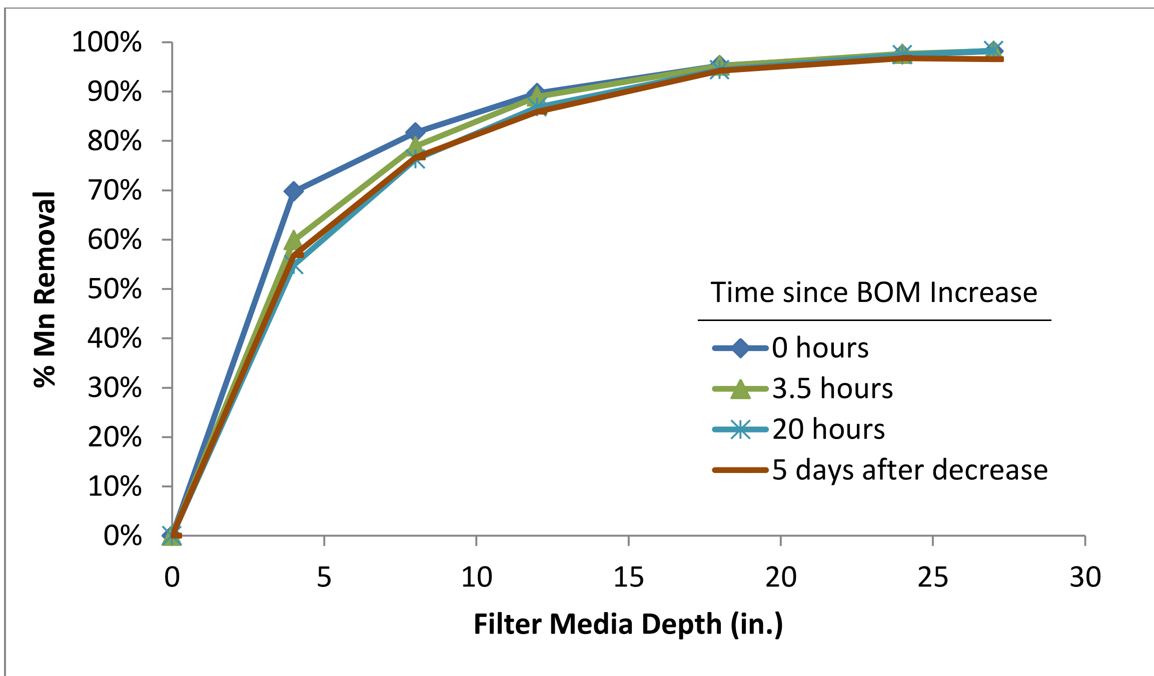

Figure 3-12. Long-term effect of influent biodegradable organic matter (BOM) increase from 0.3 to 0.6 mg/L as C on Mn removal across Column A media depth (influent pH 6.3)

3.2.4 Temperature Decrease

Decreased influent temperature corresponded to a decrease in Mn removal in Column C (pH 7.0) (Figure 3-13). This was expected as biological activity generally slows as water temperature decreases. Mn removal across the media depth also decreased (Figure 3-14) as water temperature decreased, as expected. Mn removal increased once the water temperature began to increase and was able to return to the Mn removal observed prior to the temperature decrease study. This indicated that mature MOB filters may be able to withstand winter temperatures commonly found at surface water treatment plants without requiring reinoculation once warmer temperatures return. Figure 3-15 show the relationship between Mn removal and influent water temperature during the temperature increase and decrease. The Mn removal at a given temperature varied, indicating that effect of influent water temperature on MOB was impacted by whether the temperature was decreasing or increasing.

3.3 Studies Evaluating the Cause of Limited Mn Removal in Column D

Various studies were designed to explore the cause of low Mn removal in Column D (pH 7.3). Immediate effects of pH were evaluated in a batch study. A plate inhibition study explored biological inhibition by other microorganisms present in the Column D biofilm. Finally, influent pH in the lab-scale column was decreased to pH 6.7 to determine if this would stimulate MOB activity and corresponding Mn removal. Additionally, influent BOM concentration was removed and then subsequently increased to determine the effect of BOM on Mn removal.

3.3.1 Inhibition Studies

The batch inhibition study did not show a direct, immediate effect of pH as shown in Figure 3-16. The cell suspensions were obtained from the lab-scale column media (see section 2.7.3) and included all the microorganisms present in the media biofilms, as opposed to pure cultures of MOB. The cell suspension from Column B (which was removing greater than 95% Mn in the lab-scale column at pH 6.7) was able to oxidize similar amounts of Mn at both pH 7.3 and 6.7, as indicated by the decrease in soluble Mn concentration. The cell suspension from Column D (which was not removing appreciable Mn at pH 7.3) was unable to oxidize Mn at either pH 6.7 or 7.3 in the five-hour experiment duration. These results indicated that the effects of pH on MOB activity in this pH range were not immediate.

3.3.2 Plate Inhibition

The plates that were prepared as described in section 2.4.2 were investigated for a zone of inhibition. Usually a zone of inhibition is identified by an area of decreased colony growth surrounding the chemical or isolate of interest (in this case, non-MOB isolates selected from plates of Column D media cell suspension). Either an area of decreased microbial growth or an area of decreased Mn oxidation (lack of MnO_2 deposition) would have indicated inhibition for the purposes of this study. Neither was observed for any of the colonies selected from the Column D plate. This result indicated that inhibition by other microorganisms present in Column

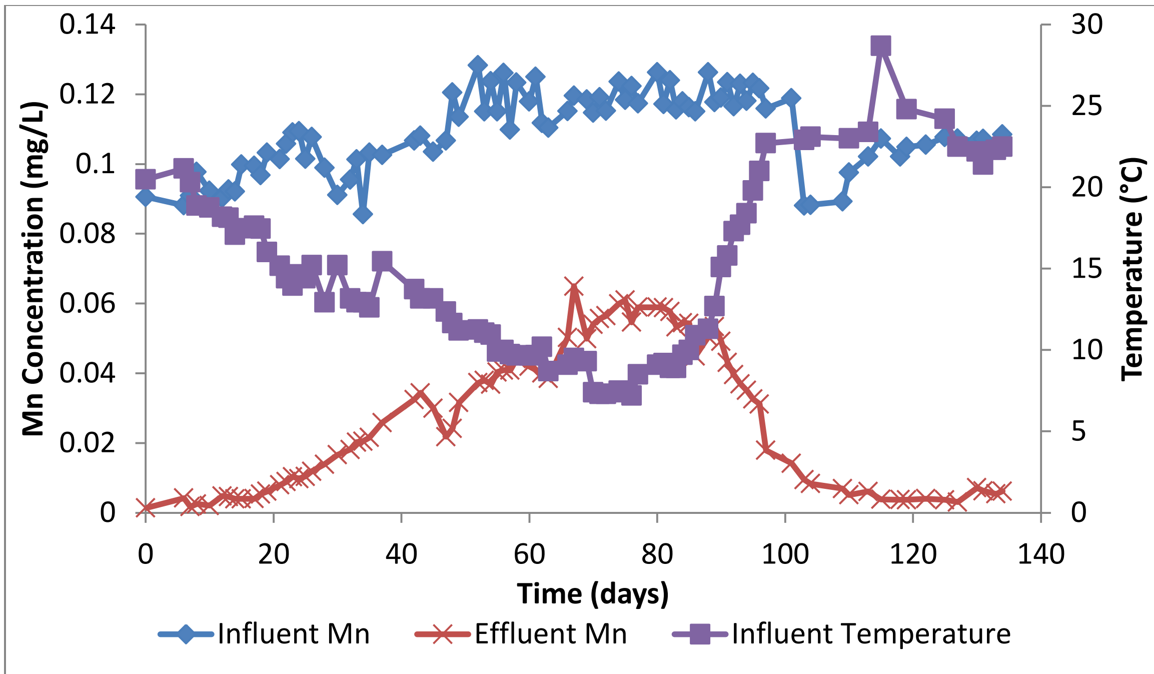

Figure 3-13. Effect of temperature on Mn removal in Column C (influent pH of 7.0). Time is displayed as time since temperature study began. (Day 0 is 226 days after Phase 1 startup).

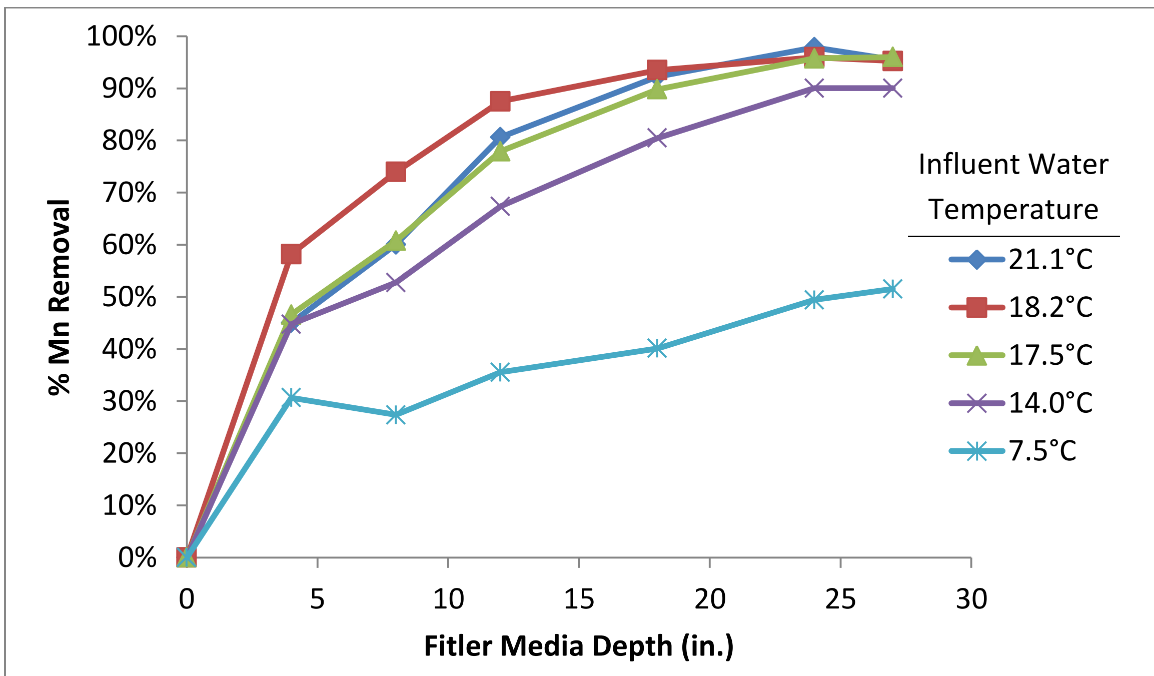

Figure 3-14. Effect of temperature on Mn removal profile across Column C media depth (influent pH of 7.0)

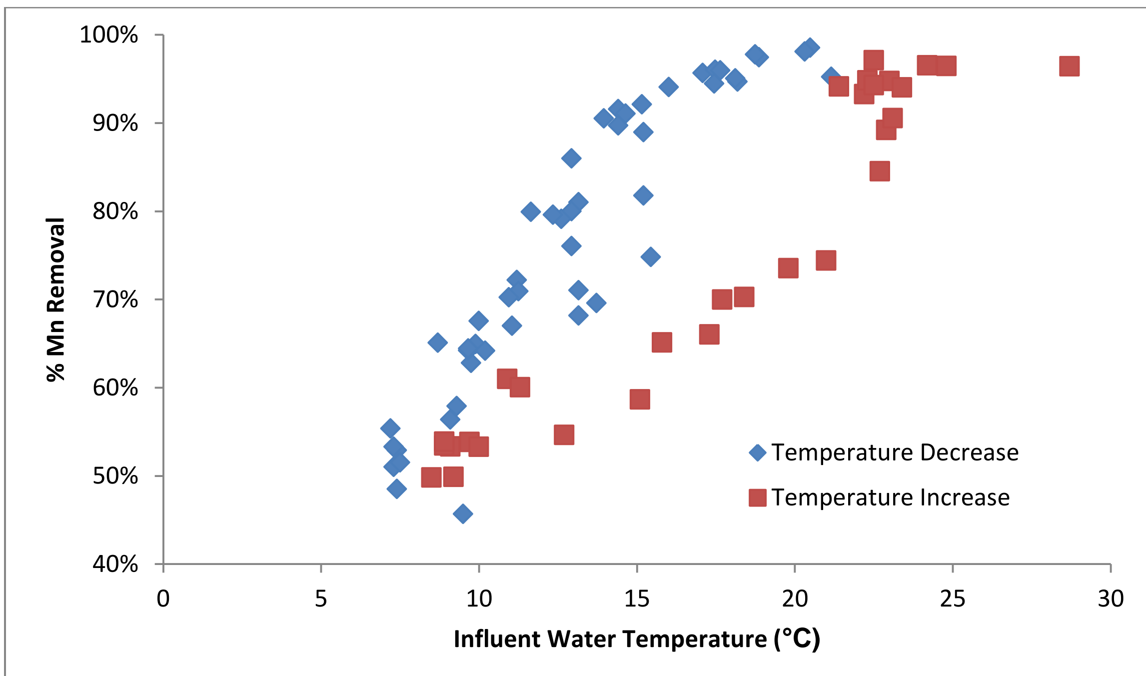

Figure 3-15. Relationship between Mn removal and influent water temperature in Column C during the temperature increase and decrease (influent pH of 7.0)

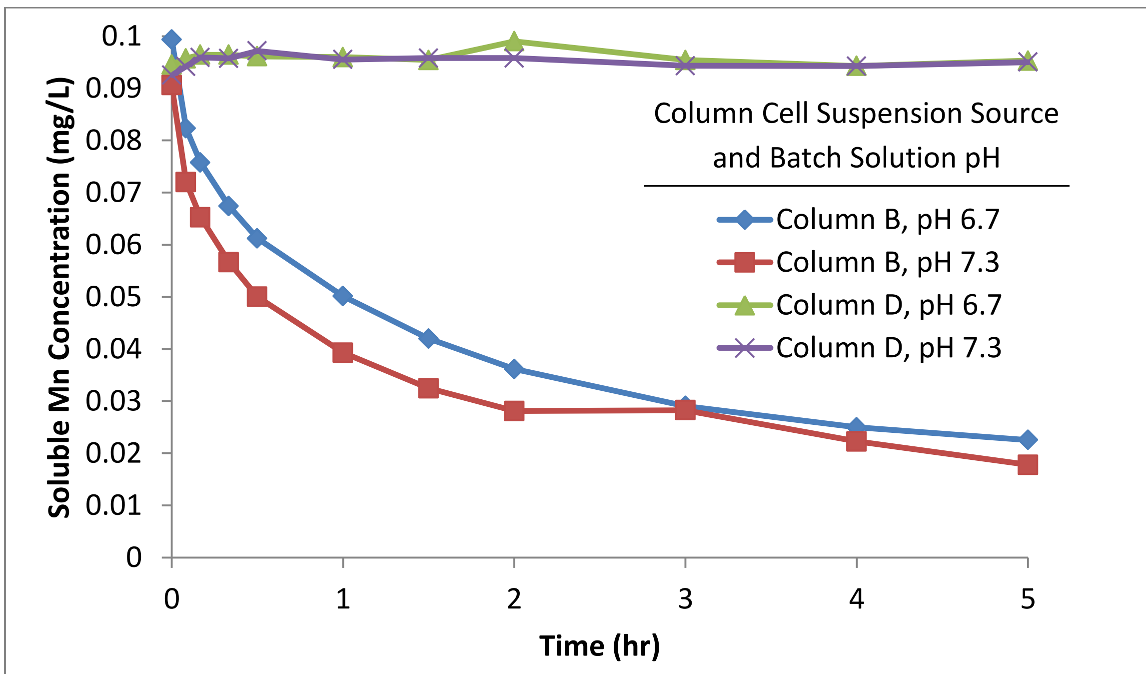

Figure 3-16. Effect of pH and column cell suspension source on soluble Mn concentration in batch study

D was probably not the cause of the lack of Mn removal; however, the possibility of inhibition by other microorganisms cannot be completely eliminated since it was not feasible to select all of the different isolates found in the Column D cell suspension for analysis.

3.3.3 Effect of Influent pH

Column D did not remove a substantial amount of Mn for the first 200+ days of operation, during which the influent to Column D was maintained at pH 7.3 (Figure 3-17). When the influent pH was decreased to 6.7, Mn removal began to increase (Figure 3-18). The decrease in effluent Mn concentration was slow (around 0.5 ppb/day) but eventually Mn removal of greater than 98% was achieved. Influent pH was increased back to 7.3 once Mn removal reached 98%, and the effluent Mn concentration began to increase slowly to a maximum of around 0.03 mg/L, corresponding to a Mn removal of 70%. The effluent Mn concentration then began to decrease slowly, with influent pH remaining at 7.3.

3.3.4 Effect of Influent BOM

The removal of BOM from Column D influent did not make an appreciable difference in the Mn removal trend (Figure 3-19). Additionally, the increase in BOM concentration to five times the original concentration also did not make a noticeable difference in Mn removal. The decreasing effluent Mn trend began before the removal of BOM and continued throughout the changing BOM concentrations. No direct effect of BOM on Mn removal by MOB was observed. The biofilm was able to remove greater than 97% BOM when at the higher influent BOM concentration (Figure 3-20).

3.4 Biological Analyses

Various biological analysis techniques were used to characterize the microbial community in the lab-scale biofilters. The presence and identification of MOB in the biofilters were also investigated.

3.4.1 Presence of MOB

The agar plating methods described in section 2.7.3 allowed for percentage MOB with respect to total heterotrophic plate count (HPC) bacteria in the media biofilm to be determined. Table 3-1 presents the results for the lab-scale columns. The days refer to time since Phase 1 startup. At Day 104 and Day 118, Columns B (pH 6.7) and C (pH 7.0) were removing greater than 98% Mn. Column A was removing around 70% Mn, and Column D was not appreciably removing Mn. MOB accounted for a very small percentage of total HPC; however, MOB entirely comprised the spores isolated from the biofilm (Table 3-1). It should be noted that the media samples were collected directly after backwashing, and the agar plating method is limited to only capturing organisms that are culturable by plating techniques. The biological community may include more organisms than can be detected by plating methods; as such, MOB may comprise an even smaller portion of the total microbial population.

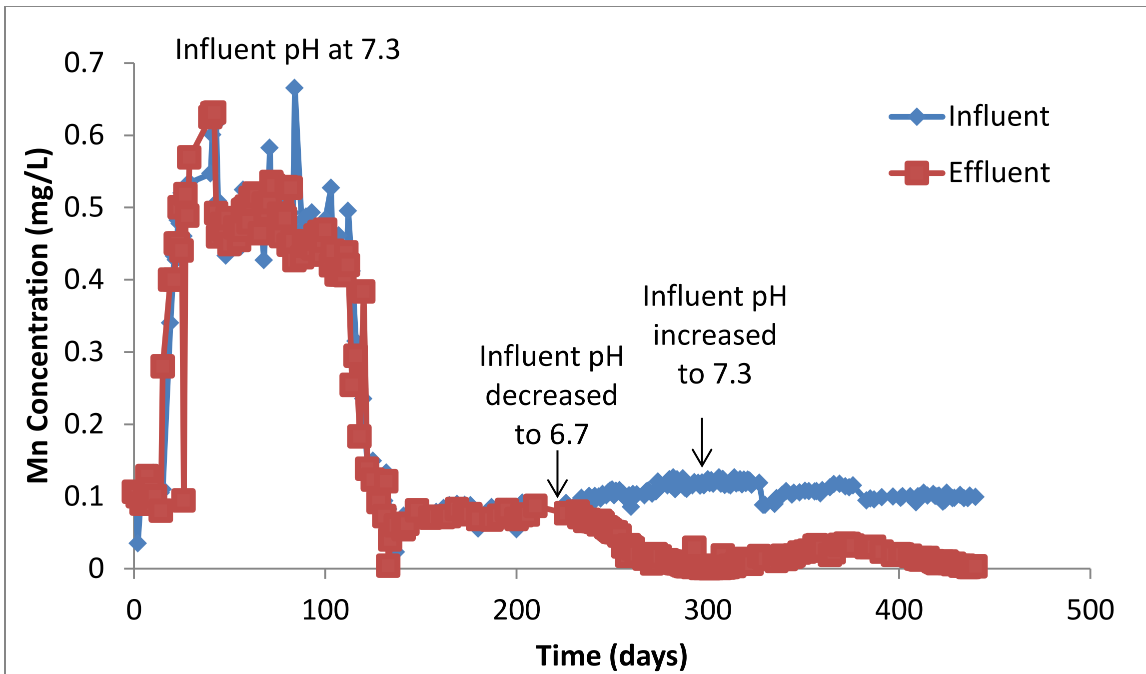

Figure 3-17. Mn removal in Column D during startup and influent pH changes

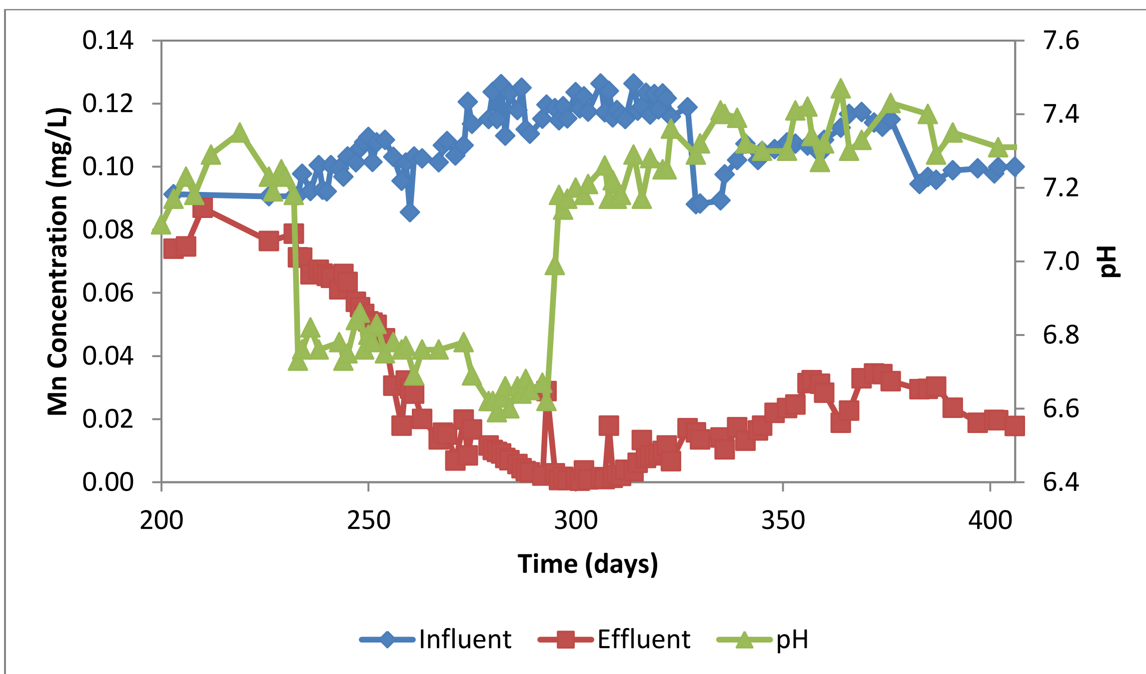

Figure 3-18. Effect of influent pH on Mn removal in Column D

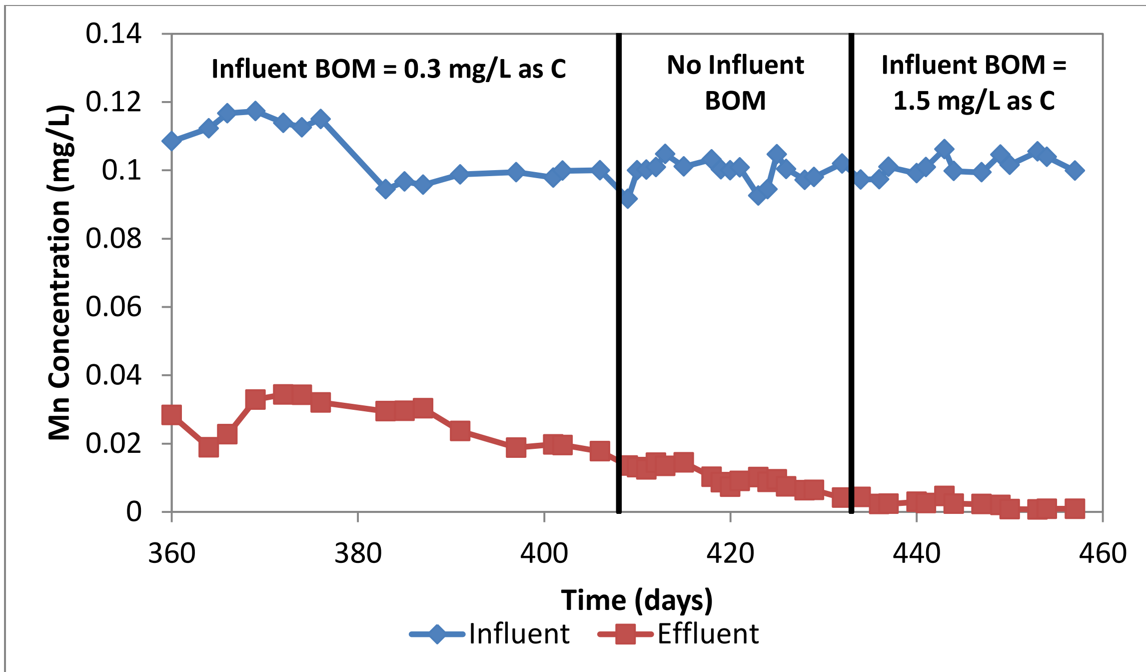

Figure 3-19. Effect of influent biodegradable organic matter (BOM) concentration on Mn removal in Column D (influent pH of 7.3)

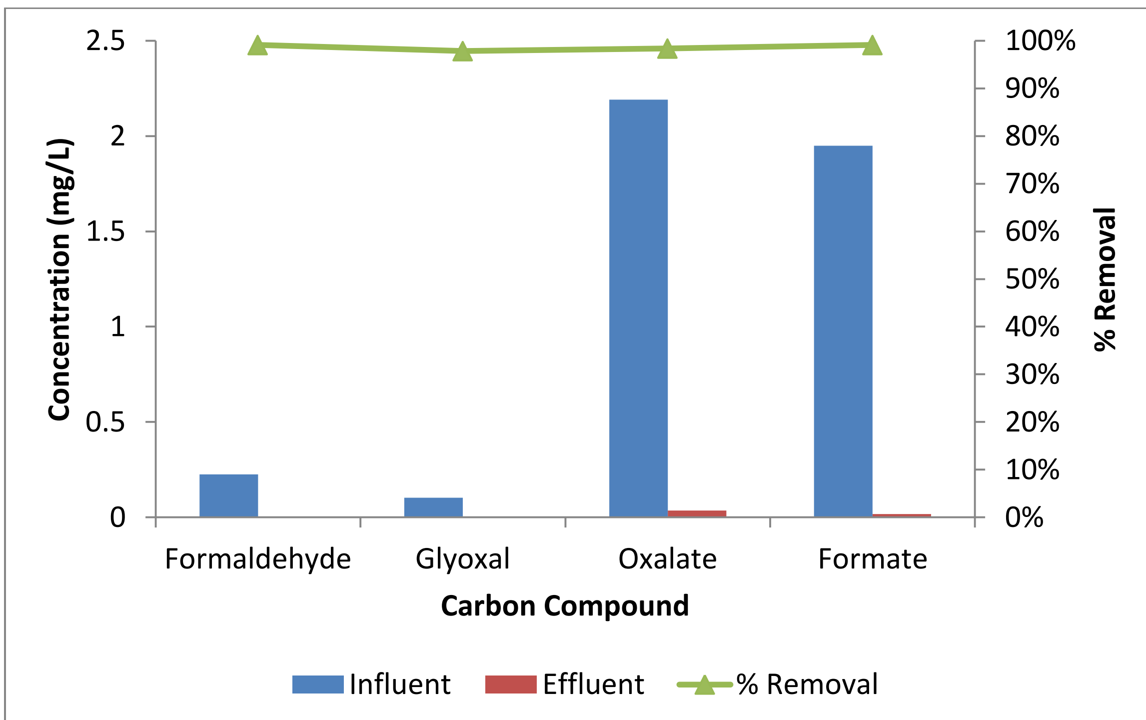

Figure 3-20. Biodegradable organic matter (BOM) removal in Column D at BOM concentrations five times greater than normal (influent pH 7.3)

Table 3-1. MOB composition of total heterotrophic plate count (HPC) on lab-scale column biofilter media (Day refers to time since Phase 1 startup)

Sample	% MOB in Total HPC
Column A Biofilm (day 118)	3.6
Column B Biofilm (day 118)	5.7
Column C Biofilm (day 118)	1.3
Column D Biofilm (day 118)	2.1
Column A Spores (day 104)	100
Column B Spores (day 104)	100
Column C Spores (day 104)	100
Column D Spores (day 104)	100

3.4.2 Repetitive-Sequence-Based Polymerase Chain Reaction (*rep*-PCR) Results

The *rep*-PCR gel images are shown in Figure 3-21 and Figure 3-22. The first well is a 1kb ladder, and the following five wells are the five MOB isolates reinoculated in Phase 2. The additional wells are spore samples (Figure 3-21) or selected colonies from the plated column media (Figure 3-22). Letters indicate the column from which the sample colony was obtained, and asterisks indicate colonies that had visible MnO_2 formation. Other colonies were selected because they had similar colony morphology to the inoculated isolates, but had no visible MnO_2 formation. Percent similarities between the samples and the original isolates are shown in Table 3-2. Similarity was evaluated based on the number of bands shared, and the relatedness between the original MOB isolates and the selected colonies was evaluated using the method outlined in Tenover *et al.* (1995). Underlined and bold values indicated the colony was closely related to the corresponding MOB isolate (2-3 bands different). Underlined values indicate that the colony was possibly related to the corresponding MOB isolate (4-6 bands different). The spores and colonies selected for analysis were most similar to MB-3. The low percentage similarities and low number of closely related isolates between the MOB isolates and the colonies with demonstrated Mn oxidation indicate that *rep*-PCR may not be an effective MOB identification method. This may also be an artifact of the 200 days that had passed from inoculation to *rep*-PCR analysis, since mutations can occur over generations. Additionally, the *rep*-PCR method is limited to analyzing only colonies selected and isolated for DNA extraction. The presence of MOB in terms of composition of total microbial population in the columns cannot be elucidated using *rep*-PCR.

3.4.3 Denaturing Gradient Gel Electrophoresis (DGGE) Results

Several of the MOB isolates were indistinguishable from each other using DGGE analysis and will be grouped together for further discussion (Table 3-3). MOB isolates within a group could not be distinguished from each other; however, DGGE could differentiate between the three groups. The samples analyzed using DGGE are described in Table 3-4. The purpose of selecting

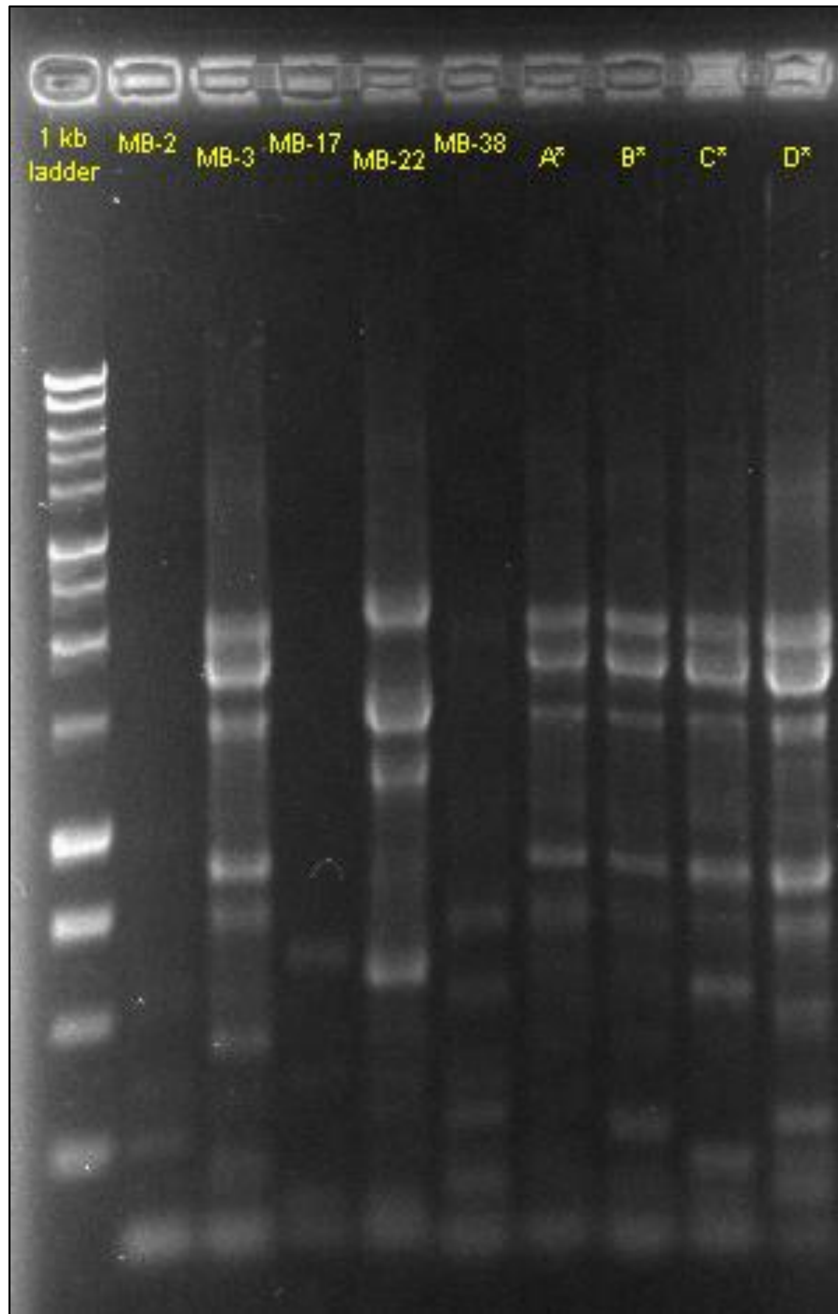

Figure 3-21. Results from *rep*-PCR gel analysis for five original MOB isolates and spore colonies from each of the lab-scale columns (Letters refer to lab-scale column, and asterisks indicate colonies with MnO_2 formation)

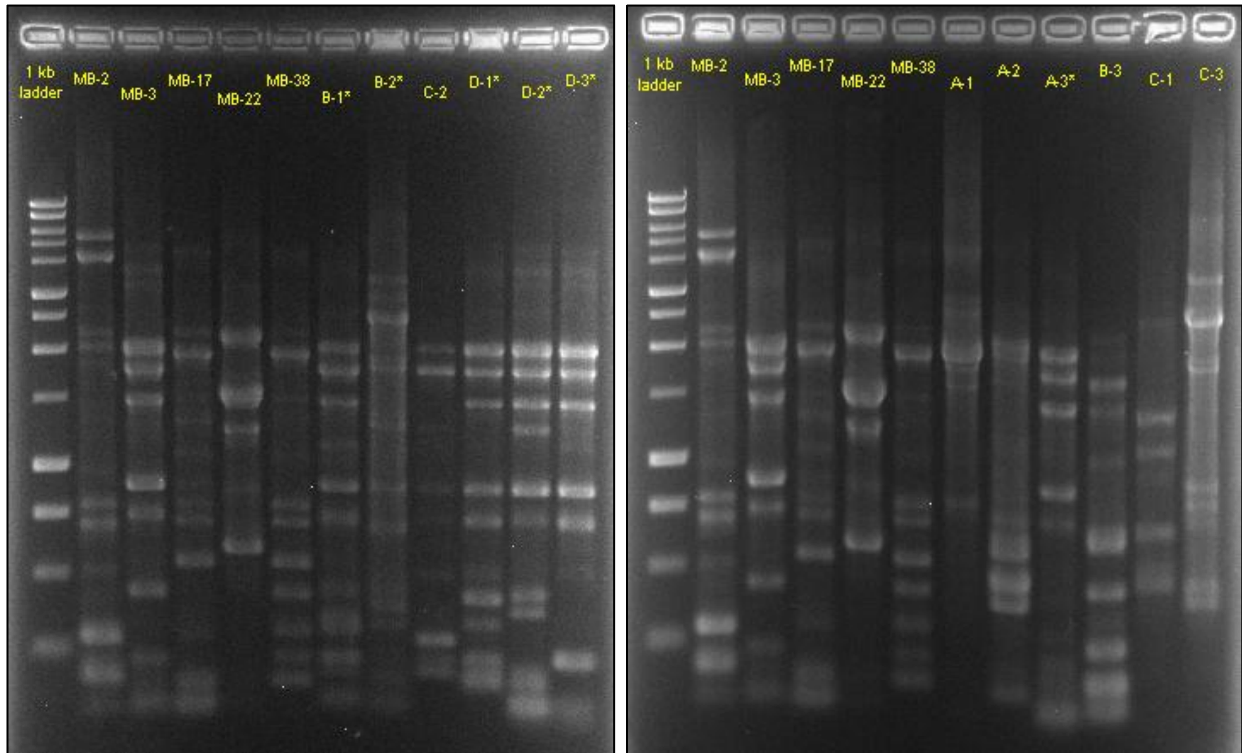

Figure 3-22. Results from *rep*-PCR gel analysis for five original MOB isolates and colonies selected from the lab-scale column media biofilms (Letters refer to lab-scale column, and asterisks indicate colonies with MnO_2 formation)

Table 3-2. Similarity between selected colonies and spores isolated from the lab-scale column media and the five original MOB isolates based on *rep*-PCR gel band analysis (Letters refer to lab-scale column, and asterisks indicate colonies with MnO_2 formation. Underlined and bold values indicated closely related isolates. Underlined values indicate possibly related isolates.)

Colony	MB-2	MB-3	MB-17	MB-22	MB-38
A Spore*	17%	**<u>82%</u>**	43%	56%	40%
B Spore*	15%	<u>78%</u>	40%	63%	63%
C Spore*	15%	**<u>89%</u>**	27%	63%	59%
D Spore*	29%	<u>74%</u>	25%	60%	59%
A-1	36%	**<u>84%</u>**	38%	38%	60%
A-2	36%	50%	30%	27%	21%
A-3*	56%	47%	47%	33%	38%
B-1*	27%	<u>76%</u>	30%	23%	40%
B-2*	19%	20%	30%	27%	11%
B-3	38%	60%	30%	40%	63%
C-1	43%	<u>82%</u>	45%	47%	48%
C-2	44%	47%	44%	17%	50%
C-3	43%	<u>82%</u>	45%	42%	38%
D-1*	38%	50%	40%	13%	63%
D-2*	43%	<u>73%</u>	45%	35%	48%
D-3*	43%	<u>73%</u>	55%	35%	38%

Table 3-3. Grouping of MOB isolates based on differentiation ability of DGGE. Isolates in the same group are indistinguishable from each other by DGGE.

Group	MOB Isolates
1	MB-3, MB-22
2	MB-33
3	MB-2, MB-17, MB-38

Table 3-4. Descriptors for lab-scale biofilter media samples analyzed with DGGE

Sample	Description
Not Inoc. 7-25	Prior to Phase 1 MOB inoculation
Inoc. 7-25	Directly after Phase 1 MOB inoculation
A 8-5, B 8-5, C 8-5, D 8-5	Column (indicated by letter) 10 days after Phase 1 inoculation; No Mn removal
A 1-26, B 1-26, C 1-26, D 1-26	Column (indicated by letter) 145 days after Phase 2 reinoculation; >98% Mn removal in A, B, and C; 20% Mn removal in D
D 4-11	Column D 27 days after pH decrease from 7.3 to 6.7; 65% Mn removal

these samples was to determine differences in the biological community on the lab-scale biofilter media when the columns were either removing or not removing Mn. The cluster analysis of the DGGE results is given in Figure 3-23, with the Bray-Curtis similarity results given in Table 3-5. Red dashed lines indicate no statistically significant difference ($p<0.05$) in the biological community profiles based on DGGE band analysis.

Based on these results, samples D 1-26 and D 4-11 were not different. Column D was not removing Mn when sample D 1-26 was collected and was removing 65% Mn when sample D 4-11 was collected. This suggests that the reason for the Mn removal increase in D 4-11 was not due to a significant change in the microbial community, but to some other interaction between pH and MOB. Alternatively, the pH change could have affected the activity of other organisms that may have been inhibiting Mn oxidation by MOB. The D 1-26 and D 4-11 samples were different from the A 1-26, B 1-26, and C 1-26 samples (all of which were statistically the same). This may indicate that differences in the microbial community in Column D may have contributed to the lack of Mn removal for most of the study. All of the influent parameters were identical across the columns except for influent pH. There were no differences in the original media placed in the columns during Phase 1 or in the MOB inoculations during Phase 1 and Phase 2. It is feasible that pH could have influenced which MOB or which other microorganisms present on the media flourished within the columns. The higher pH in Column D may have selected different organisms, and this in turn may have been responsible for the lack of Mn removal in Column D.

The observation that the biological profiles in the Inoculated 7-25 sample and the Non-inoculated 7-25 sample were different from each other and all of the other samples is not surprising. MOB inoculation can be attributed to the difference between these two samples, and changes in the biological community over time in the lab-scale columns can be attributed to

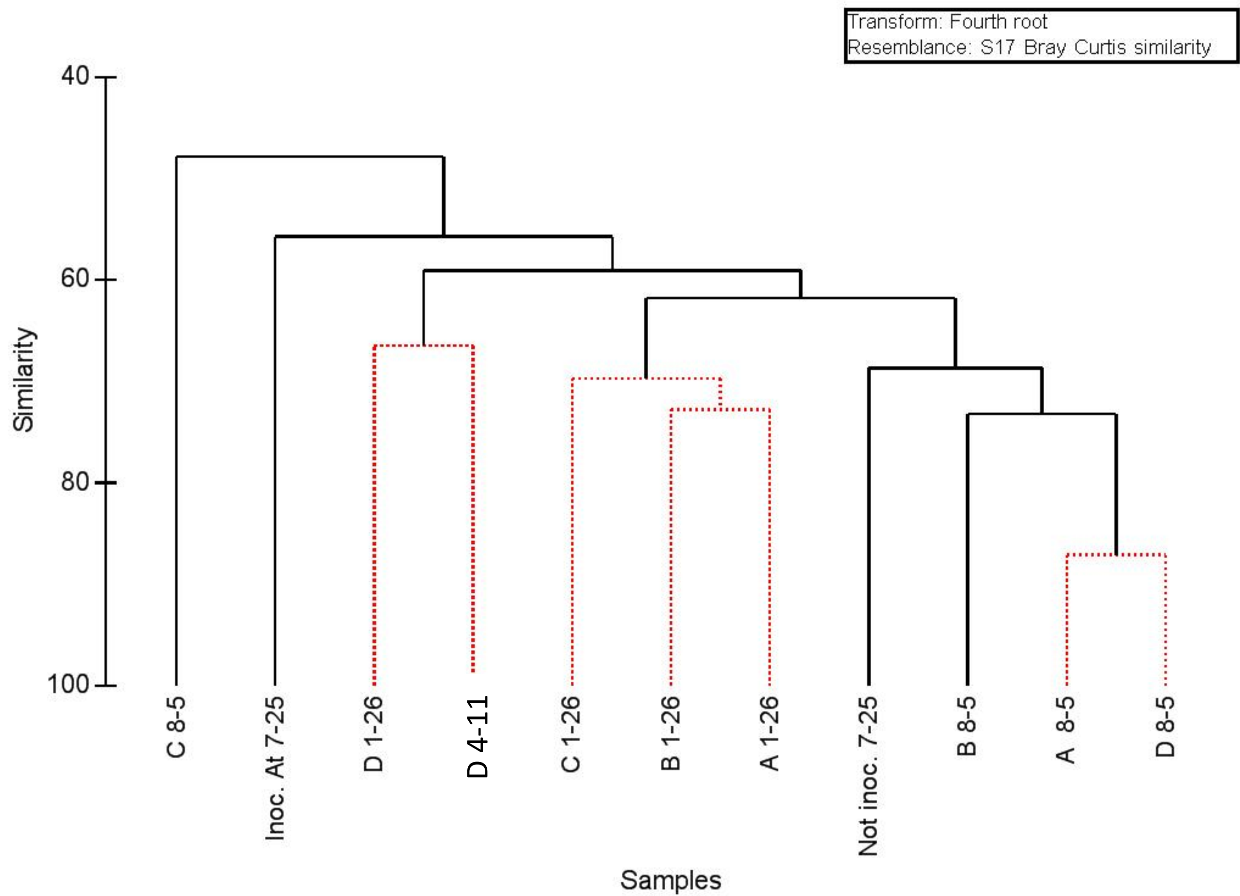

Figure 3-23. Hierarchical cluster of the biological communities present on the lab-scale biofilter column media. Sample descriptions can be found in Table 3-4. Red dashed lines indicated no statistically significant difference between the clusters ($p<0.05$). The y-axis presents the Bray-Curtis similarity where the cluster is formed.

Table 3-5. Bray-Curtis similarity between lab-scale biofilter media samples based on DGGE banding patterns.

	Not inoc. 7-25	Inoc. 7-25	A 8-5	B 8-5	C 8-5	D 8-5	A 1-26	B 1-26	C 1-26	D 1-26
Inoc. 7-25	62.9									
A 8-5	69.8	51.8								
B 8-5	64.7	55.7	71.5							
C 8-5	45.5	46.3	45.7	52.5						
D 8-5	71.6	52.6	87.1	74.9	47.1					
A 1-26	57.7	55.2	70.6	63.3	45.2	72.2				
B 1-26	57.1	60.2	59.2	68.6	51.4	63.8	72.8			
C 1-26	54.3	56.2	58.0	60.7	53.4	56.3	68.0	71.5		
D 1-26	61.8	56.9	57.6	58.8	51.7	54.6	55.0	55.6	66.9	
D 4-11	53.3	50.4	64.9	63.1	39.8	64.5	63.1	56.1	52.0	66.5

the difference between these two samples and the rest of the samples. Interestingly, the 8-5 samples were different from one another (save A and D). Whether this was due to pH or another factor is unknown. The 1-26 samples were all the same, indicating that the Phase 2 reinoculation had an effect on the biological community (save Column D).

Table 3-6 presents the relative composition of MOB within the total microbial community detectable by DGGE. The percentages are much lower than those determined by Mn-oxidation agar plating methods, but the plating methods are limited to only capturing culturable organisms. Additionally, DGGE does not differentiate between living and non-living organisms since it is only based on DNA presence. Furthermore, the plating methods, *rep*-PCR, and DGGE analysis each used media biofilm samples that were collected at different times from the lab-scale columns. The microbial communities present in the column could have changed over time in response to slight variations in influent conditions, competition from other organisms, etc. Differences in results between biological analysis techniques could be partly attributed to this time-based variation in the overall microbial community

Interestingly, MOB were detected using band migration analysis in the non-inoculated sample (Table 3-6). It is feasible that MOB may be present in the non-inoculated media samples since MB-3, MB-17, MB-22, and MB-38 were all originally obtained from a previous study at the Lee Hall Newport News Waterworks plant where the lab-scale biofilter media was obtained. It should be mentioned that organisms other than MOB could be present at the band location associated with the MOB groups, and show up in the calculation of percentage MOB.

Table 3-6. Relative presence of MOB groups in terms of total microbial population detected by DGGE.

Sample	Group 1	Group 2	Group 3
Not inoc. 7-25	0.07%	0.08%	0.01%
Inoc. 7-25	0.13%	n.d.	0.14%
A 8-5	0.03%	0.05%	n.d.
B 8-5	n.d.	n.d.	0.02%
C 8-5	n.d.	0.04%	0.21%
D 8-5	0.02%	n.d.	0.01%
A 1-26	n.d.	n.d.	0.07%
B 1-26	n.d.	n.d.	0.09%
C 1-26	n.d.	n.d.	0.03%
D 1-26	n.d.	n.d.	0.06%
D 4-11	n.d.	n.d.	0.06%

3.4 Pilot-Scale Column Results

Both soluble and insoluble Mn was present in the influent to the pilot-scale filters. Soluble Mn was supplemented to achieve a target 0.1 mg/L influent soluble Mn. Influent samples were filtered through 0.45 μm to determine soluble Mn concentration, which was used for percent Mn removal calculations. Effluent samples did not require filtration, as all of the Mn present was soluble. MOB were inoculated in the pilot-scale columns by placing media from lab-scale Columns A (pH 6.3) and B (pH 6.7) on top of the pilot-scale column media. The lab-scale columns were removing greater than 98% Mn when the media was removed. Figure 3-24 depicts Mn removal in the four pilot-scale filters throughout the four pilot study phases. The presence of MOB in Filter 3 and 4 was the only difference between the columns during Phase 1. The sharp decrease in Mn removal on Day 50 in all four filters can be attributed to an increase in hydraulic loading rate from 1.15 gpm/ft^2 to 2.0 gpm/ft$^{2.}$ to better simulate typical surface water treatment plant conditions (data from this day were not included in the statistical analysis). The hydraulic loading rate was returned to 1.15 gpm/ft^2 the following day to prevent further decreases in Mn removal. Filter 4 influent soluble Mn concentration was increased from 0.1 mg/L to 0.5 mg/L in Phase 2 in an attempt to increase Mn removal and was maintained at the higher concentration during Phases 3 and 4. Phosphorus was supplemented to the influents of all four columns in Phase 3 (0.05 mg/L as P) in a further attempt to increase Mn removal. The hydraulic loading rate increase during Phase 4 was intended to simulate typical loading rates at surface water treatment plants.

3.4.1 Statistical Analysis

Ordinary least squares regression analyses were used to analyze the pilot scale percent Mn removal data. Details of the statistical analyses performed can be found in the Appendix. The presence of MOB in Phase 1 significantly increased Mn removal ($p<0.0001$), indicating that Mn

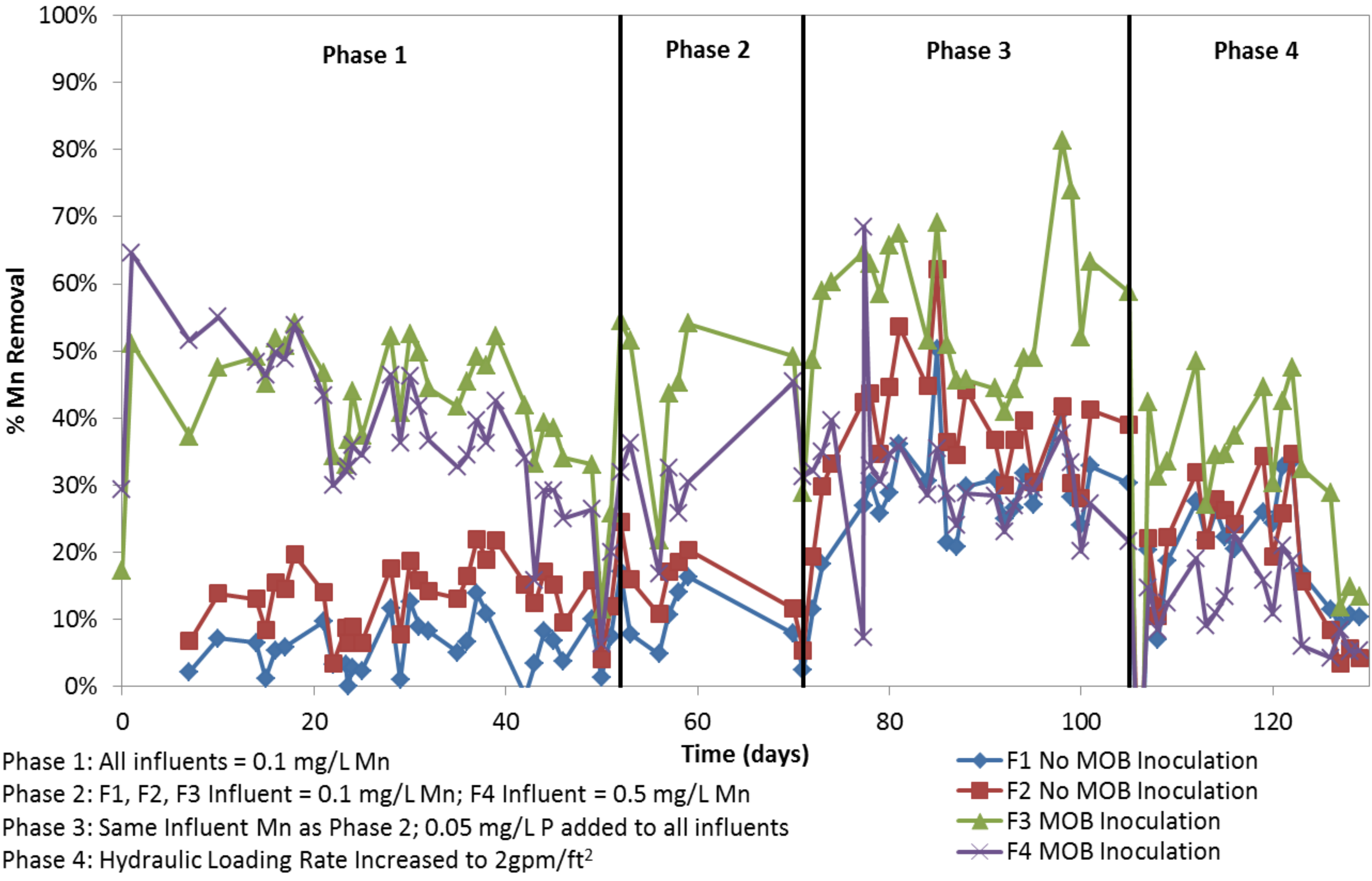

Figure 3-24. Mn removal in pilot-scale biofilters after MOB inoculation with varying influent conditions (phases)

removal was greater in the MOB-inoculated columns compared to the control columns. Time and pH both impacted Mn removal across the MOB columns in Phase 1 (Figure 3-25); however, pH decreased as time increased (Figure 3-26) so the effects of each individually may be confounded. A significant effect of time would describe continued decrease in Mn removal due to lack of MOB acclimation to the pilot-scale filter conditions. A significant pH effect would indicate that as the pH decreased towards 6.0, the ability of MOB to oxidize Mn was diminished. The effect of pH was statistically significant during Phase 1 ($p=0.0159$), and the effect of time was not statistically significant ($p=0.2384$), supporting the latter explanation. Additionally, the effect of pH on the MOB-inoculated columns was particularly pronounced ($p<0.0001$). Filter 3 Mn removal was significantly greater than Filter 4 Mn removal ($p=0.0132$) during Phase 1, and Filter 2 Mn removal was significantly greater than Filter 1 Mn removal ($p<0.0001$) during both Phase 1 and Phase 2. It should be noted that Filter 3 received media from the pH 6.3 lab-scale column, and Filter 4 received media from the pH 6.7 lab-scale column. The MOB and biological community established on the lab-scale media may have been different between columns. Additionally, native microbial populations established on the pilot-scale filter media before MOB inoculation may have differed between columns, explaining the differing Mn removals observed.

The effect of the influent Mn increase on Filter 4 in Phase 2 was analyzed by comparing the Mn removal in Filter 3 and Filter 4. pH was still a significant factor ($p<0.0001$) during Phase 2, with higher pH corresponding to higher Mn removal, as seen in Phase 1. Filter 3 had significantly greater Mn removal during both Phase 1 and Phase 2 ($p=0.0004$). The effect of increasing influent Mn did not improve Mn removal in Filter 4 compared with Filter 3, as was expected. Filter 3 was able to achieve a greater Mn removal increase during Phase 2 than Filter 4, but the difference in removal was not quite significant ($p=0.066$). It should be noted that the mass Mn removed by Filter 4 increased substantially compared to Filter 3, even though the percent Mn removal remained lower. Presence of MOB was still a statistically significant factor ($p<0.0001$) during Phase 2.

Days 72-77 were not included in the statistical analysis for evaluating the effect of supplementing phosphorus to the influent in Phase 3. Mn removal was increasing in Filters 1 and 2 during this time as the filters adjusted to the added phosphorus. The difference between the Mn removal in the filters before and after this transition was more of interest than the change during the transition. Additionally, Filter 4 data were removed for Phase 3 analysis since the influent Mn concentration to Filter 4 was higher than the other columns. Overall, Phase 3 resulted in a statistically significant ($p<0.0001$) increase in Mn removal. Filter 3 had a significantly ($p<0.0001$) lower increase in Mn removal than Filters 1 and 2 after adding phosphorus; however Filter 3 Mn removal remained significantly ($p<0.0001$) higher than Filters 1 and 2. The increases in Mn removal in Filter 1 and 2 from Phase 2 to Phase 3 were not significantly different ($p=0.3192$), but the total Mn removal in Filter 2 remained significantly higher ($p<0.0001$) than

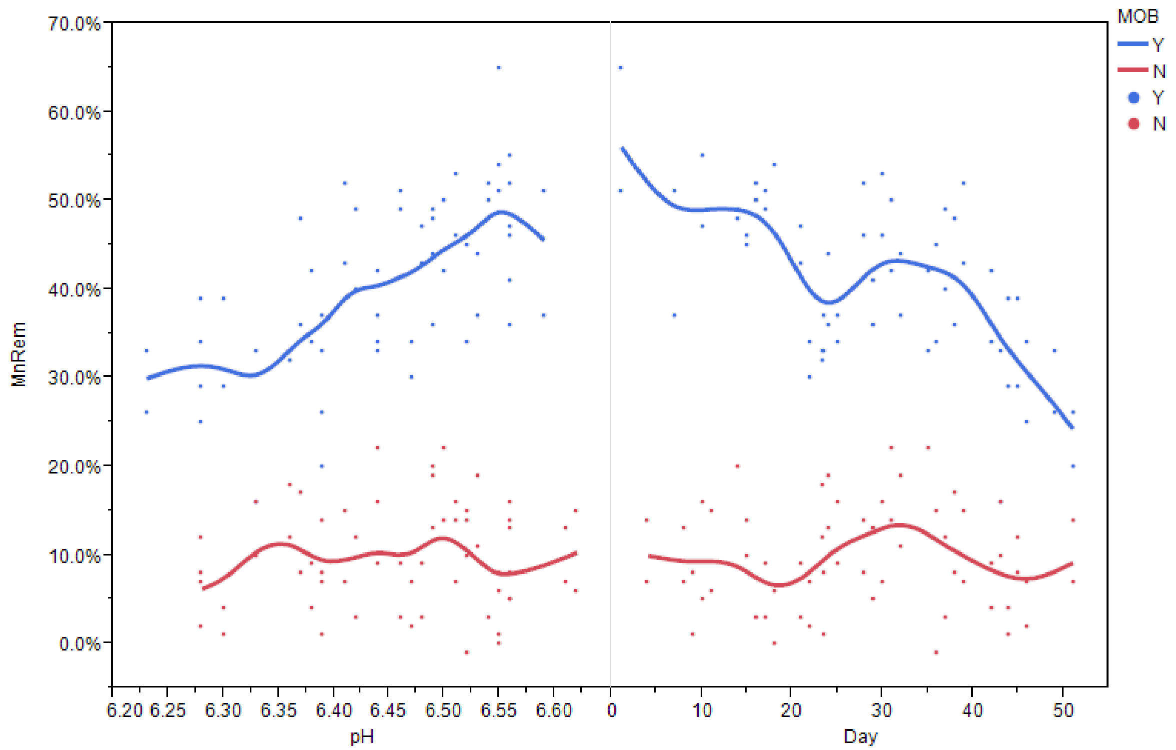

Figure 3-25. Effects of pH and time on Mn removal in MOB-inoculated and control (not MOB-inoculated) columns during pilot study Phase 1

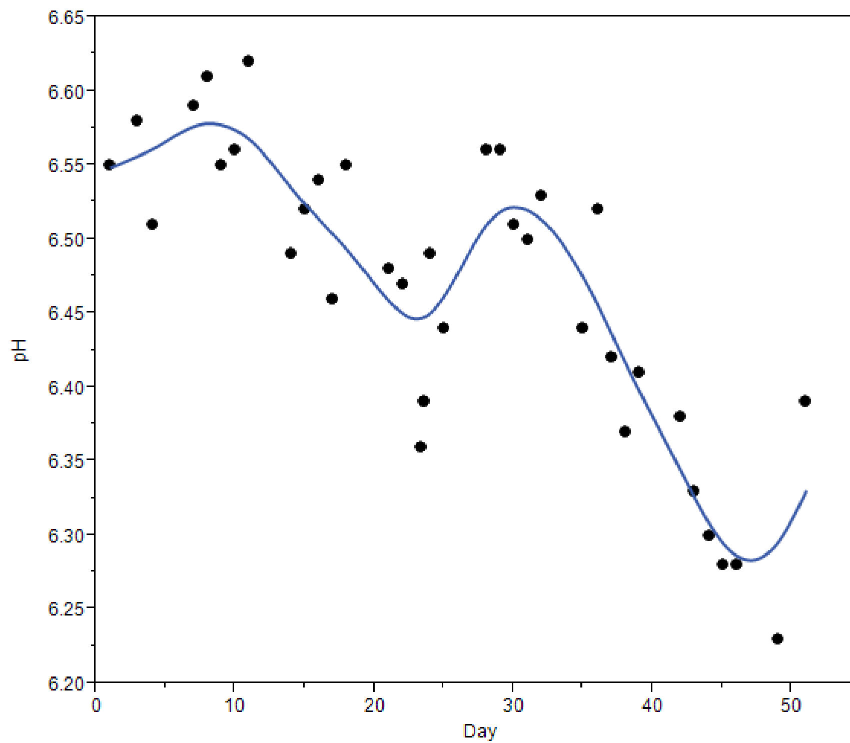

Figure 3-26. Relationship between pH and time during pilot study Phase 1

Filter 1. This means that the Mn removal response to added phosphorus was similar for Filter 1 and Filter 2, but Filter 2 continued to operate with a higher steady-state Mn removal than Filter 1. The Mn removal increase in Filters 1 and 2 is interesting because four of the original six MOB isolates inoculated into the lab-scale columns were originally obtained from Lee Hall water treatment plant (see Table 2-1 in section 2.1) where the pilot plant was located. It is feasible that MOB may already be present in the control filters from the full-scale plant, but conditions are not typically amenable to biological Mn oxidation. Phosphorus addition may have allowed MOB already present in the filters to begin oxidizing Mn.

The hydraulic loading rate increase in Phase 4 coincided with seasonal temperature decrease, as seen in Figure 3-27. This relationship makes discerning which is the more influential factor difficult. Time being a significant factor would indicate that the MOB were not able to acclimate to the higher hydraulic loading rate, and the longer the columns remained at the higher loading rate, the worse Mn removal became. Temperature being a significant factor would indicate that the MOB were possibly slowing down their biological activity and were thus removing less Mn, and that at higher temperatures, the results may have been different. Both are significant using regression analysis when analyzed independently and are too correlated to be analyzed concurrently with any meaning. Mn removal in the filters (Figure 3-28) appears to follow the same pattern as the temperature in Figure 3-27, supporting the hypothesis that the temperature is a driving factor in decreased Mn removal. This Mn removal decrease at lower influent water temperatures also correlates well to the results of the lab-scale temperature study (Figure 3-13). It may be that the increased hydraulic loading rate caused an initial decrease in Mn removal across the columns, and then the temperature decrease caused a corresponding further decrease in Mn removal over time. It should be noted Filter 3 Mn removal was still significantly greater than that of Filters 1 and 2 ($p<0.0001$).

3.4.2 Mn Removal Across Media Depth

Figure 3-29 and Figure 3-30 depict Mn removal down the depth of Filter 3 and Filter 4, respectively, at various points in the pilot-scale study. Mn removal down the filter media depth substantially increased in both Filter 3 and Filter 4 after MOB inoculation. Initially, most of the Mn removal occurred in the top portion of the filters, which was expected since the MOB-coated lab-scale media was placed on top of the pilot-scale column media. Mn removal became more distributed throughout the depth of the column after backwashing once the MOB-coated media was distributed throughout the pilot-scale column media. Mn removal was expected to increase as the MOB acclimated to the pilot-scale column conditions; however, Mn removal remained relatively constant in Filter 3 and decreased over time in Filter 4. The Mn removal profiles down the media depth decreased substantially for both filters. The higher influent Mn concentration in Filter 4 during Phase 2 slightly increased Mn removal down the column profile. The influent phosphorus addition during Phase 3 increased Mn removal down the media depth in Filter 3.

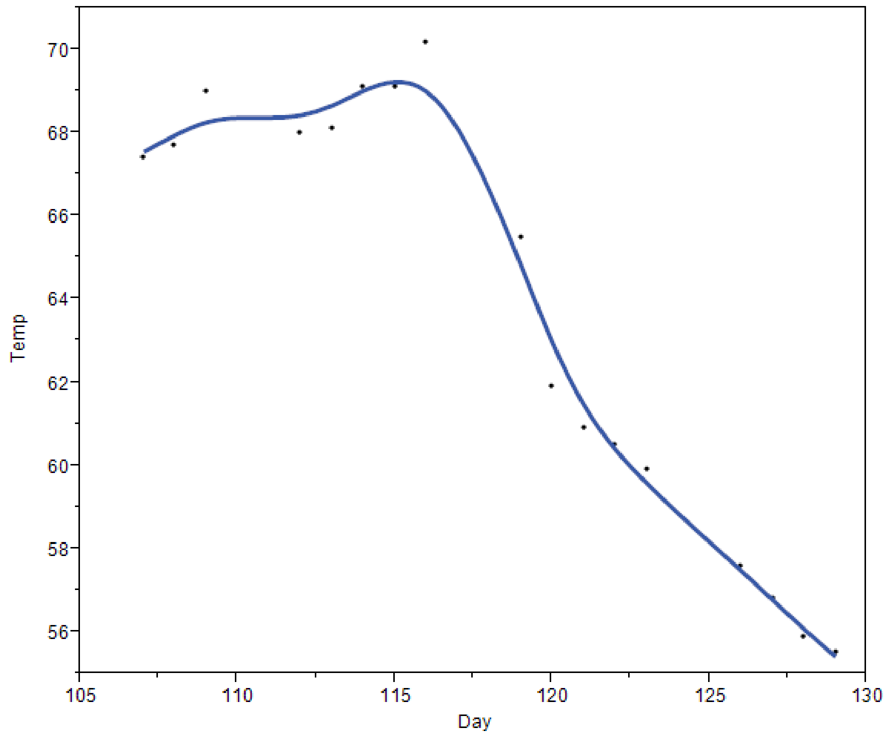

Figure 3-27. Relationship between time and temperature during pilot study Phase 4

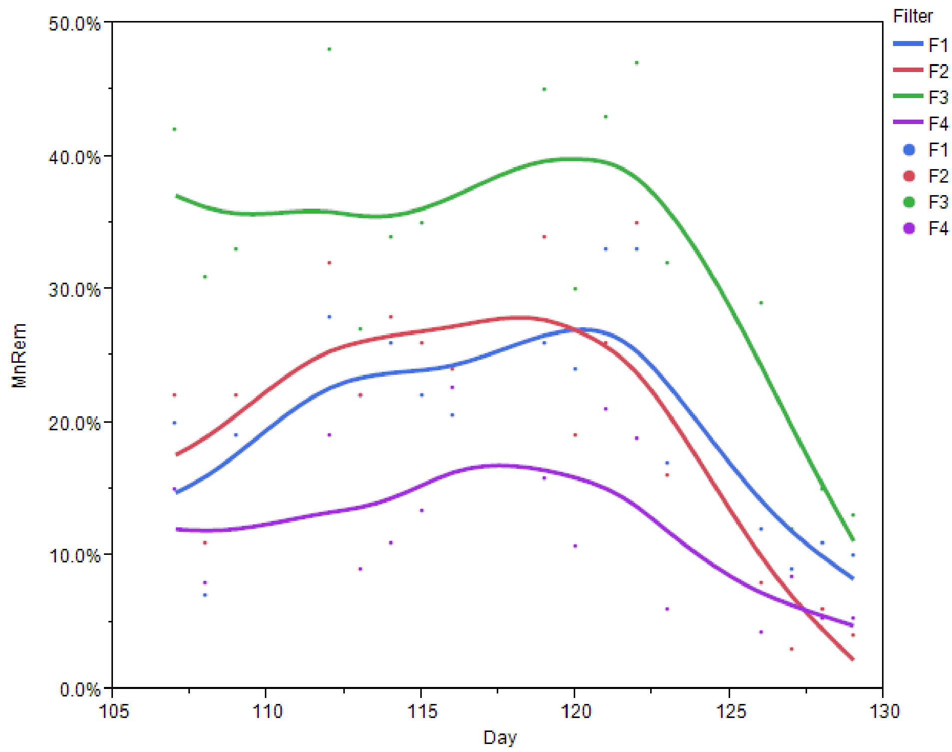

Figure 3-28. Mn removal over time in pilot-scale filters during Phase 4.

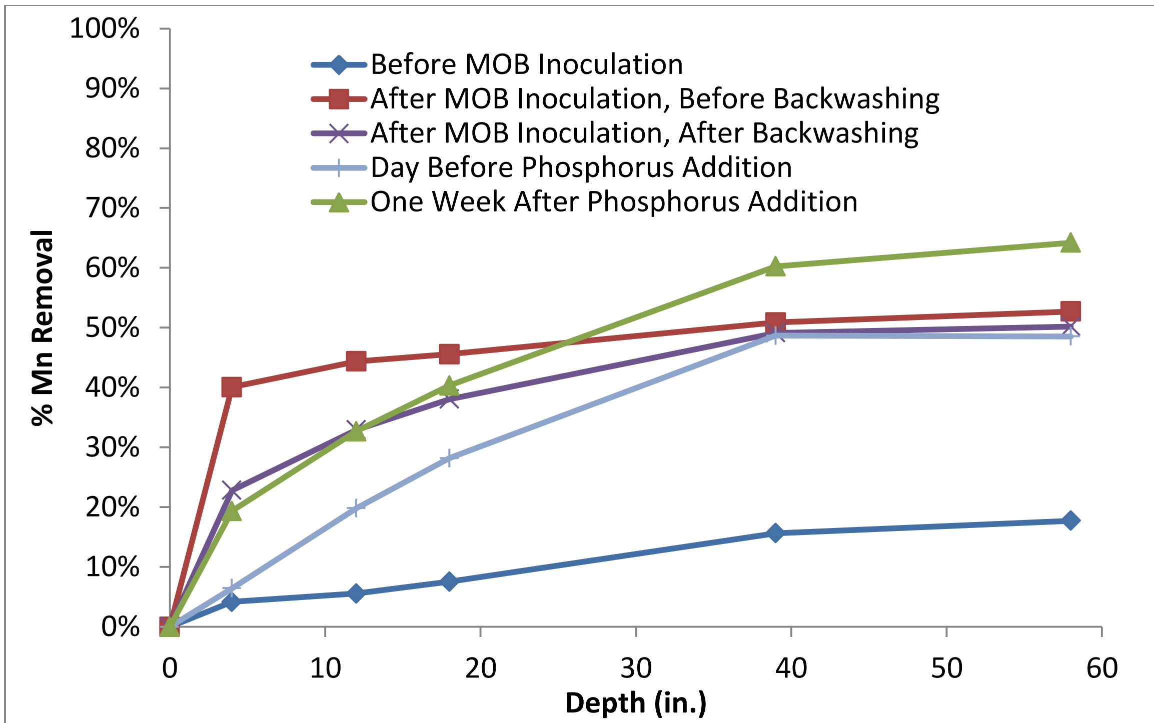

Figure 3-29. Mn removal profiles across Filter 3 media depth at various points during the pilot-scale study

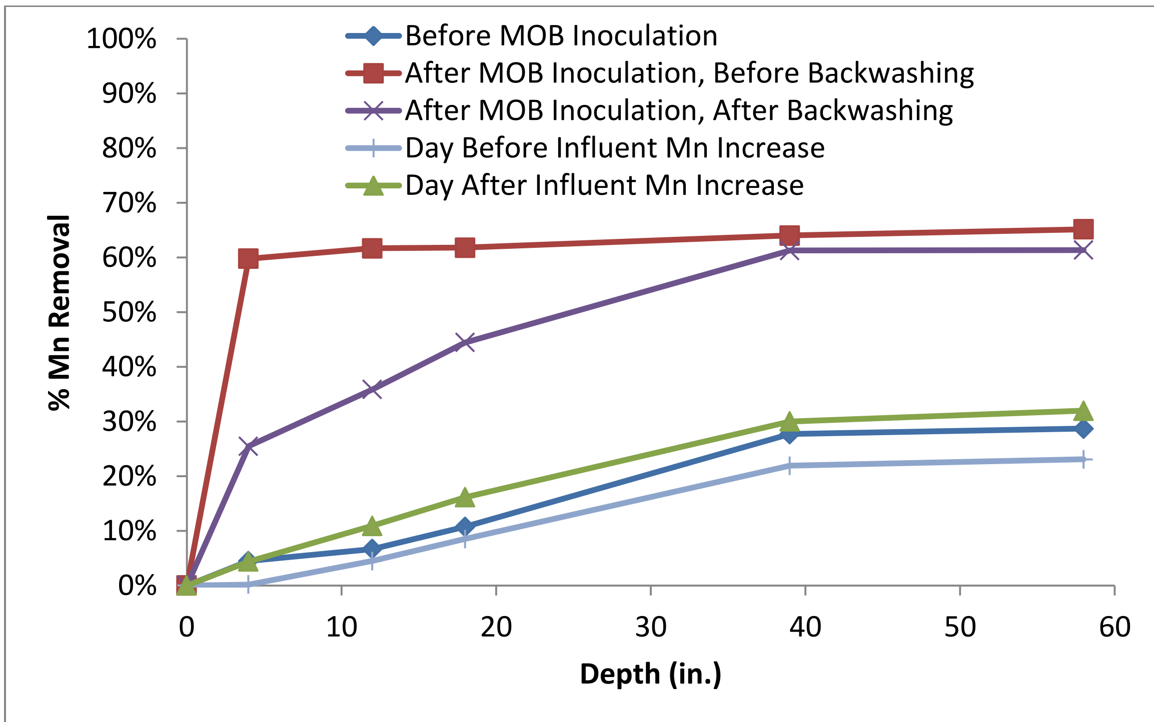

Figure 3-30. Mn removal profiles across Filter 4 media depth at various points during the pilot-scale study

CHAPTER 4: DISCUSSION

4.1 pH RANGE OF MOB ACTIVITY

One of the main objectives of this research was to explore the pH range in which MOB can effectively oxidize and remove Mn in a biofilter setup. The results indicated that MOB are able to oxidize Mn under a broader range of pH conditions than traditionally thought, as demonstrated by the laboratory-scale column startup. Mouchet (1992) asserted that pH greater than 7.4-7.5 was required for biological Mn oxidation, as this was the traditional knowledge on biological Mn removal. The finding that MOB can oxidize Mn as low as pH 6.3 supplements other research which demonstrated that MOB can oxidize Mn as low as pH 6.5 (Burger *et al.* 2008a). Additionally, the columns operating at lower pH (6.3 and 6.7) began removing Mn earlier than those at higher pH (7.0 and 7.3), further indicating that the traditional understanding of the pH requirements of MOB may be limited. In agreement with this finding, Burger *et al.* (2008b) observed better Mn removal at pH 6.5 than pH 7.5 in lab-scale studies.

The effect of pH was clearly demonstrated in the pH change study on Column D. Mn removal did not drastically increase until influent pH was decreased from 7.3 to 6.7 despite over 200 days of acclimation time at pH 7.3. Furthermore, Mn removal began to decrease when the pH was returned to 7.3, suggesting a direct effect of pH on Mn removal; however, an immediate effect of pH was not observed in the batch studies. The observed effect of pH on biological Mn removal was further confounded when the Column D Mn removal decreased to 70% when the pH was increased to 7.3 and subsequently began to increase while the influent was maintained at pH 7.3, eventually achieving Mn removal greater than 98%. This result may indicate that once acclimated, the MOB were able to adjust to pH 7.3 influent and effectively remove Mn. Alternatively, it is probable that once the MOB were acclimated at pH 6.7 and an $MnO_x(s)$ surface had formed, physical-chemical processes began to contribute to soluble Mn sorption and removal. Mn removal in pilot-scale trickling filters has been attributed to both biological and physical-chemical mechanisms (Gouzinis *et al.* 1998). Burger *et al.* (2008a) speculated on the interaction between biological and abiotic Mn oxidation when MOB were not identified in a full-scale groundwater biofilter where greater than 96% Mn removal was occurring at pH 7.2. It is possible that the role that MOB play in soluble Mn removal may be different at a higher influent pH once an $MnO_x(s)$ surface is formed than at lower pH when biological Mn removal appears to be the dominant means for Mn oxidation.

Influent pH did not play a role in biodegradable organic matter (BOM) removal. All four columns were able to achieve high levels of BOM removal. The presence of MOB did not affect BOM removal either, which was demonstrated both during the Phase 1 and 2 lab-scale column startups and during the BOM increase in Column D. The Column D result was particularly

compelling since the biofilm was able to achieve high levels of BOM removal when the influent BOM concentration was five times greater than normal. The Column D biofilm was able to accomplish this while simultaneously removing 98% Mn. The ability of a biofilter to remove BOM effectively is the primary purpose of biofiltration. As such, demonstrating that MOB do not adversely impact BOM removal was important for potential use of MOB in full-scale biofilters.

4.2 Influent Characteristics and Biological Mn Removal

Another objective of this research was to evaluate the effect of various influent characteristics on biological Mn removal. The effects of influent Mn concentration, hydraulic loading rate, biodegradable organic matter concentration, phosphorus concentration, and temperature were assessed through lab-scale stress studies and the pilot study.

4.2.1 Mn Concentration

Influent Mn concentration is variable at surface water treatment plants. The greatest variability is seasonal, with peaks usually occurring in summer during thermal stratification of reservoirs; however, short-term variability can also occur due to changes in upstream treatment. The Mn increase stress study indicated that acclimated MOB communities are resilient enough to handle these common short-term increases in Mn concentration. Burger *et al.* (2008b) noted similar results in acclimated lab-scale groundwater MOB biofilters. Breakthrough of Mn in the filter effluent did not occur in the Mn increase stress study at either pH tested; however, the Mn removal profiles did shift slightly towards higher Mn levels persisting further down the media depth, indicating there may be long-term effects on Mn removal if elevated Mn levels had persisted.

4.2.2 Hydraulic Loading Rate

Hydraulic loading rate may vary to some degree with demand in drinking water treatment plants, so it is important that the microbial community in a biofilter be able to respond to increases in hydraulic loading rate being applied to a filter without breakthrough of contaminants resulting. The lab-scale hydraulic loading rate stress studies did not result in Mn breakthrough during the 24-hour stress period. The Mn removal profile down the column depth did shift slightly over time, indicating that there may be long-term effects of a hydraulic loading rate increase. Stembal *et al.* (2005) found similar shifting of Mn removal profiles in groundwater biofilters at increasing hydraulic loading rates. Similarly, effluent Mn concentration remained unchanged in their study when the hydraulic loading rate was doubled.

The MOB in the pilot study did not respond well to the temporary increase in hydraulic loading rate on Day 50 or Phase 4, where the hydraulic loading rate was increased for the duration of the study. The MOB community in the pilot study differs from that of the lab-scale studies in that the MOB did not seem to be fully acclimated to the pilot-scale column conditions when the hydraulic loading rate increases occurred. The lack of MOB acclimation in the pilot-scale

columns was indicated by Mn removal of around 50% as opposed to the lab-scale columns achieving greater than 98% Mn removal. The observed decrease in Mn removal in the pilot-scale columns may not have been as pronounced if the MOB had been fully acclimated prior to the hydraulic loading rate increase. The concurrent decrease in temperature during Phase 4 may have also played a role in the Mn removal decrease.

4.2.3 Biodegradable Organic Matter

The effect of BOM on biological Mn removal was explored by both changing the influent BOM concentration to Column D and increasing the influent BOM concentration in the Column A stress study. The Column D study did not demonstrate a direct effect of BOM on biological Mn removal, but there were some caveats in the study that are worth discussing. One of the reasons that BOM is suspected to affect MOB communities in biofilters is that the higher BOM concentrations in surface water treatment plants utilizing ozonation may allow other microorganisms to flourish and outcompete MOB for carbon and other nutrients. The influent pH to Column D was 7.3 during this study, and it is feasible that the role that MOB play in the lab-scale filter columns may be different at pH 7.3 than pH 6.7, which would explain the Mn removal results during the pH changes. It may be difficult to draw conclusions about the effect of BOM on biological Mn removal when considering this potential difference between the two pH conditions. Additionally, a slight effect of increased BOM on Mn removal by MOB was observed in the stress study on Column A at pH 6.3; however, this study had a 24-hour duration, which may not be enough to fully capture the long-term effects.

4.2.4 Phosphorus

Phosphorus addition to the pilot-scale columns significantly increased Mn removal in the control columns and one of the MOB-inoculated columns. The reason for the Mn removal increase in the control columns has not been determined, but it is feasible that MOB may already be present in the biofilters. Four of the six MOB isolates inoculated into the lab-scale columns were originally obtained from Lee Hall water treatment plant, where the pilot plant was located. Furthermore, Cerrato *et al.* (2010) found sixteen MOB isolates in the sedimentation basins and four isolates in the filtrations basins at Lee Hall water treatment plant. It is possible that MOB present in the filters are unable to oxidize and remove Mn due to a lack of nutrients or other factors. The increase in Mn removal after phosphorus addition supports this hypothesis. A Mn removal increase resulting from phosphorus addition was observed by Lauderdale *et al.* (2012), in addition to other benefits such as reducing head loss and increasing DOC removal in the biofilters. The phosphorus concentration in the lab-scale column influent was elevated due to the corrosion inhibitor added to the tap water at the treatment plant (average 0.24 mg/l as P), so the lab-scale columns did not experience phosphorus limitation.

4.2.5 Temperature

The lab-scale column temperature decrease study indicated that well-acclimated MOB can withstand temperatures common in a mild winter. Mn removal decreases with decreasing

temperature, but this is not a concern for most surface water treatment plants. Soluble Mn in surface water sources is typically only a summer concern, as thermal stratification of reservoirs and lakes allows anaerobic reduction of oxidized Mn in the sediments. The more critical factor is Mn removal recovery after temperatures increase. The MOB were able to increase Mn removal once influent water temperature was increased, eventually achieving essentially complete Mn removal (>98%). Mn removal in the pilot-scale columns decreased to less than 10% removal during Phase 4 as the temperature decreased, although whether this was due to the MOB not being fully acclimated or the concurrent increase in the hydraulic loading rate is unknown.

4.3 MOB Acclimation Time

MOB are known to have extended acclimation times (Mouchet 1992), which may be a barrier to full-scale implementation. The lab-scale column startup required 50 days from initial Phase 1 inoculation to begin removing Mn. This time period was only two weeks after Phase 2 reinoculation and setup alteration. Thus, it is unknown whether Phase 2 changes stimulated Mn removal or if the MOB required the extended acclimation time, and the timing of Phase 2 startup was coincidental. Even once Mn removal began, nearly complete removal was not reached for an additional 50 days. Mouchet (1992) found a similar slow decline in Mn removal, with the time between initial Mn removal and near complete removal taking approximately 35 days. Additionally, Burger *et al.* (2008b) found that a lab-scale groundwater MOB biofilter required six weeks for acclimation.

The effect of influent Mn concentration on MOB acclimation time was not clearly demonstrated by the lab-scale studies. The increased Mn concentration during Phase 2 startup of the lab-scale columns may have aided the eventual stimulation of Mn removal. As previously mentioned, most studies on the use of MOB in biofiltration have involved groundwater systems which generally have higher influent Mn concentration (Mouchet 1992; Vandenabeele *et al.* 1992; Hope and Bott 2004; Katsoyiannis and Zouboulis 2004; Li *et al.* 2006; Burger *et al.* 2008a). Hope and Bott (2004) recommended an initial Mn concentration of 2.5 mg/L in a recirculation setup for fastest acclimation. The lab-scale columns were operated in this configuration during reinoculation in Phase 2 for the first week of operation before starting normal flow at 0.5 mg/L Mn. This may have contributed to the Mn removal increase shortly after Phase 2 reinoculation.

4.4 Presence of MOB in Mature Biofilters

The most prominent conclusion from the biological analyses is that MOB comprise a small percentage of the total microbial population in mature surface water treatment biofilters. The Mn-oxidation agar plating analysis yielded the highest MOB composition at 6% of the total heterotrophic plate count. This is markedly different from other studies evaluating the MOB presence in mature full-scale groundwater treatment biofilters. Vandenabeele *et al.* (1992) found that MOB can make up 25-33% of the bacteria present in biofilms of mature groundwater biofilters. The stark difference in the MOB composition can most likely be attributed to the

presence of biodegradable dissolved organic carbon (BDOC) in surface water treatment biofilters that utilize upstream ozonation. Groundwater treatment plants typically do not use ozonation for primary disinfection, so BDOC is much lower in the biofilter influent water than for surface water treatment plant biofilters. BDOC may encourage proliferation of heterotrophs other than MOB, accounting for lower MOB composition in the microbial community.

The *rep*-PCR analysis revealed the majority of the colonies selected for analysis that exhibited MnO_2 formation were related to MOB isolate MB-3. It is possible that other isolates were present, but the frequency with which MB-3 was identified indicates that MB-3 was most likely the dominant MOB isolate in the lab-scale columns. The reasons for one MOB isolate proliferating more than others are elusive because much is still unknown about the ability of MOB to oxidize Mn. MB-3 belongs to the species *Bacillus pumilus* that was originally isolated from the Newport News Lee Hall water treatment plant where the lab-scale media was obtained. *Bacilli* are known for their ability to form spores to survive environmental threats. It may be that MB-3 was ideally suited for survival in the lab-scale biofilters because it was already adapted to the biological community present in the Newport News biofilter media and could better withstand sharp changes in the lab-scale biofilter influent, which occasionally occurred. It is also feasible that *Bacilli* MOB species require stressful conditions to begin oxidizing Mn, as these species are known to oxidize Mn in their spore form (Devrind *et al.* 1986; Bargar *et al.* 2000) which could be a reason for the extended acclimation time. The MB-3 growth curve (Figure A-2 in the Appendix) may provide a further clue as to why Column D was unable to remove substantial Mn at pH 7.3. The lag phase of MB-3 is much longer at pH 7.4 than at pH 6.0 or 6.5. MB-3 may grow and oxidize Mn differently at higher pH than lower pH.

DGGE analysis determined that the samples from Column D before and after the pH change were not statistically different; however, both Column D samples from after Phase 2 inoculation were statistically different from the samples from Columns A, B, and C. The higher pH in Column D is most likely the cause of the difference in the biological communities, since all other parameters were identical across the four columns. A change in the microbial community was probably not the cause of the changes in Mn removal observed during the pH change experiments with Column D since the biological community before and after the pH change was the same. This suggests a more direct effect of pH either on the MOB or on other microorganisms present in Column D that may have been inhibiting biological Mn oxidation by MOB. The identification of MOB using DGGE analysis has limitations. Each band should represent an individual species, referred to as an operational taxonomic unit (OTU). Bands at the same location in different lanes are concluded to be part of the same OTU. Unfortunately, co-migration can occur allowing two different species to be represented in the same band location (Gafan and Spratt 2005). Thus, an indication of the presence of an MOB group may accidentally include other organisms. A lack of a band at the location where an MOB isolate is known to be

identified means that if that MOB isolate was present, the concentration of cells in the media sample was too low to detect.

Interestingly, the DGGE analysis did not identify MB-3 (Group 1) in the 1-26 samples, when the columns were achieving nearly complete Mn removal, whereas *rep*-PCR indicated that this was the dominant MOB isolate present in the columns. It should be noted that the media samples for *rep*-PCR and DGGE were collected two months apart. It is certainly feasible that the microbial population might have changed in the columns over time due to slight changes in influent conditions, as well as the stress studies. Additionally, MB-33 (Group 2) was not identified in the media sample collected directly after inoculation or in two of the four samples collected when the columns were not removing Mn in Phase 1. These results contradict Mn-oxidation agar plating results which observed MB-33 as the dominant species during Phase 1. It is possible that the species identified on the Mn-oxidation plates were not MB-33, but morphologically resembled MB-33. *Rep*-PCR analysis was not used to confirm the presence of MB-33 on the Mn-oxidation plates from Phase 1.

There were limitations to the biological analysis techniques used to identify MOB presence, and these limitations are worth discussing. MOB are phylogenetically diverse, and many different species have demonstrated the ability to oxidize Mn (Tebo *et al.* 2004). As such, detecting MOB using current biological analysis methods can be challenging. A target gene that identifies MOB has not yet been located, so DNA based analytical methods rely on matching the isolates identified in samples to known MOB isolates. This can be particularly challenging since bacteria have the ability to mutate over the course of generations which complicates matching to a known MOB isolate, as seen in the *rep*-PCR results. The colonies with visible MnO_2 formation in some cases did not match the original MOB isolates well. DGGE was able to give a broader picture of the microbial community in the lab-scale columns; however, the MOB results were limited. DNA specific techniques have been developed for a known MOB isolate, *Leptothrix discophora*; however, *Leptothrix discophora* is not always found on mature full-scale MOB biofilters (Burger *et al.* 2008a). Plating techniques using Mn-oxidation agar can be challenging as MOB comprise such a small percentage of the total microbial population. Typical dilution methods can make finding the MOB difficult. Other studies have had difficulties locating MOB in mature full-scale and lab-scale groundwater biofilters using plating methods despite substantial observed biological Mn removal (Burger *et al.* 2008a; Burger *et al.* 2008b).

4.5 Suitability of MOB for Mn Control in Surface Water Treatment Plants

The lab-scale study demonstrated that MOB have potential for use in surface water treatment plants, as previously discussed; however, the pilot study shows that further study is required to better understand how MOB function under surface water treatment plant biofilter conditions. There are clearly underlying factors that affect the ability of MOB to oxidize and remove Mn that are not yet fully understood. The pilot study demonstrated that pre-acclimated MOB can

continue to remove Mn when relocated to another filter. The Mn removal in Filter 4 decreased over time, but pH was a likely factor in the decline. Filter 3 Mn removal was statistically higher than that of Filter 4 in Phase 1, when the influent parameters to both columns were identical. This may be attributed to the pH at which the lab-scale media was acclimated. Filter 3 received media from Column A (pH 6.3), and Filter 4 received media from Column B (pH 6.7). The MOB in Column A may have been better suited to the lower pH in the pilot-scale biofilters than that from Column B. Additionally, pH was a statistically significant factor in Mn removal in the MOB-inoculated columns in Phase 1 and Phase 2, supporting the hypothesis that pH may be a driving factor in MOB acclimation. This may mean that water plant operators need to adjust filter influent pH to achieve an acceptable balance between Mn removal and organics control through enhanced coagulation.

The increase in Mn removal in the control columns during Phase 3 of the pilot plant study when phosphorus was added was particularly interesting. It is feasible that MOB were present in the control columns already from the influent water pumped from the full-scale plant. Phosphorus limitation may have been preventing the MOB from oxidizing Mn in the control columns. There may be other conditions present that are preventing the MOB from becoming fully acclimated that were not identified in the timeframe of the pilot study. Other studies have had similar issues with MOB acclimation in pilot- and full-scale studies. MOB have required eight weeks to increase Mn removal in pilot-scale groundwater roughing upflow filters from 10% to 90% (Pacini *et al.* 2005). Li *et al.* (2006) were only able to achieve a maximum of 40% Mn removal after inoculating a groundwater biofilter with MOB after four months of acclimation time. They were able to achieve an increase to approximately 55% Mn removal in month five, which finally increased to 90% removal in month seven. The pilot study at Newport News was operated for just under five months. It is feasible that more time may be required to achieve greater Mn removal, especially considering the dynamic influent characteristics present at surface water treatment plants compared with groundwater treatment plants.

CONCLUSION

Lab-scale and pilot-scale studies have demonstrated that MOB have potential for use in surface water treatment plants for soluble Mn control. The mechanism and purpose of biological Mn removal is not well understood; however, insight into the ability of MOB to oxidize Mn under surface water treatment plant conditions has been gained. The following conclusions can be drawn from the results of these experiments:

1. MOB can oxidize and remove soluble Mn in a biofilter setup at as low as pH 6.3, at which surface water treatment plants typically operate for enhanced coagulation.
2. Biological Mn removal can be achieved without adversely affecting biodegradable organic matter (BOM) removal, allowing biofilters to achieve their primary objectives: increasing finished water biostability and reducing the formation of chlorinated disinfection by-products.
3. Once acclimated, MOB can tolerate short-term (e.g. 1-2 days) changes in influent parameters commonly present at surface water treatment plants, including influent increases in Mn concentration, BOM concentration, and hydraulic loading rate without Mn breakthrough occurring. Long-term effects of these changes may be more pronounced.
4. MOB can withstand and recover from influent water temperature decreases simulating a mild winter.
5. MOB may be present in full-scale surface water treatment plants due to their ubiquity in the environment and may require changes in biofilter operational parameters, such as phosphorus supplementation, to be able to oxidize Mn effectively.

FUTURE RESEARCH

There is still much to learn about the nature of MOB and their ability to oxidize Mn effectively in surface water treatment plants. The dynamic nature of influent conditions at surface water treatment plants requires a greater understanding of MOB before successful full-scale implementation can be expected. The following areas of research would add to the current knowledge of MOB and would benefit the application of MOB to surface water treatment plants:

1. Identification of factors that reduce acclimation time and optimize soluble Mn removal by MOB including, but not limited to, nutrient supplementation and pH adjustment.
2. Development of DNA-based analysis techniques that target the identification of MOB.
3. Evaluation of the presence of MOB in full-scale biofilters.

The difficulties in acclimating MOB in pilot- and full-scale filters indicate that further research is needed to identify what operational parameters might reduce acclimation time. The potential presence of MOB in surface water treatment plant biofilters opens another area of study.

Identifying what may be preventing MOB present in full-scale biofilters from oxidizing Mn might allow surface water treatment plants to stimulate biological Mn removal by adjusting operational parameters. Future research into these areas may allow MOB to become a viable option for full-scale implementation at surface water treatment plants with chronic Mn control issues.

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APPENDIX A

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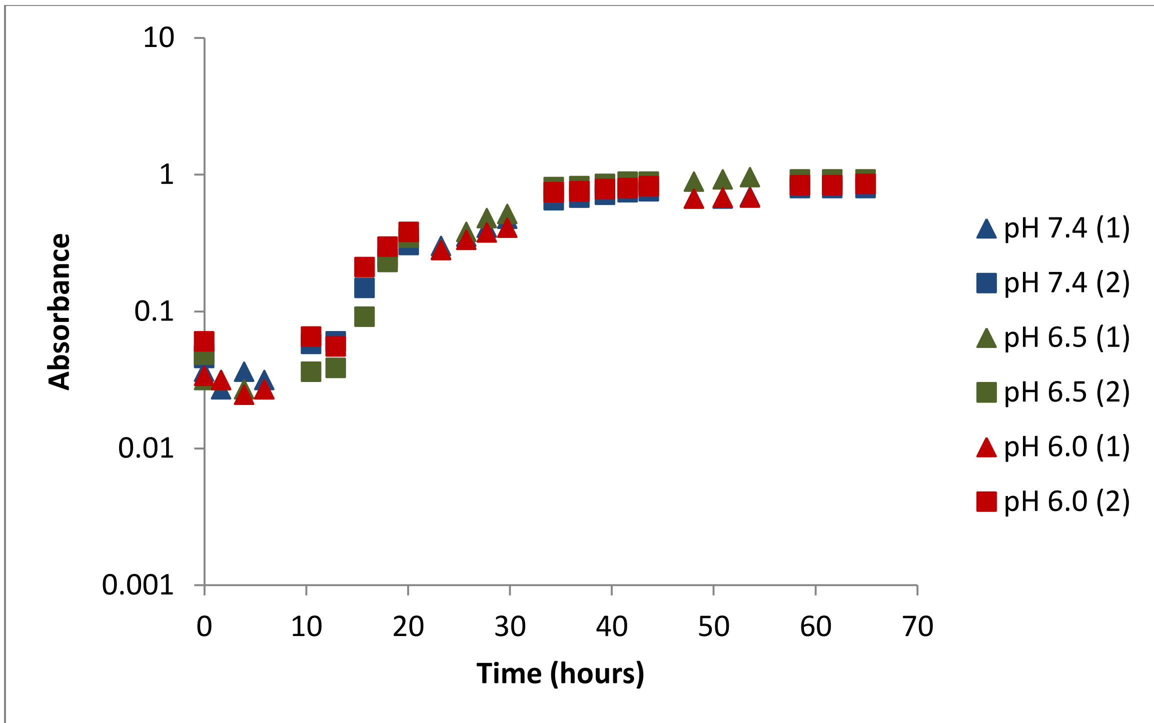

Figure A-1. Detailed growth curve for MOB isolate MB-2. Curves were developed over two trials, indicated by the numbers in parentheses.

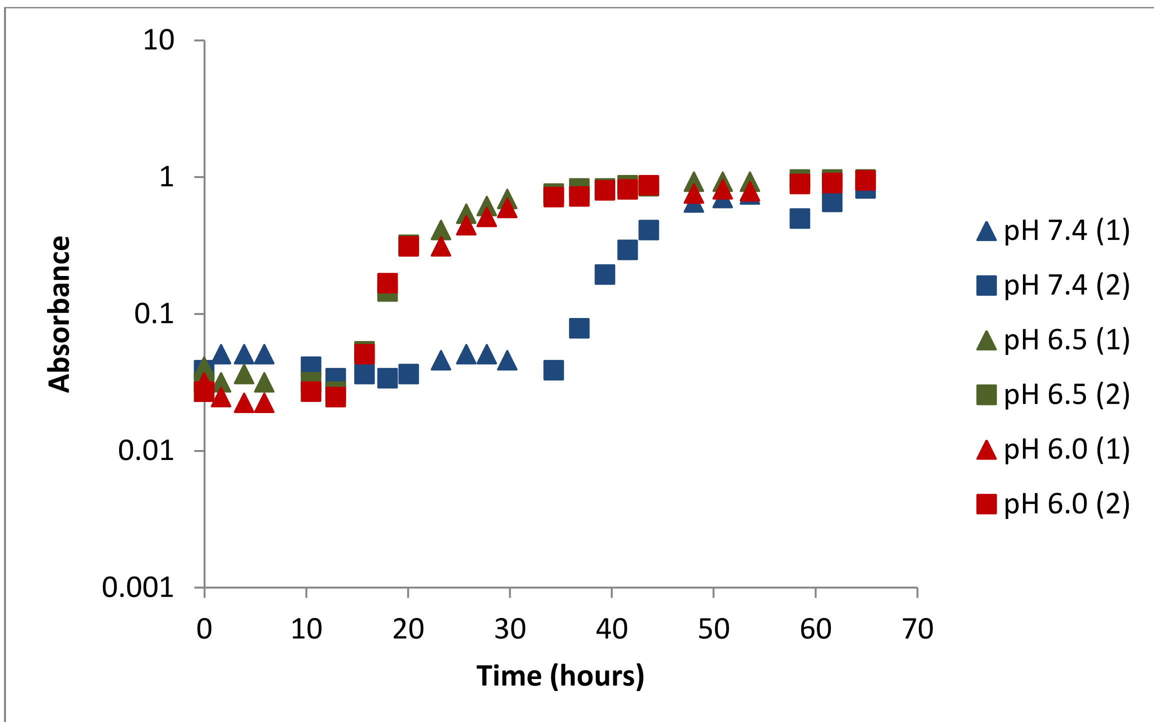

Figure A-2. Detailed growth curve for MOB isolate MB-3. Curves were developed over two trials, indicated by the numbers in parentheses.

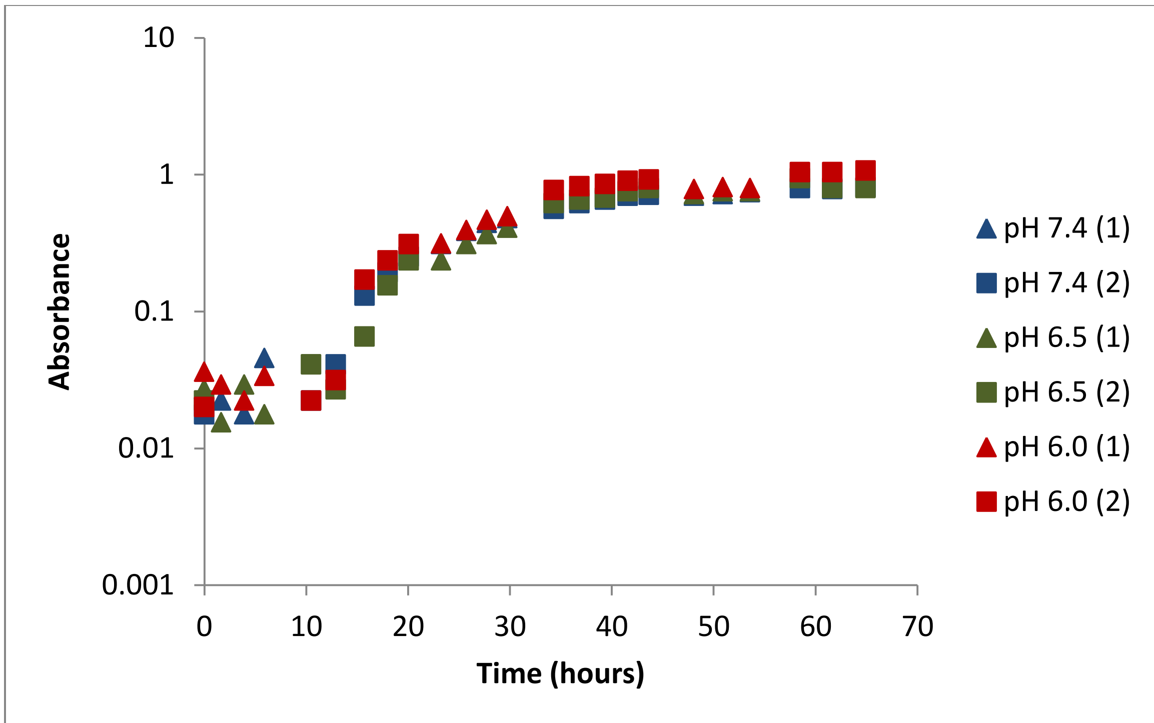

Figure A-3. Detailed growth curve for MOB isolate MB-17. Curves were developed over two trials, indicated by the numbers in parentheses.

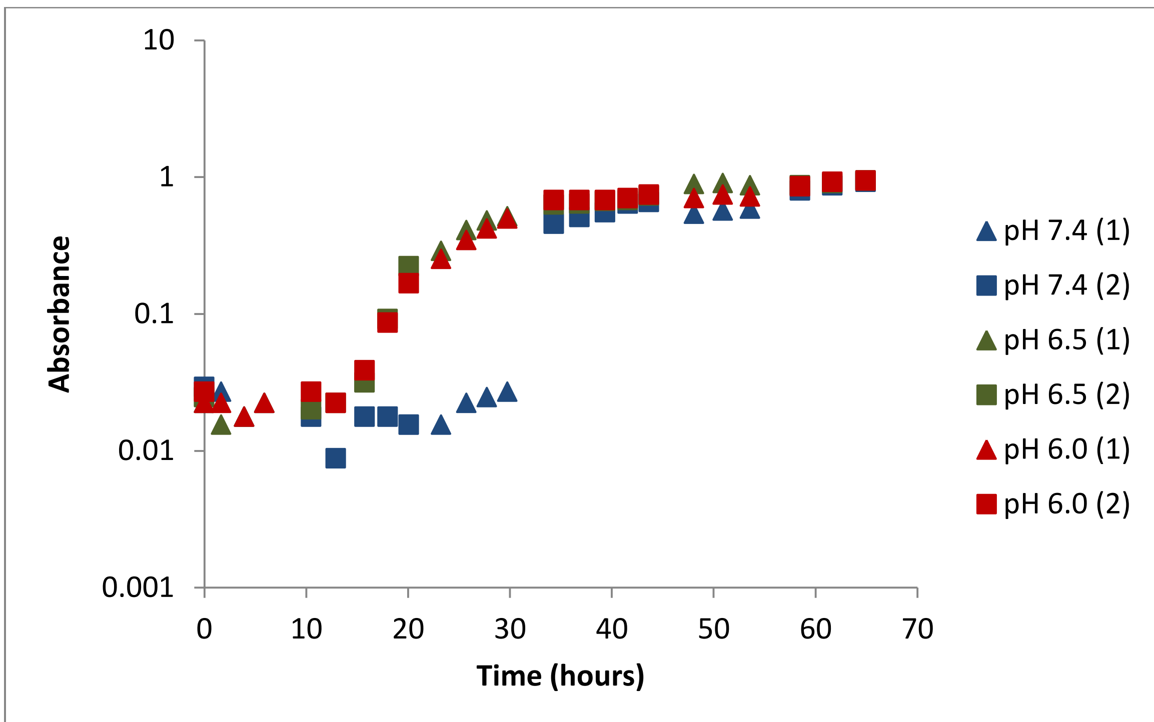

Figure A-4. Detailed growth curve for MOB isolate MB-22. Curves were developed over two trials, indicated by the numbers in parentheses.

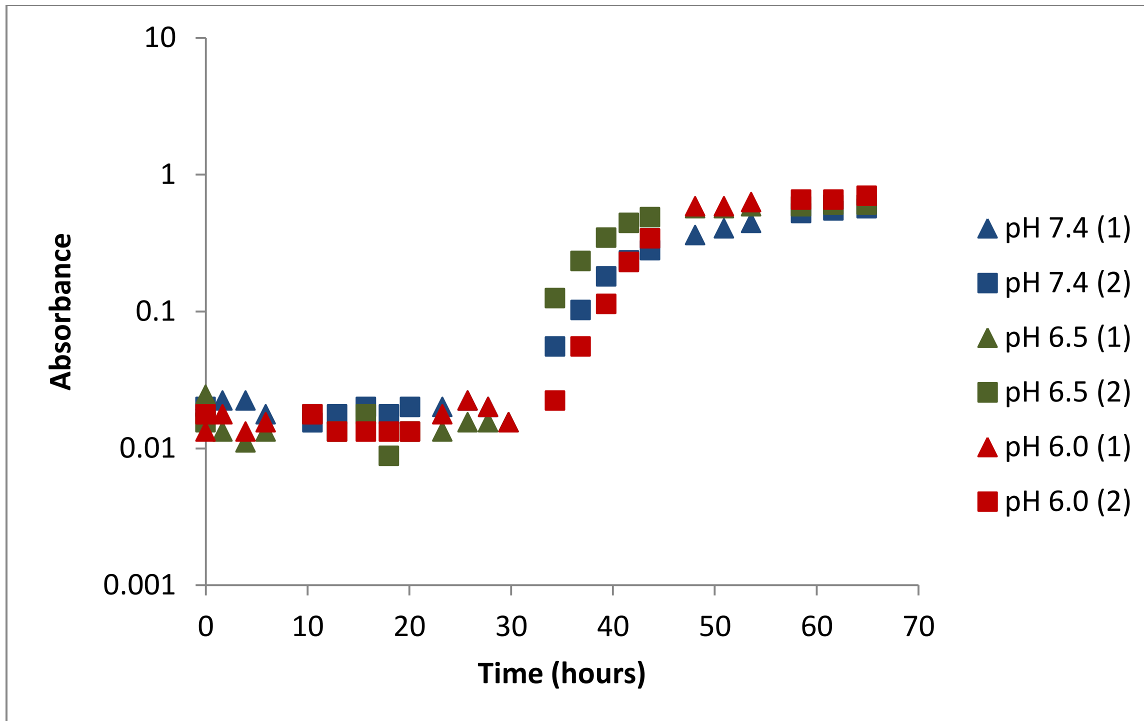

Figure A-5. Detailed growth curve for MOB isolate MB-33. Curves were developed over two trials, indicated by the numbers in parentheses.

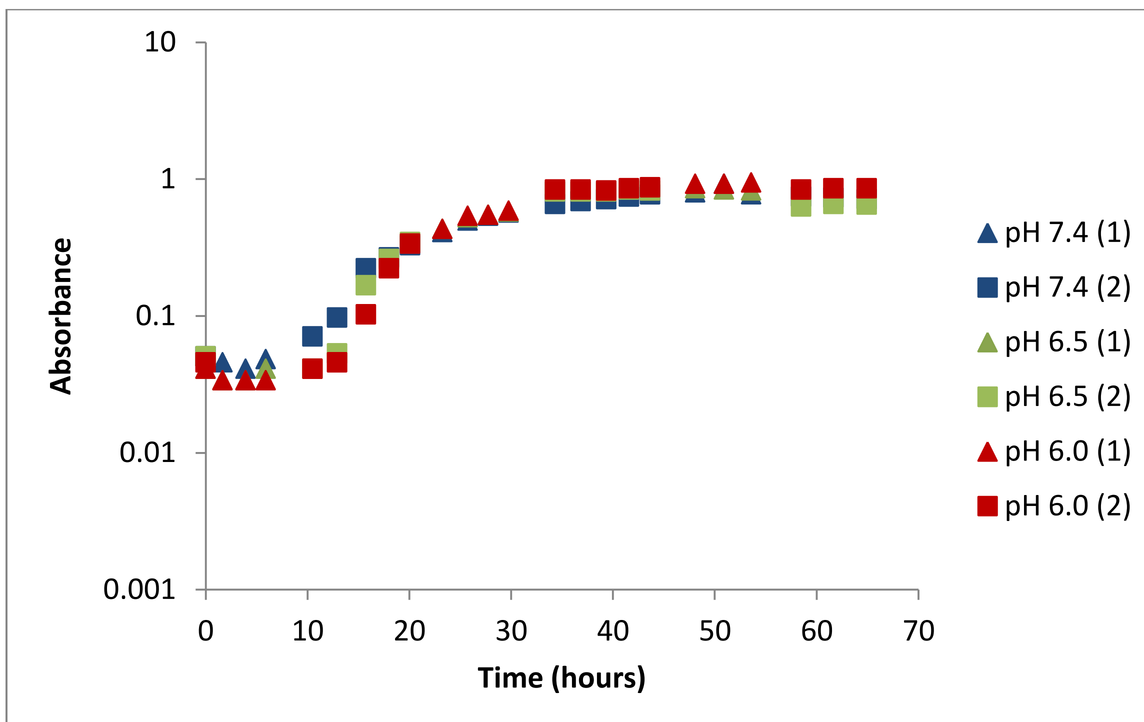

Figure A-6. Detailed growth curved for MOB isolate MB-38. Curves were developed over two trials, indicated by the numbers in parentheses.

Table A-1. Ordinary least squares regression model results for pilot study Phase 1 evaluating the effects of time, pH, and presence of MOB on Mn removal

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	-1.355807	0.683996	-1.98	0.0497*
Day	-0.000909	0.000767	-1.18	0.2384
pH	0.253128	0.103441	2.45	0.0159*
MOB[Y]	0.1590347	0.006497	24.48	<.0001*

Table A-2. Ordinary least squares regression model results for pilot study Phase 1 evaluating the effects of pH and presence of MOB on Mn removal

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	-1.914134	0.410169	-4.67	<.0001*
MOB[Y]	0.1589175	0.006002	26.48	<.0001*
pH	0.3360175	0.063527	5.29	<.0001*
(pH-6.45573)*MOB[Y]	0.2997714	0.063527	4.72	<.0001*

Table A-3. Ordinary least squares regression model results comparing pilot-scale Filter 3 and Filter 4 during Phase 1

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	-3.690459	0.604553	-6.10	<.0001*
pH	0.635789	0.093742	6.78	<.0001*
Filter[F3]	0.0229032	0.00896	2.56	0.0132*

Table A-4. Ordinary least squares regression model results comparing Filter 1 and Filter 2 Mn removal during Phase 1 and Phase 2. pH was not included in the model because it was not statistically significant for this data subset.

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	0.1020658	0.005573	18.31	<.0001*
Filter[F1]	-0.034434	0.005573	-6.18	<.0001*

Table A-5. Ordinary least squares regression model results for pilot study Phase 2 comparison between Filter 3 and Filter 4

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	-4.02723373	0.6075903894	-6.63	<.0001*
pH	0.6868473776	0.0942100161	7.29	<.0001*
Filter[F3]	0.0307692308	0.0082907545	3.71	0.0004*
(Phase1/Phase2-0.20513)*Filter[F3]	0.0383467742	0.0205320741	1.87	0.0658

Table A-6. Ordinary least squares regression model results for Phase 2 evaluating the effect of the presence of MOB on Mn removal

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	-6.919541	1.782156	-3.88	0.0005*
pH	1.1117021	0.276295	4.02	0.0004*
MOB[Y]	0.1228125	0.013394	9.17	<.0001*

Table A-7. Ordinary least squares regression model results for Phase 3 evaluating the effect of phosphorus addition on Mn removal

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	-1.935483	0.389138	-4.97	<.0001*
pH	0.3548116	0.060073	5.91	<.0001*
MOB[Y]	0.1355611	0.005555	24.40	<.0001*
MOB[N]:Filter[F1]	-0.041031	0.006399	-6.41	<.0001*
Phase2/Phase3[1]	0.0853576	0.005735	14.88	<.0001*
MOB[Y]*Phase2/Phase3[1]	-0.037232	0.005735	-6.49	<.0001*
MOB[N]:Filter[F1]*Phase2/Phase3[1]	-0.006601	0.006399	-1.03	0.3038
(pH-6.47126)*MOB[Y]	0.3199023	0.060073	5.33	<.0001*

Table A-8. Ordinary least squares regression model results for pilot study Phase 3 evaluating the difference between Filter 3 and Filters 1 and 2.

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	-1.463413	0.96926	-1.51	0.1369
pH	0.2953319	0.149066	1.98	0.0527
MOB[Y]	0.1080263	0.013467	8.02	<.0001*

Table A-9. Ordinary least squares regression model results for pilot study Phase 4 evaluating the effect of time and the presence of MOB on Mn removal

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	0.9882224	0.214219	4.61	<.0001*
Day	-0.006143	0.001809	-3.40	0.0014*
MOB[Y]	0.0630074	0.013266	4.75	<.0001*

Table A-10. Ordinary least squares regression model results for pilot study Phase 4 evaluating the effect of temperature and the presence of MOB on Mn removal

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	-0.371042	0.147235	-2.52	0.0151*
Temp	0.0099405	0.002303	4.32	<.0001*
MOB[Y]	0.0630074	0.012539	5.03	<.0001*