

Biofiltration for Manganese Removal from Groundwater: Mechanistic Insights and Operational Strategies

by

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Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Statement of Contributions

This research was conducted at the University of Waterloo and partner utility by Hemant Arora under the supervision of Dr. Peter M. Huck and Dr. Sigrid Peldszus. The funding for this project was provided by the Natural Sciences and Engineering Research Council (NSERC) in the form of an Industrial Research Chair in Water Treatment (grant no. NSERC IRC PJ#147477-17) at the University of Waterloo, Ontario, Canada. Chair partners are listed at <https://uwaterloo.ca/nserc-chair-water-treatment/partners>.

Hemant Arora was the sole author of **chapters 1 and 7**, which were written under the supervision of Dr. Peter M. Huck and Dr. Sigrid Peldszus and were not written for publication.

This thesis consists in part of six manuscripts written for publication. Exceptions to sole authorship of material are as follows:

Literature review presented in **chapter 2**: Hemant Arora contributed to the conceptualization, data curation, investigation, visualization, writing the original draft, and submission of manuscripts. Dr. Sigrid Peldszus and Dr. Peter M. Huck contributed to the conceptualization, supervision, reviewing, and editing of the manuscript. Dr. Robin Slawson contributed to the reviewing and editing of the manuscript.

Research presented in **chapter 3**: Hemant Arora contributed to the conceptualization, methodology, formal analysis, data curation, investigation, visualization, writing the original draft, and submission of manuscripts. Dr. Sigrid Peldszus and Dr. Peter M. Huck contributed to the conceptualization, methodology, supervision, reviewing, and editing of the manuscript. Dr. Robin Slawson contributed to the methodology, reviewing, and editing of the manuscript. Nicholas Lamba-Rautapuro contributed to the formal analysis (HPC and MnOB). Dr. Brian Kendall contributed to the formal analysis (ICP-MS), reviewing, and editing of the manuscript. Submitted to *Water Research* journal.

Research presented in **chapters 4, 5, and 6**: Hemant Arora contributed to the conceptualization, methodology, formal analysis, data curation, investigation, visualization, writing the original draft, and submission of manuscripts. Dr. Sigrid Peldszus and Dr. Peter M. Huck contributed to the conceptualization, methodology, supervision, reviewing, and editing of the manuscript. Dr. Robin Slawson contributed to the methodology, reviewing, and editing of the manuscript. Michelle M. McKnight and Dr. Josh D. Neufeld contributed to the formal analysis (DNA sequencing), reviewing,

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Abstract

Groundwater is an essential source of drinking water worldwide. Among various contaminants that are present in groundwater, manganese (Mn) is one of them. Manganese in drinking water can cause aesthetic and operational problems and has been associated with cognitive and neurobehavioral effects in children. In response, Health Canada has established as guidelines a maximum acceptable concentration (MAC) of 120 µg/L and an aesthetic objective (AO) of 20 µg/L for Mn in drinking water.

Biofiltration provides an environmentally friendly and effective method for removing Mn from water, as it does not require chemicals and does not produce harmful by-products. However, limitations include a prolonged start-up period with virgin media and diminished efficacy at lower water temperatures (<15°C) due to reduced microbial activity, particularly when iron (Fe) is present as a co-contaminant in groundwater. Additionally, while biofilters are typically operated continuously (24 h/d), intermittent operation (6-12 h/d) in small-scale or remote communities, depending on local demand, may affect the performance of biofilters. Despite research on Mn removal mechanisms by biofiltration, the evolution of these processes as biofilters mature requires further investigation. Consequently, this research aims to deepen the understanding of Mn removal mechanisms in biofilters from startup to maturity and to investigate the influence of filter media characteristics and operational modes (intermittent vs. continuous) on the performance of biofilters for Mn and Fe removal.

The research was conducted in three phases, utilizing a combination of pilot-scale biofilters and bench-scale batch experiments. Pilot-scale biofilters were designed and constructed at a drinking water facility in Southern Ontario, Canada and were operated under various configurations for approximately 400 days with raw groundwater containing Mn and Fe. Concurrent bench-scale batch experiments with and without inhibitors were conducted to elucidate different Mn removal mechanisms. This study employed multiple analytical techniques such as scanning electron microscopy (SEM), energy dispersive X-ray (EDX), Raman spectroscopy, adenosine triphosphate (ATP) measurements, extracellular polymeric substance (EPS) analysis, cultural plating techniques, and 16S rRNA gene sequencing.

Phase one evaluated the impact of different filter media, including granular activated carbon (GAC), sand, and anthracite, on startup, Mn removal mechanisms, and microbial community dynamics. Findings indicated that filter media characteristics influence the startup period of Mn removal; GAC

biofilters primarily initiated Mn removal through adsorption, transitioning to biological and physicochemical processes, while sand and anthracite predominantly engaged in biological processes. The batch tests confirmed these findings, with sand and anthracite media showing biological dominance at the top layer and GAC media exhibiting physicochemical dominance throughout. The presence of manganese-oxidizing bacteria (MnOB) genera varied across biofilter media types and depths, highlighting the complex interplay between biofilter media and microbial colonization patterns.

Phase two focused on the evolution of Mn removal mechanisms in a sand biofilter from startup to maturity, utilizing a combination of pilot-scale biofilter and bench-scale batch experiments. The study revealed an initial dominance of biologically generated manganese oxides (Bio-MnO_x), which gradually transitioned to physicochemical forms of MnO_x. This shift is likely due to the competitive dynamics between MnO_x and MnOB, with the influence of MnOB diminishing over time. Other contributing factors include changes in the nutrient consumption patterns of MnOB and shifts in microbial community composition. Several MnOB genera, including *Sphingopyxis*, *Sphingomonas*, *Hyphomicrobium*, *Hydrogenophaga*, and *Variovorax*, were present in the biofilter from startup to maturity. Genes associated with direct and indirect biological Mn oxidation pathways were also predicted, highlighting the complex, multi-pathway nature of biological Mn oxidation.

Phase three evaluated the performance of intermittently (6 h/d, 12 h/d) and continuously operated biofilters (24 h/d), in addition to the effects of a 10-day shutdown. The findings demonstrated that intermittently operated biofilters maintain Mn and Fe removal efficiency comparable to continuously operated biofilters, although continuous biofilters exhibited higher ATP and EPS levels. Biofilters quickly recovered after a 10-day shutdown, highlighting their robustness. The overall microbial community composition was not significantly different between continuously and intermittently operated biofilters.

Overall, the study successfully demonstrated that pilot-scale biofilters could reduce Mn levels below the Health Canada recommended AO of 20 µg/L, achieving over 90% removal efficiency in the presence of Fe at low water temperatures (15°C). The findings highlight the potential of GAC media to shorten start-up times and the feasibility of operating biofilters intermittently without compromising Mn and Fe removal efficiency.

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Dedication

I dedicate my thesis to my parents and sister for their endless love, support, and encouragement throughout my pursuit of education. To my lovely wife, without whose support, care, and love, this PhD would not have been completed.

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List of Abbreviations

Al	Aluminum
AO	Aesthetic objective
As	Arsenic
ASV	Amplicon sequencing variants
ATP	Adenosine triphosphate
B/P	Biological/physicochemical
Bio-MnO _x	Biological manganese oxides
BOM	Biodegradable organic matter
Ca	Calcium
d	day
DBP	Disinfection by-products
DO	Dissolved oxygen
DOC	Dissolved organic carbon
Dw	Dry weight
EBCT	Empty bed contact time
EDX	Energy-dispersive x-ray
Eh	Redox potential
EPS	Extracellular polymeric substances
Fe	Iron
GAC	Granular activated carbon
GWTP	Groundwater treatment plant
h	hours
h/d	hours per day
HNO ₃	Nitric acid
HPC	Heterotrophic plate counts
IOB	Iron oxidizing bacteria
KEGG	Kyoto encyclopedia of genes and genomes
MAC	Maximum acceptable concentration
MCO	Multicopper oxidase enzymes

Mg	Magnesium
Mn	Manganese
MnOB	Manganese oxidizing bacteria
MnO _x	Manganese oxides
MOG	Manganese oxidizing genes
K	Potassium
NaN ₃	Sodium azide
NH ₄ ⁺	Ammonium ion
NO ₃ ⁻	Nitrate
NOB	Nitrite oxidizing bacteria
NOM	Natural organic matter
ORP	Oxidation-reduction potential
P/C	Protein-to-polysaccharide
Pb	Lead
PCoA	Principal coordinates analysis
PERMANOVA	Permutational multivariate analysis of variance
PFO	Pseudo-first order
PICRUST2	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
PSO	Pseudo-second order
QQQ-ICP-MS	Triple quadrupole inductively coupled plasma mass spectrometry
ROS	Reactive oxygen species
S.D	Standard deviation
SEM	Scanning electron microscopy
Si	Silica
UC	Uniformity coefficient

1 Introduction

1.1 Problem statement

Manganese in the oxidized form in drinking water can cause aesthetic and operational problems, such as stains in sinks (e.g., Tobiason et al., 2016). Manganese and iron (Fe) oxides particles tend to flocculate and form sediments in distribution lines that can clog pipelines, potentially leading to increased energy loss (Vries et al., 2017). While trace levels of Mn are essential for the growth and development of humans, several studies have now linked a higher level of Mn ($>100 \mu\text{g/L}$) in drinking water with cognitive and neurobehavioral effects in children (Bouchard et al., 2007; Rahman et al., 2017; Wasserman et al., 2016). Manganese is also associated with Pb release in the distribution system (Trueman et al., 2019). Identifying the possible side effects of Mn in drinking water, countries have issued specific standards for Mn in drinking water (Table 1.1). Health Canada has established a maximum acceptable concentration (MAC) of $120 \mu\text{g/L}$ and an aesthetic objective (AO) of $20 \mu\text{g/L}$ for Mn in drinking water (Health Canada, 2019).

Table 1.1: Guidelines for Mn in drinking water

Country/Organization (Issued year)	Guideline Value (mg/L)	Standards
WHO (2020)	0.08	Provisional guideline
Health Canada (2019)	0.12	MAC
	0.02	AO
USEPA (1994, 2002)	0.30	Health advisory
	0.05	Secondary maximum contaminant levels (SMCL)
Australia (2011)	0.50	Health-based
	0.10	Aesthetic
European Union (1998)	0.05	Indicator parameter

The National Drinking Water Survey reported Mn presence in various water sources across different locations in Canada (Fig. 1.1), with concentrations exceeding the AO of $20 \mu\text{g/L}$ and MAC of $120 \mu\text{g/L}$ in multiple provinces (Health Canada, 2019). Although the average concentrations in many provinces were below the MAC threshold, the highest recorded level reached $796,000 \mu\text{g/L}$ in British Columbia, necessitating urgent attention. Manganese can be effectively removed from drinking water using physical, chemical, and biological methods, depending on water quality parameters and the oxidation state of Mn (Tobiason et al., 2016). In groundwater, Mn is usually present in the dissolved

form Mn(II), which is oxidized to MnO₂, followed by removal of the MnO₂ precipitates by physical treatment processes. A detailed description of these physical/chemical methods can be found elsewhere and is summarized in Table A1.1 (Brandhuber et al., 2013; Tobiason et al., 2016). However, applying physicochemical methods, such as catalytic oxidation with chlorine, can result in the formation of disinfection by-products (DBPs), which are carcinogenic (Basu et al., 2016; Tobiason et al., 2016). Additional challenges include the production of sludge and the continuous use of chemicals, which increases maintenance and operational costs (Marsidi et al., 2018; Mouchet, 1992). This led to the emergence of biological approaches/treatments that are sustainable, environmentally friendly, control the formation of DBPs, and lower operation & maintenance costs (Basu et al., 2016; Mouchet, 1992; Piche et al., 2019). The commonly used biological approach is aeration - rapid sand filtration, also known as biofiltration, which is the focus of this thesis. Biofiltration presents a green and sustainable treatment technology for Mn removal. In biofiltration, biofilter media become biologically active due to the attachment of microorganisms that form a biofilm and contribute to removing contaminants.

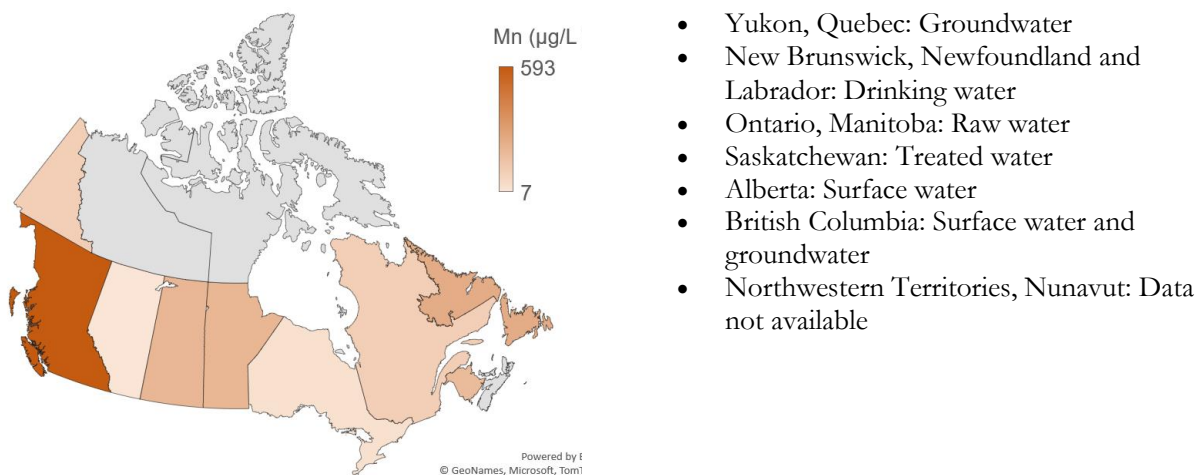


Figure 1.1: Average Mn concentration reported in various water sources across Canadian provinces and Territories (Health Canada, 2019)

Despite the numerous benefits of biofiltration compared to other treatment processes, it faces specific challenges and limitations. A significant limitation is the prolonged start-up period for biofilters using virgin media, which can extend from one to six months and is influenced by the influent water

conditions and operational parameters (Bruins et al., 2015a). The presence of Fe can further complicate Mn removal in biofilters, specifically at low water temperatures ($< 15^{\circ}\text{C}$), where the microbial activity slows down, and the efficacy of the biofilter diminishes (Ciancio Casalini et al., 2020; Ramsay et al., 2018). Various methods have been investigated to reduce the start-up time of Mn-removing biofilters, mainly by using different filter media (Araya-Obando et al., 2021; Breda et al., 2017; Evans et al., 2020; Jez-Walkowiak et al., 2015, 2017) and by applying inoculants from mature biofilters containing manganese-oxidizing bacteria (MnOB) (Cai et al., 2015; Ciancio Casalini et al., 2020; Zeng et al., 2019). However, research on media characteristics like surface area, pore size, and media size has been inconsistent, with most studies focusing on non-adsorptive media such as sand and anthracite. The potential of granular activated carbon (GAC) media, with its higher surface area and pore volume, suggests it could improve adsorption and potentially microorganism attachment during biofilter start-up, yet this area remains underexplored.

Previous studies employing non-adsorptive media like sand or anthracite have demonstrated that upon start-up, Mn removal in biofilters typically begins with a biological mechanism, which gradually shifts to being dominated by physicochemical processes as the biofilter matures (Bruins et al., 2015b; Olańczuk-Neyman & Bray, 2000; Sahabi et al., 2009). However, existing literature has often not fully explored how these removal mechanisms vary across different filter bed depths or the impact of using varied filter media, especially adsorptive ones like GAC. Furthermore, how the contributions of physicochemical and biological removals evolve needs further investigation.

Microorganisms play a crucial role in Mn removal in biofilters. Several known MnOB genera, such as *Lepothrix*, *Pseudomonas*, *Sphingobium*, *Shingomonas*, and *Sphingopyxis*, which are capable of Mn oxidation have been identified in biofilters (Breda et al., 2017; Cai et al., 2023; Dangeti et al., 2020; McCormick et al., 2022; Piazza et al., 2019; Tebo et al., 2005). However, the presence of MnOB that can potentially oxidize Mn does not guarantee substantial Mn oxidation and subsequent Mn removal in biofilters. Moreover, these identification studies were done at a pseudo-steady-state or an earlier biofilter stage. Few studies report changes in the microbial community from start-up to maturity and the relationship between the biofilter stage and the microbial community in the biofilter, especially from natural groundwater (Cheng et al., 2019; Cheng et al., 2017). Thus, the dynamic evolution of the microbial community and its role in Mn removal warrant further investigation.

Biofilters are typically operated continuously without shutdown; however, certain small-scale or remote communities and facilities with excess plant capacity may run their biofilters intermittently.

Periodic or intermittent operations may adversely affect biofilter performance as the biological activity may be diminished, decreasing contaminant removal efficiency. Therefore, the applicability of biofilters in the intermittent mode for Mn and Fe removal needs to be further evaluated.

Thus, key areas that need further investigation are:

- Impact of filter media characteristics on the start-up of biofilters
- Impact of filter media on Mn removal mechanisms
- Effect of Fe on Mn removal processes at low water temperatures ($<15^{\circ}\text{C}$)
- Mechanism of Mn removal across different stages of biofilter maturity
- Contribution of biological and physicochemical processes to Mn removal over time in biofilters
- Evolution of microbial community over time in biofilters
- Impact of intermittent operation on biofilter performance

1.2 Aims and objectives

The primary goal of this PhD research is to better understand the Mn removal mechanisms in biofilters from start-up to maturity and to study the impact of operational parameters on the performance of biofilters for Mn and Fe removal. A combination of pilot-scale biofilters and bench-scale batch experiments was carried out to achieve the following research objectives:

1. Investigate the impact of filter media characteristics on the performance of biofilters for Mn and Fe removal during start-up.
2. Examine the impact of filter media characteristics on the mechanisms of Mn removal.
3. Provide a better understanding of the Mn removal mechanisms (biological and physicochemical) during the start-up of biofilters and their evolution over time.
4. Enhance the understanding of the microbial community composition in Mn removal biofilters.
5. Assess the impact of intermittent operation and potential long-term shutdown on the performance of biofilters for Mn and Fe removal.

1.3 Outline of thesis

Chapter 1 presents the problem statement, general information about different treatment technologies for Mn removal, and the need for Mn removal based on health, aesthetic, and operational

considerations. It summarizes the identified knowledge gaps and concludes with the study's goals, research objectives, and an outline of the thesis.

Chapter 2 offers a literature review that discusses the sources of Mn in groundwater, Mn chemistry, and the processes of Mn removal in biofilters. This includes the mechanisms of Mn removal, factors affecting Mn removal such as water quality and operational parameters, and factors influencing the start-up time of biofilters. Finally, the literature review delves into the performance of biofilters under intermittent operation for Mn removal and identifies knowledge gaps.

Chapter 3 describes the impact of filter media characteristics, such as pore size distribution and media size, on the start-up of biofilters. This includes analyzing the biofilter performance for Mn and Fe removal, kinetics of Mn removal, characterization of MnO_x using SEM-EDX and Raman Spectroscopy, and establishing potential relationships between culturable MnOB, biological activity (ATP), and biofilter performance.

Chapter 4 outlines the impact of filter media characteristics (GAC, anthracite, sand) on the mechanisms of Mn removal and the microbial community composition in biofilters. A combination of pilot-scale and batch-scale studies was conducted. The kinetics of Mn removal in batch tests using biofilter media from the pilot-scale biofilters were studied using non-linear pseudo-first order (PFO) and pseudo-second order (PSO) kinetic models. The contributions of biological and physicochemical processes to Mn removal for biofilter media from different biofilter bed depths were quantified in these batch tests. The microbial community composition of the biofilm in the different biofilters was determined through 16S rRNA gene sequencing of the V3-V4 regions. Additionally, predicting potential functional genes associated with Mn oxidation pathways was performed using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2).

Chapter 5 deals with how the Mn removal process evolves in a biofilter from start-up to maturity, documenting changes in MnO_x morphology using SEM and how the contributions of different removal mechanisms shift throughout the biofilter's operational life, by carrying out batch studies using the biofilter media collected at day 70, 150, and 240. Finally, changes in microbial community composition in biofilters were analyzed, and key classes and genera involved in Mn oxidation were identified through 16S rRNA gene sequencing of the V3-V4 regions.

Chapter 6 investigates the applicability of biofiltration in the intermittent operation mode for Mn and Fe removal. In this chapter, a comparative analysis of biofilters under continuous and intermittent

operations from their inception through to maturation was conducted, and the influence of varied durations of operation (24 h/d, 12 h/d, 6 h/d) on biofilter performance was evaluated. The impacts of backwashing and long-term (10 days) shutdown on the performance of biofilters were also investigated. Additionally, the effects of intermittent operation on biological activity (ATP), biofilm composition by quantifying EPS, and microbial community composition (16S rRNA gene sequencing) were evaluated and compared with those of continuously operated biofilters.

Finally, **Chapter 7** summarizes the main research results, conclusions, and limitations. It also provides recommendations for practical application and suggests directions for future research.

2 Literature Review: Manganese, Biofiltration & Intermittent Operation

Part of this chapter will be submitted for publication as:

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2.1 Sources of Mn in groundwater

Manganese is among the most prevalent metals in the Earth's crust and can be found in several oxidative states. In subsurface environments, such as soil and rock, Mn appears as dissolved Mn(II) and as insoluble Mn(III) and Mn(IV) oxides (Brandhuber et al., 2013). The presence and thermodynamic stability of various manganese (Mn) species in groundwater are influenced by the reduction-oxidation (redox) potential (Eh), electron activity (pe), and pH (Fig. 2.1). Additional factors influencing the speciation and solubility of manganese (Mn) encompass the physicochemical characteristics of the surrounding environment. These include the content of organic carbon, cation exchange capacity, and the presence of minerals and particulates, with pH and redox conditions being the most significant (Kohl & Medlar, 2006). Under anaerobic conditions and relatively low pH, often found in anaerobic groundwater, Mn is reduced to the more soluble form, Mn(II) (Fig. 2.1). Manganese is released by the weathering of igneous and metamorphic rocks/minerals in the ground. Because dissolved oxygen (DO) concentrations in groundwater tend to decrease with depth, high Mn concentrations are more commonly found in deep wells. Anthropogenic causes of Mn in groundwater (influenced by surface water) include industrial discharges, mining activities, and landfill leaching (Kohl & Medlar, 2006; Ljung & Vahter, 2007).

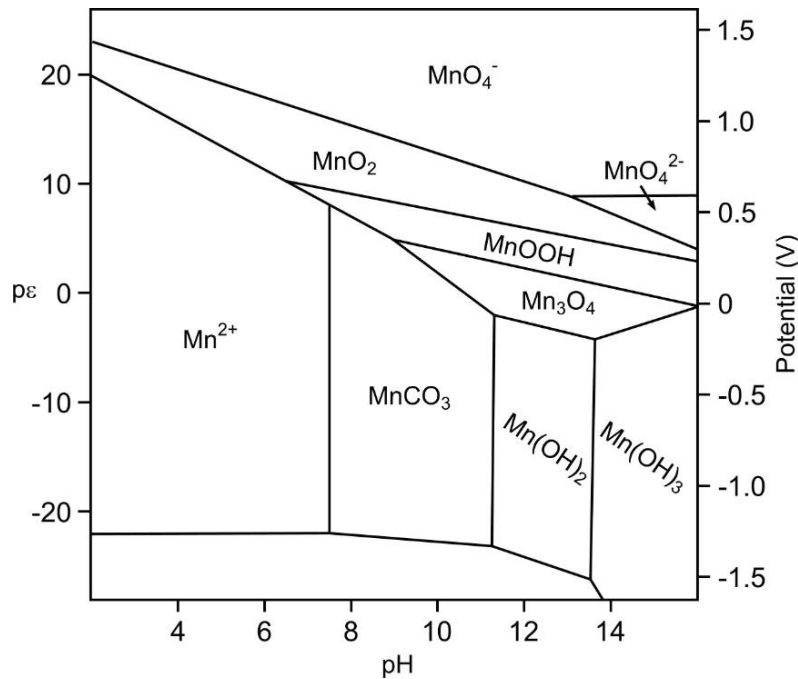


Figure 2.1: Manganese/Manganese containing compounds under different conditions of Eh, pe, and pH (adopted from Stumm and Morgan, 1996; Bruins et al., 2015c)

2.2 Biofiltration

In biofiltration, the filter media becomes biologically active as bacteria and other organisms adhere to its surface, forming a biofilm that aids in the removal of contaminants. The primary mechanism for contaminant removal in biofilters is biodegradation/biotransformation. A particle removal step is often provided before biofiltration in the case of surface water, where biofiltration can serve the dual purpose of physical particle removal and biodegradation of many organic constituents. For many groundwater, direct biofiltration is used, with no prior particle removal step. When using adsorptive filter media, such as GAC, biofilters can also remove contaminants through adsorption during the initial stages of biofilter operation. Microorganisms break down or alter biodegradable organic contaminants through two metabolic pathways: direct catabolism (primary substrate utilization) or co-metabolism (secondary substrate utilization) (Benner et al., 2013).

In aerobic biofiltration, which is common in drinking water biofilters, the biological oxidation of substrates is carried out in the presence of DO, which serves as an electron acceptor. Bacterial enzymes catalyze the redox reaction to oxidize biodegradable electron donor substrates. The most relevant electron donor substrate material in drinking water is biodegradable organic matter (BOM), which often serves as a primary substrate (Liu & Huck, 2001). Primary substrates exist in concentrations sufficient to provide energy for essential cellular processes without the need for an additional substrate (Benner et al., 2013). Examples of primary substrates includes Mn, Fe, ammonia (NH_4^+), nitrate (NO_3), dissolved hydrogen (H_2), and several sulfur species (Rittmann & Huck, 1989). On the other hand, secondary substrates, such as micropollutants (e.g., endocrine-disrupting compounds, pharmaceuticals, personal care products, pesticides) and algal metabolites (e.g., 2-methylisoborneol and geosmin), are present in concentrations too low to directly support primary cellular functions. These are biodegraded by bacteria using enzymes produced through the metabolism of primary substrates (Brown et al., 2020; Rittmann & McCarty, 2001). In the context of groundwater, which is the focus of this study, BOM is usually present at very low concentrations, and inorganics like Mn, Fe, and NH_4^+ act as primary substrates.

2.3 Mn transformation in a biofilter

Manganese removal in a biofilter can be broadly classified into physicochemical and biological removal (Fig 2.2) (Barboza et al., 2016). The dominant Mn removal mechanism will depend upon the influent water characteristics, operational parameters, and biofilter operating stage (start-up or matured).

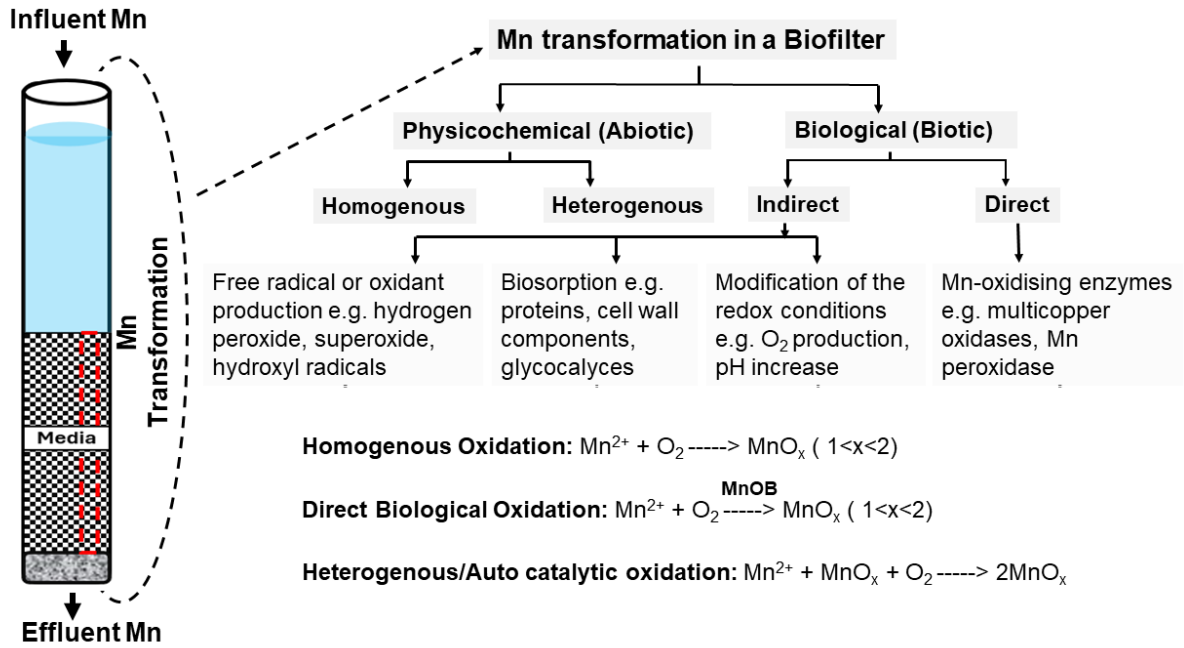


Figure 2.2: Manganese transformation in a drinking water biofilter (Adopted and modified from Tebo et al., 2005; Barboza et al., 2016)

2.3.1 Homogenous Mn removal

The homogeneous oxidation of Mn by oxygen is thermodynamically favored at a neutral pH (Diem & Stumm, 1984) but is very slow in the absence of metal oxide surfaces at a pH below 9, which is usually encountered in drinking water biofilters (pH: 6-8) (Morgan, 2005). However, in natural environments such as lakes, marine, and freshwater ecosystems, the rate of Mn oxidation is comparatively high, suggesting the importance of biological Mn oxidation (Godwin et al., 2020).

2.3.2 Biological Mn removal

Biological Mn oxidation can occur due to indirect or direct processes (Fig 2.2). Indirect processes include modification of pH and redox potential by bacterial growth (Chaput et al., 2019; Tebo et al., 2005) or release of metabolic end products such as hydrogen peroxide (H_2O_2) (Godwin et al., 2020; Tebo et al., 2005) or production of superoxide radicals ($O_2^{\cdot-}$) (Godwin et al., 2020; Learman et al., 2011). Direct oxidation occurs via Mn oxidizing enzymes such as multicopper oxidase (MCO) or heme peroxidases (Dick et al., 2008; Nakama et al., 2014; Tebo et al., 2005). Microorganisms like *Pseudomonas*,

Streptomyces, *Crenothrix*, *Gallionella*, *Leptothrix*, and *Hydrogenophaga*, which are capable of direct biological Mn oxidation, have been identified in drinking water biofilters (Table A2.1) (Bruins et al., 2017; Burger et al., 2008a; Cheng et al., 2017; Ciano Casalini et al., 2020; Dangeti et al., 2020; McCormick et al., 2022; Piazza et al., 2019). Mouchet (1992) suggested that these microorganisms have the unique property of causing oxidation and precipitation of dissolved Mn under certain pH (7.2-8.0) and redox potential ($E_h >$ about 300-400 mV) conditions. The presence and diversity of MnOB in the source water and their ability to form an active biofilm on biofilter media greatly influence Mn removal in biofilters. Manganese removal in biofilters can also occur through extracellular adsorption, where soluble Mn binds to the negatively charged EPS (Tobiason et al., 2016). Microorganisms, including species of *Pseudomonas*, *Bacillus*, and *Mycobacterium*, are known to facilitate this process by promoting biofilm formation through EPS production (Tondo et al., 2020).

Three commonly found MnOB, *Leptothrix discophora* SS-1, *Pseudomonas putida* MnB1 and GB-1, and *Bacillus* sp. SG-1, belonging to phylogenetically distinct MnOB, was studied in detail to determine the mechanism of direct Mn oxidation. The genes involved in the Mn(II) oxidation share sequence similarity to MCO enzymes (Brouwers et al., 2000; Butterfield et al., 2013; Dick et al., 2008). Several Mn oxidation genes such as *mnxG* (Van Waasbergen et al., 1996), *moxA* (Morales Medina et al., 2023; Ridge et al., 2007), *cumA* (Brouwers et al., 1999), and *mofA* (Corstjens et al., 1997; Morales Medina et al., 2023), are known to produce MCO enzymes. MCO enzymes utilize multiple copper (Cu^{2+}) ions as cofactors to oxidize a broad range of substrates, including Mn (Solomon et al., 1996). Wang et al. (2010) suggested that MCOs lower the activation energy required for the oxidation of Mn during the oxidation process. However, other enzymes, such as peroxidases (Barboza et al., 2016; Kohl & Dixon, 2012) and a two-component regulatory protein (Geszvain & Tebo, 2010), have also been implicated. Several genes can also facilitate Mn oxidation either directly through enzymatic reactions or indirectly via chemical reactions involving reactive oxygen species (ROS) such as $\text{O}_2^{\cdot-}$, H_2O_2 , and hydroxyl radicals ($\text{OH}^{\cdot}$) (Anpo et al., 1999; Hayyan et al., 2016; Liu et al., 2022). However, these genes have not yet been identified within Mn-removing biofilters, suggesting that Mn removal in biofilters can take place through multiple pathways, which have yet to be fully elucidated.

The potential benefits for microorganisms oxidizing Mn include energy gain to sustain bacterial growth (Yu & Leadbetter, 2020). Other possible benefits include the protection of cells from harmful UV, toxicity from heavy metals, predation, or viral attacks (Brouwers et al., 2000), oxidation of complex humic substances (Sunda & Kieber, 1994), and co-metabolic degradation of micropollutants

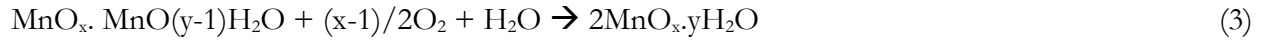
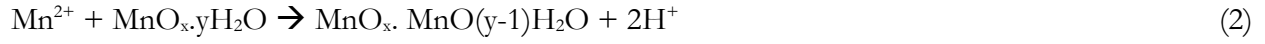
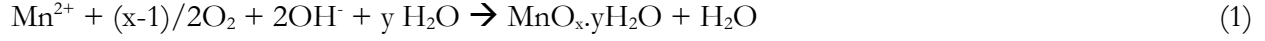
by MnO_x (Meerburg et al., 2012). However, the physiological roles of these activities remain unclear (Tebo et al., 2005). The MnOB such as *Pseudomonas*, *Bacillus*, *Leptothrix*, and *Pedomicrobium* are heterotrophic and organotrophic and can grow in cultures with or without Mn(II) , suggesting that the presence of MnOB does not necessarily guarantee Mn oxidation (Tebo et al., 2005). Several researchers have also reported that MnOB does not always correlate with effective Mn control in biofilters (Burger et al., 2008a; Kohl & Dixon, 2012). The organisms capable of Mn oxidation, such as *Leptothrix sp.* and *Pseudomonas sp.* can also oxidize Fe^{2+} , NH_4^+ , and As, which commonly occur with Mn in groundwater (Fleming et al., 2011; Van Le et al., 2022). Depending on the process conditions, species of *Bacillus* and *Pseudomonas* can also reduce Mn instead of oxidizing it (Cerrato et al., 2010; Tebo et al., 2005).

The kinetics and dynamics of Mn oxidation and reduction processes mediated by bacteria depend on water quality parameters, which can vary between biofilters (Kohl & Dixon, 2012). Burger et al. (2008a) observed significant differences in the microbiology within four biofilters treating different groundwater despite a similar filter design. Moreover, the commonly found Mn-oxidizing organism *Leptothrix* was only found in one biofilter, even though all filters achieved 100% Mn removal. This suggests that biological Mn removal is attributed to a microbial consortium rather than a single species of organisms (Vandenabeele et al., 1992). The majority of MnOB identification studies are conducted at a single point in time during biofilter operation (Breda et al., 2017; Cheng et al., 2019; Cheng et al., 2017; Dangeti et al., 2020; McCormick et al., 2022). There is a notable gap in research tracking changes in the microbial community from the start-up phase to maturity, particularly in biofilters treating natural groundwater.

2.3.3 Heterogenous Mn removal

Manganese oxidation, whether through homogeneous or biological processes, results in the formation of MnO_x . MnOB enzymatically oxidizes Mn(II) , forming poorly crystalline oxides with a high specific surface area, which indicates a strong adsorption capacity. These oxides display an intermediate oxidation state between 3 and 4, suggesting autocatalytic properties (Katsoyiannis & Zouboulis, 2004; Nelson et al., 1999; Tebo et al., 2004). One such MnO_x identified in biofilters is birnessite (Bruins et al., 2015b,c; Dangeti et al., 2020; Jez-Walkowiak et al., 2017). Birnessite belongs to a group of Mn minerals with layered structures, cation exchange capacities, and redox properties (oxidation-reduction reaction) (Post, 1999). Due to these properties, birnessite has a high adsorption capacity for Mn(II) and promotes further autocatalysis of Mn(II) oxidation (Post 1999). Birnessite's average Mn oxidation

state ranges from +3.5 to +3.9 (Cui et al., 2009), unlike pyrolusite (MnO_2), which is the most stable form of MnO_x and possesses only adsorptive properties. The formed MnO_x further contributes to Mn removal by autocatalysis, a two-step process (Eqs. 1-3). Birnessite and similar MnO_x compounds, with their autocatalytic properties, significantly enhance the efficiency of biofilters in removing Mn.



Birnessite, observed to be of biological origin in the early stages of biofilter operation (Bruins et al., 2015b; Dangeti et al., 2020), undergoes a transition in its formation mechanism as the biofilter matures. With the development of a coating on the filter media, the formation of birnessite shifts to being predominantly physicochemical (Bruins et al., 2015b). The exact reasons for this transformation in birnessite formation mechanisms remain elusive. Potential factors include changes in the microbial community composition responsible for Mn oxidation, crystallization phenomena over time, changes in the functional behavior of heterotrophic MnOB, or variations in process conditions that may become less conducive to biological Mn oxidation at later stages. Additionally, the surface morphology of MnO_x evolves significantly with the aging of the filter, with older media displaying a mineral-rich surface composition of Al, Ca, Si, and Mn (Bernstein et al., 2022). This evolution in structural characteristics of MnO_x is further influenced by the presence of cations in the water, such as K^+ , Mg^{2+} , Ca^{2+} , and Cu^{2+} , affecting their oxidative activity and adsorptive or catalytic performance (Li et al., 2019). The elemental composition of the biofilter media also plays a critical role in dictating the formation of various MnO_x phases (Vallejo, 2015). Thermodynamic modeling using PHREEQC has predicted the potential formation of diverse MnO_x , including manganite, nsutite, pyrolusite, and bixbyite, within ten full-scale biofilters, suggesting different types of MnO_x can be formed in biofilters under varying biofilter conditions but was not confirmed experimentally (Earle et al., 2021).

2.3.4 Biological vs. physicochemical

Several batch studies using biofilter media have been conducted to assess the relative contributions of the biological and physiochemical processes to Mn removal in biofilters using sodium azide (NaN_3) as an inhibitor (Breda et al., 2019; Olańczuk-Neyman & Bray, 2000; Sahabi et al., 2009; Vandenabeele et al., 1992). Manganese removal was attributed to both physicochemical and biological processes during start-up, but as the filter matures, the physicochemical process dominates (Breda et al., 2019).

In a separate study, biofilter media from a 3-year-old biofilter exhibited a lower adsorption capacity and greater dependence on microbial activity than 15-year-old biofilter media (Sahabi et al., 2009). A similar conclusion was drawn by Olańczuk-Neyman & Bray (2000), Mn removal mainly occurs due to adsorptive and auto-catalytic properties of the MnO_x in the presence of inhibitors.

The effect of inhibitors should be considered as diminishing microbial activity rather than total inhibition. The inhibitory effect of NaN_3 on biological Mn removal is primarily limited to direct pathways, allowing indirect biological removal processes to continue contributing to Mn removal (Breda et al., 2019). During simultaneous removal of Fe, NH_4^+ , and Mn, NaN_3 only inhibited nitrifying bacteria, while heterotrophic MnOB remained active. Consequently, the efficiency of Mn removal in biofilters improved (Olańczuk-Neyman & Bray, 2000). Therefore, co-constituents and inhibitors can influence these studies' relative contributions to biological and physicochemical removal processes. Moreover, distinguishing between MnO_x formed biologically or physiochemically, which contribute to Mn removal through autocatalytic adsorption and oxidation, becomes challenging once the biofilter matures. Nonetheless, these studies offer baseline data, suggesting that the dominant mechanism for Mn removal in biofilters can change as the filter matures.

The contribution of biological/physicochemical Mn removal in biofilters also depends on the biofilter's age and the filter media's characteristics. Previous studies employing non-adsorptive media like sand or anthracite have demonstrated that Mn removal in biofilters typically begins with a biological mechanism, which gradually shifts to being dominated by physicochemical processes as the biofilter matures (Breda et al., 2019; Bruins et al., 2015b). However, the existing literature has often not fully explored how these removal mechanisms vary across different filter bed depths or the impact of using various types of filter media, especially adsorptive media like GAC.

2.4 Factors affecting Mn removal in a biofilter

2.4.1 Water quality characteristics

2.4.1.1 pH

Homogenous oxidation of Mn with oxygen in water without a catalyst is very slow at pH values below 8.6 (Graveland, 1971). Therefore, in biofilters, Mn removal is likely to occur through either biological means or heterogeneous removal by autocatalytic oxidation at the pH levels encountered in drinking water (6-8) (Katsoyiannis & Zouboulis, 2004). Granger et al. (2014) observed higher biological Mn removal at pH 6 compared to pH 9-11 using different types of filter media (GAC and anthracite).

Similarly, Hoyland et al. (2014) observed more than 98% removal in the anthracite columns inoculated with a mixture of six MnOB at a pH as low as 6.5. Burger et al. (2008b) reported similar findings; with inoculation of the *Leptothrix* genus at pH 6.2–6.3 in biofilters, greater than 98% removal was observed. The above results contradicted the study conducted by Kohl & Dixon (2012), who found more effective removal at a higher pH (7.2–7.6) than at pH 6.5. Possible reasons for ineffective manganese removal at pH 6.5 might be the unavailability of MnOB that can acclimatize to a pH of 6.5 in the influent water compared to the previous two studies where the filters were inoculated with MnOB. Bruins et al. (2014) recommended pH > 7.1 for effective biological Mn oxidation. This suggests that pH is an important parameter for biological Mn oxidation in biofilters.

2.4.1.2 Temperature

Temperature variations, both seasonal and diurnal, notably influence the kinetics of biological Mn oxidation (Ciancio Casalini et al., 2020). Although this review primarily focuses on groundwater, where temperature variation from a single source tends to be minimal, the differences in temperature across various geographical locations can be considerable, making temperature a critical factor in the effectiveness of biofiltration. The biological activity of MnOB can significantly decrease below 15°C, affecting the performance of biofilters for Mn removal (Berbenni et al., 2000). An increase in effluent Mn concentration occurred when water temperatures fell below 15°C in anthracite bench columns fed with modified tap water. However, as temperatures increased, the effective oxidation of Mn resumed (Hoyland et al., 2014). Similarly, Piazza et al. (2019) noted differences in the oxidizing capacity of MnOB isolated from a groundwater treatment plant (GWTP) at various temperatures. Moreover, temperature has been shown to influence the start-up time of biofilters, with faster acclimation observed at 20° C compared to 12° C in both anthracite and GAC biofilters (McCormick et al., 2021). Ghiorse and Chapnick (1983) studied the influence of temperature on *Leptothrix*, a commonly found Mn-oxidizing organism in biofilters. With a decrease in temperature from 30° C to 10° C, the growth rate reduced by 75%, and at temperatures above 30° C, growth rates declined steeply, dropping to zero at 37° C. Boogerd & de Vrind (1987) observed similar results, the oxidation activity of Mn(II) by *Leptothrix discophora* (MnOB) decreased by 50–80% when incubated at 8° C compared to room temperature (20° C). Conversely, some MnOB genera, such as *Hydrogenophaga sp.*, can oxidize Mn at lower temperatures (8 to 15° C) (Dangeti et al., 2017). This suggests that different molecular mechanisms or differential gene expression may trigger Mn oxidation in MnOB under varying temperature conditions (Piazza et al., 2019).

2.4.1.3 Dissolved oxygen

DO is essential for both the chemical oxidation of Mn and the metabolic processes of MnOB. (Mouchet, 1992) indicated that MnOB requires DO concentrations greater than 5 mg/L. Olańczuk-Neyman & Bray (2000) demonstrated that Mn concentrations in effluent significantly increased when DO levels in a biofilter dropped below 2 mg/L. In contrast, Guo et al. (2019) found that Mn could still be effectively removed with influent DO concentrations around 1.0–1.5 mg/L, using ripened sand coated with an iron-manganese co-oxide (Fe-MnO_x) as the filter media. These differences suggest varying removal mechanisms: the Fe-MnO_x coating likely facilitates Mn removal predominantly through adsorption onto the filter media and autocatalytic oxidation, compared to the biological process, which might require higher DO levels.

Similarly, Cheng et al. (2020) observed that filter media coated with a birnessite-type MnO_x achieved higher Mn removal under anaerobic conditions, likely due to a larger contact surface area and pore volume provided by the MnO_x coating. In a separate study, Cheng et al. (2019) observed that MnO_x formed at higher DO concentrations exhibited greater Mn removal activity, attributed to abundant lattice oxygen. Furthermore, the crystallization of MnO_x increased over time in filters with higher DO levels (DO = 6.5 ± 0.5 mg/L). In contrast, it remained constant in filters with lower DO (DO = 1.5 ± 0.5 mg/L), emphasizing the critical role of DO in both chemical and biological Mn removal processes (Cheng et al., 2019). This illustrates that while DO is a crucial factor in both chemical and biological processes, the effectiveness of Mn removal can vary significantly based on the operational conditions and the specific characteristics of the filter media used.

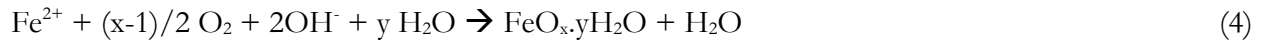
2.4.1.4 Nutrients

An increase in the removal of both Mn and DOC with enhanced nutrients (phosphorus at concentrations of 20 and 200 µg/L) on the removal efficiency of biofilters in a bench-scale setup was observed (Granger et al., 2014). Similarly, Lauderdale et al. (2016) observed that C: N: P ratios ranging from 100:12:4 to 100:8:7 improved Mn removal in pilot-scale anthracite biofilters with chemically induced MnO_x coatings, compared to a phosphorus-limited control. However, confounding results have been reported. Lauderdale et al. (2014) reported that nutrient addition did not improve the quality of filter effluent Mn. Tani et al. (2003) demonstrated that media with limited organic nutrients more effectively promote Mn-oxidizer growth than nutrient-rich environments. Most studies examining nutrient addition have been conducted on a bench or pilot-scale due to the challenges associated with

managing nutrient levels in full-scale biofilters. This underscores the complexity of optimizing nutrient levels to enhance biofilter performance.

2.4.1.5 Presence of co-constituents

Fe^{2+} , NH_4^+ , and Mn are commonly present co-constituents in groundwater, significantly affecting the biofilters' start-up period for Mn removal. The presence of Fe in the influent water can interfere with biological Mn removal (Gouzinis et al., 1998). Cheng et al. (2017a) evaluated the simultaneous removal of Fe, Mn, and NH_4^+ in pilot-scale biofilters and observed that Mn (II) removal could only occur after iron was oxidized. Similar results were observed in trickling filters, and influent iron concentration affected biological Mn oxidation (Tekerlekopoulou & Vayenas, 2008). Fe oxidation requires low Eh (100-400 mV) and pH (6-8) values and is thermodynamically more favorable than Mn oxidation (Mouchet, 1992). In addition, iron bacteria can more readily establish iron-oxidizing biofilms than MnOB biofilms, leading to Fe removal before Mn in biofilters. The presence of Fe and NH_4^+ will also affect the chemical oxidation demand (Eqs. 4, 5, and 6).



Bruins et al. (2014) suggested that effective Mn removal can only be achieved when NH_4^+ removal efficiency exceeds 85%. Biological Mn removal requires a specific ORP, achievable only after complete nitrification (Abu Hasan et al., 2013). Manganese removal was not affected when the NH_4^+ concentration in the influent was lower than 2.5 mg/L, and Mn removal can occur only once Fe is completely oxidized (Cheng et al., 2017b). Similar results were observed by (Gouzinis et al., 1998); at low NH_4^+ concentrations (< 1 mg N/L), there is minimal impact on biological Mn removal, whereas (Vandenabeele et al., 1995) observed both negative and positive effects of NH_4^+ on Mn removal efficiency. Inorganic nitrogen species may also interfere with biological Mn oxidation (Kohl & Dixon, 2012; Vandenabeele et al., 1995). Nitrite inhibits Mn(II) removal by half, while nitrate increases Mn(II) removal by 38 percent (Cheng et al., 2017). Nitrite has also been observed to reduce Mn oxides to dissolved Mn(II).

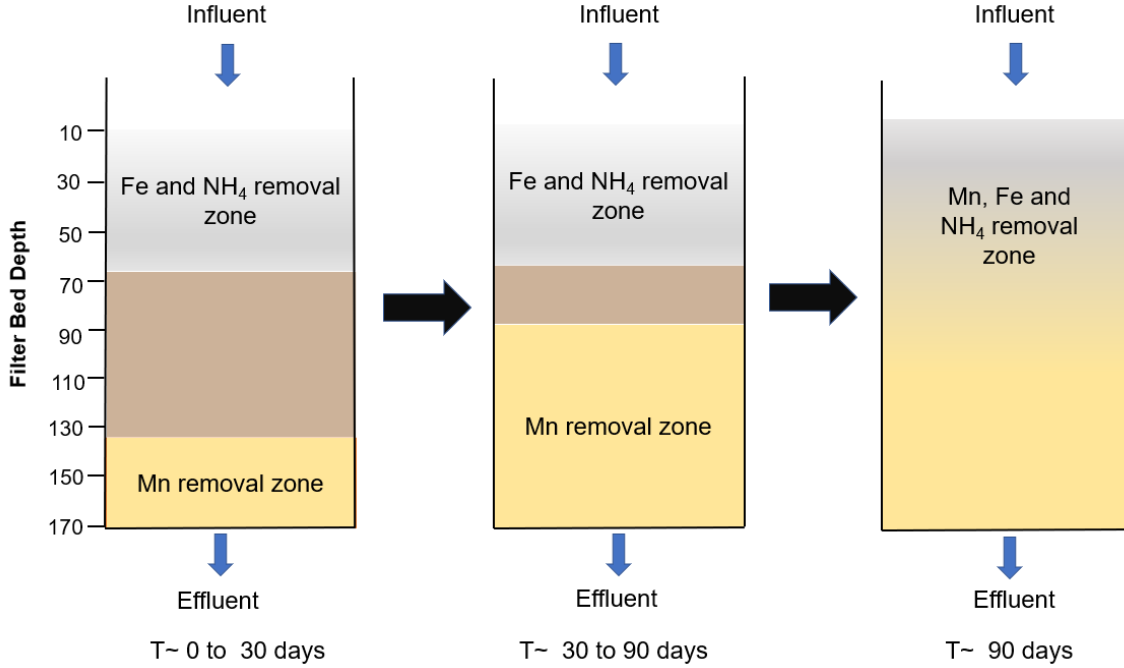


Figure 2.3: Stratification in biofilters simultaneously removing Mn, Fe, and NH_4 (Here, T = Biofilter operating days and zone of contaminant removal is used as a reference and does not represent the actual time and zone. The actual T and zone of removal will depend on factors such as water quality characteristics, operational and design parameters)

Biofilters are characterized by oxygen gradients on a macro-scale from top to bottom and a micro-scale inside biofilms (Vandenabeele et al., 1995). Transitions between oxic and anoxic conditions could occur during filter runs, leading to stratification within the filter bed where iron and ammonia are removed at the top, followed by Mn (Fig. 2.3) (Cheng et al., 2019; Cheng et al., 2017; Cheng et al., 2017b; Ramsay et al., 2018). This stratification tends to be more pronounced in the early stages of biofilter operation, with more homogenous removal of Mn throughout the filter bed expected as the filter matures (Fig. 2.3).

Natural organic matter (NOM) can also impact Mn removal in biofilters (Tebo et al., 2005). Since MnOB are known heterotrophs and organotrophs, the concentration of biodegradable NOM may influence the overall growth of the microbial community, including MnOB (Evans et al., 2020; Tebo et al., 2005). The bacterial population in a rapid sand biofilter for removing Fe and Mn could also degrade organic substances from groundwater (Korth et al., 2002).

2.4.2 Operational parameters

2.4.2.1 Mode of biofilter operation: Intermittent vs. continuous

Drinking water biofilters are typically operated continuously without shutdown. However, certain small-scale or remote communities and facilities with excess plant capacity may intermittently run their treatment plants (6-18 h/d), regardless of biofiltration. Additionally, under emergencies, such as water intake shutdowns caused by deteriorating source water quality or plant failures, treatment plants may also experience shutdowns (7-15 d). Periodic or intermittent biofilter operations involving regular shutdowns can lead to substrate and oxygen deprivation periods, depending on local conditions.

Biological filtration is governed by mass transport between the bulk liquid and the biofilm, substrate diffusion inside the biofilm, substrate utilization, growth, and decay of microorganisms (Rittmann, 1995). However, for readily biodegradable carbon, both internal and external mass transfer limitations can be ignored (Huck et al., 2015). Compared to continuous biofiltration, which benefits from a constant source of substrates, intermittent operations are characterized by a periodic supply of substrates. Since the only source of the substrate is in the bulk liquid, it must be transported by diffusion across the stagnant layer into the biofilm matrix, to be utilized by the microorganisms. This process creates a substrate concentration gradient within the biofilm, acting as a driving force for diffusion. With periodic substrate supply, the concentration gradient is likely to decrease during off periods, resulting in a lower diffusion rate into the biofilm and thereby impacting the biological removal of contaminants (Liao et al., 2013; Prest et al., 2016).

Moreover, intermittent operation can lead to anoxic conditions or low oxygen levels within biofilters, impacting biomass levels (Niquette et al., 1998). Although a substantial amount of biomass may be retained, this operation type applies selective pressure on the microbial community, likely influencing biofilm composition, thickness, and the extent of EPS production. Consequently, the performance of biofilters under intermittent mode may adversely affect biofilter efficacy, as biological activity may diminish, potentially leading to decreased contaminant removal efficiency. Due to potentially slower biofilm development, more extended start-up periods may also be observed for intermittently operated biofilters. Given these challenges, the performance of biofilters operating intermittently may not be uniform but may vary significantly depending on several factors, including water quality and operational parameters (Fig. 2.4).

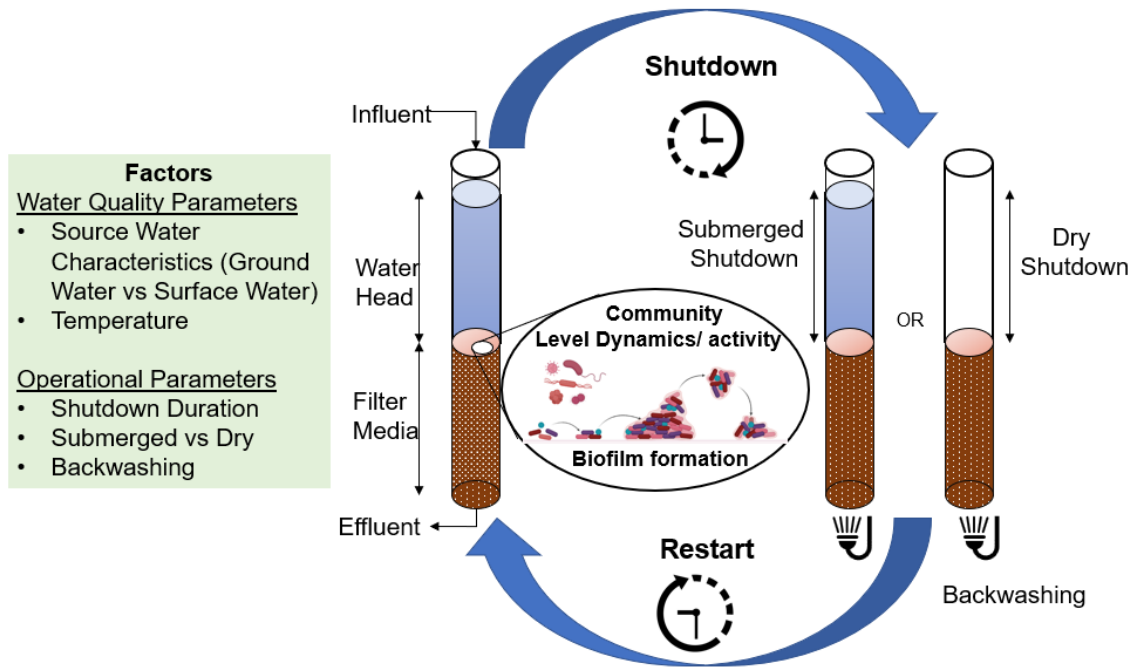


Figure 2.4: Factors potentially affecting the intermittent operation of biofilters

2.4.2.1.1 Impact of water quality parameters on Mn removal

Microorganisms in drinking water biofilms derive energy and nutrients from primary or secondary substrates in the influents. Since the only source of the substrate is in the influent, this supply is likely not continuous in intermittent operation. If the nutrients are severely depleted during biofilter shutdown periods, microorganisms may consume EPS (Brown et al., 2020; Zhang & Bishop, 2003). This may affect the biofilm's physical properties and lead to thinner biofilms (Liao et al., 2014). Conversely, nutrient limitations can prompt excess EPS secretion, producing filamentous biofilm morphology, especially during high-stress periods like substrate-limiting conditions (Lauderdale et al., 2011). This can subsequently impair the hydraulic performance of biofilters and diminish the efficiency of contaminant removal (Brown et al., 2020; Keithley & Kirisits, 2019). Excess EPS production could also lead to a thicker biofilm, which might act as a diffusion barrier to substrates and dissolved oxygen across the biofilms (Lazarova & Manem, 1995; Leung et al., 2006; Simpson, 2008). Lauderdale et al. (2011) observed that when the biofilters were no longer nutrient-limited, the microorganisms responsible for EPS production were reduced, leading to increased contaminant removal from the biofilters.

Mn removal in biofilters requires the presence of MnOB and the formation of catalytic MnO_x (Bruins et al., 2015b,c). MnOB requires a DO content $> 5 \text{ mg/L}$, an Eh $>$ about 300-400 mV (depending on the pH), and a pH > 7.1 (Mouchet, 1992). In cases of intermittent operation, the absence of a continuous oxygen supply can lead to anoxic conditions, favoring the activity of bacteria that use Mn(IV) oxides as an electron acceptor for respiration instead of oxygen. This situation can chemically reduce MnO_x , releasing soluble Mn(II) into the water and thus increasing effluent Mn concentration (Gregory & Carlson, 2003). Evans et al. (2020) observed that during a winter shutdown, two biofilters receiving different source waters exhibited significantly varied Mn removal efficiencies upon start-up. While biofilters at one utility did not show any decrease in total Mn removal, the other experienced a significant reduction in Mn removal across biofilters, including Mn release, potentially due to anoxic conditions in the biofilters. Although the operating conditions at these utilities were markedly different, it is challenging to attribute the performance discrepancies solely to differences in their influent water characteristics. Nonetheless, the impact of water quality characteristics on intermittent biofilters remains significant and warrants further investigation. Further research into characterizing the microbial community, understanding biofilm formation, and examining the role of EPS could provide deeper insights into the operation of drinking water biofilters in intermittent modes.

2.4.2.1.2 Impact of operational parameters on Mn removal

In the case of intermittent biofilter operation, additional parameters such as duration of biofilter shutdown, filter media status (submerged vs. drained), backwashing frequency, and technique influence biofilters' performance. The duration of the shutdown period is expected to influence the concentration of contaminants in the effluent after restart. It will also determine if other actions are required to mitigate the potential negative impacts of the biofilter shutdown. In the case of intermittent operation, subjecting biofilters to backwash before restart has been shown to negate the negative impacts and improve filter performance (Niquette et al., 1998). The effect of a long-term shutdown (two weeks vs. four weeks) was studied on biofilters removing Mn from surface water during cold water conditions ($< 15^\circ \text{C}$) with GAC and anthracite as biofilter media. Manganese removal across the GAC biofilters decreased after the four-week shutdown, but no differences were observed in the two-week shutdown. In contrast, Mn removal across the anthracite biofilter decreased following both shutdowns (Evans et al., 2020).

During the shutdown period, biofilters can be kept either drained or submerged. If biofilter media drying occurs, this might cause the loss of biological activity. If the filter remains submerged, the

decreasing amount of substrate in the surrounding stagnant water may compromise the biofilm communities of organisms. Evans et al. (2020) observed that the biofilters could maintain active biomass through the two-week shutdown and suggested the possibility that leaving the biofilters submerged and backwashing before bringing the biofilters back online may have contributed to maintaining the biomass. Zeng et al. (2019) observed that submerging biofilters during shutdown negatively impacted the performance of biofilters removing Mn, whereas drained biofilters showed better Mn removal. A decrease in the DO was observed, making the biofilter anaerobic, thus possibly inhibiting the growth and reproduction of MnOB and resulting in an increase in effluent Mn concentration after restart. Further research is required to optimize operational strategies for effective Mn removal in biofilters under intermittent conditions.

2.4.2.2 Biofilter media

Greenstein et al. (2018) observed that GAC pilot-scale biofilters could achieve nearly 100 percent removal of influent Mn, while anthracite pilot columns only removed an average of 12 percent of the influent Mn. Despite similar levels of biological activity measured as ATP on both media types, GAC filters exhibited greater microbial diversity than anthracite biofilters. This might be due to GAC having a more mature biofilm and more established MnO_x than anthracite. However, in another study, there was no significant difference in Mn removal efficiency between bench-scale GAC and anthracite biofilters (Granger et al., 2014). Huck et al. (2000) suggested that after the initial adsorption capacity is exhausted, anthracite and GAC biofilters remove dissolved organic carbon, and by extension, Mn would likely show no substantial performance difference.

Several researchers have explored the use of alternative media such as quartz, calcium carbonate, polystyrene, manganese oxides (Breda et al., 2017), oxide-coated auto-activated silica sand, gabon Mn (Jez-Walkowiak et al., 2017), chalcedonite (Jez-Walkowiak et al., 2015), and virgin pumice (Araya-Obando et al., 2021). The use of virgin pumice, silica sand, and anthracite as biofilter media does not impact the start-up time of biofilters removing Mn (Araya-Obando et al., 2021), whereas Breda et al. (2017) observed differences in the start-up times of biofilters using quartz, calcium carbonate, polystyrene, and manganese oxides. This suggests that the choice of biofilter media can affect the start-up phase of biofilters. Standard biofilter media include anthracite, sand, GAC, or a combination of these as dual media for Mn removal. However, comparative data on anthracite, sand, and GAC biofilters operated under similar conditions are scarce, indicating a need for further research.

In studies investigating the impact of filter media on biofilter performance for Mn removal, the effect of media size is often not addressed. The available surface area for biological growth depends on media size, a critical parameter for the optimized performance of traditional filters. However, the impact of decreased contact time associated with increased media size on Mn removal in biofilters is currently unknown. The selection of filter media can influence the organisms present in the biofilter and their functional potential and activity (Kirisits et al., 2019). Dymaczewski et al. (2020) studied the impact of the microstructure of filtration material (silica sand, chalcedonite sand, and GAC) on autocatalytic properties for Mn removal from groundwater. It was observed that auto-activation times were influenced by the mesopore volumes and specific surface area of the filtration material, with shorter auto-activation times for GAC and chalcedonite (24 and 32 days, respectively) compared to silica sand (50 days). The presence of mesopores in chalcedonite sand might facilitate microbial growth and oxide deposition within the pores (Jez-Walkowiak et al., 2017), although this study did not confirm it. Further investigations are required to determine the impact of filter media characteristics, such as pore volume distribution and surface area, on biofilter performance for Mn removal.

2.4.2.3 Empty bed contact time (EBCT)

EBCT represents the time the influent water to be treated is in contact with the biofilter media. EBCT is a critical parameter in the biological oxidation of Mn, especially when Fe and NH_4^+ are present in the influent water, which can lead to stratification (Fig. 2.3). The required EBCT to achieve specific performance levels depends on the kinetics of the process; thus, kinetics directly influence the minimum EBCT needed for optimal performance. Liu et al. (2001) observed that biodegradation kinetics improved with longer EBCT under cold-water conditions. However, with first-order kinetics, an increase in EBCT will give diminishing returns beyond a certain threshold. Lauderdale et al. (2016) did not observe a clear relationship between decreasing EBCT (from 6.4 min to 3.2 min) and Mn removal efficiency under cold-water conditions while spiking Mn. The authors suggested that a higher loading rate may have contributed to inadequate Mn removal during cold-water testing, as the two EBCTs were not tested in parallel. In another study, EBCT was lowered from 13.2 to 8.5 min, and no reduction in Mn removal was observed across the biofilters, with both removing Mn below 50 $\mu\text{g/L}$ (Lauderdale et al., 2014). However, Bruins et al. (2014) proposed that an EBCT of more than 11.5 minutes is essential for effective Mn removal in groundwater biofilters containing Fe and NH_4^+ .

Hoyland et al. (2014) examined the impact of media depth (i.e., the EBCT available) on Mn removal in bench-scale biofilters. The study showed that around 60 to 90 percent of Mn removal occurred in

the top 10 cm of the biofilter. They also noted that Mn removal extended deeper into the biofilter bed with higher influent Mn concentrations, suggesting the possibility of incomplete Mn removal with shallower media depths (i.e., shorter EBCTs). Similar results were observed when Mn concentration was increased from 1 to 10 mg/L in a pilot-scale biofilter while simultaneously removing Mn, Fe, and NH_4^+ (Cheng et al., 2019). Manganese removal occurred in deeper layers of the biofilter bed, and the kinetics of Mn oxidation did not adhere to first-order kinetics at a concentration of 10 mg/L. Therefore, the EBCT necessary for effective Mn removal is likely influenced by water quality characteristics, including Mn, Fe, and other co-contaminant concentrations.

2.4.2.4 Backwashing

Key factors such as backwash frequency/duration and the use of air scour are among the conditions that can affect Mn removal in biofilters (Lauderdale et al., 2011). Bruins et al. (2017) noted that the impact of backwashing frequency on Mn removal was more pronounced during the start-up phase of new anthracite biofilters. After 14 days of continuous operation, the Mn(II) removal efficiency was decrease from 28% to <10% (Bruins et al., 2017). However, as the biofilters matured, the influence of backwash frequency on Mn removal became less significant, a change attributed to the growth of biomass and the accumulation of MnO_x on the biofilter media. Furthermore, more frequent backwashing could hinder the formation of a catalytic layer on the biofilter media, which is essential for effective Mn removal (Bruins et al., 2017; Cai et al., 2016). The intensity and duration of backwashing also affected the presence of MnOB and could alter the density of microorganisms within the biofilters (Cai et al., 2016; Li et al., 2006). Thus, the optimal management of backwashing is essential for maintaining the balance between a healthy microbial community and the effective removal of Mn in biofilters.

2.5 Importance of biofilter start-up period

The start-up period is defined as the time taken by the biofilters to achieve the treatment objective. Manganese removal in biofilters depends on the acclimation of MnOB and the development of MnO_x , which can affect the start-up period. The primary cause of concern with a longer start-up time for a new biofilter includes wastage of produced water, energy costs associated with pumping and backwashing, and alternate options to meet the production needs during the start-up time. The start-up period of biofilters can range from a few days to more than six months, depending on the biofilter media, water quality characteristics, and operational parameters (Araya-Obando et al., 2021; Breda et al., 2017). The presence of co-constituents such as NH_4^+ and Fe can significantly affect the start-up

process of Mn removing biofilters (Katsoyiannis & Zouboulis, 2006; Pacini et al., 2005; Tekerlekopoulou & Vayenas, 2008). The co-precipitation of MnCO_3 can also hinder the start-up process as it has no adsorptive or autocatalytic properties and can cover the already formed MnO_x (Buamah et al., 2009).

Various methods to reduce the start-up time of Mn-removing biofilters have been explored, including using different filter media (Araya-Obando et al., 2021; Breda et al., 2017; Evans et al., 2020; Jeż-Walkowiak et al., 2015), applying inoculants containing MnOB (Ciancio Casalini et al., 2020; Guo et al., 2019), adding a layer of matured media to biofilters (Bruins et al., 2015a; Guo et al., 2019; Jia et al., 2015; Piispanen & Sallanko, 2010), using oxidants or adjusting Fe/Mn ratio to develop Fe- MnO_x (Cheng et al., 2018; Štembal et al., 2005; Yang et al., 2021), and adjusting pH (Granger et al., 2016; McCormick et al., 2021) (Table A2.2). However, the effectiveness of these strategies is site-specific depending on initial Mn concentration, presence of co-contaminants, temperature, pH, and operational parameters.

2.6 Knowledge gaps

Key areas that need further investigation are:

- Impact of filter media characteristics such as pore size distribution, media size, and surface area on the start-up of biofilters and Mn removal mechanisms.
- Applicability of biofiltration for Mn removal in low water temperatures ($< 15^\circ\text{C}$) in the presence of Fe.
- Enhance understanding of how contributions of different Mn removal mechanisms (biological and physicochemical) evolve from start-up to maturity and how these contributions change with increasing biofilter depth.
- Provide a better understanding of how the microbial community composition evolves in biofilters and identify potential Mn removal pathways.
- Investigate the impact of intermittent operation on biofilter Mn removal performance, evaluate the influence of different intermittent shut-down periods and start-up methodologies on the performance of biofilters, and determine the impact of intermittent operation on biological activity, biofilm development, and microbial community composition.

3

Evaluating Manganese Removal in Groundwater Using Pilot-Scale Biofilters: The Role of Filter Media Characteristics during Start-up

Part of this chapter has been submitted for publication as:

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3.1 Summary

This study investigated the influence of filter media characteristics on Mn removal in groundwater biofilters during the start-up phase. Six pilot-scale biofilters containing three different GAC, two anthracite, and a sand media were run at a drinking water utility for 133 days to examine their Mn removal performance while monitoring ATP and bacterial growth. Key findings demonstrate the critical role of media characteristics, especially for GAC, which, with its higher surface area and total pore volume, showed early-stage Mn adsorption and high extents of Mn removal. Adsorption initially contributed to Mn removal during the start-up of the GAC media, followed by a transition to biological and physicochemical oxidation over time. In contrast, biological processes dominated Mn removal on anthracite and sand during start-up. Additionally, the presence of MnOB bacteria was detected in biofilters. ATP levels were correlated to Mn removal up until the biofilters were acclimated, showing the potential of ATP as an acclimation monitoring metric. Once acclimated, all biofilters consistently reduced Mn levels from $60.9 \pm 4.5 \mu\text{g/L}$ to below $5 \mu\text{g/L}$ (>90% removal) while concurrently removing Fe, thereby highlighting the biofilter's effectiveness at low water temperatures (<15°C). This study demonstrates the advantage of GAC media for Mn removal in biofilters during start-up in regions with lower water temperatures.

Keywords: Iron, ATP, manganese oxides, pore size distribution, manganese-oxidizing bacteria

3.2 Introduction

Manganese in drinking water, traditionally linked to aesthetic and operational issues, has recently gained attention due to emerging health concerns. Multiple studies have demonstrated a significant correlation between elevated levels of Mn in drinking water ($> 100 \mu\text{g/L}$) and cognitive and neurobehavioral effects in children (e.g., Bouchard et al., 2007; Rahman et al., 2017). Furthermore, Mn has been identified as a key driver of Pb release in distribution systems (Trueman et al., 2019) and exacerbates health issues related to As exposure (Wright et al., 2006). In response, the World Health Organization has set a provisional guideline of $80 \mu\text{g/L}$ for Mn in drinking water (WHO, 2020), while Health Canada has established a MAC of $120 \mu\text{g/L}$ and an AO of $20 \mu\text{g/L}$ (Health Canada, 2019).

In groundwater, Mn predominantly exists as dissolved Mn(II), which can undergo oxidation through various processes within a biofilter, including homogeneous, heterogeneous, and biological pathways (Tebo et al., 2005; Vries et al., 2017). Dissolved Mn(II) can also be removed through adsorption depending on the filter media properties (Buamah et al., 2008). While homogeneous oxidation of Mn(II) by oxygen is thermodynamically favored at neutral pH (Diem & Stumm, 1984), it is slow in the absence of metal oxide surfaces like MnO_x , which act as a catalyst (Morgan, 2005). Biological Mn oxidation occurs through both indirect and direct processes. Indirect processes involve bacterial growth-induced pH and redox modifications, while direct oxidation involves specific enzymes (Tebo et al., 2005). Manganese oxidation, regardless of the process, results in MnO_x formation. The Mn removal is further facilitated by the adsorption of Mn onto Mn oxides, catalyzing the transformation of Mn(II) to MnO_x through oxidation. During the start-up of biofilters, the primary formation of MnO_x is a consequence of biological Mn oxidation in sand and anthracite biofilters (Bruins et al., 2015b; Dangeti et al., 2020).

However, the effectiveness of biofilters is challenged by low water temperatures ($7\text{--}17^\circ\text{C}$), which can slow down the start-up process, particularly in the presence of Fe, a common co-contaminant in groundwater (Ciancio Casalini et al., 2020; Ramsay et al., 2018). Various methods have been investigated to reduce the start-up time of Mn-removing biofilters, mainly by using different filter media (Araya-Obando et al., 2021; Breda et al., 2017; Evans et al., 2020; Jez-Walkowiak et al., 2015) and applying inoculants from mature biofilters containing MnOB (Cai et al., 2015; Ciancio Casalini et al., 2020).

Despite numerous studies investigating the impact of different filter media on start-up, essential characteristics like surface area, pore size distribution, and media size have often been insufficiently addressed, leading to conflicting findings (Araya-Obando et al., 2021; Evans et al., 2020; Granger et al., 2014). Moreover, these studies have primarily focused on non-adsorptive media like sand and anthracite. GAC media provide a higher surface area, pore volume, and increased availability of functional groups, which may contribute to microorganisms' adsorption and potential attachment during the initial stages of biofilter operation. The role of biofilter media is critical during start-up, as it offers surfaces for microorganism attachment and biological Mn oxidation. The extent of available external surface area for biological growth depends on the media size, and a higher specific surface area may also enhance Mn removal through direct adsorption on the media. These factors may influence the Mn removal mechanism, especially during start-up. Dymaczewski et al. (2020) observed that mesopore volumes and specific surface area of the filter media were dominant factors affecting the start-up time for Mn removal but did not explore the contributions of MnOB and MnO_x to Mn removal. Overall, there is limited information on the role of media characteristics in Mn-removing biofilters and a limited understanding of the dominant processes for Mn removal in biofilters, particularly across different filter materials.

This study aims to bridge these gaps by examining the relationship between filter media properties and Mn removal efficiency in biofilters used for groundwater treatment during the start-up phase, addressing challenges such as low water temperatures and the presence of Fe while also investigating the roles of MnOB and MnO_x. The specific objectives are: 1) to investigate the influence of pore size distribution and media size on the biofilter performance and the kinetics of Mn removal, 2) to characterize MnO_x on the ripened filter medium coating to gain insights into the mechanism of the Mn removal processes, and 3) to investigate the relationship between culturable MnOB, biological activity, and biofilter performance. To achieve these objectives, six pilot-scale biofilters were set up and operated for 133 days, thus providing a comprehensive understanding of the factors influencing Mn removal and offering practical insights for water utilities.

3.3 Materials and methods

3.3.1 Experimental setup and operating conditions

The pilot-scale biofilters were operated at a water treatment plant in Southern Ontario. Raw water from groundwater wells was collected in two 1000 L tanks, where it was aerated before pumping into

the biofilters. The average groundwater DO was less than 1.8 mg/L. Raw water quality parameters were analyzed weekly (Table 3.1).

Table 3.1: Influent water characteristics to pilot scale biofilters

Parameter	Mean $\pm$ S.D (number of data points)	Max	Min	Units
Temperature	11.6 $\pm$ 2.3 (40)	14.1	8.6	$^{\circ}\text{C}$
pH	7.5 $\pm$ 0.3 (40)	7.9	7.1	-
Manganese	60.9 $\pm$ 4.5 (20)	68.4	51.4	$\mu\text{g/L}$
Iron	71.7 $\pm$ 17.4 (20)	127.2	56.3	$\mu\text{g/L}$
TOC*	0.8 $\pm$ 0.1 (4)	0.6	0.9	mg/L C
Hardness*	248 $\pm$ 38 (4)	301	195	mg/L as CaCO_3
Alkalinity*	182 $\pm$ 23 (4)	213	75	mg/L as CaCO_3
ORP	158 $\pm$ 30 (40)	204	107	mV
DO (after aeration)	8.7 $\pm$ 0.9 (68)	10.1	7.8	mg/L

*Analyzed monthly; TOC = total organic carbon

The pilot-scale setup consisted of six PVC columns (PVC SCH 80, Fabco plastics), each with an inner diameter of ~ 0.1 m, height of 3 m, and media depth of 1.2 m over 0.2 m gravel support (Fig. 3.1 & Text A3.1). Three different types of commercially sourced coal-based GAC (GAC1, GAC2, GAC3), two types (media size) of anthracite (A1, A2), and one type of sand (S1) were used (Text A3.1.3).

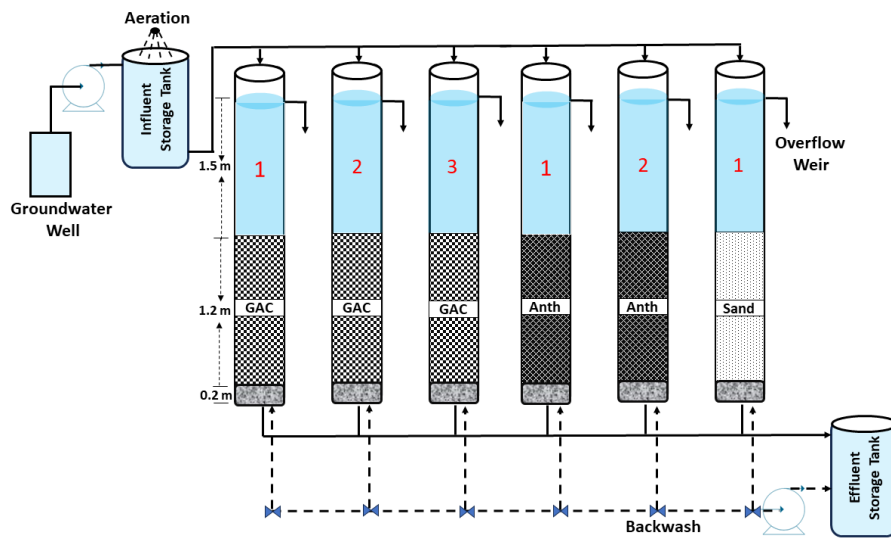


Figure 3.1: Schematics of pilot-scale biofilter setup

All media were sieved to ensure an effective size of 0.85-1.00 mm and uniformity coefficient (UC) of 1.8, except for A1, which had a size of 1.10-1.25 mm. The effluent flow rate in each column was maintained at 5 m/h, resulting in an empty bed contact time of 14.4 minutes or less due to a reduction in filter bed volume over time as filter media was removed for sampling. Backwashing was done once per week with a flow rate of 10-20 m/h using the effluent from the biofilters, resulting in bed expansions of 20-30%, which was performed until clear water (5-10 min) was observed in the supernatant and backwash effluent.

3.3.2 Analytical methods

Influent and effluent water samples were collected weekly for Mn and Fe analysis. In addition, samples were collected from different bed depths for analysis on days 18, 28, 56, 84, 126, and 133. The water samples were collected in acid-cleaned (7% HNO_3) 50 mL plastic tubes (Falcon®, VWR™) and transported on ice to the laboratory for immediate processing. The samples were acidified with 2% HNO_3 (ARISTAR® PLUS, VWR Chemicals BDH®) to $\text{pH} < 2$ and stored at 4°C before their analysis using triple quadrupole inductively coupled plasma mass spectrometry (QQQ-ICP-MS, Agilent 8800). Helium was used as a collision cell gas, and scandium was used as an internal standard to correct instrument drift. United States Geological Survey water standards (T211, T227, T231) were analyzed multiple times during each analytical session to verify instrument accuracy. The average measured Mn and Fe concentrations of these standards were $94 \pm 5 \%$ and $98 \pm 5 \%$ (SD, $n = 122$), respectively, of their certified values. Data reproducibility was also verified by duplicate analysis of a subset of water samples during each analytical session. The average deviation between the duplicate and original analysis was $1 \pm 7 \%$ and $1 \pm 8 \%$ for Mn and Fe, respectively ($n = 156$). The average detection limits for Mn and Fe were $0.016 \pm 0.006 \mu\text{g/L}$ and 0.345 ± 0.160 ($n = 28$), respectively. The pH, ORP, DO, and temperature were determined on-site using a YSI professional plus multimeter (Xylem, USA).

3.3.2.1 Filter media characterization

The surface area was determined according to the Brunauer–Emmett–Teller (BET) method with adsorption/desorption isotherms that were measured at 77 K using a Tristar II 3020 apparatus (Micromeritics). The t-plot method was used to analyze the micropore volume of the GACs (Dymaczewski et al., 2020). Based on the adsorption data, the mesopore size distributions were derived from the desorption branches of isotherms using the Barrett–Joyner–Halenda (BJH) model. Macropore volume was determined through mercury intrusion porosimetry (MIP) using an AutoPore

IV 9500 (Lu et al., 2020). The total pore volume was calculated as the sum of micropore, mesopore, and macropore volumes (Text A3.2). Raman spectroscopy was performed to distinguish different types of MnO_x on the biofilter media (Bruker Senterra Raman, WATLab, University of Waterloo). SEM was performed to determine the structure of the filter media coating and the MnO_x . EDX provided information regarding the trace elements present in these structures (FEI Quanta FEG 250 ESEM, WATLab, University of Waterloo). SEM-EDX and Raman spectroscopy were performed following the procedure outlined in Bruins et al. (2015b,c).

3.3.2.2 Microbiological analysis

The biofilter media samples (~3 g) were collected from the top of the biofilters (0-5 cm bed depth) once per week for ATP analysis, which was performed using a deposit and surface analysis kit (DSA™, LuminUltra Technologies, Canada). The media samples were collected using a sterilized spatula, stored in sterile 50 mL plastic tubes (Falcon®, VWR™), and transported on ice to the University of Waterloo for immediate processing (Text A3.3.1). The biofilter media samples (~3 g) were collected at days 28, 56, and 128 from the top of the filter bed and at 40 cm bed depth to perform heterotrophic plate counts (HPC) and MnOB counts using three different culture media (Mn supp, Mn min, and R2A+Mn) following the procedure outlined in Breda et al. (2017) (Text A3.3.2). The media samples were stored in sterile 50 mL plastic tubes (Falcon®, VWR™) and transported on ice to Wilfrid Laurier University for immediate processing. The ability of heterotrophs to oxidize Mn was verified using the leucoberbelin blue (LBB) assay (65 % dye, Sigma Aldrich, Canada) following the procedure outlined in Araya-Obando et al. (2021) (Text A3.3.2).

3.3.2.3 Kinetics analysis

Manganese removal kinetics are expressed using a first-order kinetic model (Dangeti et al., 2017; Katsoyiannis & Zouboulis, 2004) as follows:

$$d \frac{\{Mn(II)\}_t}{dt} = -k \in \{Mn(II)\}_t$$

In the equation above: k is the first-order rate constant (min^{-1}) for the overall removal of Mn(II), which may include biological and physicochemical removal; ϵ is the bulk porosity of the biofilter media, $\{Mn\}_t$ is the concentration of Mn(II) at the EBCT; and EBCT is the amount of time for influent to pass through the empty space occupied by the biofilter. Water samples collected from each 20 cm depth interval of the biofilters were used to calculate k at days 28, 56, 84, 126, and 133. The first-order removal constant k (min^{-1}) was determined for each of the biofilters as the slope from the

linear regression between $\ln[\{Mn(II)_t\} / \{Mn(II)_o\}]$ and $\epsilon * EBCT$ and is reported in Table S1. The values of porosity were taken from the literature: for GAC, a value of 0.45 (Reed et al., 1996); for anthracite, a value of 0.5 (Anthracite supplier, Greenbird Canada); and for sand, a value of 0.45 (Hwang et al., 2017).

3.3.2.3 Statistical analysis

All statistical analysis was conducted using the Origin Pro 2024 software. A significant level of $p = 0.05$ was used for Spearman correlations, Kruskal Wallis, and ANOVA tests.

3.4 Results and discussion

3.4.1 Filter media characteristics

Table 3.2 highlights the filter media characteristics. GAC1 has a total pore volume of 0.391 mL/g with a low proportion of mesopores compared to micropores and macropores; GAC2 has the highest pore volume of 0.639 mL/g with micropores > mesopores > macropores, whereas GAC3 has a pore volume of 0.376 mL/g with lower levels of macropores and mesopores than GAC2, but a similarly high volume of micropores. The pore volumes of the anthracite and sand media were very low compared to GAC media, and the pore volumes were concentrated in the macropore range, as expected.

Table 3.2: Filter media characteristics

Media	Media Size (mm)	Total Pore volume (mL/g)	Macropores (mL/g) (> 50 nm)	Mesopores (mL/g) (2-50 nm)	Micropores (mL/g) (< 2 nm)	Surface Area (BET) (m ² /g)
GAC1	0.85 - 1.00	0.391	0.174	0.091	0.125	1021.90
GAC2	0.85 - 1.00	0.639	0.169	0.210	0.260	1256.97
GAC3	0.85 - 1.00	0.376	0.047	0.067	0.262	1146.90
A1	1.10 - 1.25	0.004	0.004	0.000	0.000	0.22
A2	0.85 - 1.00	0.009	0.009	0.000	0.000	0.88
S1	0.85 - 1.00	0.002	0.002	0.000	0.000	0.19

3.4.2 Start-up and steady-state functioning of biofilters

3.4.2.1 Mn removal across biofilters

Fig. 3.2 shows the influent and effluent Mn concentrations in six pilot-scale biofilters over 133 days. The average influent Mn concentration was $60.9 \pm 4.5 \mu\text{g/L}$. The start-up or acclimation period was complete when the biofilters consistently removed more than 90% of the average influent Mn concentration. The Day 0 measurement represents the data collected 8-12 hours after the start of biofilter operations. On day 77, all biofilters met this treatment objective. However, the time to reach this objective varied substantially ($p < 0.05$). The start-up periods for GAC1, GAC2, and GAC3 were 21, 28, and 35 days respectively, whereas A1, A2, and S1 took 56, 42 and 77 days, respectively. The higher BET surface area and inherent adsorption capacity of GAC media for Mn(II) might have resulted in early Mn removal, leading to shorter start-up times for the GAC filters. The anthracite media (A2) with the smaller effective size reached the treatment objective levels earlier than its larger counterpart A1. The increase in filter grain size leads to a decrease in the external specific surface area, which might have contributed to slower acclimation. Although sand (S1) and anthracite (A1) have comparable surface areas, sand media took longer to reach acclimation; this might be due to the different filter media characteristics.

The influent raw water temperature was $11.6 \pm 2.3^\circ\text{C}$, and more than 90% Mn removal was observed for all biofilters within 11 weeks of operation. Ciancio Casalini et al. (2020) reported that acclimation of new Mn-removing biofilters was slower at water temperatures below 15°C . However, the GAC filters in this study displayed a robust performance despite the suboptimal temperature range, reaching operational efficiency quickly. This indicates that GAC media may have inherent properties that counteract the negative impact of lower temperatures on the biological mechanisms responsible for Mn removal. Mouchet (1992) reported that a $\text{pH} > 7.5$ and an Eh of 300 to 400 mV are required for effective biological oxidation. During the start-up, the effluent ORP was below 300 mV. However, there was a gradual increase in the ORP in the biofilters over time, correlating with a decrease in Mn(II) concentrations in the biofilter effluent. This correlation was statistically significant based on a Spearman correlation test (Fig. A3.1, $n = 126$, Spearman correlation coefficient (ρ): -0.62, $p < 0.05$).

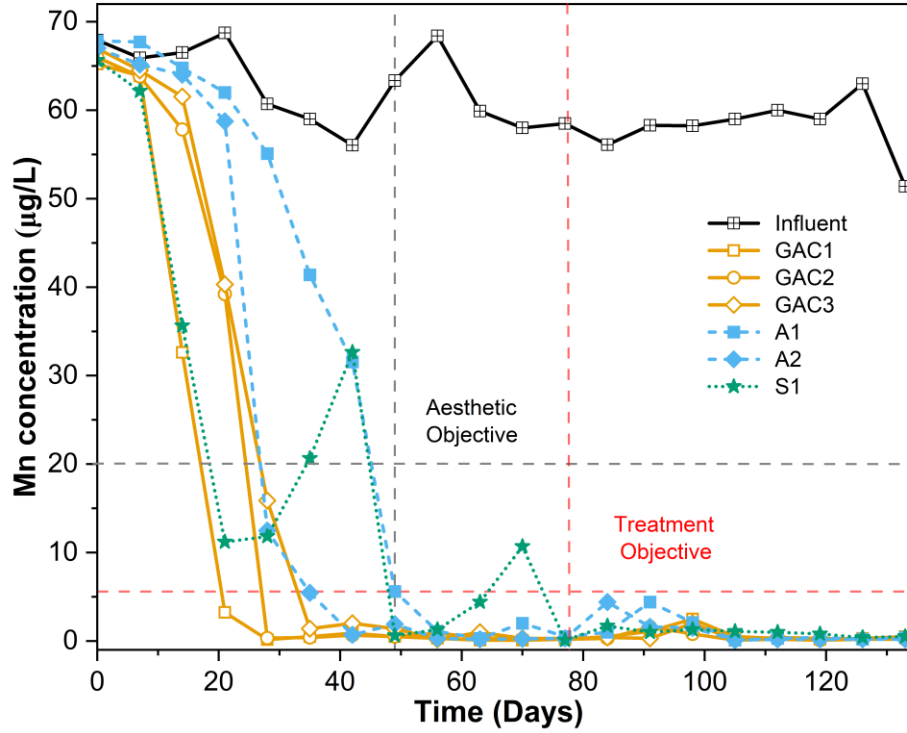


Figure 3.2: Biofilter influent and effluent Mn concentrations. The vertical black and red dashed lines represent the time taken by all biofilters to consistently remove Mn below the aesthetic objective ($20 \mu\text{g/L}$) and treatment objective ($>90\%$ removal) levels.

Fig. 3.3 indicates the variation in Mn concentration along the filter bed depth at different points during the experimental period. At day 28, a 100-120 cm filter bed depth was required to reach the AO level of $20 \mu\text{g/L}$ by all media except GAC1 and GAC2, which only needed 20 cm bed depth, and A1 did not meet AO levels on day 28. This shorter distance is attributed to the high adsorption capacity of the GAC media. By day 56, GAC1, GAC2, GAC3, and A2 reached the aesthetic objective at 0-20 cm, but A1 and S1 required 40-60 cm and 60-80 cm, respectively (Fig 3.3b). By days 126 and 133, all biofilters effectively met the objectives within the top 20-40 cm (Fig 3.3d,e). This shift in the Mn removal zone towards the upper filter layers aligns with findings from previous studies utilizing non-adsorptive media such as sand and anthracite (Breda et al., 2019; Dangeti et al., 2017). A particularly notable comparison can be made with Zhao et al. (2021), who observed that over 93% of Mn removal was accomplished in the top 25 cm of a Mn sand biofilter after 120 days. These results suggest that this phenomenon is not exclusive to non-adsorptive media but is also present in GAC media,

indicating strong biological activity and MnO_x accumulation in biofilters' upper regions, contributing to Mn removal from groundwater.

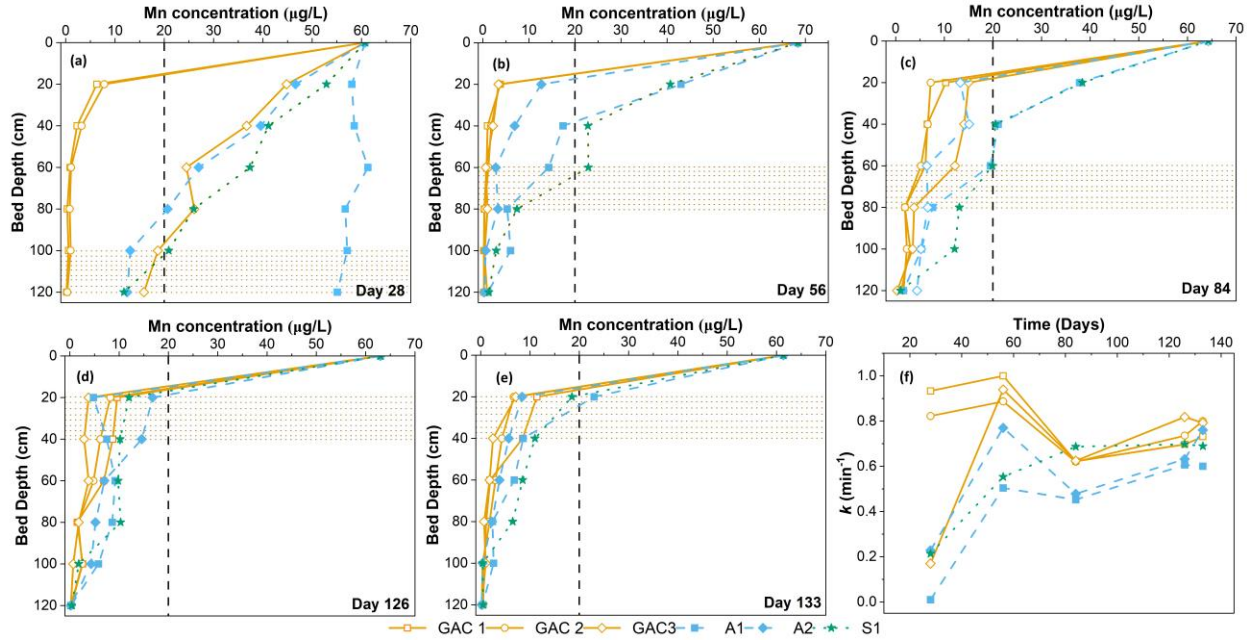


Figure 3.3: Mn depth profile for different biofilter media at (a) Day 28, (b) Day 56, (c) Day 84, (d) Day 126, and (e) Day 133 (the vertical black lines represent the aesthetic objective level of 20 $\mu\text{g/L}$ and horizontal brown lines represent the bed depth zone that is required to achieve those levels by all media; (f) Variation in the first order removal constant k (min^{-1}) over time

These results also highlight that filter media characteristics and the operational stage of the biofilter influence the Mn depth profile. Except for the GAC3 biofilter on day 28, GAC biofilters perform better than anthracite and sand, especially early in the start-up until day 84. This can probably be related to the adsorption of Mn(II) in the internal pores of the GACs, though these differences diminish over time. A disparity in performance between different GAC media can be observed. For example, on day 28 (Fig. 3.3a), GAC3 media required a filter bed depth of 100 cm to achieve the treatment objective, whereas GAC1 and GAC2 needed only a depth of 20 cm. This suggests that not just the total pore volume but also the distribution of pore size impacts the adsorption of Mn(II) and, consequently, the performance of the filter media during the early stages of biofilter operation.

Additionally, the comparison between A1 and A2 (non-adsorptive media) shows that biofilter A2, with its smaller effective media size and, therefore, higher specific surface area, may be characterized

by a more effective adsorption process or biofilm development, leading to a more effective Mn removal at shallower depths during start-up. Nevertheless, these differences gradually decrease beyond day 84 with the likely development of MnO_x and biofilm. Beyond media characteristics and operational stages, influent water quality can impact the Mn removal process in biofilters. The concentration of hydrogen carbonate in the influent water can result in the precipitation of MnCO_3 which does not have any auto-catalytical adsorption and oxidation properties (Bruins et al., 2014; Buamah, 2009). The influent water to the biofilter had a pH below 8 (Table 3.1), making the significant formation of manganese carbonate unlikely. This assumption was confirmed by Raman spectroscopy analyses of the matured media (section 3.4.3), where no MnCO_3 formation was observed.

3.4.2.2 Kinetics of Mn removal in biofilter

Manganese removal kinetics were only studied previously for non-adsorptive media such as sand and anthracite (Dangeti et al., 2017; Katsoyiannis & Zouboulis, 2004). The present study assessed the kinetics of Mn removal using a first-order kinetic model for all biofilters, differentiating between adsorptive and non-adsorptive media. For all biofilters, the first-order removal constant k (min^{-1}) ranged from 0.005 to 1.1 min^{-1} at different stages of biofilter operation (Fig. 3.3f & Table A3.1). The k values observed here were higher than in other studies (Dangeti et al., 2017; Ramsay et al., 2018), which might result from the low Mn influent concentration and the high extents of Mn removal observed in this study. Moreover, previous studies (Dangeti et al., 2017; Katsoyiannis & Zouboulis, 2004) used EBCT in the calculation of k , whereas the actual time that the water spends reaching a given depth is EBCT to that depth multiplied by bulk porosity.

On day 28, the adsorptive media, except GAC3, exhibited markedly higher k values than non-adsorptive media (Fig. 3.3f). Specifically, GAC1 and GAC2 had k values of 1.026 and 0.904 min^{-1} , respectively, compared to GAC3's significantly lower value of 0.186 min^{-1} . The biofilters A1, A2, and S1 recorded k values of 0.01, 0.228, and 0.213 min^{-1} , respectively, on day 28. The initially higher k values for GAC1 and GAC2 likely reflect their high adsorption capacity (Omri & Benzina, 2012) and higher total pore volume than the other media (Table 3.2). In contrast, the initially much lower rate constants for sand and anthracite media could be due to a limited number of active sites. This trend persisted on day 56, with all GACs displaying high k values, surpassing those of the anthracite and sand media. The lower k values observed for non-adsorptive media on days 28 and 56 suggest an incomplete maturation of these filter media, indicative of biofilters in an early start-up phase. By day 84, the k values began to converge, with GAC values decreasing. This converging trend continues

from here forward, and by day 133, the k values across the biofilters converged, yielding mean k values of 0.767 ± 0.099 (SD) min^{-1} , indicative of biofilter maturation.

The non-linear progression of the k values over time (Fig. 3.3f) suggests a transition from adsorption as the dominant removal mechanism to heterogeneous and biological oxidation in the GAC biofilters. Biofilters A2 and, to a lesser extent, A1 exhibited similar patterns, albeit with lower values. Biofilter S1 showed an increasing trend in k values, eventually converging to levels similar to GAC and anthracite biofilters. This trend signifies that, following acclimation and maturation, the impact of the filter media on k values diminishes. Furthermore, this kinetic study indicates that while the treatment objectives were met at day 77 for all biofilters, the Mn removal efficiency observed in the biofilter effluent at this time does not fully represent the maturation of the filter media. This is evidenced by the continued evolution of the k values past day 77. These findings underscore that filter media characteristics influence the kinetics of Mn removal within biofilters during start-up. Monitoring changes in the rate constant can thus serve as a crucial indicator for discerning different operational phases of biofilter operation.

3.4.2.3 Concurrent Fe removal and its impact on Mn removal

The average influent Fe concentration was 71.7 ± 17.4 $\mu\text{g/L}$ (Fig. A3.2). In the first week of operation, GAC biofilters demonstrated a Fe removal efficiency of over 90%, significantly higher than the 75-80% observed in anthracite biofilters and 30% in the sand biofilter. By the second week, more than 80% Fe removal was observed in all biofilters. However, following these initial high removal rates, an increase in effluent Fe concentrations for all biofilters was observed, potentially linked to a sudden rise in influent Fe concentration during the third and fourth week. Despite this increase, by day 133, the effluent Fe concentrations in all biofilters were reduced to below 10 $\mu\text{g/L}$, achieving more than 88% Fe removal with no significant differences in Fe removal efficiencies among the various biofilters ($p > 0.05$).

In the initial stages of biofilter operation, Fe removal is predominantly facilitated by homogeneous oxidation processes and adsorption (for GAC media). Supernatant levels of 1.5 m above the biofilter media in this study extend the residence time, thereby enhancing homogeneous oxidation. Vries et al. (2017) noted that an 80 cm supernatant layer achieved 10% Fe removal, while a 27 cm layer achieved 5% removal, underscoring the impact of residence time on oxidation processes. Iron oxidized to particulate form will be removed from the biofilters during backwashing.

Particularly in GAC media, the early phases of biofiltration exhibited enhanced Fe removal, which may be attributed to the adsorptive capacity of the media. This observation is consistent with previous studies that reported competitive adsorption between Fe(II) and Mn(II) on filter media (Jusoh et al., 2005; Hu et al., 2004) and the reported interference of Fe with biological Mn removal (Kohl & Dixon, 2012). The thermodynamic preference of Fe oxidation over Mn oxidation, especially at lower Eh (100-400 mV) and pH (6-8) values (Mouchet, 1992), is supported by the observed average influent conditions (Eh: 158 ± 30 mV, pH: 7.5 ± 0.3) in this study. These conditions facilitated higher initial Fe removal, leading to a stratified filter bed, where Fe was predominantly removed in the top 20 cm, in contrast to the more homogeneous Mn removal (Fig. A3.3a). As filtration progressed, influenced by regular backwashing, the Mn removal zone shifted upwards, with over 80% removal of both Fe and Mn observed in the top 40 cm of the filter bed (Fig. A3.3b). This observed stratification, with effective Fe and Mn removal primarily in the upper layers of the biofilter media, underscores a significant implication for biofilter design. This study demonstrates that reduced bed depths can potentially be effectively utilized for Fe and Mn removal, contingent on the influent water characteristics. This stratification pattern agrees with the observations made by Ramsay et al. (2018) in full-scale drinking water biofilters, suggesting that such a phenomenon may be broadly applicable across various biofiltration systems.

3.4.2.4 Impact of filter media characteristics on Mn removal

Fig. 3.4 illustrates the relationship between the time taken by biofilters to achieve the Mn removal target (> 90%) and the initial pore volumes and BET surface areas of media in those biofilters. A strong inverse Spearman correlation between macropore volume and time ($\rho = -0.99$, $p < 0.05$) was observed, with GAC1 (macropore volume = 0.174 mL/g) achieving the target in 21 days, followed by GAC2 (0.169 mL/g, 28 days) and GAC3 (0.047 mL/g, 35 days). This trend was also evident in non-adsorptive media such as A2 (0.009 mL/g, 42 days) compared to A1 (0.004 mL/g, 56 days) and S1 (0.002 mL/g, 77 days). These findings suggest that larger macropore volumes may expedite the biofilter start-up process, likely due to their suitability for bacterial attachment (Walker & Weatherley, 1998) and MnO_x accumulation. Mesopore volume also negatively correlated with time ($\rho = -0.886$, $p < 0.05$), but its impact was less pronounced than macropores. GAC2 had the highest mesopore volume, yet its time to target was longer than GAC1, indicating other factors at play.

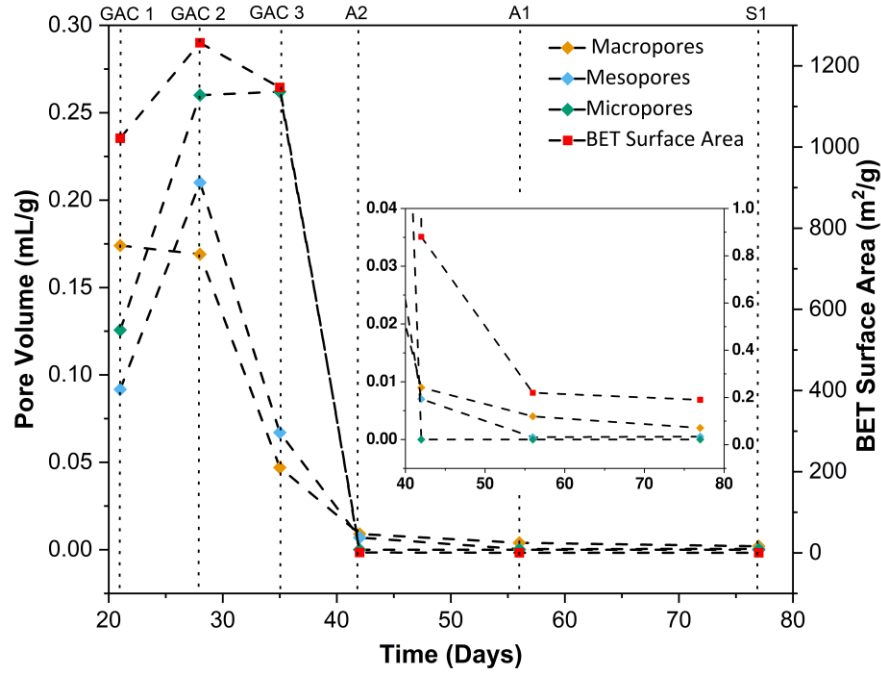


Figure 3.4: Impact of pore size distribution and BET surface area of the media on the time taken for the respective biofilters to reach Mn concentrations below the treatment objective

The ionic radius of Mn(II) with six coordinated hydrated complexes in aqueous solution is 0.084 nm, which can easily penetrate the micropores of GAC for adsorption. This facilitates the adsorption of Mn(II) ions onto the internal surfaces of the GAC through both physical and chemical mechanisms. Physical adsorption occurs via van der Waals forces, where Mn(II) ions are attracted to the GAC surface due to weak intermolecular forces. This type of adsorption is non-specific and reversible, primarily relying on the large surface area and high porosity of GAC. In contrast, chemical adsorption involves groups such as hydroxyl, carboxyl, and carbonyl groups present on the GAC surface. This results in the formation of stable surface complexes. However, despite these effective adsorption mechanisms, the GAC's capacity for Mn(II) adsorption can be quickly exhausted, leading to an initial breakthrough where Mn(II) concentrations in the effluent start to rise. The initial rapid adsorption was not observed in the current study, likely because the Mn effluent measurements were performed only once a week. Once Mn(II) ions are adsorbed, they can undergo either homogeneous or biological oxidation, forming MnO_x . The formation of MnO_x aids in further removal of Mn(II) due to its adsorptive and autocatalytic properties. Micropore volume, while important for adsorption on GAC (Lorenc-Grabowska & Gryglewicz, 2005), showed a weaker and non-significant correlation ($\rho = -$

0.698, $p > 0.05$). This suggests that not all micropores contribute effectively to Mn removal, possibly due to blockage by MnO_x (>10 nm) accumulation over mesopores and macropores, thus reducing the adsorption capacity of GAC (Suresh Kumar et al., 2019). This phenomenon could also explain the reduction in k values at day 84 for GAC media (Fig. 3.3f), where the development of MnO_x might have reduced access to pores, thus contributing to the transition of the dominant mechanism of removal from adsorption to heterogenous and biological oxidation. Overall, the role of micropores appears complex, affecting the adsorption capacity of GAC media for Mn removal.

The BET surface area, predominantly influenced by micropores, was strongly correlated with time ($\rho = -0.82$, $p < 0.05$). GAC samples with higher BET areas reached the Mn removal target levels faster than anthracite and sand, underscoring the importance of surface area in adsorption. However, GAC3, despite a higher BET area, took longer than GAC1 to reach the target, implying limitations in Mn diffusion into micropores and the potential inefficiency of micropores in Mn removal.

In summary, these results highlight the significance of macropore volume and BET surface area in the start-up efficiency of biofilters for Mn removal. Macropores, in particular, play a crucial role, likely due to their suitability for microbial colonization and provide greater space for MnO_x accumulation. The findings also suggest that the contribution of micropores to overall adsorption capacity may be limited, warranting further investigation into the dynamics of Mn(II) adsorption in biofilters.

3.4.3 Physical characteristics of MnO_x accumulated on biofilter media

The Raman spectra of all mature filter media at day 126 (Fig. 3.5a) consistently show the characteristic bands of the birnessite group of MnO_x around 510 cm^{-1} (a small peak), 580 cm^{-1} (a very strong peak) and 630 cm^{-1} (a strong peak), aligning with findings from literature (Bernardini et al., 2019; Bruins et al., 2015b,c) and reference birnessite spectra (Fig. 3.5a). Birnessite has very high adsorptive properties for Mn(II) and leads to subsequent autocatalytic oxidation of Mn(II) (Bruins et al., 2015b,c). Due to substantial background noise, the spectra for GAC1 and S1 were inconclusive, so they are not further discussed. Peak sizes at 630 cm^{-1} and 580 cm^{-1} varied for the different media, which may be attributed to the concentration of different counterions (Bruins et al., 2015c), as supported by SEM-EDX results (Fig. 3.5b). Additional weaker peaks at $\sim 1080\text{ cm}^{-1}$, $\sim 770\text{ cm}^{-1}$, and $\sim 247\text{ cm}^{-1}$ for A1 and $\sim 443\text{ cm}^{-1}$ for GAC3 were observed, which are resonant with the Raman spectra of manganosite, pyrolusite, manganite, and chalcophanite, respectively (Bernardini et al., 2019; Vermeersch et al., 2023).

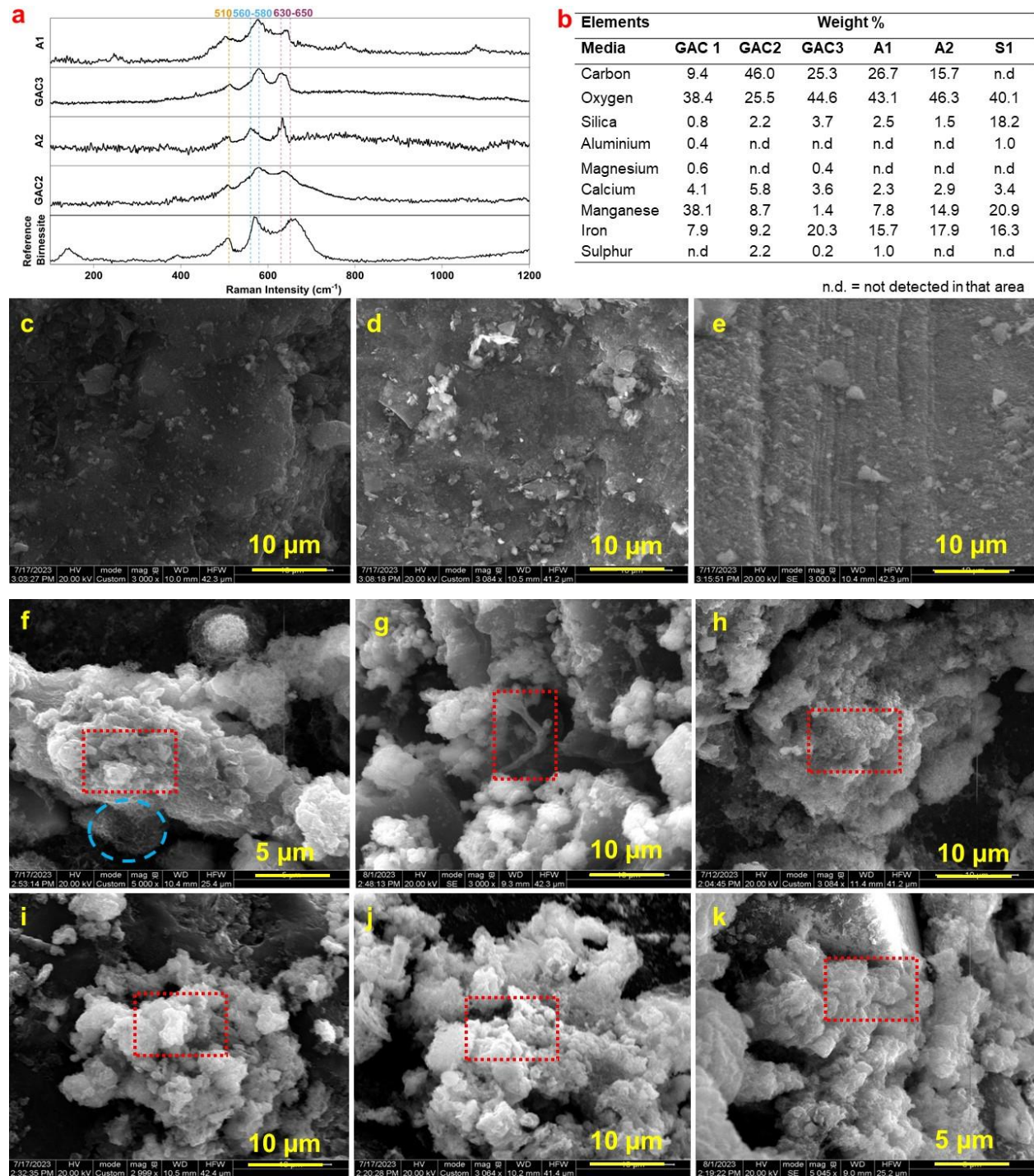


Figure 3.5: (a) Raman spectra of MnO_2 ; (b) Elemental composition from EDX analysis (average from multiple spots, marked in red); SEM images of the virgin (c) GAC, (d) Anthracite, (e) Sand and matured media, (f) GAC1, (g) GAC 2, (h) GAC 3, (i) A1, (j) A2, and (k) S1 (media was collected at day 126 from the top of the biofilters)

Two potential explanations exist for these additional peaks: One is the susceptibility of MnO_x to thermal degradation from the high laser power and prolonged exposure during Raman analysis, leading to the formation of pyrolusite and hausmannite (Bruins et al., 2015c). The other explanation involves the transformation of initially formed birnessite into manganite under alkaline conditions (pH 7 to 8.5), with further transformations into feitknechtite and hausmannite occurring more rapidly in the presence of Ca^{2+} or Mg^{2+} ions (Elzinga, 2011). The presence of Ca^{2+} and Mg^{2+} in the MnO_x coating, as confirmed by EDX analysis (Fig. 3.5b), supports this hypothesis. Moreover, the elemental composition of the media itself influences the formation of different MnO_x phases (Vallejo, 2015). While we lack direct experimental evidence, it is likely that surface roughness and granular morphology play significant roles in the formation of MnO_x coatings (Vallejo, 2015).

To visualize the structure of the coating and the MnO_x , SEM micrographs of ripened media (Fig. 3.5f-k) are presented next to those of virgin media (Fig. 3.5c-e). The SEM micrographs (f-k) show the presence of fluffy structures (highlighted in red), similar to biologically formed birnessite (Bruins et al., 2015b; Dangeti et al., 2020), thereby confirming the Raman spectroscopy results. Fig. 3.5f shows the presence of both physicochemical (coral or sponge structures, marked in blue) and biological Mn oxides on the GAC1 media. This suggests that the MnO_x and Mn(II) removal mechanism during start-up might differ for GAC media compared to sand and anthracite.

3.4.4 Microbiological analysis

3.4.4.1 Biological activity

ATP levels were quantified to investigate the potential correlation between biofilter efficacy in Mn removal and biomass accumulation during biofilter start-up, with ATP serving as a key biofilm activity indicator. On day 126, ATP levels ranged between 100 and 140 ng/cm³ dry weight of media, except for sand (Fig. 3.6a). This is at the lower range of 100-1000 ng ATP/cm³ reported for biofilters acclimatized for organics removal (Pharand et al., 2014), likely due to the minimal organic content in the groundwater treated by the biofilters in this study. Evans et al. (2020) indicated suboptimal Mn removal efficiency when ATP concentrations fell below 200 ng ATP/cm³. However, this phenomenon was not observed in the present study, likely due to the lower organic load in the biofilter feedwater. Some studies have reported that due to the irregularity and more abundant macropores in GAC, a greater biomass accumulation would take place in biofilters investigating organic carbon removal (Urfer & Huck, 2001), but GAC media exhibited ATP levels comparable to those of anthracite and sand media ($p > 0.05$), which is consistent with literature values summarized by Pharand

et al. (2014). Notably, anthracite biofilter A1 displayed higher ATP levels than A2 and, occasionally, GAC media (Fig. 3.6a).

Fig. 3.6b reveals a negative correlation between effluent Mn concentration and ATP levels with a Spearman coefficient (ρ) of -0.58 over 126 days. Notably, the correlation coefficient was stronger during the acclimation phase (days 0 to 77) at -0.70. In contrast, from day 77 to 126 (pseudo-steady-state phase), the coefficient decreased to -0.22. This observation aligns with Pharand et al. (2014), who reported no direct relation between ATP levels and organics removal performance for biofilters at steady-state. The moderate correlation of -0.70 during the acclimation phase implies that Mn removal efficacy is enhanced with biomass accumulation during the start-up phase. Following day 77, effluent Mn concentrations remained consistently below 5 $\mu\text{g/L}$, irrespective of ATP levels, which initially increased and then remained steady for most media. This is consistent with the previous observation for full-scale GAC biofilters (removing organics) that biomass attached to the filter media accumulated for 90 days and then maintained a steady state for 200 days (Velten et al., 2011).

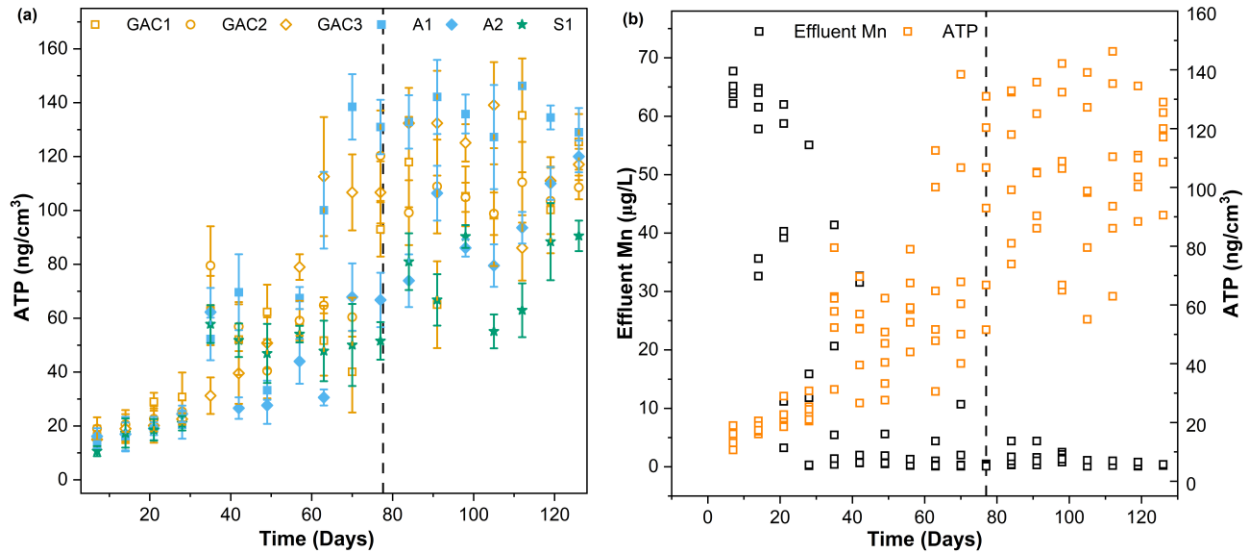


Figure 3.6: (a) ATP levels from the top of the filter bed (error bars represent the standard deviation of duplicate measurements, the vertical black line represents the time taken by all biofilters to meet treatment levels of 90% removal); (b) Correlation between ATP level and effluent Mn concentration

3.4.4.2 Impact of HPC and MnOB on biofilter performance

3.4.4.2.1 HPC

HPCs confirm the presence of heterotrophic bacteria in biofilters, ranging from 10^3 - 10^7 CFU/g for Mn Supp, 10^5 - 10^8 CFU/g for Mn min, and 10^6 - 10^8 CFU/g for R2A+Mn culture media (Fig. 3.7a-c). HPC decreased over time in all biofilters, with a more pronounced reduction observed in Mn Supp media. No significant difference was observed in HPC at the top of the biofilter compared to 40 cm bed depth ($p > 0.05$). The values reported here align with those reported in the literature for R2A+Mn media of 10^7 CFU/g (Araya-Obando et al., 2021; Granger et al., 2014).

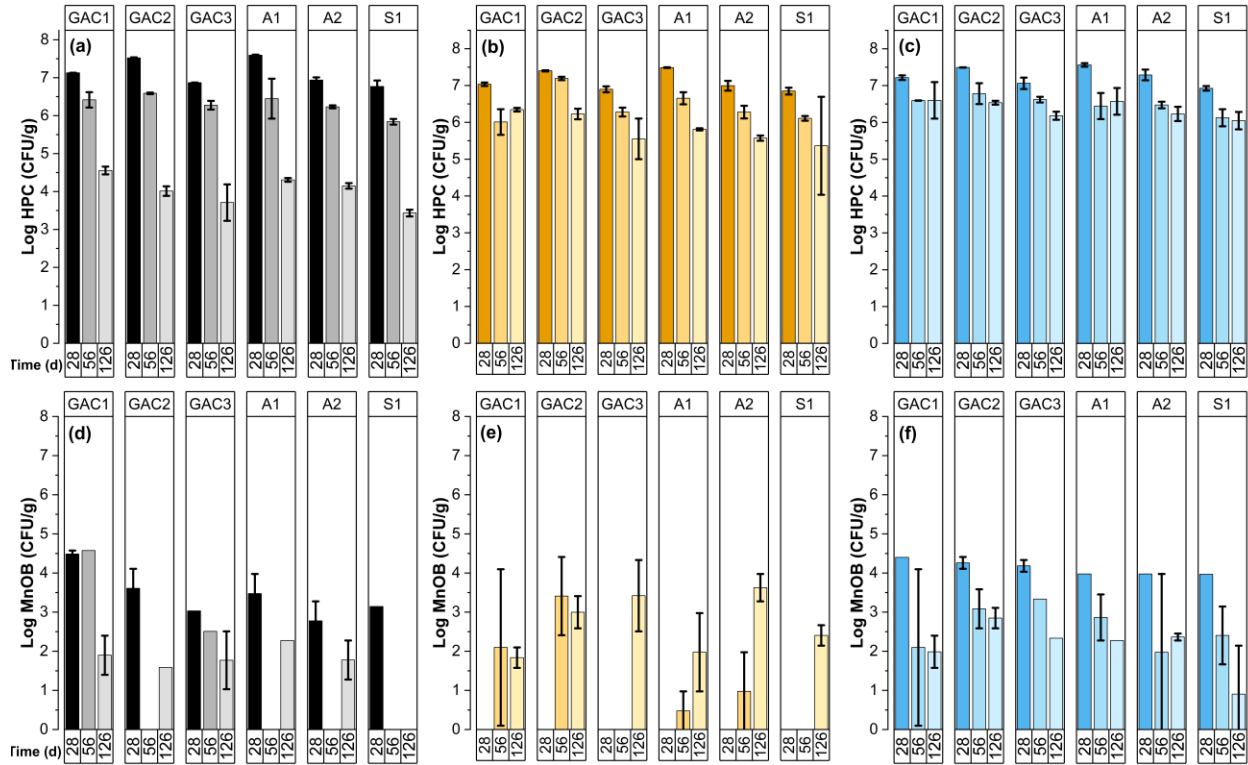


Figure 3.7: Microbial abundance on biofilter media sampled at different times. Average log HPC for (a) Mn supp, (b) Mn min, and (c) R2A+ Mn culture media; Average log MnOB for (d) Mn supp, (e) Mn min, and (f) R2A+ Mn culture media (time refers to biofilter media age; error bars represent the standard deviation between biofilter media collected from the top and at 40 cm bed depth)

ANOVA tests confirmed significant effects of time (biofilter media age) ($p < 0.05$) and culture media type ($p < 0.05$) on HPCs. In contrast, HPC values across biofilter media types varied, but biofilter

media types did not show a consistent significant impact ($p > 0.05$). Interaction analysis highlighted a significant interaction between the time (days) and culture media ($p < 0.05$), suggesting a synergistic effect of these factors on HPC.

3.4.4.2.2 MnOB

MnOB counts varied from non-detectable levels up to 10^4 CFU/g across the tested culture media (Fig. 3.7, d-f). MnOB abundances were consistently two to three orders of magnitude lower than HPC, with some instances yielding no detectable MnOB, as indicated by negative LBB tests. Similar to HPC trends, no significant difference was observed in MnOB counts at the top of the biofilter and 40 cm bed depth ($p > 0.05$). Zheng et al. (2023) observed a decrease in the culturable MnOB abundance within deeper layers of the filter bed, possibly because of a higher Mn concentration in the influent water for that study. Higher Mn loading has been reported to increase the biological removal of Mn (Kohl & Dixon, 2012), thus possibly increasing MnOB levels and resulting in a gradient within a filter bed. The values reported for the present study are comparable to those reported in the literature, i.e., 10^2 - 10^3 CFU/g for R2A+Mn media (Araya-Obando et al., 2021; Granger et al., 2014).

Statistical analysis using ANOVA revealed that, while variability in MnOB counts was noted across different biofilter media, there was no significant trend between MnOB and biofilter media ($p > 0.05$). Time (biofilter media age) was a significant factor ($p < 0.05$) across all culture media. This suggests that as biofilters matured, MnOB counts on the biofilter media decreased, similarly to HPCs. The exception was MnOB counts on Mn Min culture media. Furthermore, the interaction between the biofilter media age (time) and the type of culture media used had significant effects ($p < 0.05$), corroborating the importance of these factors in MnOB proliferation.

Correlation analysis between macropores in the biofilter media and MnOB revealed a strong positive correlation for Mn Supp at day 28 ($r = 0.79$, $p > 0.05$) and a very strong correlation for R2A+Mn at day 28 ($r = 0.93$, $p < 0.05$), indicating a potential link between biofilter media structure and bacterial abundance. The correlations weakened over time for both Mn Supp and R2A+Mn. By contrast, Mn Min showed a significant positive correlation at day 56 ($r = 0.86$, $p < 0.05$) but not at other time points. These correlations suggest that biofilter media properties may have a time-dependent influence on MnOB proliferation.

The differences in MnOB counts across culture and biofilter media suggest that these bacteria have specific nutrient and environmental preferences. For Mn Supp and R2A+Mn, there is a decrease in

MnOB counts with time, whereas for Mn Min, an increase was observed. This shift in the nutrient consumption patterns of MnOB may be attributed to the dynamic conditions within biofilters. MnOBs, being heterotrophs, can utilize a broad spectrum of available nutrients beyond Mn (Tebo et al., 2005). This broader nutrient utilization would lead to decreased microbial Mn oxidation as the biofilter matures. Conversely, an increase in MnOB counts was observed with Mn Min media, which solely provides Mn. These results suggest that a combination of culture media (Mn Min and Mn Supp/R2A+ Mn) should be used to fully understand the functional potential of MnOB. It is also crucial to note that culturable MnOB represents only a subset of the total MnOB population in the biofilters. This underscores the complexity of MnOB as a group and their varied roles in biofilter performance.

3.4.5 Mn removal mechanism in biofilters

The initiation of Mn removal in biofilters is complex. It has been established that Mn removal in biofilters is initiated biologically, and as filtration progresses, physicochemical removal dominates (Bruins et al., 2015b; Dangeti et al., 2020). However, these studies used non-adsorptive media such as sand and anthracite. Unlike these media, GAC possesses a higher surface area with an intricate pore structure and functional groups, which play a pivotal role in adsorbing Mn(II) during the early stages of biofilter operation. This adsorptive capacity, coupled with the media's surface characteristics, may accelerate the onset of Mn removal within GAC biofilters, as evidenced by the shorter start-up time (Fig. 3.2) and the presence of both physiochemically and biologically formed MnO_x on one of the GAC media (Fig. 3.5f).

The ability of GAC to adsorb Mn(II) within its internal pores can lead to a higher initial Mn removal. Once Mn is adsorbed, biological and possibly homogenous oxidation will occur, forming MnO_x, such as birnessite (Fig. 3.5a). These oxides then serve a dual function: they act as sites for further Mn(II) adsorption and as catalysts for the oxidation process. The adsorbed Mn(II) undergoes catalytic/heterogeneous oxidation on the surface of the MnO_x, facilitating the formation of a new MnO_x layer. This autocatalytic mechanism underpins the self-propagating nature of the MnO_x layer, which is integral to the sustained removal of Mn(II) within the biofilter system. However, as biofiltration progresses, the potential clogging of pores by MnO_x and biofilm reduces the accessibility to adsorption sites in the GAC, thereby transitioning the dominant mechanism from adsorption to catalytic and biological oxidation. Concurrently, the development of a biofilm contributes to the generation of biologically formed MnO_x, which, over time, likely transforms into physiochemically-

formed MnO_x , as evidenced by a decrease in MnOB counts. This shift underscores the dynamic nature of the Mn removal process in GAC media.

Electrostatic interactions significantly influence the adsorption process, where a positive net surface charge on GAC media under specific pH conditions may promote the development of active MnO_x , which further adsorbs and oxidizes additional Mn(II) (McCormick et al., 2021). With the point of zero charge (PZC) of GAC media ranging between 6 and 10 (Derylo-Marczewska et al., 2019; Fijolek et al., 2021), anthracite between 5 and 7 (Sahabi et al., 2009; Zhang et al., 2020), and sand between 6 and 7 (Kong et al., 2020), and given that the pH of the groundwater in this study is 7.5 ± 0.3 , the sand and anthracite media were likely negatively charged, whereas GAC media may have been positively charged. A negative surface charge on media may not favor accumulation of MnO_x , thereby impacting the efficacy of Mn removal (Sahabi et al., 2009). Therefore, adsorption capacity is not solely a function of internal surface area and pore structure but is also determined by the media's surface chemistry.

Thus, we propose that the mechanism of Mn removal during the start-up phase of biofilters is markedly distinct in GAC media, where first adsorption and then biological and physicochemical oxidation processes contribute to Mn(II) removal. In contrast, the removal process in sand and anthracite media is predominantly biological, as these media lack initial adsorptive properties. This is evidenced by the formation of MnO_x , which structurally resembles biologically formed birnessite (Fig. 3.5i-k), and by the decrease in MnOB counts over time in Mn Supp and R2A+Mn media (Fig 3.7). These observations are consistent with previous findings reported for sand and anthracite media (Bruins et al., 2015b; Dangeti et al., 2020).

3.6 Conclusions

This pilot study examines six biofilter media for low-concentration Mn(II) removal from groundwater and highlights the importance of filter media characteristics for removal efficiency and acclimation time. Key findings include:

- All the biofilters effectively removed Mn below the aesthetic objective level of $20 \mu\text{g/L}$.
- The filter media characteristics impact the start-up period of the biofilter. Higher surface area and pore volume of GAC media resulted in high adsorption of Mn, leading to the early onset of Mn removal.
- The lower effective media size for two anthracite media resulted in a shorter start-up time owing to its higher external surface area.

- The research confirms the potential for simultaneous removal of Fe and Mn in biofilters, particularly under low water temperature conditions.
- The study underscores the importance of different Mn removal mechanisms in biofilters during start-up, with adsorption followed by biological/physicochemical oxidation predominating in GAC media. In contrast, biological removal predominates in sand and anthracite media.
- The correlation between ATP levels and Mn removal efficiency during start-up further enhances our understanding of biofilter function, suggesting a potential indicator for monitoring biofilter acclimation.

4 Impact of Filter Media on Manganese Removal Mechanism and Microbial Communities in Biofilters

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4.1 Summary

This study investigated the impact of filter media types such as GAC, sand, and anthracite on the Mn removal mechanisms in drinking water biofilters, utilizing media from acclimated pilot-scale biofilters in bench-scale batch tests and conducting microbial analysis. The dynamics of Mn removal in batch tests were assessed using PFO and PSO non-linear kinetic models, with the PSO model having a better fit, particularly for GAC media samples. The analysis revealed that, in the top layer of acclimated biofilters, sand and anthracite primarily utilize biological processes for Mn removal whereas GAC favors physicochemical processes. This indicates that the choice of media can influence the removal mechanisms. Microbial community analysis highlighted the diversity of MnOB genera across biofilters, suggesting a link between biofilter media and microbial patterns. *Pelomonas* and *Reyranella* were exclusive to sand and anthracite biofilters, while *Noviherbaspirillum* was only present in sand biofilters. Furthermore, predictive functional genomics indicated genes involved in direct and indirect Mn oxidation pathways, such as *cueO* and *cotA* for direct oxidation, and others related to reactive oxygen species (ROS), fatty acid metabolism, and electron transport. This highlights the involvement of multiple pathways in facilitating biological Mn oxidation in biofilters.

Keywords: Kinetics, inhibition, genes, manganese-oxidizing bacteria, PICRUSt2

4.2 Introduction

Biofiltration has been established as a suitable treatment technology for removing Mn from drinking water, involving mechanisms such as adsorption and oxidation. The oxidation of Mn, which can occur through physicochemical (both homogenous and heterogenous) and biological processes, leads to the formation of MnO_x , contributing to further Mn removal through adsorption and autocatalytic oxidation. Previous studies employing non-adsorptive filter media like sand or anthracite have demonstrated that Mn removal in biofilters typically begins with a biological mechanism, which gradually shifts to being dominated by physicochemical processes as the filter matures (Bruins et al., 2015b; Olańczuk-Neyman & Bray, 2000; Sahabi et al., 2009). However, existing literature has not fully explored how these removal mechanisms may vary at different filter bed depths or how these are impacted by using various filter media, especially adsorptive ones like GAC, given its high surface area and superior adsorption capacity, notably during the initial stages of biofilter operation.

Furthermore, the composition of the microbial community within biofilters, which plays a vital role in the biological oxidation of Mn, may be influenced by the choice of filter media. Organisms such as *Pseudomonas*, *Leptothrix*, and *Streptomyces*, recognized for their Mn oxidation capabilities, have been extensively identified in biofilters utilizing sand or anthracite media (Burger et al., 2008a; Cheng et al., 2019; Cheng et al., 2017; Dangeti et al., 2020; Piazza et al., 2019). However, the unique properties of GAC might affect these microbial communities. Breda et al. (2017) observed that the type of filter media (quartz, calcium carbonate, polystyrene, MnO_x) influences the bacterial community that forms on the media coatings during the start-up period of Mn-removing biofilters. It was suggested that variations in properties like the porous structure, surface chemistry, and particle size of the filter media could impact microbial compositions (Lu et al., 2020; Xu et al., 2021). However, conclusive evidence remains elusive in current research.

Bacteria facilitate Mn(II) oxidation through direct and indirect processes, including environmental pH and redox condition modifications, or by releasing metabolic end products that chemically oxidize Mn(II) (Tebo et al., 2005). Direct oxidation involves producing polysaccharides or proteins that catalyze the reaction. The enzymatic pathways involved in bacterial Mn oxidation, including MCO enzymes (Dick et al., 2008; Su et al., 2013; Tebo et al., 2005), heme peroxidases (Anderson et al., 2011), and two-component regulatory proteins (Geszvain & Tebo, 2010), indicate that Mn removal in biofilters can proceed through multiple pathways, which have yet to be fully elucidated.

In summary, while biofiltration is a key technique for Mn removal from drinking water, the influence of filter media type on Mn removal mechanisms along the filter bed depth, particularly for GAC media, and the relationship between microbial communities and Mn removal requires further research. This study aims to fill these gaps by (a) quantifying the contributions of biological and physicochemical processes to Mn removal across different biofilter bed depths, using sand, anthracite, and GAC as media; (b) exploring how filter media characteristics affect its kinetics for Mn removal and the composition and diversity of microbial communities in biofilters; and (c) elucidating the functional capabilities of microbial communities by identifying their specific pathways for Mn removal. A combination of pilot-scale and bench-scale batch experiments was conducted to achieve the stated objectives.

4.3 Materials and methods

4.3.1 Experimental setup and operating conditions

The pilot-scale biofilters were set up at a water treatment plant in Southern Ontario, Canada. The biofilters utilized source water drawn from raw groundwater wells, with the analyzed water quality parameters presented in Table 3.1. The detailed design and operation of the six pilot-scale biofilters are described in section 3.3.1.

4.3.2 Analytical methods

Manganese concentration in the water samples was analyzed using the procedure outlined in section 3.3.2. For microbial community analysis, the biofilter media (~1 g) were collected using a sterilized spatula (Cole Parmer, Canada) at day 126 from two different bed depths (top: 0-5 cm, middle: 40 cm). The media sample was stored in sterile 50 mL plastic tubes (Falcon®, VWR™) and transported on ice for immediate processing. The FastDNA™ SPIN Kit for Soil (MP Biomedicals) was used for DNA extraction following the manufacturer's protocol. Using the extracted DNA, 16S rRNA gene sequencing of the V3-V4 region was performed at the Genomics Facility, University of Guelph, to assess the microbial community composition of the biofilters (Fig. A4.1). Demultiplexed sequences were analyzed using QIIME2 (version 2020.6) using the AXIOME3 pipeline (Bolyen et al., 2019; Min et al., 2021), which performed sequence quality filtering, merging of paired reads, chimera removal, and denoising with DADA2 (Callahan et al., 2016). Taxonomic classification was performed as part of the AXIOME3 pipeline using the SILVA database (release 138) (Quast et al., 2013). A final amplicon sequencing variants (ASV) table was generated from the pipeline for further downstream

analysis. All raw DNA sequences were deposited in the National Center for Biotechnology Information (NCBI) sequence read archive.

Additionally, PICRUSt2 analysis of the ASVs was performed to determine metagenome functional prediction, generating a table of Kyoto Encyclopedia of Genes and Genomes (KEGG) ID (Douglas et al., 2020). Before analysis, all singleton ASVs were removed from the ASV table. The complete PICRUSt2 pipeline (v. 2.5.2) was run using the `picrust2_pipeline.py` script, with the ASV table and corresponding ASV sequences as input. The entire pipeline performs sequence placement, hidden-state prediction of genomes, metagenome prediction, and pathway-level prediction (Douglas et al., 2020). The KEGG ID corresponding to each target gene was obtained from the output analysis, after which the genes were mapped onto the KEGG pathway map.

4.3.3 Batch test

Batch experiments were conducted to assess the roles of biological and physicochemical processes in Mn removal. NaN_3 was used to inhibit biological activity and thereby ascertain the physicochemical removal (Breda et al., 2019; Rosson et al., 1984; Sahabi et al., 2009). Biological contributions were determined by subtracting physicochemical removals from total Mn removals. Biofilter media from different bed depths (0-5 cm, 20 cm, 40 cm, and 100 cm) were collected using a sterilized spatula (Cole Parmer, Canada) on day 126 to conduct batch experiments. The media samples were stored in sterile 50 mL plastic tubes (Falcon®, VWR™) and transported on ice to the University of Waterloo for immediate processing. Three batch experiments were designed as follows: test 1 used 6 g of untreated media as a control to measure total Mn removal; test 2 involved 6 g of media pretreated with 5 % (w/v) NaN_3 (VWR Chemicals BDH®) overnight (12 h at 200 rpm in a shaker with deionized water) to inhibit biological activity, measuring physicochemical removal; and test 3 observed Mn desorption from inhibited biofilter media in 300 mL of deionized water.

In tests 1 and 2, flasks (Fisherbrand™ Erlenmeyer Flasks) with 300 mL of non-sterilized and sterilized influent groundwater containing 0.85 ± 0.05 mg/L Mn(II) were incubated at 200 rpm respectively. Manganese residual content was monitored by withdrawing 5 mL aliquots at set intervals, filtering them through a 0.45 μm nylon filter (Chromspec, Canada) and analyzed for Mn (Section 3.3.2). Duplicate analysis was performed for a selected subset of samples. Control bottles, both with and without NaN_3 but lacking filter medium, were utilized to adjust for potential Mn precipitation or adsorption onto the glass surfaces of the incubation bottles.

4.3.4 Kinetics analysis

In the batch tests, adsorption kinetics of Mn removal via biofilter media were analyzed using PFO and PSO models in Origin Pro, 2024. The PFO model assumes adsorption rate dependence on solute concentration and adsorbent capacity (Lagergren, 1898). The PSO model assumes the rate is proportional to the square of the number of unoccupied sites, with chemisorption being the rate-controlling step (Blanchard et al., 1984). The non-linear model was employed to fit the experimental data (Wang, 2009; Perrin, 2017; Tran et al., 2017).

$$\text{PFO: } q = q_e(1 - e^{-k_1 t}); \text{ PSO: } \frac{t}{q} = \frac{1}{k_2 q_e^2} + \frac{t}{q_e}$$

Here, q_e and q represent the maximum Mn removed at equilibrium and at any time t per gram of media, respectively. k_1 and k_2 are the first-order and second-order rate constants. The Mn removal at any given time is determined by subtracting the Mn concentration in the flasks at that time from the initial Mn concentration of 0.85 mg/L. The amount of Mn removed per gram is then calculated by dividing the total Mn removed by the mass of the biofilter media used in the batch study.

4.3.5 Statistical analysis

The Shannon and Simpson indices were calculated via Primer7 software using ASV data to assess alpha diversity. The Shannon index evaluates community diversity by considering species richness (presence) and evenness (relative abundance). In contrast, the Simpson index focuses on dominance or the probability of encountering individuals from the same species within the community. Beta diversity was analyzed using the Bray–Curtis dissimilarity matrix on ASV data and plotted using the principal coordinates analysis (PCoA) matrix in Primer7 to visualize differences in microbial community structures. Permutational multivariate analysis of variance (PERMANOVA) tests (999 permutations) were conducted on the ASU and MnOB data in Primer7 to observe microbial community differences between biofilter media types and depths. Post hoc analysis of pairwise comparisons was also performed in Primer7. A significance level of $p = 0.05$ was used for Spearman correlations, pairwise comparisons, and PERMANOVA. Hierarchical Cluster analysis was performed on the MnOB relative abundance data using Origin Pro, 2024. All figures were generated using Origin Pro, 2024.

4.4 Results and discussion

4.4.1 Mn removal across biofilters

The influent and effluent Mn concentrations across six biofilters over 133 days are shown in Fig. 3.2. The average influent Mn concentration was recorded at $60.9 \pm 4.5 \mu\text{g/L}$. The time taken to achieve a 90% Mn removal varied among the biofilters: GAC1, GAC2, and GAC3 met this goal within 21, 28, and 35 days, respectively, while filters A1, A2, and S1 achieved it in 56, 42, and 77 days, respectively. These differences can be attributed to the filter media characteristics (Section 3.4.2). After day 77 of operation, all biofilters consistently attained 90% Mn removal ($<5 \mu\text{g/L}$), achieving levels below the Health Canada AO of $20 \mu\text{g/L}$. The acclimated biofilter media was collected at day 126 from different bed depths to perform the subsequent batch test.

4.4.2 Kinetics of Mn removal in batch test

4.4.2.1 Effect of filter media characteristics

The efficacy of biofilter media at varying depths for Mn removal was assessed using PFO and PSO kinetic models in a batch test to understand the mechanism of Mn removal. The PSO kinetics offered a better fit for the GAC media, with R^2_{adj} values peaking at 0.97 for GAC3 at the top layer compared to 0.83 for GAC1 with the PFO kinetic model (Table 4.1). The Mn removal capacity of virgin GAC media varied from 20.21 ± 0.40 to $20.41 \pm 0.36 \mu\text{g/g}$ (PSO model), which is much lower than the Mn removal capacity of biofilter GAC media, thus highlighting the role of MnO_x in Mn removal (Table 4.1). MnO_x accumulated on the biofilter media has adsorption and catalytic properties contributing to sustained Mn removal in biofilters (Buamah et al., 2008; Post, 1999). The observed higher rate constants (k_2) at the top of the biofilter suggest a rapid engagement of Mn with the GAC media, which then decreases with depth. This is most likely due to a higher accumulation of MnO_x on the top of the biofilter media, which decreases with depth. A small increase in the q_e value with bed depths (PSO model) suggests that Mn can be removed at varying rates throughout the biofilter bed depth. The adsorption capacities (q_e) and rate constants (k_1) decreased with the bed depth in the PFO kinetic model.

The PSO model shows an improvement in the fit for anthracite media, especially for deeper layers, with A2 reaching an R^2_{adj} of 0.97 at 100 cm (Table 4.1). This enhancement might reflect the gradual saturation of adsorption sites, better modeled by PSO kinetics, underscoring the cumulative effect of Mn adsorption over time. Biofilter anthracite media, A1 and A2, displayed equal Mn adsorption 42.96 ± 0.01 respectively, from the top of the biofilter media (PSO model). The virgin anthracite media A1

and A2 had q_e values of 7.08 ± 0.20 and 7.20 ± 0.16 $\mu\text{g/g}$, respectively, which is very low as compared to that of biofilter anthracite (A1 and A2) media (Table 4.1), thus confirming the role of MnO_x in Mn removal. The q_e value increased with bed depth for biofilter media A2, whereas it decreased for biofilter A1 (PSO model). These differences could be a result of media size (Table 3.2). Biofilter media A1 has an effective size of 1.1-1.25 mm, whereas A2 has an effective size of 0.85-1.00 mm. The increase in filter grain size leads to a decrease in the external specific surface area, which could lead to lower MnO_x accumulation on the biofilter media. Despite differences in the virgin media's intrinsic properties, such as lower surface area and porosity in anthracite vs. GAC media (Table 3.2), comparable q_e values and rate constants were observed for both media from the top of the biofilters. This could be attributed to the accumulation of MnO_x on the biofilter media in GAC and anthracite biofilters. Biofilter sand media (S1) showed the lowest Mn removal efficiency among the tested media types, with lower q_e values and significantly lower rate constant (k_f) than those for GAC and anthracite. The PSO kinetic model was a better fit for sand media than the PFO kinetic model, particularly in deeper layers (Table 4.1). The very high k_f and lower q_e value reported in this study compared to 0.245 h^{-1} and 132 $\mu\text{g/g}$, respectively, reported by Sahabi et al. (2009), may potentially be attributed to several factors, the larger quantity of biofilter media used in this study (2 g/100 mL as opposed to the 0.5 g/100 mL), the use of 0.85 mg/L Mn(II) in our batch tests compared to 2.4 mg/L Mn(II), and the age difference of the biofilter media (126 days in this study compared to 3 years and 15 years anthracite media).

Table 4.1: Kinetic parameters of Mn removal for biofilter media at day 126 in an uninhibited batch test

Biofilter	Depth	Pseudo First Order (PFO)			Pseudo Second Order (PSO)		
		q_e ($\mu\text{g/g}$)	k_f (h^{-1})	R^2_{adj}	q_e ($\mu\text{g/g}$)	k_2 ($\text{g}/\mu\text{g}\cdot\text{h}$)	R^2_{adj}
GAC1	Virgin	19.94 ± 0.04	5.63 ± 0.07	0.99	20.38 ± 0.34	0.64 ± 0.13	0.85
	Top	42.95 ± 0.01	25.87 ± 0.55	0.83	42.96 ± 0.00	58.10 ± 5.94	0.91
	20	42.82 ± 0.10	14.12 ± 1.07	0.58	43.01 ± 0.04	2.60 ± 0.24	0.93
	40	42.65 ± 0.31	10.26 ± 1.18	0.49	43.09 ± 0.19	0.96 ± 0.14	0.84
	100	42.48 ± 0.60	6.63 ± 0.86	0.60	43.31 ± 0.41	0.40 ± 0.06	0.85
GAC2	Virgin	19.7 ± 0.39	3.84 ± 0.42	0.87	20.41 ± 0.36	0.37 ± 0.06	0.92
	Top	42.95 ± 0.01	27.46 ± 1.80	0.30	42.96 ± 0.01	61.89 ± 10.73	0.78

GAC3	20	42.89±0.06	15.10±0.81	0.71	43.01±0.02	3.58±0.26	0.95
	40	42.80±0.15	11.72±0.83	0.69	43.08±0.07	1.52±0.13	0.94
	100	42.76±0.21	10.49±0.84	0.68	43.13±0.12	1.11±0.11	0.92
	Virgin	19.52±0.57	3.86±0.64	0.72	20.21±0.40	0.37±0.06	0.90
	Top	42.96±0.01	24.81±0.55	0.84	42.97±0.00	43.71±2.50	0.97
A1	20	42.86±0.07	16.02±1.15	0.54	42.98±0.03	4.10±0.39	0.92
	40	42.74±0.18	11.79±1.02	0.58	43.04±0.09	1.46±0.15	0.92
	100	42.59±0.50	6.42±0.67	0.73	43.38±0.34	0.39±0.05	0.90
	Virgin	6.79±0.11	3.08±0.26	0.95	7.08±0.20	0.74±0.16	0.89
	Top	42.94±0.01	25.42±1.51	0.40	42.96±0.01	39.19±5.01	0.88
A2	20	40.92±1.25	2.71±0.47	0.74	42.80±0.70	0.10±0.01	0.93
	40	40.57±1.40	1.99±0.35	0.77	42.77±0.82	0.07±0.01	0.94
	100	39.16±1.86	1.28±0.29	0.73	41.70±1.39	0.04±0.01	0.91
	Virgin	6.97±0.01	3.74±0.04	0.99	7.20±0.16	1.01±0.19	0.90
	Top	42.95±0.01	25.34±1.46	0.42	42.96±0.01	38.93±4.78	0.88
S1	20	42.64±0.29	10.34±1.12	0.52	43.07±0.16	0.98±0.12	0.89
	40	42.31±0.81	3.90±0.48	0.79	43.62±0.50	0.17±0.02	0.94
	100	42.27±1.03	0.94±0.11	0.95	45.38±1.09	0.03±0.01	0.97
	Virgin	6.05±0.34	1.63±0.40	0.71	6.39±0.25	0.41±0.09	0.89
	Top	39.50±1.26	4.09±0.86	0.50	41.14±0.82	0.17±0.03	0.89
	20	34.89±0.38	1.51±0.38	0.66	37.29±1.36	0.05±0.01	0.87
	40	31.38±2.23	1.41±0.49	0.53	34.38±2.06	0.04±0.01	0.75
	100	31.53±1.70	1.03±0.25	0.77	34.58±1.35	0.03±0.01	0.92

The variability in rate constants among different biofilter media underscores the role of MnO_x accumulation on biofilter media and media characteristics such as pore structure, surface area, and chemical composition (Table 4.1). The decrease in the rate constant at deeper bed depths in the biofilter media is likely due to the development of biofilm and MnO_x at the top of the biofilter, thus lowering MnO_x formation in the deeper layers of the biofilter bed and subsequently lowering the rate constant. The superior fit of the PSO model might be attributed to its ability to account for the decrease in available adsorption sites as the batch test progresses. The PSO kinetics better fit suggests

that the rate-limiting step might not be the mere physical availability of Mn(II) near the adsorbent surface (as assumed in PFO) but rather the physicochemical/biological interactions occurring at the surface due to the presence of MnO_x accumulation (Sahabi et al., 2009).

4.4.2.2 Mn desorption from biofilter media

Mn desorption from the inhibited biofilter media was examined to elucidate the mechanism of Mn removal in biofilters. Less than 1% desorption (< 8 µg/L) was observed from all biofilter media (Fig. A4.2). This suggests that while some Mn may simply be adsorbed, a significant portion is likely oxidized and indicates that the Mn has been incorporated into the biofilter media coating through processes such as catalytic or biological oxidation, rather than being loosely adsorbed. These processes result in a more permanent integration of Mn into the biofilter media, making it resistant to desorption under the given conditions. This strong binding point to the effectiveness of the biofilter in retaining Mn and preventing its release back into the water.

4.4.3 Estimation of contributions from different Mn removal mechanisms in acclimated biofilters

The total and physicochemical Mn removal by media samples from different biofilter depths was assessed in microbiologically uninhibited and inhibited batch tests (Fig. 4.1). A notable trend emerged, showing an increase in both total and physicochemical Mn removal over time, from 0.25 h to 24 h. GAC media from the top achieved more than 99.9% of total Mn removal within the first 15 minutes, contrasting with 83.7%, 87.7%, and 68.1% for A1, A2, and S1, respectively (Fig. 4.1a). After 24 h, total Mn removal for A1 and A2 exceeded 99.9%, whereas S1 reached 98.5%. This rapid and high removal efficiency by GAC, particularly within the initial 15 minutes, is likely attributable to a higher accumulation of Mn on MnO_x on the GAC media or inherent properties of GAC media.

Physicochemical removal contributions (removals in microbiologically inhibited batch test) also increased. At 15 min, the physicochemical removal by GAC media was 71.9% for GAC1, 78.1% for GAC2, and 63.1% for GAC3, compared to 44.6% for A1, 47.6% for A2, and 33.3% for S1. After 24 h, this removal increased by 19.2%, 14.1%, and 23.1% for GAC1, GAC2, and GAC3, respectively, and by 36.4%, 33.3%, and 47.6% for A1, A2, and S1, respectively (Fig. 4.1a). The observed disparities in physicochemical versus total Mn removal between GAC and anthracite/sand at 15 min suggest different underlying removal mechanisms. Over time, an increase in the physicochemical contribution was observed, likely due to the low initial Mn concentration used in the batch study or due to the properties of NaN₃. The biofilter media were inhibited using NaN₃ before the batch test and not

continuously during the experiments. Rather than completely inhibiting microbial activity, NaN_3 induces a resistance in microbial activity that may reduce over time, thus contributing to Mn removal, which could add to physicochemical contributions (Breda et al., 2019). Nonetheless, using inhibitors provides a baseline for comparing different Mn removal mechanisms, as consistently demonstrated in several studies (Breda et al., 2019; Olańczuk-Neyman & Bray, 2000; Sahabi et al., 2009; Vandenabeele et al., 1992; Zheng et al., 2023).

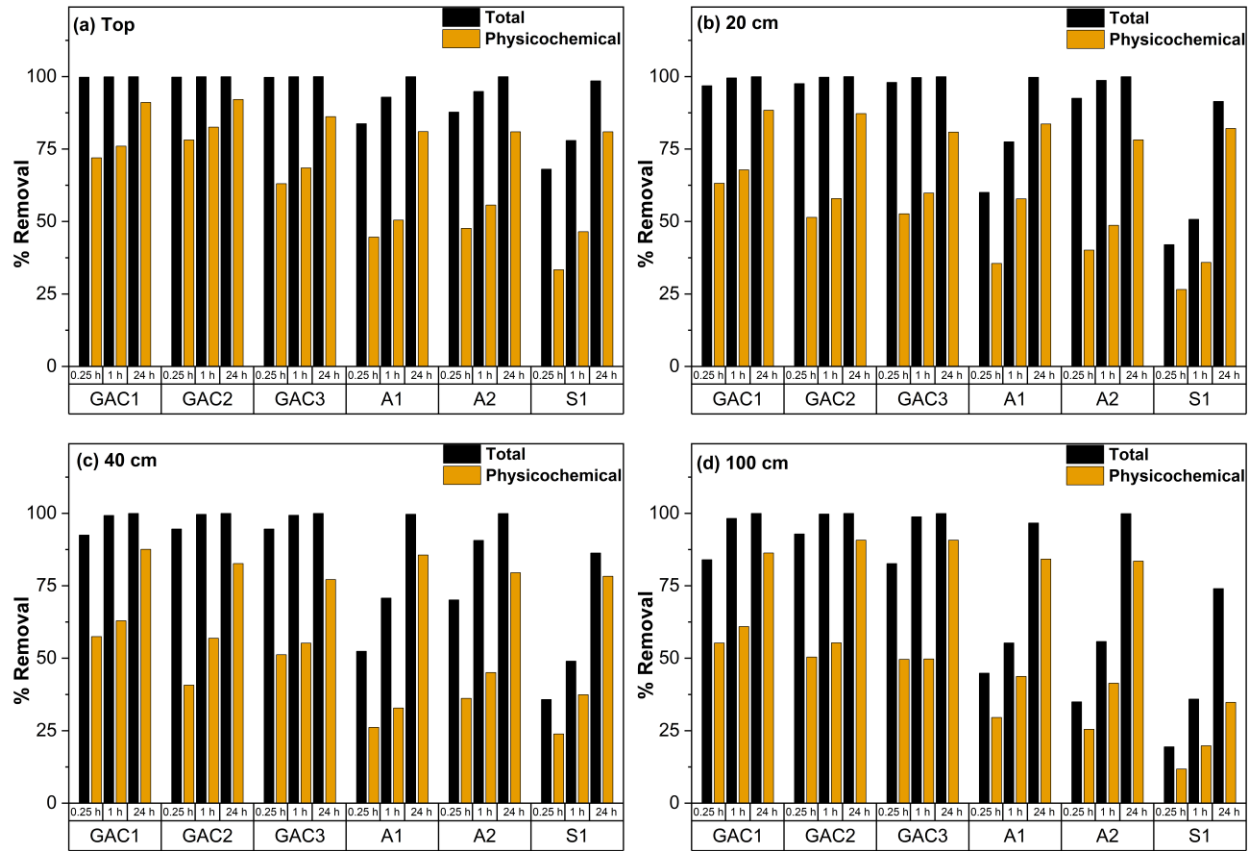


Figure 4.1: Percentage of total and physicochemical Mn removal based on an initial Mn(II) concentration of 0.85 mg/L over time ($t = 0.25, 1, 24 \text{ h}$) for different biofilter media samples from various depths: (a) Top, (b) 20 cm, (c) 40 cm, (d) 100 cm

Deeper within the biofilter bed, the time required to achieve comparable total and physicochemical Mn removal levels increased (Fig. 4.1b-d). GAC media from 100 cm bed depth showed 83.9%, 92.8%, and 82.6% total Mn removal for GAC1, GAC2, and GAC3, respectively at 15 min (Fig. 4.1d). To

achieve more than 99.9% removal, GAC media took 24 hours, in contrast to 15 minutes for media collected from the top of the biofilter. Similarly, initial physicochemical removal rates were lower in deeper layers of the bed across all media, with marked improvements over 24 h. Comparatively, A1, A2, and S1 achieved 44.8%, 34.9%, and 19.4% total Mn removal from the 100 cm bed depth, which was 38.9%, 52.8%, and 48.6% less from the top layer at 15 min, respectively. Correspondingly, lower physicochemical Mn removal rates were also observed, reaching only 29.6%, 25.5%, and 11.7% for A1, A2, and S1, respectively (Fig. 4.1d). A decrease in both total and physicochemical Mn removal was observed with depth. Similar patterns were observed in the media collected at 20 cm and 40 cm bed depths (Fig 4.1b,c).

The interaction between biofilter media, operational depth, and Mn removal mechanisms (B/P) was significant, likely due to Mn accumulation and biofilter media characteristics (PERMANOVA, $p = 0.001$). The interaction between biofilter media and depth on Mn removal also demonstrated statistical significance ($p = 0.003$), indicating that biofilter media performance depends on the specific depth. Specifically, significant differences were observed between GAC and anthracite media (GAC1 vs. A1: $p = 0.001$; GAC1 vs. A2: $p = 0.01$; GAC2 vs. A1: $p = 0.01$), GAC compared to sand media (GAC1 vs. S1: $p = 0.001$; GAC3 vs. S1: $p = 0.003$) and anthracite and sand (A2 vs. S1: $p = 0.01$) among other comparisons. Further analysis at different depths through pairwise comparisons revealed significant disparities in Mn removal efficiencies, emphasizing the impact of depth on removal performance for the age of the media tested (133 days). The differences were notably significant between the 0 and 40 cm ($p = 0.003$), 0 and 100 cm ($p = 0.001$), and 20 and 100 cm ($p = 0.003$) depths, underscoring that the biofilter depth is a determinant factor in Mn removal efficacy. This highlights the significant roles that biofilter media, depth, and their interactive effects play in the Mn removal process. The observed superior performance of GAC media across several comparisons, coupled with the notable impact of operational depth, provides insights into Mn removal within deeper layers of biofilter with different media characteristics.

4.4.4 Biological vs. physicochemical Mn removal in acclimated biofilters

The ratio of biological/physicochemical (B/P) Mn removal was calculated to further understand the dominant process for Mn removal in biofilters, and their correlations with virgin biofilter media characteristics were established (Fig. 4.2a). The B/P ratio indicates the dominant process within the biofilter: ratios greater than 1 indicate biological domination, whereas ratios less than 1 imply physicochemical domination. In the sand and anthracite media, the highest B/P ratios (> 1) were

observed at the top layer, except for A2, which peaked at a 20 cm bed depth. The diminishing B/P ratios with increasing depth in S1, A2 (except at 20 cm), and A1 (except at 40 cm) (Fig. 4.2a) indicate the growing dominance of physicochemical processes within these deeper layers, which is consistent with Breda et al. (2019).

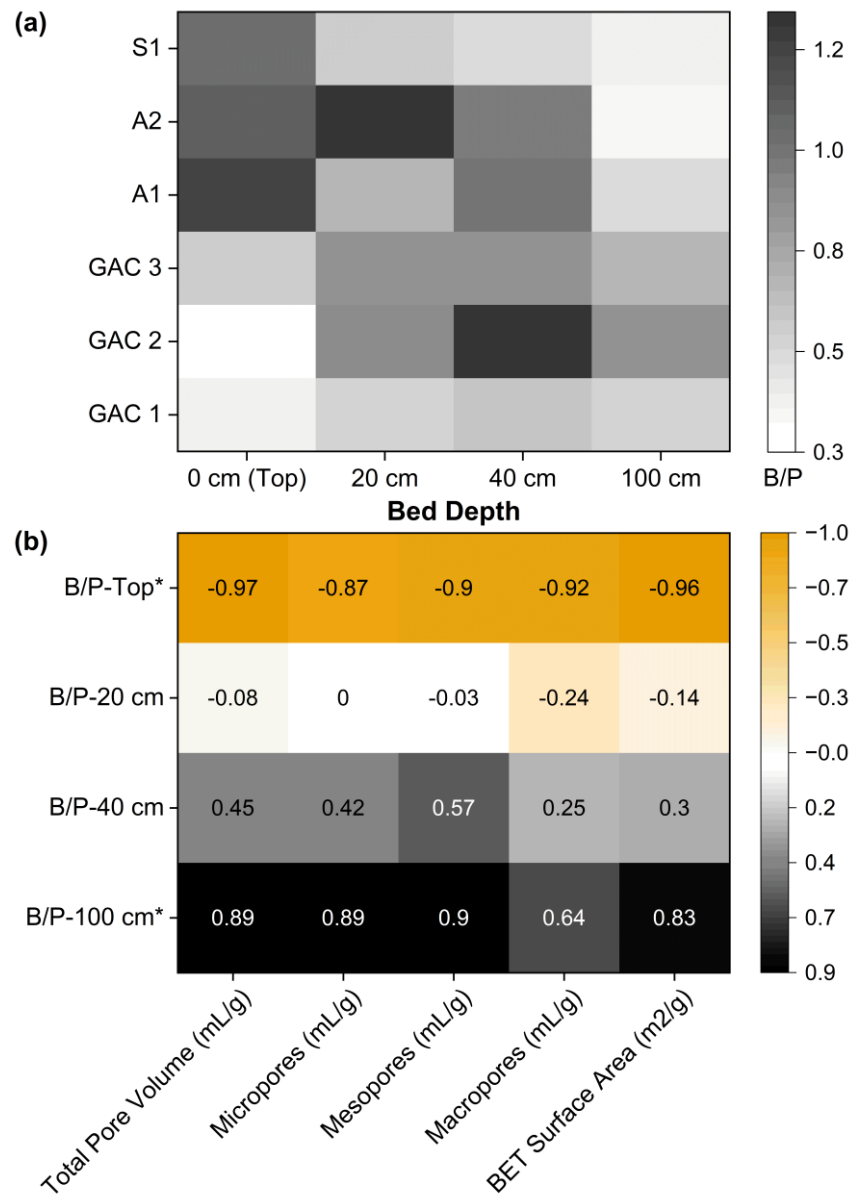


Figure 4.2: (a) Biological/Physicochemical (B/P) Mn removal ratios at $t = 15$ min (corresponding to the EBCT of the biofilters); (b) Correlation coefficient between B/P ratio and virgin biofilter media characteristics, *represents a significant value ($p < 0.05$)

In stark contrast, the GAC media demonstrate the lowest B/P values at the surface, indicating a stronger influence of physicochemical processes. However, a peak in the B/P ratio is observed at a 40 cm depth, particularly in GAC2, suggesting a zone where biological processes are maximized. Despite an increase in the middle layers, the B/P ratios remained below 1 (except GAC2 at 40 cm) and decreased again at 100 cm biofilter depth overall, indicating a physicochemical dominance in GAC media (Fig. 4.2a). This underscores the variability in the contributions of different Mn removal mechanisms depending on the biofilter media characteristics and indicates that biological processes were maximized at 40 cm depth for GAC media. In contrast, biological processes were most significant at the top of the biofilter for sand and anthracite media.

When correlating B/P ratios to media properties, a significant inverse correlation ($p < 0.05$) is noted at the top of the biofilter media, suggesting that smaller media, having greater external surface area per unit filter volume, tend to favor physicochemical removal mechanisms (Fig. 4.2b). Conversely, at a 100 cm bed depth, a positive correlation ($p < 0.05$) is observed, indicating that media size plays a more influential role in promoting biological processes at this depth (Fig. 4.2b). The differential B/P ratios observed in sand and anthracite media in comparison to GAC media (Fig. 4.2a) underscore the distinct removal mechanisms at play, likely affected by MnO_x accumulation and filter media characteristics, and illustrate how the contributions of these mechanisms vary within the acclimated biofilters.

4.4.5 Impact of filter media type on microbial communities in acclimated biofilters

Proteobacteria were the most abundant genera in all the biofilters, followed by *Nitrospirae* (Fig. A4.3). These results are in line with other observations of high relative abundance of *Proteobacteria* and *Nitrospira* in drinking water biofilters removing Mn and other contaminants (Gülay et al., 2016; Palomo et al., 2016; Piazza et al., 2019; White et al., 2012). A higher relative abundance of *Proteobacteria* was observed at the 40 cm bed depth compared to the top of all biofilters. Specifically, for GAC media, the relative abundance of *Proteobacteria* was 37.5%, 39.9%, and 42.9% at the top, which notably increased to 57.4%, 50.1%, and 55.5% at the 40 cm bed depth for GAC1, GAC2, and GAC3, respectively. Sand and anthracite media demonstrated a lower relative abundance of *Proteobacteria* in comparison to GAC, with 32.5%, 37.9%, and 39.2% at the top, and 38.4%, 42.9%, and 36.2% at the 40 cm depth for A1, A2, and S1, respectively. This phenomenon of higher *Proteobacteria* relative abundance in GAC media compared to sand agrees with previous findings in drinking water biofilters (Lautenschlager et al., 2014).

For *Nitrospirae*, the opposite trend was observed, with a higher relative abundance at the top compared to the 40 cm bed depth. Similar trends for *Proteobacteria* and *Nitrospirae* have been reported in the literature (Bai et al., 2022; Palomo et al., 2016). In GAC media, the relative abundance of *Nitrospirae* varied from 20.3% to 36.1% at the top and from 12.2% to 14.2% at 40 cm depth. In contrast, sand and anthracite media showed a relative abundance of 34.1% to 40.1% at the top and 15.8% to 20.8% at the 40 cm depth. *Nitrospirae* are involved in nitrogen cycling (nitrification) which predominantly occurs in the upper layers of the biofilter due to higher oxygen availability. Vignola et al. (2018) also observed a higher presence of *Nitrospirae* in sand biofilters than in GAC drinking water biofilters. Other notable phyla in the biofilters included *Actinobacteriota*, *Bacteroidota*, *Patescibacteria*, *Verrucomicrobiota*, *Bdellovibrionota*, and *Planctomycetota*, indicating a diverse microbial ecosystem within these biofilters (Fig. A4.4). A notable difference was the higher relative abundance of *Bacteroidota* in sand and anthracite (non-adsorptive media) biofilters of 24.1%, 26.1%, and 26.4 % A1, A2, and S1, respectively, compared to 9.5%, 9.2%, and 15.1% in GAC1, GAC2 and GAC3 respectively at 40 cm bed depth.

Collected bacterial communities on the biofilter media showed Shannon Index values (species richness and evenness) of 4.53 ± 0.09 , 4.52 ± 0.01 , and 4.52 ± 0.06 in GAC, anthracite, and sand biofilters, respectively. Simpson diversities (dominance) varied from 0.97 to 0.98 across all the biofilters, indicating a highly diverse community. Both Shannon Index and Simpson diversity increased with depth in the biofilters, though the increase was not substantial (Table A4.1). Beta diversity was analyzed using the Bray–Curtis dissimilarity matrix and plotted as PCoA to observe variability in the microbial community composition between different biofilters. The samples can be broadly categorized into four clusters based on the biofilter media and depth (Fig. 4.3).

The PCoA1 and PCoA2 collectively accounted for 76% of the variation in biofilter media samples. The variation along PCoA1 explains the largest proportion of variance (47.8%), highlighting the substantial differences in community composition at the 40 cm bed depth between the GAC and the other types of biofilter media. Within the adsorptive (GAC1, GAC2, GAC3) and non-adsorptive media (A1, A2, S1), there is a visible grouping between the top and 40 cm samples, particularly evident in the GAC group where the 40 cm samples tend to have higher PCoA1 values (Fig. 4.3). This suggests depth-related variations in the microbial communities, potentially indicating stratification of microbial diversity or activity within the biofilters (Gülay et al., 2016; Moll et al., 1998; Palomo et al., 2016).

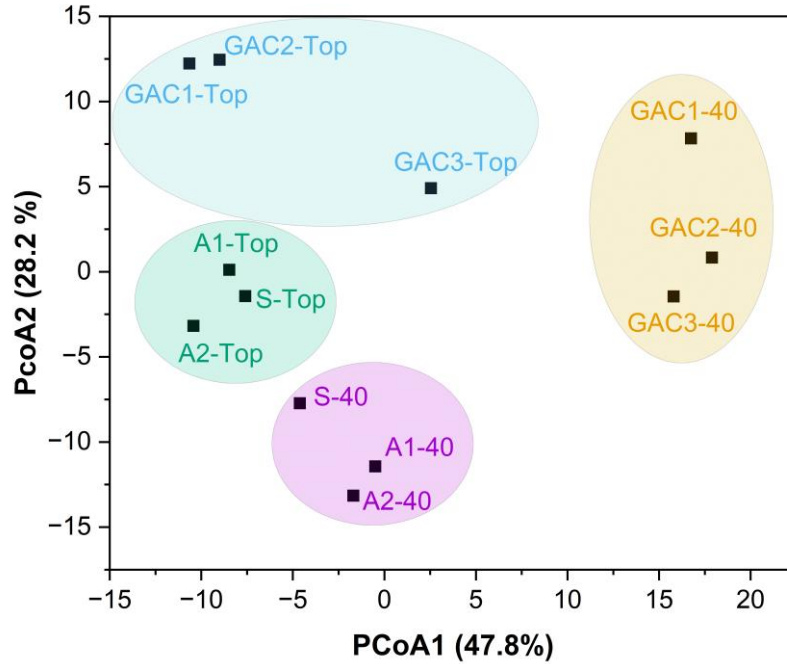


Figure 4.3: PCoA generated using Bray Curtis dissimilarity analysis for the biofilter media sampled on day 126 (colored ellipses represent different clusters)

PERMANOVA results indicated a significant influence of depth on microbial diversity ($p = 0.01$), with no significant effect of biofilter media type ($p = 0.06$). However, subsequent pairwise comparisons (post hoc) between the biofilters revealed significant differences both between biofilter types ($p < 0.005$) and across depths ($p = 0.01$). The observed differences in microbial community composition between biofilter media GAC3 and GAC1/GAC2 at the top (Fig. 4.3) might be a result of the pore size distribution. GAC3 has the lowest proportion of macropores (0.047 mL/g) as compared to GAC1 (0.174 mL/g) and GAC2 (0.169 mL/g) (Table 3.2) and macropores can provide surface area for bacterial attachment (Walker & Weatherley, 1998). In conclusion, the differences in bacterial communities between GAC and anthracite/sand media are probably due to the distinct filter media characteristics (e.g., roughness, surface chemistry, pore volume, and hydrophobicity), which may affect initial colonization by microorganisms and nutrient availability (Ma et al., 2020; Vignola et al., 2018; Yapsakli et al., 2009).

4.4.6 Presence of MnOB in acclimated biofilters

MnOB genera such as *Leptothrix*, *Pseudomonas*, *Sphingopyxis*, *Shingomonas*, *Hyphomicrobium*, *Hydrogenophaga*, *Variovorax*, *Acidovorax*, *Flavobacterium*, *Caulobacter*, *Rhodococcus*, and *Bacillus* were detected in the biofilters

(Fig. 4.4). These MnOB have been reported in various other Mn-removing biofilters (Breda et al., 2017; Cai et al., 2023; Dangeti et al., 2020; Evans et al., 2020; McCormick et al., 2021; Piazza et al., 2019; Tebo et al., 2005). The most abundant taxonomic group in the biofilters associated with MnOB were *Betaproteobacteria*, which contain the genera *Hydrogenophaga*, *Acidovorax*, *Variovorax*, and *Leptothrix*, and *Alphaproteobacteria* containing the genera *Sphingopyxis*, *Sbingomonas*, and *Caulobacter*, among others. Members of *Betaproteobacteria* are also known to contribute indirectly to Mn removal through co-occurrence relationships (present together) with other MnOB and nitrifying bacteria, in addition to direct Mn oxidation (McCormick et al., 2023). The relative abundance of *Alphaproteobacteria* and *Betaproteobacteria* decreased with bed depth in the biofilters (except Biofilter A1 for *Alphaproteobacteria*). The biofilters also detected the *Gammaproteobacteria* containing the MnOB genera *Legionella* and *Pseudomonas*.

The most dominant MnOB-associated genera detected throughout all biofilters was *Hydrogenophaga*, with its highest relative abundance at 7.6 % in GAC3-Top and the lowest at 2.7% in A1-40 (Fig. 4.4). *Hydrogenophaga* has been reported as a dominant genus in drinking water biofilters (Dangeti et al., 2020; Ma et al., 2020). The other dominant MnOB genera detected in the biofilters were *Sphingopyxis*, *Rhodococcus*, and *Pseudonocardia* (Fig. 4.4). A notable finding was the higher relative abundance of *Pseudomonas* in sand biofilters ($2.6 \pm 0.1\%$), in comparison to GAC ($0.1 \pm 0.1\%$) and anthracite ($0.07 \pm 0.03\%$) biofilters. This might indicate a potential selective advantage or preferential conditions within the sand media that support the proliferation of *Pseudomonas*.

A distinct distribution pattern of certain MnOB genera that were exclusive to specific biofilters was also observed (Fig. 4.4). *Ralstonia* was absent in both GAC1 and S1 biofilters, and *Flavobacterium* was not detected in the A2 biofilter. Conversely, *Pelomonas* and *Reyranella* were detected solely in sand and anthracite biofilters, suggesting a preference or selective advantage in these media types. Furthermore, *Noviherbaspirillum* was only present in sand biofilters, demonstrating microbial niche preferences and the influence of filter media composition on bacterial community structure. Similar to the overall microbial community, subsequent pairwise comparisons (post-hoc) between the MnOB in biofilters revealed significant differences both between biofilter media types ($p < 0.005$) and across depths ($p = 0.005$).

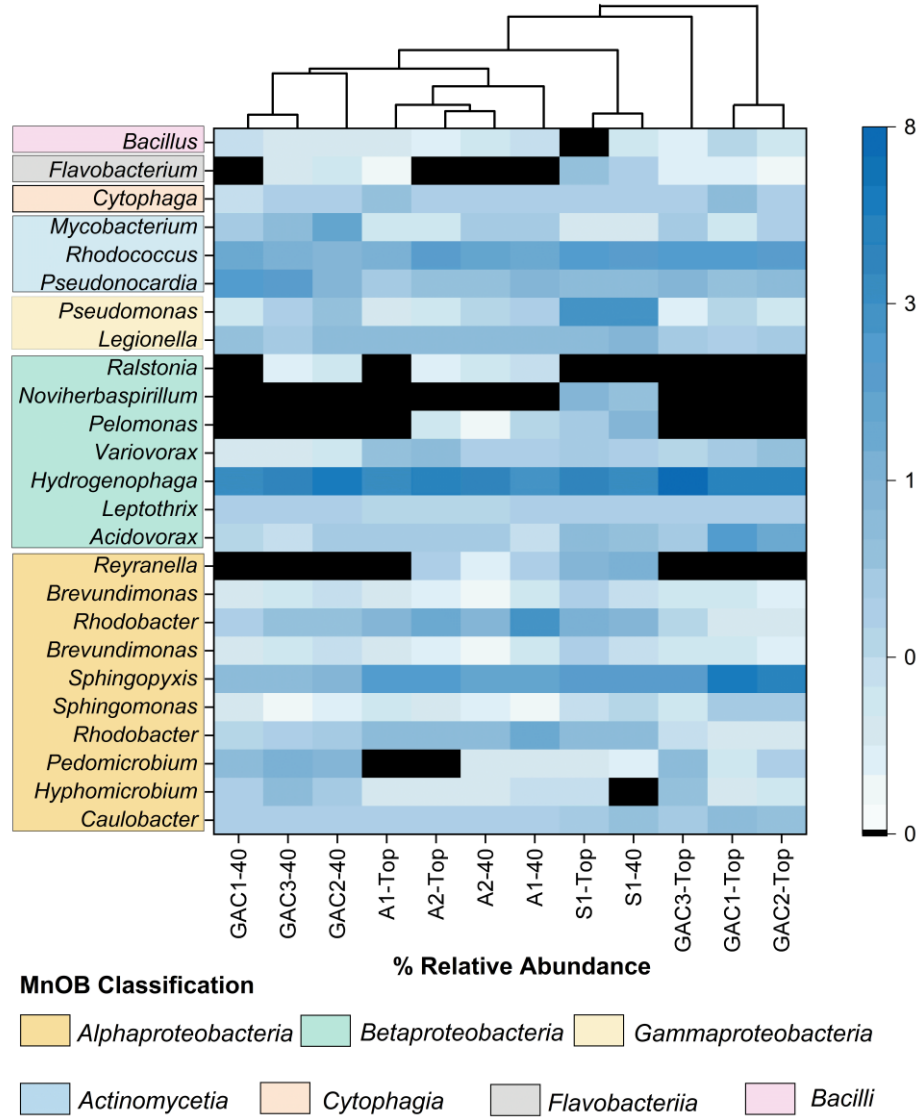


Figure 4.4: Heatmap of the relative abundances of known MnOB genera in the biofilters on day 126. Samples are grouped based on a hierarchical cluster analysis shown at the top of the heatmap

Hierarchical cluster analysis further reveals distinct clustering patterns that correspond closely to the biofilter media type and depth, thereby offering a macroscopic view of the microbial distribution influenced by the filter media characteristics (Fig. 4.4). Specifically, the clustering of GAC1-Top with GAC2-Top, GAC1-40 with GAC3-40, A2-Top with A2-40, and S1-Top with S1-40, demonstrates the influence of media on microbial community composition. The grouping of sand samples across both depths highlights a distinct distribution pattern of MnOB-associated genera in sand compared to other media types, aligning with our earlier observations of specific MnOB genera preferences. Moreover,

the hierarchical analysis illustrates less similarity among particular samples, such as the pairing of GAC3-Top with the combined node of GAC1-Top and GAC2-Top, reflecting the differences in MnOB composition, which might be attributed to different characteristics of the GAC media (Table 3.1). This differential presence and absence of specific bacterial genera across various biofilters highlights the complex interplay between biofilter media and MnOB colonization patterns, contributing to the diversity of microbial ecosystems in biofilters.

4.4.7 Predicting Mn removal pathways in acclimated biofilters

Potential functional genes associated with Mn oxidation pathways in biofilters were examined in this study using the PICRUSt2 and mapping findings onto KEGG pathways (Table A4.2). More than 15 genes associated with Mn oxidation were predicted based on 16S rRNA gene sequences in the biofilters (Fig. 4.5). Notably, typical Mn oxidation genes such as *mnxG* (Van Waasbergen et al., 1993), *moxA* (Morales Medina et al., 2023; Ridge et al., 2007), *cumA* (Brouwers et al., 1999), and *mofA* (Corstjens et al., 1997; Morales Medina et al., 2023), which are known to produce MCO enzymes, were not predicted in the biofilters. However, genes like *cueO* and *cotA*, both encoding MCO enzymes found in *Escherichia coli* and *Bacillus subtilis*, respectively (Hu et al., 2020), were predicted at a very low relative abundance across all biofilters (Fig. 4.5). The *cueO* gene has been implicated in the production of biogenic oxides and the oxidation of Mn(II) to Mn(III) or Mn(IV) (Su et al., 2014).

The presence of the *poxB* gene, encoding pyruvate oxidase, was also predicted (Fig. 4.5). This enzyme facilitates the generation of H₂O₂, an ROS that can chemically transform Mn(II) into its more oxidized forms, Mn(III) or Mn(IV), under specific catalytic or environmental conditions (Wang et al., 2017). This observation aligns with studies demonstrating the enhanced efficiency of Mn removal from biofilters supplemented with H₂O₂ (Granger et al., 2016; McCormick et al., 2021), supporting the potential indirect role of these genes in Mn oxidation via ROS production. Furthermore, genes, namely *fabA*, *fabD*, and *fabZ*, linked to fatty acid metabolism were predicted (Fig. 4.5). The presence of these genes within the microbial communities of all biofilters points to their potential role in shaping metabolic activities that, while primarily focused on fatty acid synthesis and degradation, may also influence redox conditions, or produce metabolites that facilitate Mn oxidation (Wang et al., 2017). Cytochrome c-related genes (*ccmE*, *ccmA*, *ccmG*) were also detected in the biofilters (Fig. 4.5). Given the role of cytochromes in electron transfer processes, it is plausible to hypothesize that the *ccm* genes may contribute indirectly to Mn oxidation through their involvement in the electron transport chain, potentially influencing redox reactions that facilitate Mn(II) to Mn(III) or Mn(IV) oxidation (Caspi et

al., 1998; De Vrind et al., 1998; Liu et al., 2014). However, the exact pathways and mechanisms through which *ccm* genes may influence Mn oxidation remain to be fully elucidated and would likely require further investigation. Similarly, the *cydB* gene, encoding for cytochrome bd oxidase, and *fdx*, encoding ferredoxin, are implicated in controlling the redox environment and participating in electron transfer reactions (Wang et al., 2017). Both genes might indirectly influence Mn oxidation through their roles in cellular respiration and redox regulation.

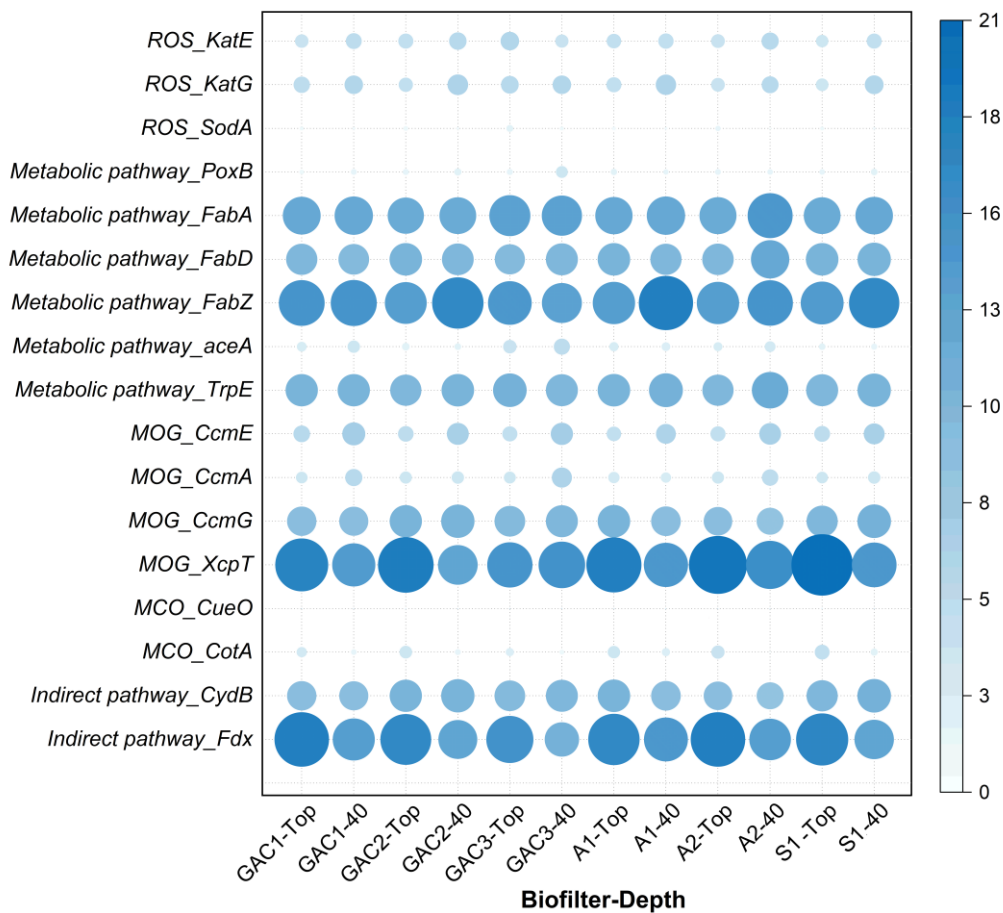


Figure 4.5: Potential functional pathways in biofilters and genes involved in Mn oxidation (Each circle represents the relative abundance in percent; ROS: Reactive oxygen species, MOG: Manganese oxidizing genes, MCO: Multicopper oxidase enzymes)

These findings predicted the indirect pathways by which genes involved in the management of ROS, fatty acid metabolism, and electron transport can impact Mn oxidation (Fig. 4.5). These insights reveal

the complex interplay of microbial processes and interactions within biofilters, highlighting the capacity of broader metabolic functions to influence Mn oxidation dynamics indirectly. Although numerous studies have demonstrated the ability of the PICRUSt2 method to reveal functional profiles of microbial communities accurately and effectively, it has limitations (Douglas et al., 2020). It should be noted that the functions of microorganisms are not directly reflected by the 16S rRNA data used for prediction. The PICRUSt2 method was used to predict functional enzymes, but these predictions may be limited by the relatively small number of generic genes that can be analyzed using 16S rRNA gene sequencing (Gu et al., 2022). Therefore, employing metagenomics, proteomics, and other omics approaches is necessary to study microorganisms more comprehensively.

4.5 Conclusions

This study examined the impact of filter media characteristics on Mn removal using media from acclimated pilot-scale biofilters in batch tests and microbial analysis. Key findings are:

- Analysis of biological versus physicochemical Mn removal mechanisms revealed that, while sand and anthracite media exhibit a biological dominance at the top layer, GAC media shows a physicochemical dominance, indicating the influence of media type on removal mechanisms.
- The maximum biological activity for GAC media was observed at a 40 cm bed depth, though physicochemical processes dominated the Mn removal in GAC media.
- The PSO model provides a better fit than PFO in describing Mn removal kinetics in batch tests of biofilter media samples, underscoring the importance of filter media characteristics and the role of Mn oxides in Mn removal.
- The presence of MnOB genera varied across biofilter media types and depths, highlighting the complex interplay between biofilter media and microbial colonization patterns.
- PICRUSt2 predicted genes linked to two types of Mn oxidation pathways: directly through *cueO* and *cotA* genes and indirectly via *katE*, *poxB*, and *cmmE* genes, among others. The indirect pathways involve interactions with reactive oxygen species, fatty acid metabolism, and electron transport. This highlights the complexity of Mn oxidation, involving multiple biological pathways.

5

Spatial and Temporal Changes in the Microbiome and Manganese Oxides Contributing to Manganese Removal in a Biofilter

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5.1 Summary

A 240-day pilot-scale study was conducted to investigate the mechanism of Mn removal in sand biofilters from start-up to maturation, focusing on how the Mn removal process evolves. Initially, Mn removal was primarily driven by biologically generated manganese oxides (Bio-MnO_x), which typically have high adsorption capacities, leading to a rapid Mn accumulation on the biofilter media. Over time, a shift to the physicochemical form of MnO_x was observed, as evidenced by SEM-EDX analysis and inhibition batch experiments. Despite an increase in the relative abundance of MnOB, a transition towards domination of physicochemical removal mechanisms was observed with time. This transition is likely linked to competitive dynamics between MnO_x and MnOB, with the high adsorptive and autocatalytic properties of MnO_x potentially overshadowing MnOB, leading to a decrease in microbial oxidation and Bio-MnO_x formation. Nonetheless, biological processes persist in contributing to Mn removal, albeit with a diminishing role as the biofilter ages. A decrease in the relative abundance of bacteria such as *Pseudomonas*, *Leptothrix*, and *Bacillus*, known for Bio-MnO_x production, was observed as the biofilter matured. Despite differences in the relative abundance within the microbial community, the overall microbial community composition was not significantly different as the biofilter matured ($p > 0.05$). However, specific microbial communities varied from startup to maturity.

Keywords: Mechanism, inhibitors, manganese-oxidizing bacteria, start-up, maturity

5.2 Introduction

Biofiltration has emerged as a robust technique for the removal of Mn from groundwater, leveraging a synergy of physicochemical and biological processes (Bruins et al., 2015b,c; Dangeti et al., 2020; McCormick et al., 2021). The effectiveness of this process varies depending on the characteristics of the filter media, including the capacity for adsorption and the oxidation processes leading to MnO_x formation. MnO_x is crucial in this context due to its strong adsorptive properties and high surface reactivity, facilitating the binding and removal of Mn(II) from water (Han et al., 2006; Kim et al., 2009). These oxides also act as catalysts, aiding in the oxidation of adsorbed Mn to higher oxidation states (Post, 1999). This auto-catalytic process is further enhanced by specific microbial communities (MnOB) that mediate Mn oxidation, resulting in the accumulation of MnO_x on the biofilter media (Tebo et al., 2005; Vandenabeele et al., 1992; Villalobos et al., 2003). Compared to abiotic Mn oxidation, MnOB can accelerate the oxidation rate by several orders of magnitude (Nealson et al., 1988; Tebo et al., 2005).

Research by Bruins et al. (2015b) indicates that the MnO_x formed initially in biofilters is predominantly of biological origin, evolving to a more physicochemical form as the biofilter matures. This suggests a shift in the primary formation of MnO_x and subsequent Mn removal mechanisms over time. However, the reasons behind this change and the possible continued contribution of biological processes remain unclear. A variety of MnOBs, which are capable of Mn oxidation, have been identified, typically through studies conducted at a single point in time during biofilter operation (Breda et al., 2017; Cheng et al., 2019; Cheng et al., 2017; Dangeti et al., 2020; Marcus et al., 2017; McCormick et al., 2022; Piazza et al., 2019). However, there is a notable gap in research tracking changes in the microbial community from the start-up phase to maturity, particularly in biofilters processing natural groundwater. To the best of our knowledge, there are no long-term studies examining the dynamic relationship between biofilter aging, MnO_x morphology, Mn accumulation, and possible changes in microbial community composition.

This study aims to bridge these gaps by focusing on how Mn removal evolves in a biofilter from start-up to maturity. Our objectives are threefold: first, to document and track any changes in MnO_x morphology and Mn accumulation; second, to analyze the composition and dynamics of microbial communities within a biofilter, identifying key genera involved in Mn oxidation; and third, to assess how the contributions of different removal mechanisms may shift throughout a biofilter's operational

life. The study aims to provide a comprehensive understanding of Mn removal in biofilters by addressing these objectives.

5.3 Material and methods

5.3.1 Experimental setup and operating conditions

The pilot-scale biofilter was set up at a water treatment plant in Southern Ontario, Canada. The biofilter utilized source water drawn from raw groundwater wells, with the analyzed water quality parameters presented in Table 5.1. The detailed design and operation of these biofilters are described elsewhere (section 3.3.1). Briefly, the biofilter was packed with sand media (effective size: 0.85-1.00 mm, UC: 1.7) to a depth of 1.2 m, underlain by 20 cm of gravel. The biofilter operated continuously for approximately 240 days, from January 26, 2023, to September 24, 2023, with an effluent flow rate of 5 m/h. This setup achieved an empty bed contact time of 14.4 minutes or less, attributed to a decrease in the volume of the filter bed over time as media was removed for sampling. The average DO level in the groundwater was less than 2 mg/L before aeration. Prior to being pumped to the biofilters, the water was aerated.

Table 5.1: Influent water characteristics to pilot scale biofilters

Parameter	Average $\pm$ S.D. (number of observations)	Max	Min	Units
Temperature	12.7 $\pm$ 2.3 (68)	14.1	7.6	$^{\circ}\text{C}$
pH	7.5 $\pm$ 0.4 (68)	8.1	7.2	-
Manganese	64.7 $\pm$ 3.2 (35)	72.2	56.1	$\mu\text{g/L}$
Iron	89.3 $\pm$ 43.9 (35)	259.6	62.6	$\mu\text{g/L}$
TOC	0.8 $\pm$ 0.1 (4)	0.6	0.9	mg/L C
Hardness	248 $\pm$ 38 (4)	301	195	mg/L as CaCO_3
Alkalinity	182 $\pm$ 23 (4)	213	75	mg/L as CaCO_3
ORP	149 $\pm$ 27 (68)	209	113	mV
DO (after aeration)	8.1 $\pm$ 1.2 (68)	10.4	7.2	mg/L

Initially, the biofilters were backwashed after operating for three weeks. Subsequently, backwashing occurred weekly at a flow rate of 10-20 m/h, utilizing effluent from the biofilters and air scour to achieve a bed expansion of 20-30%. This process was continued until the water in the supernatant and backwash effluent appeared clear, typically within 5-10 minutes.

5.3.2 Analytical methods

Influent and effluent water samples were analyzed for Mn analysis using the procedure mentioned in section 3.3.2. Manganese accumulated on sampled biofilter media from the top layer (0-5 cm) was quantified using acid digestion and hydrogen peroxide (30% ACS, VWR Chemicals BDH®) additions, following the protocols specified in EPA Method 3050B (Evans et al., 2020) (Text A5.1). SEM was utilized to analyze the coating's structure and the MnO_x morphology, while EDX was employed to identify trace elements within these structures (in duplicate) from the top (0-5 cm) of the biofilter media on days 70 and 150 (FEI Quanta FEG 250 ESEM, WATLab, University of Waterloo) (Section 3.3.2).

For microbial community analysis, the biofilter media (~1 g) were collected using sterilized spatula (Cole Parmer, Canada) tubes from three different bed depths (top: 0-5 cm, middle: 40-60 cm, and bottom: 100-120 cm) at days 70, 150 and 240 and analyzed following procedures in Section 4.3.2.

5.3.3 Batch test

Batch experiments were carried out to understand the contribution of biological and physicochemical processes to Mn removal. Media samples were collected from the biofilter's top (0-5 cm) on days 70, 150, and 240 following the procedure mentioned in section 4.3.3. Two batch experiments were designed as follows: Test 1 employed 1 g of untreated filter media as a control, with Mn removal quantified as total Mn removal. In Test 2, 1 g of mature filter media was first pretreated with 5% (w/v) NaN_3 (VWR Chemicals BDH®) overnight (12 hours at 150 rpm in a shaker with deionized water) to inhibit biological activity. Following this treatment, experiments were conducted, and the Mn removal was termed physicochemical removal.

Test 1 and 2 utilized conical flasks (Fisherbrand™ Erlenmeyer Flasks) containing 0.5 ± 0.04 mg/L Mn(II) in 200 ml of non-sterilized and sterilized influent groundwater (pH: 7.3 ± 1.1) at 150 rpm respectively. Water samples were collected from flasks at pre-determined time intervals and analyzed for Mn (Section 3.3.2). All experiments were conducted in duplicate with control bottles (section 4.3.3), and the results were reported as average values with standard deviations.

5.3.4 Statistical analysis

Statistical analysis and methods for data visualization are detailed in section 4.3.5.

5.4 Results and discussion

5.4.1 Mn removal in the biofilter

Fig. 5.1a illustrates the influent and effluent Mn concentrations in the pilot-scale biofilter over 240 days. The influent's average Mn concentration was measured at $64.7 \pm 3.2 \mu\text{g/L}$. The biofilter was considered fully operational once it consistently achieved over 90% removal of the average influent Mn. The removal efficiency of Mn gradually improved, surpassing 70% after 21 days (approximately week 3). The start-up phase was deemed concluded on day 54, when the biofilter succeeded in removing more than 90% of the average influent Mn. The start-up period of biofilters for Mn removal typically takes between 1 and 4 months, depending on operational parameters and water quality parameters (Araya-Obando et al., 2021; Bruins et al., 2015a; Štembal et al., 2005). The result was in this range, though within the first three weeks of operation, the biofilter successfully met Health Canada's AO of $20 \mu\text{g/L}$ for Mn in drinking water. A restart caused the sudden increase in the effluent concentration of Mn at day 220 after a 10-d shutdown. However, the levels quickly returned to within the acceptable range after 6 h of operation.

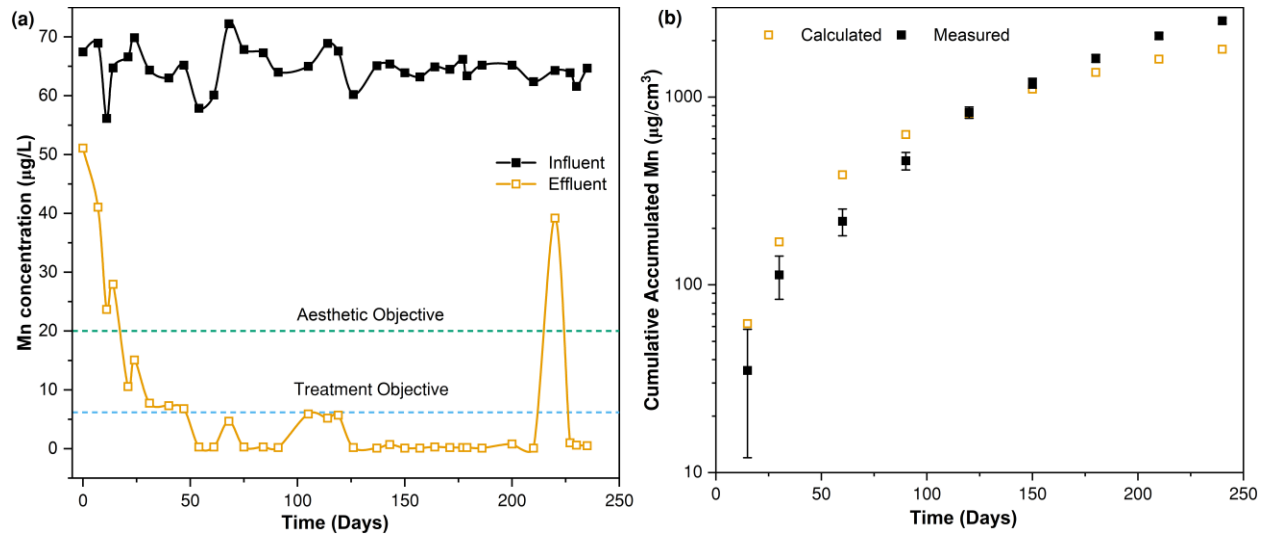


Figure 5.1: (a) Biofilter influent and effluent Mn concentrations; (b) Temporal accumulation of Mn. The calculated value represents the average Mn accumulation in the biofilter. The measured value represents the accumulation at the top of the biofilter media, and the error bars represent the standard deviation of duplicate measurements.

Fig. 5.1b outlines the temporal cumulative accumulation of Mn from the top 0-5 cm of the biofilter media contrasting with calculated cumulative accumulation based on influent and effluent concentrations. Calculated accumulations are based on the average Mn accumulated over the period (~30 days) under consideration multiplied by the number of days of that period. The measured Mn demonstrated an upward trajectory ranging from 35 ± 23 to 510 ± 19 $\mu\text{g}/\text{cm}^3$ dry weight of media, while calculated accumulations ranged from 62 to 282 $\mu\text{g}/\text{cm}^3$ dry weight. The rising Mn accumulation, as indicated by both the calculated and measured values, implies a significant role for MnO_x -coated surfaces within the biofilters. These surfaces facilitate Mn adsorption, which is then followed by oxidation, contributing to the progressive build-up of MnO_x . The accumulation pattern was characterized by an initial rapid increase followed by a steady incremental gain. Statistical analysis using a two-sample t-test revealed no significant difference between the calculated and measured values, confirming the consistency of the accumulation trend ($p > 0.05$).

The initial higher calculated Mn, surpassing measured values until day 120, could be attributed to uniform Mn removal along the filter bed during the initial stages of biofilter operations, leading to lower accumulations at the top of the biofilter surface compared to later stages. The uniform distribution of Mn removal during the initial stages of biofilter operation could result from Fe in the influent (89.3 ± 43.9 $\mu\text{g}/\text{L}$). The presence of Fe can lead to stratification in the biofilter media, with Fe primarily being removed at the top layers and Mn at lower depths (Section 3.4.2.3). Furthermore, this disparity may also result from immature biofilms during the initial stages, where MnO_x could be released into backwash waste, diminishing Mn accumulation on the media (Ramsay et al., 2018).

Post day 120, the measured Mn exceeded calculated values, aligning with findings that, upon maturation, Mn removal predominantly occurs in the top 20 cm of the biofilter media, hence indicating higher accumulation of Mn in this region (Section 3.4.2.3) (Breda et al., 2019; Zhao et al., 2021). The calculated accumulated Mn represents the average accumulation within the filter bed, which would be lower than the maximum accumulation at the top of the biofilter and decrease with depth. This was also supported by Evans et al. (2020), who found significant differences ($650 \pm 1,020$ $\mu\text{g}/\text{cm}^3$ dw) in Mn concentration between samples from just below the surface (~10 cm) and those deeper (~86 cm) within the biofilter.

5.4.2 Characterization of MnO_x on biofilter media

The morphological characteristics of MnO_x coatings formed on the sand media grains during the filtration process are shown in figure 5.2. These images capture the virgin media's initial condition and

the progressive development of MnO_x coatings after 70 and 150 days of operation. The virgin media (Fig. 5.2a-c) exhibits a baseline texture for comparison, showing that the surface of virgin sand was not entirely smooth. Subsequent images (Fig. 2d,f) illustrate the evolution of the media's surface, marked by distinctive structural changes likely attributable to biotic and abiotic processes. The presence of fluffy structures on day 70 reflects the biological origin of MnO_x formation, consistent with the birnessite type as reported in the literature (Bruins et al., 2015b; Dangeti et al., 2020). This biologically induced birnessite contrasts with the denser morphology observed on day 150, suggesting a transition toward physicochemical MnO_x formation as the biofilter matures (Bruins et al., 2015b).

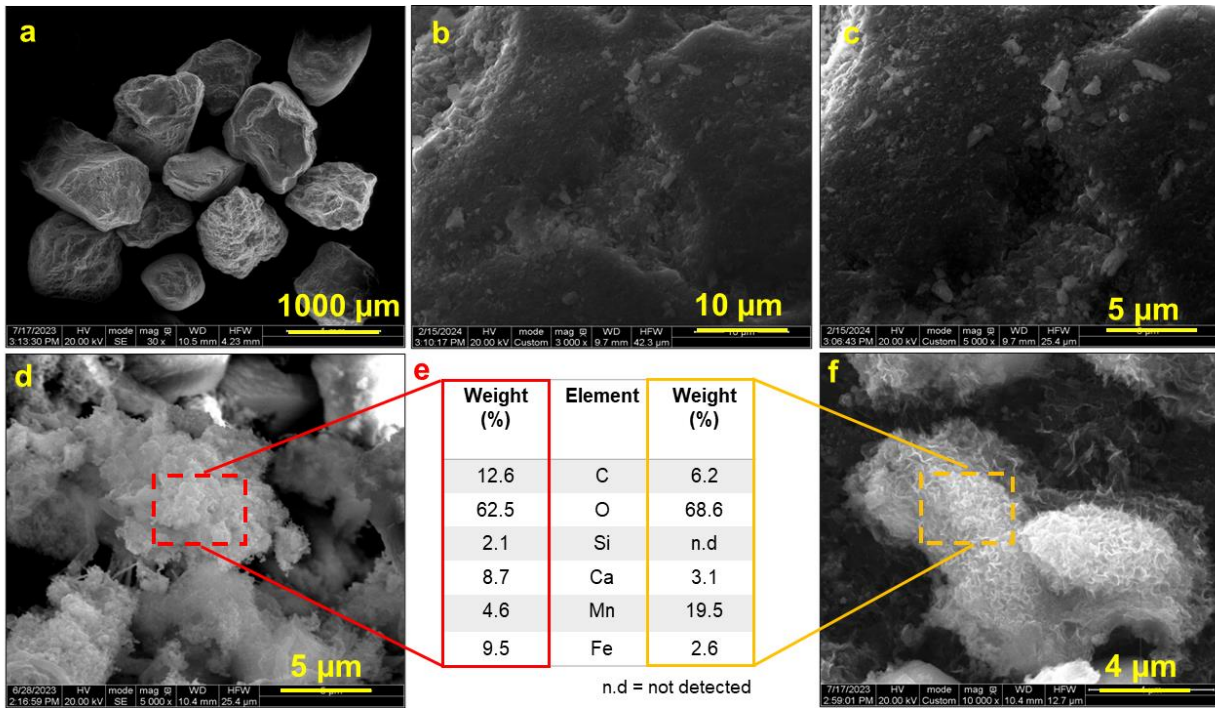


Figure 5.2: SEM images of virgin media (a) 30 $\times$ (b) 3000 $\times$ (c) 10000 $\times$; biofilter media collected from the top at (d) day 70 (f) day 150; (e) Elemental composition from EDX analysis

The observed change in MnO_x morphology over time is crucial and can be elucidated as follows: The MnO_x initially formed originates from biological processes. This is because biological Mn oxidation occurs more rapidly than abiotic Mn oxidation. The initially formed MnO_x has high adsorptive properties and possesses a negative charge, which arises either from vacancy defects or from the inclusion of heterovalent cations, including Mn(III) and Mn(II) within their octahedral layers or

mineral structures (Tebo et al., 2004). This leads to Mn(II) sorption to neutralize the negative charge, which leads to subsequent oxidation and formation of new MnO_x. As biofilters mature, the MnO_x tends to accumulate on surfaces, as observed in this study (Fig. 5.1b) and supported by elemental analysis of the biofilter media, potentially impeding mass transfer processes (Fig. 5.2e). Due to its strong adsorption capacity and affinity, MnO_x will likely compete with MnOB for Mn(II), potentially decreasing biologically generated MnO_x.

Moreover, this ongoing MnO_x formation may transition into various mineral forms that cannot be directly identified as microbial in origin (Bargar et al., 2005; Templeton et al., 2005). This complex interplay highlights the shift between biological and physicochemical processes in MnO_x morphology and composition over time. Nonetheless, these MnO_x oxides formed either biologically or physicochemically play a significant role in the sustained Mn removal in biofiltration.

5.4.3 Microbiome of Mn-removing biofilter

5.4.3.1 Spatial and temporal distribution of microbial community

A heterogeneous microbial consortium was observed in the biofilter, marked by a substantial representation of several key phyla, including *Proteobacteria*, *Bacteroidota*, *Verrucomicrobiota*, *Actinobacteria*, and *Nitrospirae* on days 70, 150, and 240 (Fig. A5.1). Among the phyla, *Proteobacteria* were the most prevalent, with mean relative abundances of $33.23 \pm 1.80\%$, $32.04 \pm 3.44\%$, and $36.90 \pm 4.26\%$, followed by the *Nitrospirae* at $20.30 \pm 7.01\%$, $12.02 \pm 6.60\%$, and $11.92 \pm 4.18\%$ on days 70, 150, and 240, respectively. These phyla thrive due to the unique environmental conditions of the biofilter, such as nutrient availability and operational parameters like aeration (Flemming & Wingender, 2010). *Proteobacteria*, in particular, are known for their metabolic versatility, being capable of processing both organic and inorganic compounds through oxidation-reduction reactions (Ghosal et al., 2016). They adapt remarkably well to various environmental changes, including fluctuations in pH and temperature (Fierer & Jackson, 2006). Their proficiency in forming biofilms also contributes to their prevalence in biofilter systems (Flemming & Wingender, 2010). These findings are in alignment with previous research underscoring the pivotal role of *Proteobacteria* in Mn removal within sand filtration systems (Cai et al., 2023; Gülay et al., 2016; Nitzsche et al., 2015; Piazza et al., 2019). *Nitrospirae*-affiliated bacteria are also capable of Mn oxidation through chemolithoautotrophy, linking this process to energy conservation (Yu & Leadbetter, 2020). Further, culture-dependent methodologies have demonstrated the capabilities of certain *Proteobacteria* and *Actinobacteria* strains to synthesize birnessite-type MnO_x (Lin et al., 2008). These oxides contribute to sustained Mn removal in biofilters.

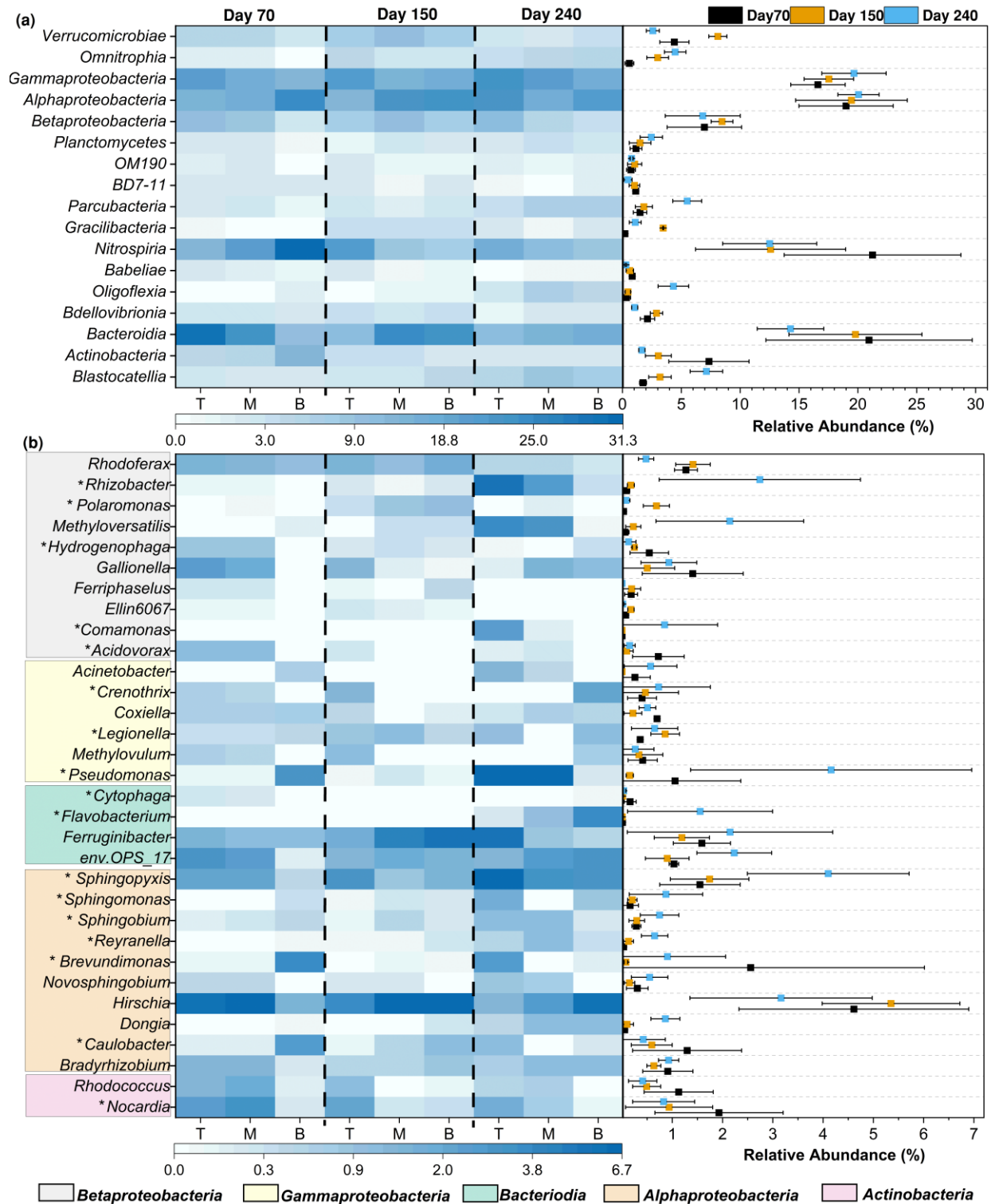


Figure 5.3: Heatmap (and average) of microbial relative abundance ($> 0.1\%$) in biofilters at day 70, 150, and 240 (a) Class level (b) Genera level (*Known MnOB genera) (T: Top, M: Middle, B: Bottom), the rectangular dot represents the mean and error bars represent the standard deviation from top, middle, and bottom

Subsequent analysis at the class level (Fig. 5.3a) revealed a predominant presence of *Alphaproteobacteria*, *Gammaproteobacteria*, *Betaproteobacteria*, *Nitrospira*, and *Bacteroidia*, together constituting $84.76 \pm 2.72\%$, $77.82 \pm 2.62\%$, and $73.32 \pm 8.06\%$ of the microbial population on days 70, 150, and 240, respectively (Fig. 5.4a). A slight increase in the relative abundance of *Alphaproteobacteria* ($18.99 \pm 4.01\%$ to $20.05 \pm 1.74\%$) and *Gammaproteobacteria* ($16.66 \pm 2.31\%$ to $19.66 \pm 2.73\%$) was observed from day 70 to day 240. *Betaproteobacteria* relative abundance ($6.96 \pm 3.16\%$ to $6.82 \pm 3.18\%$) remained consistent as the biofilter matured. Conversely, *Nitrospirae* ($21.24 \pm 7.51\%$ to $12.50 \pm 3.99\%$), *Actinobacteria* ($7.34 \pm 3.40\%$ to $1.65 \pm 0.27\%$), and *Bacteroidia* ($20.94 \pm 8.76\%$ to $1.65 \pm 0.26\%$) showed a decrease in relative abundance with the maturation of the biofilter media.

Depth stratification further revealed variances, such as the decrease of *Gammaproteobacteria* with increasing depth, while *Alphaproteobacteria* exhibited an incremental trend on days 70 and 150, followed by a marginal reduction on day 240. *Betaproteobacteria* exhibited a decrease in the relative abundance with greater depth, except on day 240, where an increase was noted at the mid-depth level before decreasing at the lower depths. McCormick et al. (2023) highlighted the crucial role of the *Betaproteobacteria* class in Mn-removing biofilters, a finding consistent with the biofilter examined in this study. The *Betaproteobacteria* class showed variations of 9.69%, 7.64%, and 11.08% at the biofilter surface on days 70, 150, and 240, respectively, and was consistently present throughout the depth of the filter bed, emphasizing its significant role in Mn removal in a biofilter. Noteworthy is the presence of various MnOB genera within the *Betaproteobacteria* class, among others (Fig. 5.3b). Additionally, the presence of IOB was also observed in the biofilter.

5.4.3.2 Presence of MnOB in biofilter

Over 15 recognized genera of MnOB were detected in the biofilter, each displaying different relative abundances (Figure 5.3b) with notable representatives such as *Sphingomonas*, *Nocardia*, *Caulobacter*, *Pseudomonas*, *Sphingobium*, *Shingomonas*, *Rhizobacter*, and *Sphingopyxis* from different phyla and classes (Cai et al., 2023; Cheng et al., 2019; Cheng, et al., 2017; Evans et al., 2020; Marcus et al., 2017; McCormick et al., 2022; Piazza et al., 2019). Consistent with existing studies, MnOB has been documented across a broad spectrum of bacterial phylogenetic lineages (Piazza et al., 2019; Tebo et al., 2005). The average relative abundance of MnOB increased with biofilter maturation, ranging from $9.11 \pm 1.21\%$, $6.20 \pm 0.91\%$, and $18.16 \pm 8.21\%$ at day 70, 150, and 240, respectively. A spatial dynamic was also apparent; at day 70, MnOB relative abundance increased from 7.86% at the top of the biofilter to 10.77%

towards the bottom. However, on day 150, there was a decline from 7.45% at the top to 5.80% at the bottom, and by day 240, a reduction from 29.95% at the top to 10.29% at the bottom was observed.

The spatial distribution of MnOB followed a similar pattern to that observed for Mn removal in the biofilters in the presence of Fe. During the start-up of biofilters, Fe is removed at the top, followed by Mn removals in deeper layers (Fig A3.2). Similarly, on day 70, a lower MnOB relative abundance was observed at the top and abundance was higher at deeper layers. However, as the biofilter matures, MnOB shifts upwards, similar to the Mn removal zone. The change in the relative abundance of *Gallionella*, a well-recognized IOB, further corroborates this observation. Initially, on day 70, the relative abundance of *Gallionella* fluctuated from 2.28% at the top to 0% at the bottom of the biofilter. By day 240, this variation shifted, ranging from 0.18% at the top to 1.08% at the bottom. This shift underscores the adaptive nature of bacterial communities in response to changing biofilter conditions and their integral role in the biofiltration process.

Sphingopyxis was identified as the predominant MnOB genera in the biofilter, with relative abundances of 2.12%, 2.78%, and 6.36% at the top, which decreased to 0.41%, 1.55%, and 2.75 % at the bottom on day 70, 150 and 240 respectively (Fig 5.3b). In contrast, *Pseudomonas* showed more variation, with relative abundances of 0.14%, 0.10%, and 6.01% at the top on days 70, 150, and 240, respectively. Notably, its relative abundance increased with depth to 2.91% on day 70 and then, to a lesser extent, 0.11% on day 150, while a decline to 0.20% was observed on day 240. Various *Pseudomonas* strains are known for their ability to generate biogenic MnO_x (Bio-MnO_x) that are poorly crystalline and have a high surface area (Cömert & Tepe, 2020; Parikh & Chorover, 2005; Villalobos et al., 2003). Other recognized producers of Bio-MnO_x include strains of *Leptothrix* (Loganathan & Bureau, 1973; Nelson et al., 1999; Tebo et al., 2004) and *Bacillus* (Bargar et al., 2000; Tebo et al., 2004; Webb et al., 2005), which were detected in the biofilter only on day 70 with an abundance of less than 0.05%. Therefore, it is plausible that the occurrence of these genera (*Pseudomonas*, *Leptothrix*, and *Bacillus*) could have led to the production of Bio-MnO_x similar to birnessite on day 70 (Fig. 5.2d).

Another notable difference was observed in the presence of *Crenothrix*, with initial relative abundances of 0.67% and 1.39% at the top layer on days 70 and 150, respectively. However, by day 240, *Crenothrix* was detected exclusively at the bottom of the biofilter, with an abundance of 2.18% (Fig. 5.3b). Given that *Crenothrix* is an IOB, its greater prevalence on the surface in the biofilter on days 70 and 150 likely played a role in facilitating the biological Fe removal. However, the primary removal of Fe is homogeneous oxidation aided by the favorable characteristics of the influent water (Eh: 149 ± 27 mV,

pH: 7.5 ± 0.4) in this study. Similarly, the genus *Nocardia* demonstrated a high initial presence that subsequently decreased as the biofilter matured, with relative abundances diminishing from $1.92 \pm 1.27\%$ on day 70 to $0.82 \pm 0.61\%$ on day 150 and further to $0.82 \pm 0.80\%$ on day 240.

Conversely, the genera *Comamonas* showed an increase in relative abundance with biofilter maturation. *Comamonas* were not detected on days 70 and 150; however, by day 240, they exhibited a relative abundance of 2.33% at the biofilter top, 0.18% at the middle, and 0.02% at the bottom. *Comamonas* has also been found to contribute to the formation of Bio-MnO_x (Tsuji et al., 2017). These observations suggest that the biofilter shifted its microbiome composition over time, developing a distinct microbial community as it matured.

Subsequent analysis revealed that the predominant MnOB (Fig. 5.3a) and those not displayed due to their relative abundances below 0.1% primarily belonged to several orders. These included *Sphingomonadales* (encompassing genera such as *Sphingopyxis*, *Sphingobium*, *Sphingomonas*), *Rhizobiales* (including *Bosea*, *Hyphomicrobium*, *Pedomicrobium*), *Caulobacterales* (comprising *Brevundimonas*, *Caulobacter*), and *Burkholderiales* (featuring *Rhizobacter*, *Acidovorax*, *Gallionella*, *Comamonas*, *Hydrogenophaga*, *Polaromonas*, *Variovax*) among others. This analysis suggests that specific taxonomic orders are more influential in the Mn removal process within biofilters than others likely due to their metabolic flexibility and ability to form biofilms, which are advantageous traits for adapting to the environmental conditions within biofilters.

5.4.3.3 Impact of biofilter maturity on the microbiome

As the biofilter aged, changes in the relative abundance within the microbial community were observed. This reflects the community's adaptive response to biofilter maturity. Spatial analysis revealed that the relative abundance of MnOB distribution varied uniquely, initially increasing from the top to the bottom of the biofilter and subsequently increasing from bottom to top at later stages of biofilter operation. The bacterial communities on the biofilter media exhibited Shannon diversity indices of 3.65 ± 0.34 , 3.94 ± 0.04 , and 4.93 ± 0.25 on days 70, 150, and 240, respectively (Table A5.1). The greatest diversity was recorded at the top of the biofilter on day 240. Meanwhile, Simpson diversity indices were 0.93 ± 0.03 , 0.95 ± 0.01 , and 0.98 ± 0.01 on days 70, 150, and 240, respectively, demonstrating high diversity, which suggests that no single species dominated the microbial ecosystem. Such high levels of diversity and evenness indicate a stable and resilient microbial community within the biofilters.

Beta diversity was analyzed using the Bray-Curtis dissimilarity matrix and plotted as PCoA, which accounted for 64.2% of the variation among biofilter media samples, with PCoA1 explaining 43.8% of this variance (Fig. A5.2). The greatest dissimilarity in microbial communities was noted between samples 70-B and 240-T, while the least dissimilarity was observed between samples 70-T and 70-M (Figure A5.3). These findings indicate shifts in microbial community composition from day 70 to day 240. Nonetheless, PERMANOVA analysis revealed that neither the day of sampling nor the depth within the biofilter significantly affected microbial diversity ($p > 0.05$). While there were changes in the relative abundances within the microbial communities, the overall composition of the microbial community did not show significant differences as the biofilter aged. However, specific microbial communities did show variations from the startup phase to maturity.

5.4.4 Contributions from different removal mechanisms

5.4.4.1 Biological vs Physicochemical

Batch experiments using media from the top of the biofilter were conducted to investigate the contribution of biological and physicochemical processes in Mn removal. From day 70, the average Mn removal efficiencies in the uninhibited batch tests were $20.1 \pm 2.8\%$, $35.2 \pm 4.6\%$, and $77.3 \pm 2.9\%$ at 0.5 h, 1 h, and 24 h respectively. The physicochemical Mn removals obtained from inhibited batch tests were $10.3 \pm 1.9\%$, $22.9 \pm 3.1\%$, and $53.6 \pm 1.6\%$, while calculated biological removals were 9.8%, 12.3%, and 23.7% for media from the same time intervals (Fig. 5.4a). Over time, an increase in the biological contribution was observed, likely due to the properties of NaN_3 as an inhibitor (section 4.4.3).

A similar pattern of removal was observed for media from day 150. Manganese removal efficiency reached $39.5 \pm 1.5\%$, $69.5 \pm 3.8\%$, and $98.9 \pm 1.7\%$ at 0.5 h, 1 h and 24 h respectively. The physicochemical removals increased to $31.8 \pm 3.2\%$, $57.5 \pm 2.5\%$, and $84.8 \pm 2.1\%$. In contrast, calculated biological removals were only 7.7%, 12.0%, and 14.1% for the media from the same time intervals, highlighting a reduction of biological contributions compared to day 70 (Fig. 5.4a). For media from day 240, the Mn removal efficiency increased to $45.2 \pm 2.9\%$, $77.1 \pm 1.6\%$, and $99.1 \pm 0.4\%$ at 0.5 h, 1 h and 24 h respectively. Here the physicochemical process dominated, accounting for $39.6 \pm 2.3\%$, $72.6 \pm 1.9\%$, and $96.2 \pm 0.1\%$ Mn removal, while biological processes removed 5.6%, 4.5%, and 2.9% at 0.5 h, 1 h and 24 h respectively (Fig. 5.4a).

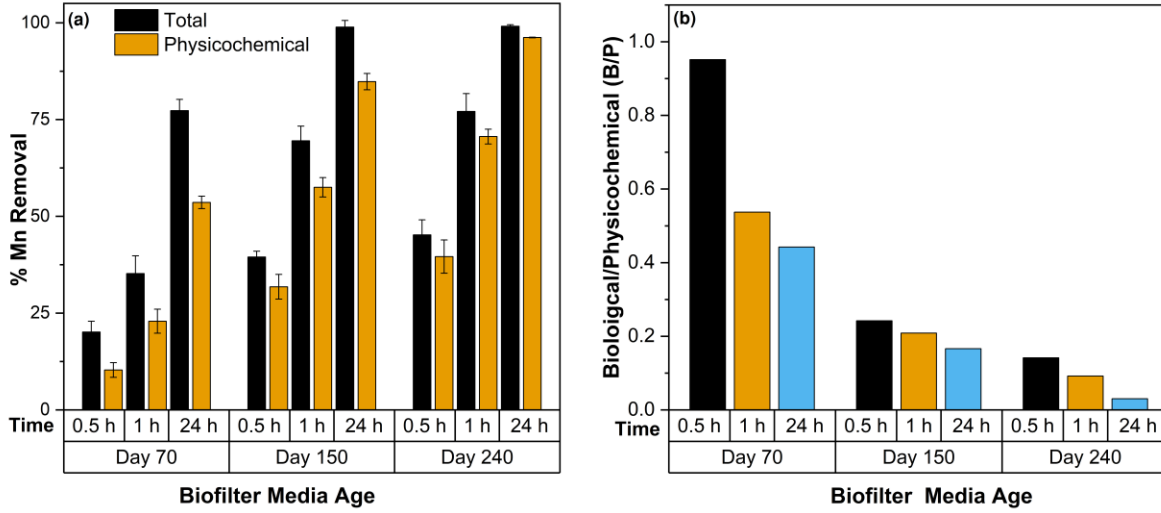


Figure 5.4: (a) Total and physicochemical Mn removals from a batch test using biofilter media from the top on days 70, 150, and 240 (Percentage removal is based on the initial Mn(II) concentration and at time $t = 0.5, 1, 24$ h), error bars represent the standard deviation of duplicate analysis; (b) B/P for biofilter media on days 70, 150 and 240

As the biofilter media aged, a decline in the biological/physicochemical (B/P) ratio was observed, with a maximum of 0.95 for media on day 70 (0.5 h), indicating that during the early stages of biofilter operation, biological processes contribute nearly equally to Mn removal (Fig. 5.4b). However, as the filter matures, physicochemical processes become more dominant, with B/P ratios decreasing to 0.24 and 0.14 for media on day 150 (0.5 h) and day 240 (0.5 h), respectively (Fig. 5.4b). Despite an observed increase in biological removal efficiency on day 70 at 1 h and 24 h, the B/P ratio continued to decline, underscoring the increasing dominance of physicochemical processes in Mn removal over time. This trend persisted through days 150 and 240. Breda et al. (2019) observed similar phenomena, with physicochemical processes dominating the filter bed except for the top 10 cm on day 48. Furthermore, in a study by Sahabi et al. (2009), biological processes had a higher contribution to Mn removal on 3-year-old matured anthracite media but no significant role on 15-year-old mature anthracite media, highlighting a shift towards physicochemical dominance as the biofilter ages. However, those studies did not address how the contribution of different mechanisms evolves from start-up to maturation in biofilters.

5.4.4.2 Shift in the removal mechanism

The decrease in the B/P ratio and the dominance of physicochemical processes over time in several studies, including this one, can be explained by a shift in the mechanism of Mn removal. For a non-

adsorptive media like sand used in this study, Mn removal in a biofilter can occur through biological or physicochemical processes (homogenous oxidation, adsorption, and autocatalytic oxidation by MnO_x). Although homogeneous Mn oxidation is thermodynamically viable, it is very slow at neutral pH (Diem & Stumm, 1984; Morgan, 2005), which was likely the case in this study. During the initial stages of biofilter operation, microorganisms played a pivotal role in removing Mn. This is supported by the detection of MnOB genera, known for their capability to produce Bio- MnO_x (Fig. 5.3b). This claim is further substantiated by the MnO_x morphologies observed in this study at day 70 (Fig. 5.2d) and reported by Bruins et al. (2015b) during the start-up of biofilters. During the start-up phase of the biofilter, microbial activity led to the production of Bio- MnO_x , probably by strains of *Pseudomonas*, *Leptothrix*, and *Bacillus* identified on media from day 70 (Fig 5.5). The adsorption capacity of Bio- MnO_x is higher than that of abiotic MnO_x (Cai et al., 2023; Zhou et al., 2015), which could explain the early onset of Mn removal in sand biofilters and initial rapid accumulation of Mn on the biofilter media (Fig. 5.1b). As the biofilter matures, the accumulation is steady, coinciding with the change in the morphology of MnO_x to physicochemical (Fig. 5.2f).

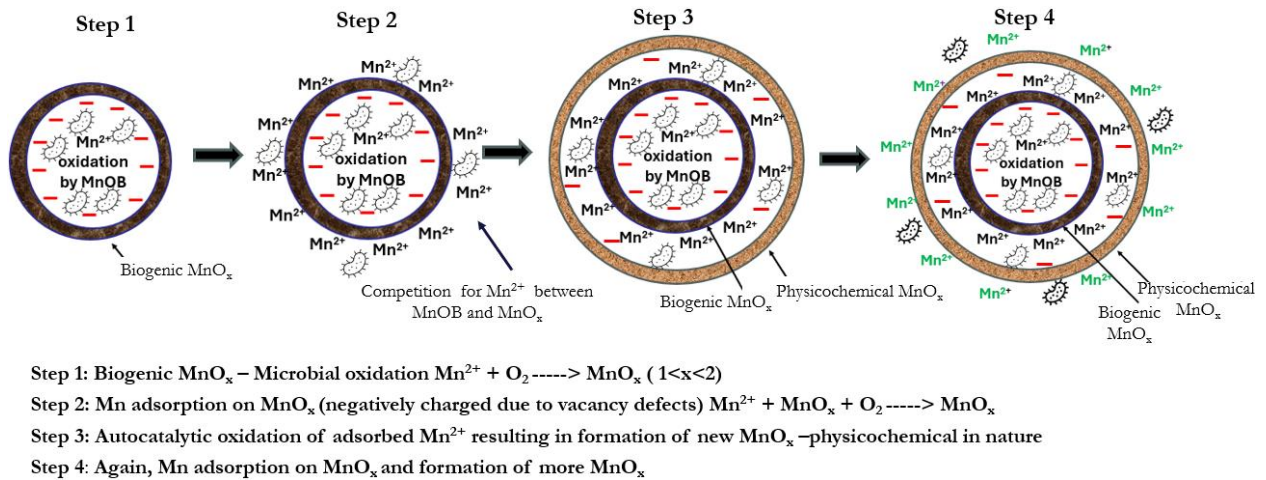


Figure 5.5: Mechanism of Mn removal in sand biofilter from startup to maturity

Despite an observed increase in the relative abundance of MnOB with the maturation of the biofilter (Fig. 5.3b), a notable shift in the Mn removal mechanism was observed. This transition could be influenced by several factors. MnOB are heterotrophs capable of growth without Mn, so not all MnOB are directly involved in manganese oxidation (Tebo et al., 2005). These bacteria can exhibit specific nutrient preferences and environmental adaptabilities which evolve as the biofilter matures.

In chapter 3, it was observed that MnOB counts decreased over time in Mn Supp and R2A+Mn media, while an increase was noted in Mn Min media. The Mn Min media, primarily composed of agar and manganese sulfate, appears to favor MnOB strains that thrive in conditions where Mn is the predominant nutrient, facing minimal competition from other nutrients. This media is particularly supportive of bacteria that can effectively utilize Mn as a major growth factor. Conversely, the more nutrient-rich conditions of the Mn Supp and R2A+Mn media support a broader spectrum of microbial life, including more fastidious organisms. This environment provides multiple growth factors, potentially favoring bacterial species that utilize a wider range of nutrients, thereby outcompeting MnOB specialized in Mn utilization. Such dynamics suggest that MnOB in the Mn Supp and R2A+Mn media might adapt to utilize available nutrients in the groundwater, leading to observed decreases in their counts. Thus, despite an increase in relative abundance of MnOB, the proportion of MnOB actively participating in Mn oxidation likely decreased.

Furthermore, not all MnOB can generate Bio-MnO_x with autocatalytic properties (Bargar et al., 2005). The prevalence of *Pseudomonas*, *Leptothrix*, and *Bacillus* genera recognized for Bio-MnO_x production diminished as the biofilter aged. This reduction could be attributed to the competitive dynamics for Mn(II) between MnO_x and MnOB. Owing to its significant adsorption capability and autocatalytic properties, MnO_x competes with MnOB for Mn(II) adsorption (Fig 5.5). This competition may reduce microbial Mn oxidation and, subsequently, Bio-MnO_x formation. The negatively charged MnO_x continues to attract more Mn (Fig. 5.5), leading to the further production of Mn oxides through physicochemical processes (Fig. 5.5). Webley (1960) emphasized Mn as a crucial nutrient for the growth of various *Nocardia* species. In our study, a decline in the relative abundance of *Nocardia* over time was noted, suggesting a scarcity of Mn(II) necessary for their proliferation, and likely indicating competition for Mn(II) between MnO_x and MnOB. As the biofilter matures, the significance of chemical and autocatalytic oxidation of MnO_x increases, marking a progressive transition from biological to physicochemical pathways for Mn removal, although biological processes continued to contribute to Mn removal as observed by the inhibition batch study.

5.5 Conclusions

This study provided a comprehensive understanding of Mn removal in biofilters from start-up to maturation by analyzing the relationship between biofilter aging and MnO_x morphology, Mn accumulation, changes in microbial community composition, and contributions of biological/physicochemical processes. Key findings are:

- Mn accumulation on the biofilter media was characterized by an initial relatively rapid increase, attributed to biological Mn oxidation that results in the formation of Bio-MnO_x with a high adsorption capacity. This was followed by a slower, steady incremental gain in Mn accumulation, reflecting a shift towards a physicochemical morphology of MnO_x.
- An increase in the relative abundance of MnOB was observed as the biofilter matured.
- Maximum dissimilarity among microbial communities was observed on day 70 from the bottom of the biofilter and on day 240 from the top. In contrast, the lowest dissimilarity was noted between samples of biofilter media from the top and middle on day 70.
- Despite differences in the relative abundance of the microbial community, the overall microbial community composition was not significantly different as the biofilter matured. However, specific microbial communities varied from startup to maturity.
- This study highlighted the initial dominance of biological processes in Mn removal, driven by MnOB-producing Bio-MnO_x with high adsorption capacities. Over time, a shift towards the predominance of physicochemical removal mechanisms became evident, influenced by competitive interactions for Mn between MnO_x and MnOB. Nonetheless, biological processes continued to play a role in Mn removal even when the biofilters were acclimated.

6 Comparative Analysis of Intermittent and Continuous Biofilter Operations for Mn and Fe Removal in Groundwater: Insights from a Pilot-Scale Study

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6.1 Summary

This pilot-scale study evaluates the performance of intermittently operated (12 h/d and 6 h/d) and continuously operated (24 h/d) biofilters over 240 days from start-up to maturation, treating groundwater containing Mn and Fe. Biofilters were also shut down for 10 days at day 220 to compare any effects of extended shutdown. Post acclimation intermittently operated biofilters (12 h/d and 6 h/d) performed similarly to continuously operated biofilters (24 h/d) ($p > 0.05$), effectively maintaining Mn and Fe levels below 20 µg/L (> 90% removal). A temporary rise in Mn and Fe levels following the 10-day shutdown normalized within 6 and 2 hours, respectively, upon restarting. Implementing backwashing before restarting during intermittent mode slightly improved biofilter performance. The 10-day shutdown period impacted biofilm stability, lowering EPS and ATP levels. Although continuous operation yielded higher ATP and EPS levels, these did not directly correlate with Mn and Fe removal effectiveness ($p > 0.05$). Over 15 known MnOB genera were identified in the biofilters, including prevalent genera such as *Sphingopyxis*, *Pseudomonas*, and *Comamonas*, alongside IOB such as *Gallionella* and *Crenothrix*. Microbial community composition showed no significant difference between continuous biofilters and intermittently operated biofilters ($p > 0.05$). This study highlights the applicability of intermittently operated biofilters treating groundwater for small-scale and remote communities or at utilities where drinking water treatment plant operations are not continuous.

Keywords: Drinking water, shutdown, backwashing, biological activity, extracellular polymeric substance, microbial community composition

6.2 Introduction

Biofiltration systems in drinking water treatment plants are conventionally designed for continuous operation to maintain biological activity and consistent contaminant removal. Small-scale and remote communities or facilities with excess plant capacity may find their water treatment systems, including biofiltration units, subjected to non-continuous operation due to local demands. The periodic or intermittent shutdown of biofilters could potentially impact biofilter performance (Niquette et al., 1998; Upadhyaya et al., 2019, 2022). Any diminished biological activity during shutdowns poses a risk to the efficacy of contaminant removal, such as Mn and Fe, which are common co-contaminants in groundwater. Manganese is a contaminant of health concern, whereas Fe is mainly associated with aesthetic and operational problems in drinking water (Barbeau et al., 2011; Bouchard et al., 2007; Vries et al., 2017). Health Canada has established a MAC of 120 µg/L and an AO of 20 µg/L for Mn and recommended an AO of 100 µg/L for Fe (Health Canada, 2019 & 2023).

Many studies have investigated the impact of short-term intermittent operation or shutdowns on biofilter efficacy (Anderson et al., 2008; Hess et al., 2020; McKie et al., 2019; Nemani et al., 2018; Niquette et al., 1998; Upadhyaya et al., 2019, 2022). However, investigations into the long-term effects of intermittent operation are scarce, particularly those examining the initial phase of biofilter use, where such operational patterns could have a pronounced impact. The performance of biofilters under intermittent operation is influenced by various factors, including the condition of the filter media during shutdown periods (Niquette et al., 1998; Zeng et al., 2019), the length of these shutdowns (Evans et al., 2020; Upadhyaya et al., 2022), the frequency of backwashing (Niquette et al., 1998), and the specific contaminants being targeted for removal (Evans et al., 2020; Hess et al., 2020; McKie et al., 2019; Upadhyaya et al., 2019, 2022).

This research focuses on the removal of Mn and Fe. The biological oxidation of Mn necessitates specific conditions: a DO concentration greater than 5 mg/L, a redox potential (Eh) above approximately 300-400 mV (dependent on pH), and a pH > 7.1 (Mouchet, 1992). Intermittent operations may hinder the maintenance of these conditions, potentially leading to anoxic environments conducive to bacteria utilizing Mn(IV) oxides as electron acceptors in the absence of oxygen (Gregory & Carlson, 2003). This can result in the chemical reduction of MnO₂, releasing soluble Mn(II) into the effluent and increasing its Mn concentration. Similarly, the leaching of Fe from their oxides within the biofilter could increase effluent Fe concentration. Accordingly, this study aims

to elucidate the effects of intermittent pilot-scale operation over 240 days on the efficacy of biofilters in treating groundwater for Mn and Fe removal under varying operational conditions.

The objectives of this study were: (1) to compare the removal efficiency of Mn and Fe by biofilters under continuous and intermittent operation from their inception through to maturation; (2) to evaluate the influence of varied intermittent operation durations (12 h/d, 6 h/d) and start-up methodologies on the performance of biofilters; (3) to determine the impact of intermittent operation on biological activity and biofilm development; and (4) to explore the microbial community composition across biofilters to identify potential changes in composition due to operational variability. Additionally, the biofilters underwent a 10-day shutdown period to explore the implications of extended operational interruptions on biofilter performance.

6.3 Materials and methods

6.3.1 Experimental setup and operating conditions

The design of the pilot-scale biofilter is detailed in Section 3.3.1. Three biofilters, each utilizing sand (effective size: 0.85-1.00 mm, uniformity coefficient: 1.7) as the media, were used. The effluent flow rate of each biofilter was kept constant at 5 m/h by adjusting the effluent valves. This led to an EBCT of 14.4 minutes or less, a result of the diminishing filter bed volume over time as filter media was extracted for sampling.

The biofilters were operated for 240 days in four distinct phases (Fig. 6.1). In phase one, Biofilter A ran for 12 (h/d), while B and C operated continuously (24 h/d). In the second phase, biofilters A and B switched to 12-hour operation, with biofilter C remaining continuous. In phase two, biofilters A & B were restarted without backwashing, except on day 90. Manganese and iron effluent concentrations were measured for an extended period following restart at days 71 and 90. In the third phase, biofilter A continued at 12 h/d, biofilter B was reduced to 6 h/d, and biofilter C remained at 24 h/d. Biofilters A and B were restarted without backwashing except on day 170 and extended monitoring of Mn and Fe effluent concentrations occurred on days 141 and 170. In phase four following the extended shutdown of 10 days, Mn and Fe effluent concentrations were measured.

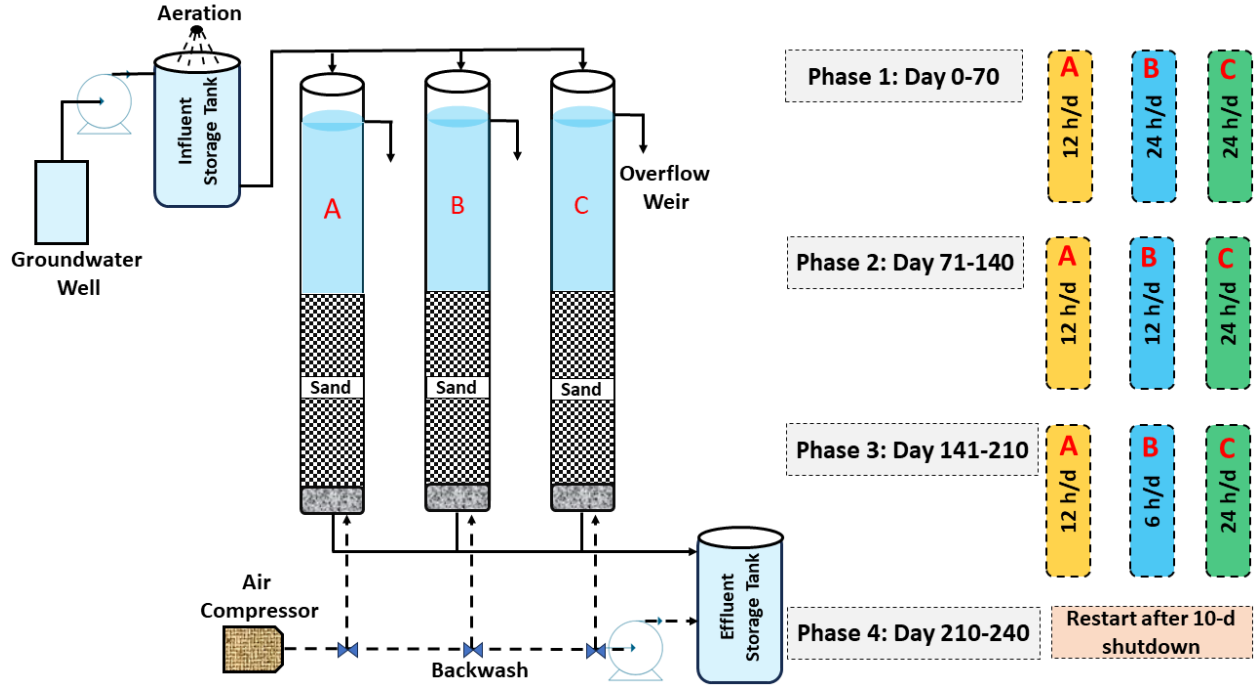


Figure 6.1: Process flow diagram and operating conditions of biofilters

Throughout intermittent operations, biofilters A and B were kept submerged, except media sampling, and operations were automated via solenoid valves that controlled start/shutdown times and logged data. Backwashing was performed with a flow rate of 10-20 m/h using the effluent from the biofilters and air scour, resulting in a bed expansion of 20-30%, and was performed until clear (5-10 mins) water was observed in the supernatant and backwash effluent. Biofilter C underwent weekly backwashing, and biofilter A once every two weeks. For biofilter B, the frequency of backwashing was adjusted depending on the operating phase to ensure the volume filtered between backwashing cycles remained consistent across all the biofilters.

6.3.2 Analytical methods

Influent and effluent water samples were collected once per week from each biofilter for Mn and Fe analysis. In addition, multiple effluent samples were collected on days 71, 90, 141, 170, and 220 and analyzed using QQQ-ICP-MS, Agilent 8800 (Section 3.2.2). The biofilter media samples (~5 g) were collected from the top 0-5 cm of the biofilters for ATP and EPS analysis using a sterilized spatula (Cole Parmer, Canada). The media samples were stored in sterile 50 mL plastic tubes (Falcon®, VWR™) and transported on ice for immediate processing. The ATP analysis of the biofilm was

performed using a deposit and surface analysis kit (DSA™, LuminUltra Technologies, Canada) (Section 3.3.2.2). EPS extractions followed the procedure outlined in Keithley & Kirisits (2018). The total carbohydrate content of the extracted EPS was determined using the phenol sulfuric acid method described by Dubois et al. (1956). The total protein content of the extracted EPS was determined using the bichronic acid method (Pierce micro-BCA protein kit, Thermo Scientific) (Text A6.3).

For microbial community analysis, the biofilter media (~1 g) was collected from three different bed depths (top: 0-5 cm, middle: 40-60 cm, and bottom: 100-120 cm) on day 240 using a sterilized spatula (Cole Parmer, Canada) and analyzed using procedure mentioned in section 4.3.2.

6.3.3 Statistical analysis

Statistical analysis and methods for data visualization are detailed in section 4.3.5.

6.4 Results and discussion

6.4.1 Mn and Fe removal across biofilters

Fig. 6.2a depicts the Mn concentrations in the influent and effluent from the three pilot-scale biofilters over 240 days, with influent Mn averaging $64.7 \pm 3.2 \mu\text{g/L}$. The biofilters operating continuously (B and C) achieved the treatment objective of 90% Mn removal by day 54, whereas the intermittently operated biofilter A reached the same level of removal by day 60. Within the first three weeks of operation, all biofilters successfully met Health Canada's aesthetic objective of $20 \mu\text{g/L}$ for Mn in drinking water. After the initial acclimation phase, the performance of the biofilters for Mn removal did not show significant differences ($p > 0.05$). This finding suggests that under the tested conditions biofilters are robust against operational changes, including intermittent versus continuous operation, without affecting their Mn removal efficiency upon acclimation. This can be attributed to the development of MnO_x and MnOB, which contribute to sustained Mn removal in biofilters (Chapter 5). Hoyland et al. (2014) also observed that well-acclimated MnOB in biofilters could withstand variations in Mn concentration, hydraulic loading rate, and temperature. Although their stress tests were short-term (1-2 days), their results underscore the resilience of biofilters to operational fluctuations. Post a 10-day shutdown period, on day 220, an increase in effluent Mn concentrations above the $20 \mu\text{g/L}$ aesthetic objective level was observed across all biofilters. However, the levels quickly returned (~ 6 h) to within the acceptable range.

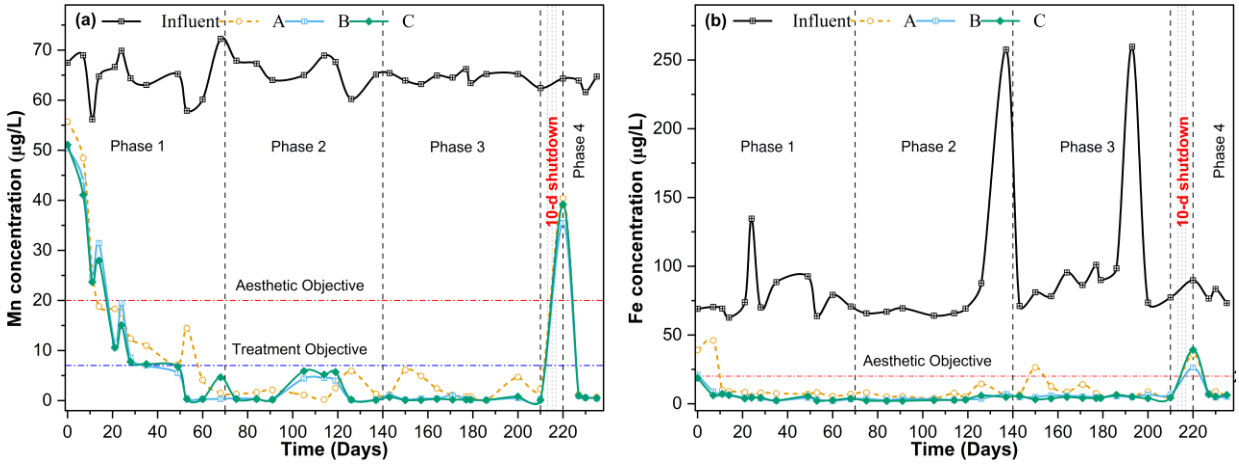


Figure 6.2: Biofilter influent and effluent concentrations (a) Mn (b) Fe (Red line represents the aesthetic objective levels of 20 µg/L)

Fig. 6.2b shows the Fe concentrations in influent and effluent of the same biofilters over 240 days, with influent Fe averaging 89.3 ± 43.9 µg/L, exceeding the influent Mn concentrations. This higher variability, indicated by the substantial standard deviation, is primarily attributed to significant spikes in influent Fe concentrations observed on days 24, 137, and 193. Given that Health Canada's recommended aesthetic objective for Fe in drinking water is set at 100 µg/L, and the average influent concentration fell below this threshold, our study pursued a more stringent treatment objective of 20 µg/L for Fe, equivalent to the aesthetic objective for Mn. During the initial week of operation, the continuously operated biofilters B and C achieved the set treatment objective for Fe removal. In contrast, the intermittent biofilter A required two weeks to meet the same standard. All biofilters consistently attained the treatment objective post-acclimation, except for biofilter A on day 149. Performance across the biofilters showed no significant differences in Fe removal efficiency ($p > 0.05$), and intermittent biofilter operation did not impact Fe removal performance. An observed increase in effluent Fe concentrations above the 20 µg/L treatment and aesthetic objective levels was noted in all biofilters following a 10-day shutdown at day 220. However, concentrations returned to acceptable levels after ~2 h.

The higher percentage of Fe removal than Mn in biofilters, particularly during start-up, can be attributed to different removal mechanisms. Vries et al. (2017) highlighted that the elevated water levels (1.5 m in this study) above the biofilter media prolonged the water residence time, enhancing the opportunity for homogeneous Fe oxidation. In contrast, homogenous Mn oxidation is slow

without Mn oxide catalyst below pH 9, despite being thermodynamically viable at neutral pH (Diem & Stumm, 1984; Morgan, 2005). The influent conditions of low Eh and pH in this study (Eh: 149 ± 27 mV, pH: 7.5 ± 0.4) further favored Fe oxidation over Mn.

6.4.2 Impact of intermittent operation on biofilter performance

6.4.2.1 Effluent Mn concentration

Biofilter A, operating intermittently (12 h/d), exhibited a pattern of increasing effluent Mn concentrations immediately following each system restart. The Mn concentration increased to 10.4, 3.8, 3.6, and 2.4 $\mu\text{g/L}$ from the baseline levels of 1.5, 1.7, 2.3, and 1.9 $\mu\text{g/L}$ for days 71, 90, 141, and 170, respectively (Fig. 6.3a). A noticeable trend in the data is the diminishing magnitude of the Mn spikes post-restart as the biofilter matures, suggesting an acclimation effect. The subsequent return to pre-shutdown Mn levels generally occurred 60-90 mins post-restart, except on day 71 event, which took longer (~ 400 min). Biofilter B demonstrated a more consistent effluent Mn concentration after restarts, with recorded levels of 4.1, 3.8, 2.8, and 2.1 $\mu\text{g/L}$ from pre-shutdown effluent concentrations of 1.8, 2.9, 3.1, and 2.6 $\mu\text{g/L}$ on days 71, 90, 141, and 170, respectively (Fig. 6.3b). Despite the changes in biofilter B operation from 24 h/d to 12 h/d (on day 70) and finally to 6 h/d (on day 140), consistent Mn removal was observed.

The difference in responses of biofilter A (intermittently operated throughout) and biofilter B (initially continuously operated then switched to intermittent operation post-day 70) after restarts, especially after day 70, highlight the impact of operational history on biofilter performance (Fig 6.3a,b). The initial continuous operation of biofilter B likely contributed to its stable performance, suggesting that continuous operation during start-up phases enhances biofilter maturation and resilience to fluctuations in effluent quality post-operational interruptions. The observed decrease in the magnitude of Mn concentration spikes over time supports the hypothesis that biofilter maturity is a crucial factor in stabilizing effluent quality. This is also evident from the fact that at day 140, biofilter B operation was changed to 6 h/d, but no substantial impact was observed on the effluent Mn concentration. Despite temporary increases in Mn levels following restarts in both biofilters, concentrations stayed below the 20 $\mu\text{g/L}$ aesthetic objective, indicating that, for the condition tested, the biofilters effectively maintain Mn levels within acceptable ranges despite temporary disruptions.

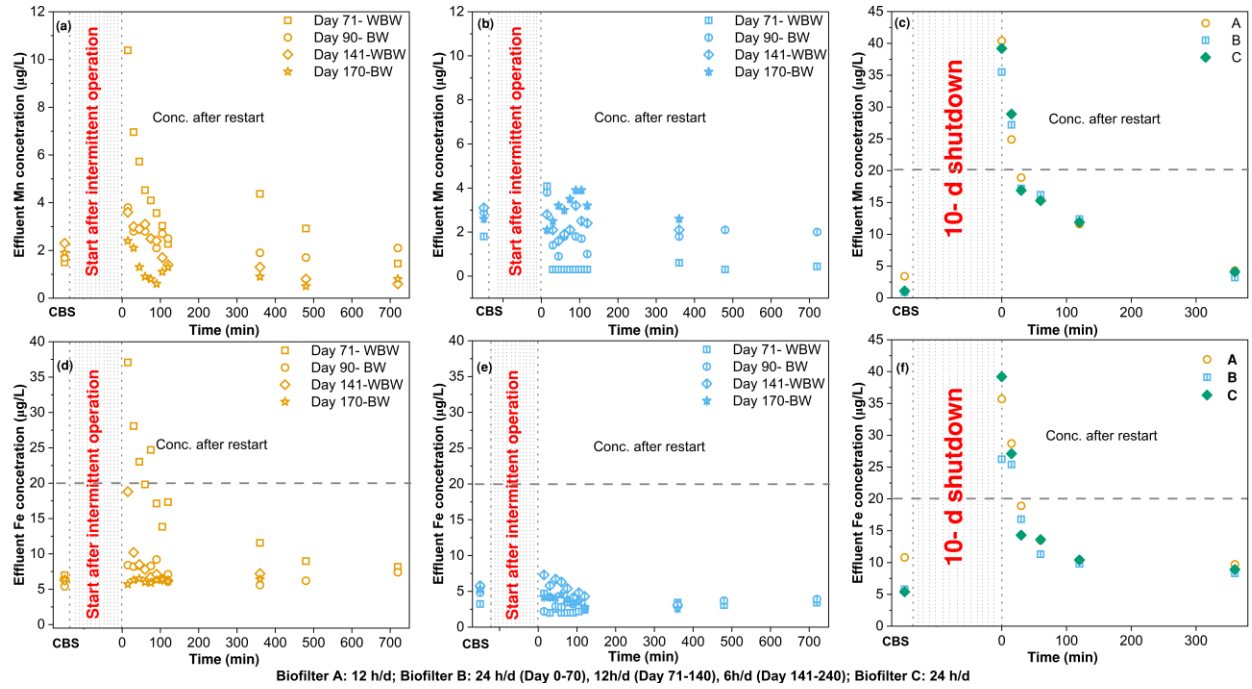


Figure 6.3: Effluent Mn concentration following restart in (a) Biofilter A, (b) Biofilter B, and (c) after a 10-day shutdown; Effluent Fe concentration following restart in (d) Biofilter A, (e) Biofilter B, and (f) after a 10-day shutdown. CBS: Concentration Before Shutdown, BW: Backwash, and WBW: Without Backwash before biofilter restarts. The horizontal dashed line indicates the aesthetic objective of 20 µg/L.

6.4.2.2 Effluent Fe concentration

Biofilter A showed a notable increase in effluent Fe concentration after restart, with values reaching 37.1, 8.4, 18.8, and 5.7 µg/L from the baseline measurements of 7.0, 5.4, 6.3, and 6.4 µg/L on days 71, 90, 141, and 170 (Fig. 6.3d). The most substantial increase occurred on day 71, with the Fe concentration reaching over five times the baseline level. This pronounced spike suggests a potential release of accumulated Fe from the biofilter media or an imbalance in the redox conditions that stabilize Fe. However, it is essential to note that after restarts on days 90 and 170, when biofilter A underwent a backwashing process before start-up, the fluctuations in Fe concentration were markedly reduced, with a minimal increase and a slight decrease in concentration, respectively. The rapid normalization of Fe levels within an hour post-restart, except for day 71, indicates a quick re-establishment of the system's equilibrium. Biofilter B's response to operational restarts was characterized by smaller variations in effluent Fe concentration, which adjusted to 4.7, 2.2, 7.3, and

4.2 µg/L from initial levels of 3.2, 4.8, 5.8, and 5.3 µg/L at days 71, 90, 141, and 170, correspondingly (Fig. 6.3e).

The significant increase in effluent Fe concentration in biofilter A compared to biofilter B, especially at day 71, could be linked to their operational history, similarly to the observations made for Mn concentrations. However, post-acclimation, the influence of their operational history appears to diminish, highlighted by no substantial changes in effluent Fe concentration when biofilter B operation changed to 6 h/d on day 140. The decreasing trend in Mn and Fe spikes post-restart accentuates the biofilters' maturation impact on effluent quality, suggesting a robust capacity to mitigate short-term shutdown effects. This resilience might stem from microbial community adaptation and biofilm stabilization, ensuring contaminant removal.

6.4.2.3 Impact of backwashing

On the commencement of operations on days 90 and 170, biofilters A and B underwent a backwashing procedure, an operational step evaluated for its influence on the post-restart effluent concentration of Mn and Fe. The effect of backwashing on Mn levels was negligible for biofilter B, whereas for biofilter A, a reduction in Mn concentration was observed post-restart after backwashing on day 90. Notably, biofilter B slightly reduced Fe concentration post-restart following backwashing. Similar results were also observed in biofilter A, where backwashing led to minimal increases or sometimes decreases in effluent Fe concentration, demonstrating its utility in maintaining effluent quality following operational interruptions (Fig. 6.3d,e). This emphasizes the distinct impact of backwashing on the removal efficiency of various contaminants. Furthermore, these backwashing effects might be more significant in scenarios where the feedwater contains higher Mn and Fe concentrations.

Backwashing is a crucial intervention to mitigate the adverse effects of system shutdowns on biofilter performance, a strategy supported by several studies. Niquette et al. (1998) demonstrated significant enhancements in biofilter functionality for NH_4^+ and DOC removal efficiencies following a restart after backwashing. This finding aligns with Anderson et al. (2008), who recommended the implementation of backwashing after brief shutdown periods or discharging the initial effluent volumes to waste as a method to return endotoxin levels to their pre-shutdown conditions.

6.4.2.4 Effluent Mn and Fe concentration following a 10-d shutdown

Effluent Mn and Fe levels measured after a 10-d shutdown in all three biofilters (Fig. 6.3c,e) have revealed significant changes. Upon restarting the biofilters, an increase in effluent Mn concentration was observed, exceeding the established aesthetic objective level of 20 µg/L. Specifically, biofilters A,

B, and C showed an initial spike in Mn concentrations of 40.4, 35.5, and 39.2 µg/L, from 3.4, 0.9, 1.1 µg/L respectively, immediately after the restart (Fig. 6.3c). Notably, within 30 minutes of operation, Mn concentrations rapidly decreased to below the aesthetic objective level. However, all biofilters recovered to pre-shutdown baseline levels within approximately 6 hours. This indicated a more extended stabilization phase than the shorter shutdown (6 or 12 h/d) when operated intermittently. Brandhuber et al. (2013) found that the Mn removal capacity of a biofilter could be re-established in approximately five days, even after a two-month shutdown period.

Evans et al. (2020) observed that GAC biofilters maintained their performance after a two-week shutdown, unlike anthracite biofilters, which observed an increase in Mn concentration following restart. Nonetheless, an increase in effluent Mn concentration following an extended shutdown of four weeks was observed in GAC and anthracite biofilters. This decline in performance was potentially linked to the biofilters being drained during the shutdown and alterations in conditions within the upstream biofilter. The treated water quality can deteriorate under shutdown conditions, possibly due to a lack of oxygen (Niquette et al., 1998). Anoxic conditions ($\text{DO} < 1 \text{ mg/L}$) can develop rapidly ($< 2 \text{ h}$) following the shutdown of biologically active filters (McKie et al., 2016). MnOB, which typically immobilizes Mn(II) through biological oxidation or adsorption onto cellular structures, may release Mn(II) upon cell lysis during such anoxic interruptions (Van Veen et al., 1978). Additionally, the presence of *Bacillus* and *Pseudomonas* species, which are capable of Mn reduction in anoxic conditions, can contribute to Mn(II) release into the effluent under certain conditions (Cerrato et al., 2010; Swain et al., 2018; Tebo et al., 2005).

The effluent Fe concentration dynamics exhibited a similar trend, albeit with a faster recovery. Initial concentrations post-restart increased from pre-shutdown levels of 10.8, 5.8, and 5.4 µg/L to 35.7, 26.2, and 39.2 µg/L for biofilters A, B, and C, respectively. Recovery to pre-shutdown Fe levels occurred within approximately 2 hours, demonstrating a quicker stabilization for Fe than Mn (Fig. 6.3f). This rapid stabilization of Fe, relative to Mn, points to different removal mechanisms. Similar to Mn, the presence of Fe-reducing bacteria, such as *Ferribacterium* (Cardenas et al., 2008; Cummings et al., 1999), might explain the initial rise in measurable Fe levels. A shift in redox potential during shutdowns and subsequent pH changes can also escalate Fe solubility, leading to its elevated presence in the effluent (Stumm and Morgan, 1996). Furthermore, the leaching of Mn and Fe from their respective deposits within the biofilter could further exacerbate changes in effluent quality, highlighting the complex interplay of biological and chemical processes influencing biofilter

performance after a restart. Statistical analysis of the data revealed that the observed increases in Mn and Fe concentrations were significant ($p < 0.05$).

6.4.3 Impact of biofilter operation on metabolic activity and biofilm EPS

6.4.3.1 Metabolic activity as reflected by ATP

ATP, which indicates metabolic activity in the biofilters, ranged between 10-170 ng ATP/cm³ (Fig. 6.4a), which is notably on the lower side of 100-1000 ng ATP/cm³ typically reported for biofilters acclimated for organic removal (Pharand et al., 2014). This reduction is likely attributable to the low organic content in the treated groundwater. Biofilters operating continuously exhibited elevated ATP levels relative to those operating intermittently (Fig. 6.4a). At day 220, the ATP levels were 22 % and 27.5 % lower in biofilters A (intermittently 12 h/d), and B (intermittently 6h/d) compared to biofilter C (continuously 24h/d). This observation aligns with McKie et al. (2019), who reported 50% lower ATP in a cyclically operating biofilter (12-14 h/d) than in continuously operated filters.

Following a 10-day shutdown, a pronounced decline in ATP concentrations was observed across biofilters A, B, and C, with reductions of 22%, 31%, and 19%, respectively (Fig. 6.4a). This decline may be attributed not only to the exposure of bacteria to potential anoxic conditions during the shutdown but also to the diminished availability of essential nutrients (carbon, phosphorus, and nitrogen) for the microorganisms residing on the filter media (McKie & Andrews, 2022). A previous publication by McKie et al. (2019) highlights that such conditions can lead to a marked reduction in the density of active biomass, as indicated by ATP measurements, within a mere two hours of a shutdown. Supporting this observation, Niquette et al. (1998) reported a 17% decrease in fixed bacterial biomass after a 24-hour shutdown period. McKie & Andrews (2022) observed a 30% reduction in biomass, as measured by ATP, for a shutdown of 16 to 36 hours. However, ATP levels returned to their pre-shutdown values within two weeks of resuming operations in the present study.

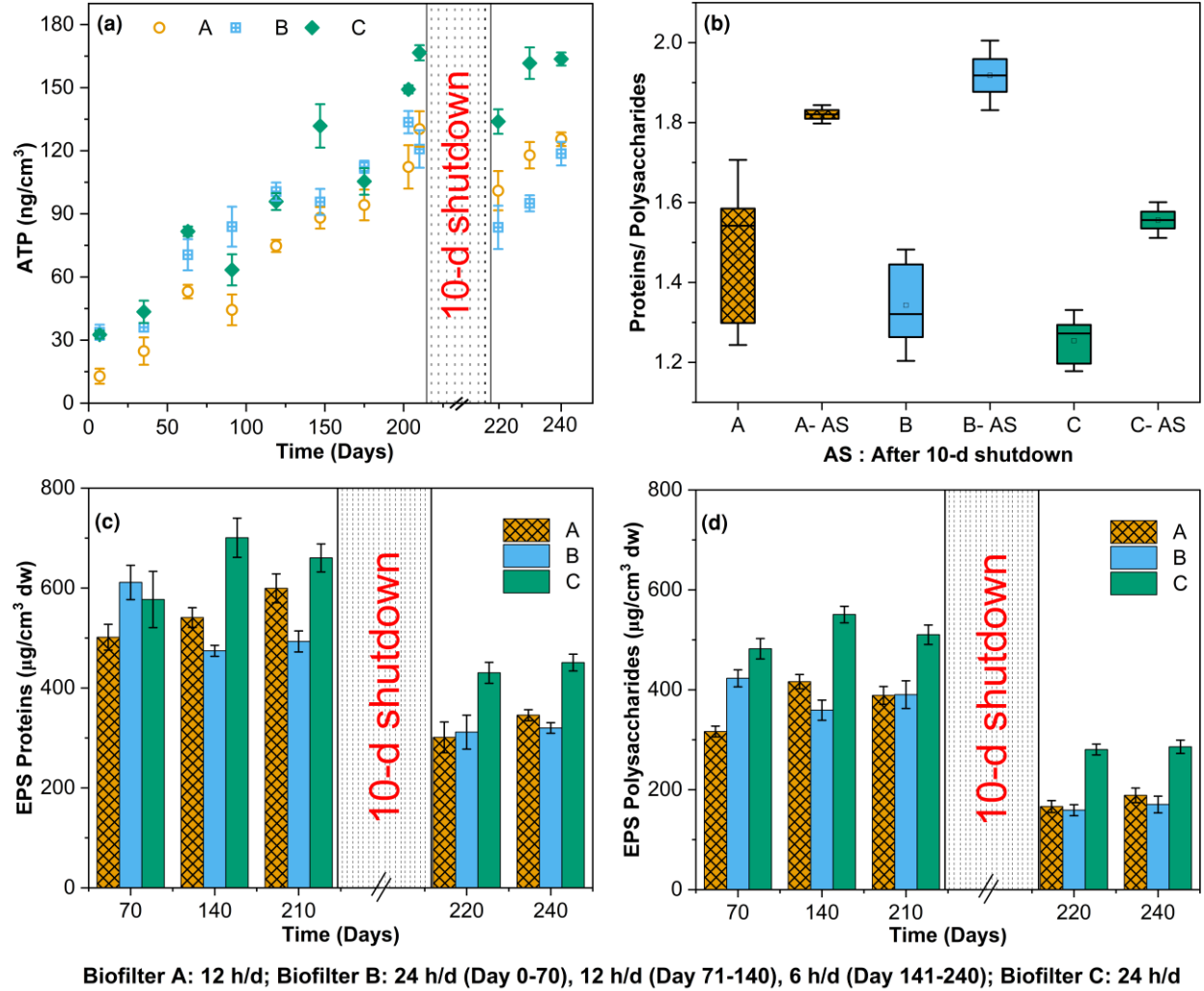


Figure 6.4: (a) ATP levels (error bars represent the standard deviation of the duplicate measurements), (b) Proteins/Polysaccharides (The horizontal line within the box indicates the median, the upper and lower bounds of the box indicate the third and first quartiles, respectively, and the whiskers extend to the largest value no greater than 1.5 times the standard deviation), (c) EPS protein, and (d) EPS polysaccharide concentration (Biofilter media collected from top 0-5 cm)

6.4.3.2 Biofilm EPS

Mn removal in biofilters can also occur through extracellular adsorption, where soluble Mn binds to the negatively charged EPS (Tobiason et al., 2016). The quantity and composition of EPS, particularly the ratio of carbohydrates to proteins, are thus crucial for understanding the efficacy of Mn removal via biofiltration. In our study, EPS protein concentrations ranged from 300 to 700 $\mu\text{g}/\text{cm}^3$ (188 - 438 $\mu\text{g}/\text{g}$) dw, and polysaccharides varied from 160 to 550 $\mu\text{g}/\text{cm}^3$ (100 - 344 $\mu\text{g}/\text{g}$) dw (Fig. 6.4c,d),

reflecting the EPS matrix within biofilters. This aligns with Keithley & Kirisits (2018), who reported EPS protein concentrations between 270 to 3,380 $\mu\text{g/g dw}$ and polysaccharide levels from 20 to 600 $\mu\text{g/g dw}$ across 11 full-scale biofilters. Continuous biofilter operation was notably linked to elevated EPS protein and polysaccharide concentrations. Despite operational variances, EPS concentrations were relatively stable throughout the study period for each biofilter. A 10-day shutdown led to a noticeable decline in both EPS proteins and polysaccharides across all biofilters, with levels failing to return to pre-shutdown concentrations after three weeks of resumed operation. This observation is consistent with findings by Evans et al. (2020), who noted similar trends following a four-week shutdown in biofilters designed for Mn removal using surface water.

In the current study, the shutdown period affected not only the levels of EPS but also led to temporary spikes in Mn and Fe concentrations in the effluent, which normalized quickly within a few hours. No direct correlation was found between the rise in EPS concentrations and the efficiency of Mn and Fe removal ($p > 0.05$), nor was there any observed correlation between ATP and EPS concentrations ($p > 0.05$). This indicates that while EPS plays a critical role in biofiltration, its relationship with Mn and Fe removal efficiency and active biomass, as measured by ATP, is complex and influenced by multiple factors beyond mere concentration levels.

The protein-to-polysaccharide (P/C) ratio, indicative of EPS composition, showed average pre-shutdown values of 1.47, 1.34, and 1.25 for biofilters A, B, and C, respectively (Fig. 6.4b). These results align with Stoquart et al. (2014), who reported a comparable protein-to-polysaccharide ratio for EPS in a drinking water treatment biofilter. Post-shutdown, the P/C ratios increased to 1.83, 1.87, and 1.58, respectively (Fig. 6.4b). The initial higher P/C ratios in biofilters A and B, relative to biofilter C, suggest that intermittent operation may influence EPS production or utilization by microbial communities. The post-shutdown rise in the P/C ratio, despite overall decreases in protein and polysaccharide levels, hints at a preferential consumption of polysaccharides by microorganisms, likely as an accessible energy source during downtime (Brown et al., 2020). This pattern aligns with the microbial need to tap into energy reserves when the external nutrient supply is limited. Alternatively, the observed increase in the P/C ratio post-shutdown could reflect a stress response, shifting towards higher relative protein content in the EPS, possibly as microbes adapt to the operational disruptions (Lauderdale et al., 2011). These findings further illustrate that shutdown periods may impact biofilm integrity, as observed by a reduction in EPS and ATP levels following a 10-day shutdown (Fig. 6.4).

6.4.4 Impact of biofilter operation on the microbial community composition

Each biofilter exhibited a rich microbial ecosystem with notable prominence of *Proteobacteria*, *Patescibacteria*, and *Nitrospirae*, underscoring their significant roles in biofilter functionality and the presence of *Actinobacteriota*, *Acidobacteriota*, *Firmicutes*, *Chloroflexi*, *Planctomycetes*, *Cyanobacteria*, *Bacteroidetes*, and *Verrucomicrobiota* among others (Fig. 6.5a). In biofilter C, running continuously, *Proteobacteria* were most abundant at the top, comprising 42.85% of the community, and had decreased relative abundance towards the bottom (33.11%). This distribution contrasts with those of biofilters A and B, operating intermittently, where *Proteobacteria* had a higher relative abundance at the bottom, comprising 30.05% and 42.85%, respectively, compared to 19.13% and 24.78% of the microbial community at the top of the biofilter (Fig. 6.5a). The difference in the spatial distribution of the microbial community of biofilter C in comparison to biofilter A and B might be influenced by its continuous operation. A similar distribution of proteobacteria was also observed in intermittently (12 h/d) operated biologically active carbon filters (Nemani et al., 2018).

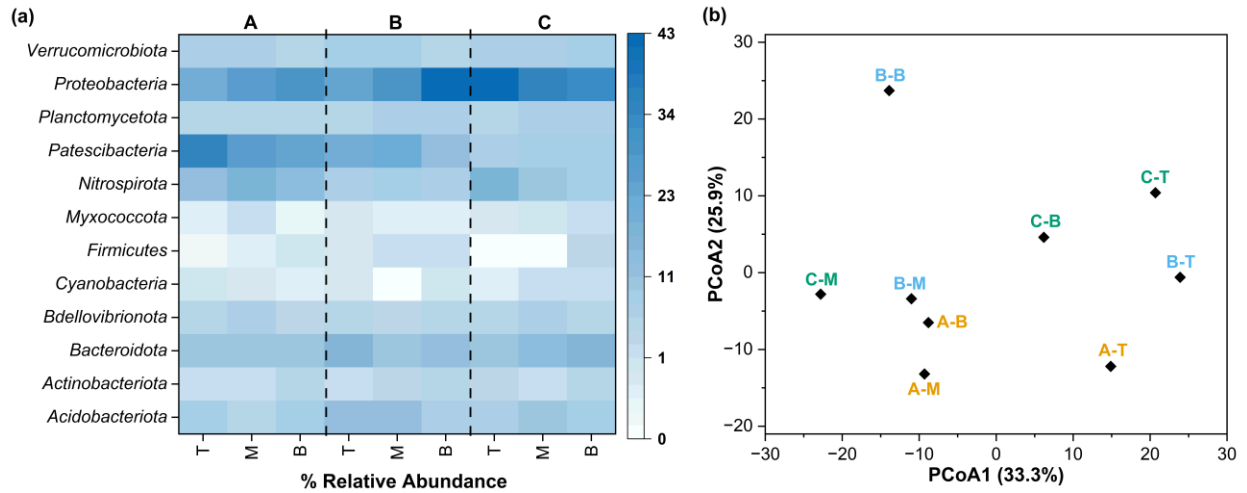


Figure 6.5: (a) Heatmap depicting the relative abundance ($> 0.25\%$) of microbial communities within biofilms at the phylum level (T: Top, M: Middle, B: Bottom); (b) PCoA generated using Bray-Curtis dissimilarity for the biofilter media

Patescibacteria, indicative of resource-limited environments (Tian et al., 2020), were found in higher relative abundances at the top of biofilters A and B (34.54% and 20.59%, respectively), compared to

only 5.35% in biofilter C (Fig. 6.5a). *Patescibacteria* can adapt to environments with limited resources by maintaining essential functions, such as genetic information processing, while significantly simplifying their metabolism, cellular activities, and stress response to eliminate unnecessary functions (Tian et al., 2020). This pattern could suggest a preference for biofilters A and B conditions, potentially reflecting adaptation to the intermittent operational conditions and limited nutrient availability (Liao et al., 2013, 2014). *Nitrospirae*, whose members are known for their involvement in nitrogen cycling (Zhao et al., 2022), also showed a higher relative abundance in the top sections of all biofilters (12.06%, 5.62%, and 17.60% for A, B, and C, respectively) (Fig. 6.5a).

Bacterial communities on the biofilter media showed a Shannon Index of 4.77 ± 0.03 , 4.92 ± 0.16 , and 4.93 ± 0.25 in biofilters A, B, and C, respectively (Table A6.1). The highest diversity within biofilter A was observed at the bottom (4.81). In contrast, peak diversity was found at the top for biofilters B (5.15) and C (5.15), suggesting spatial variation in microbial community structure. Simpson diversity across the biofilters stood uniformly high at 0.99, emphasizing not just the diversity but the equitable distribution of species, indicating no single species dominates the microbial ecosystem. Beta diversity, analyzed using the Bray-Curtis dissimilarity matrix and visualized through PCoA, accounted for 59.2% of the variation among biofilter media samples, with PCoA1 alone explaining 33.3% of this variance (Fig. 6.5b). This suggests that microbial communities at the biofilter's top share closer relations to each other than those at the top/bottom, regardless of the operational mode of the biofilter. Overall, there was no clear separation between the microbial communities among different biofilters based on the operational mode of biofilters (Fig. 6.5b). Further, PERMANOVA results indicated no significant influence of depth and biofilter operation type on microbial diversity ($p > 0.05$). This suggests that despite differences in the operational mode of biofilters and relative abundance, overall microbial community composition is significantly not different in biofilters running continuously (24 h/d) and intermittently (12 h/d and 6 h/d).

6.4.5 Presence of MnOB and IOB genera in the biofilters

MnOB genera such as *Sphingomonas*, *Hyphomicrobium*, *Caulobacter*, *Pseudomonas*, *Flavobacterium*, *Reyranella*, and *Sphingopyxis*, which are known for Mn oxidation (Breda et al., 2017; Cai et al., 2023; Cheng et al., 2017; Dangeti et al., 2020; McCormick et al., 2022; Piazza et al., 2019; Tebo et al., 2005) were detected across all biofilters. In addition, several genera potentially involved in Fe oxidation notably *Gallionella*, *Crenothrix*, and *Pseudomonas* (Cheng et al., 2019; Hallbeck et al., 1993; McCormick et al., 2022; Mouchet, 1992) were identified (Fig. 6.6). The presence of IOB genera was relatively less, with relative

abundances of 2.34%, 2.86%, and 6.20% at top which decreased to 1.58%, 1.88% and 3.47% at the bottom in biofilters A, B, and C respectively, suggesting that the majority of Fe removal occurred through physicochemical oxidation processes rather than biological Fe oxidation (Vries et al., 2017). The distribution of detected MnOB genera varied across biofilters, with relative abundances of 10.58%, 15.43%, and 31.32% at the top sections of biofilters A, B, and C, respectively, which increased to 15.29% and 42.01%, at the bottom of the biofilters A and B respectively while decreasing to 11.05% in biofilter C (Fig. 6.6).

Among the identified MnOB genera, *Sphingopyxis* emerged as the dominant genus, with relative abundances of $3.70 \pm 0.63\%$, $5.76 \pm 1.86\%$, and $4.10 \pm 1.60\%$ in biofilters A, B, and C, respectively (Fig. 6.6). The presence of *Sphingopyxis* has been reported in several Mn-removing biofilters (Araya-Obando et al., 2021; Breda et al., 2017; Piazza et al., 2019; Zheng et al., 2023). A particularly interesting observation was the elevated relative abundance of *Pseudomonas* in biofilter C in the top layer (6.01%), in stark contrast to the much lower abundances in biofilters A and B (0.20% and 0.94%, respectively). *Pseudomonas* has Mn oxidase genes (*mnxG*), which encodes an MCO enzyme contributing to direct biological Mn oxidation (Geszvain et al., 2016; Wright et al., 2018). Similarly, *Comamonas* showed a higher presence in biofilter C (3.10%) at the top, compared to biofilter A and B (0.16% and 0.10%, respectively). *Comamonas* belonging to the family *Comamonadaceae* were found to be a key MnOB in Mn removing biofilters contributing to biological Mn oxidation (McCormick et al., 2023; Shoiful et al., 2020). For both *Pseudomonas* and *Comamonas*, the relative abundance decreases with depth for biofilter C. In contrast, an increase for biofilters A and B was observed, likely due to differences in the operating conditions.

MnOB not only directly oxidizes Mn but also indirectly contributes to its oxidation by altering local pH and redox conditions and production of EPS (Chaput et al., 2019; Tebo et al., 2005). Genera such as *Pseudomonas*, *Bradyrhizobium*, and *Bacillus*, known for their ability to produce polysaccharides within biofilm EPS (Limoli et al., 2015; Quelas et al., 2006) were present in biofilters. These EPS components can capture Mn(II), which is then oxidized by enzymes (Tebo et al., 2005). Liang et al. (2017) have shown that *Sphingopyxis* can trigger Mn oxidation in other microbes via cell contact (in the presence) by inducing the microbial physiological stress leading to Mn(II) oxidation and extracellular superoxide production. This suggests that MnOB can contribute to Mn oxidation via multiple pathways.

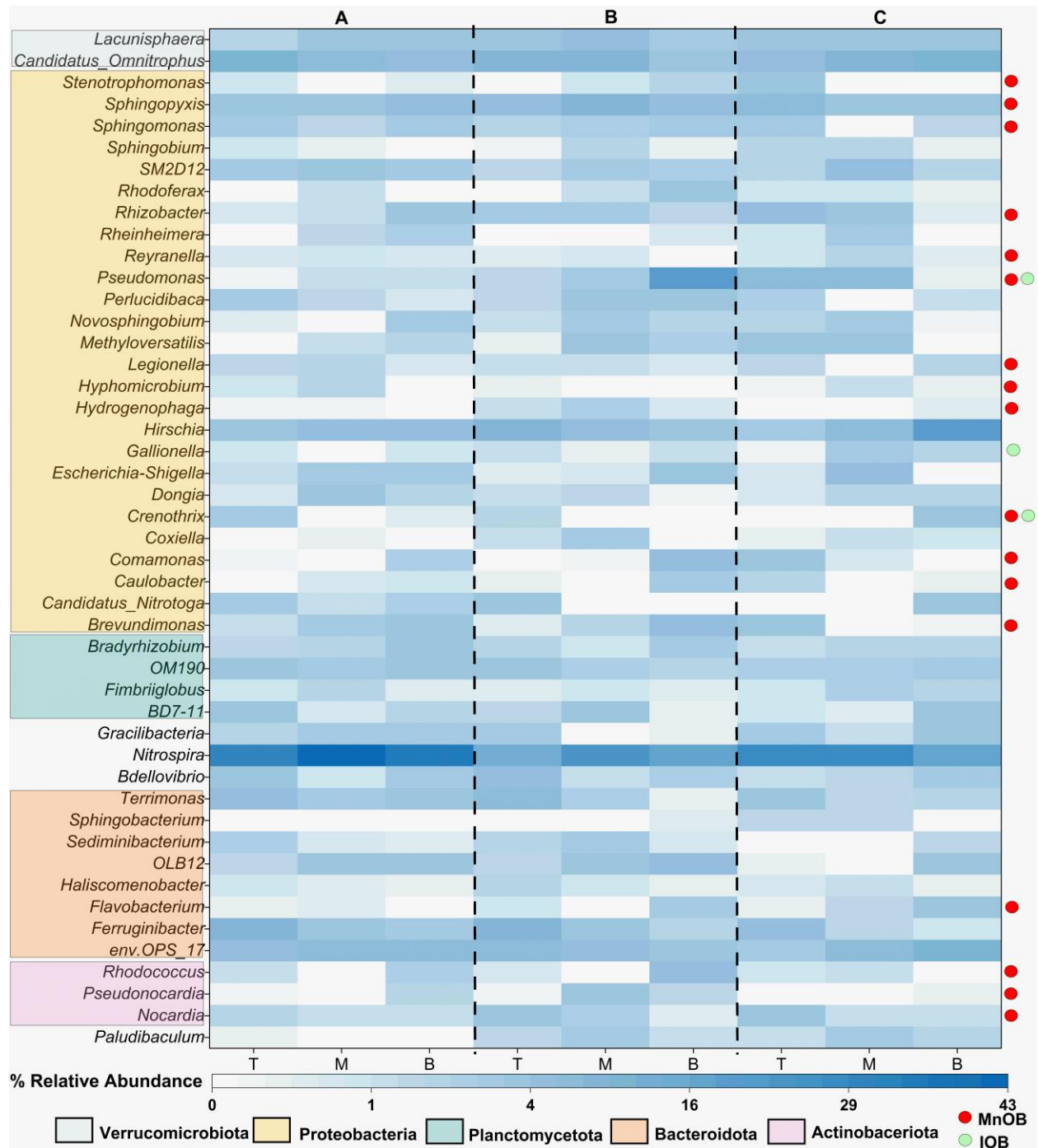


Figure 6.6: Heatmap of relative microbial abundance (> 0.25%) at genus levels (T: Top, M: Middle, B: Bottom), the red and green dot indicates known MnOB and IOB genera, respectively

6.4.6 Comparison of Mn and Fe removal with other contaminants in intermittently operated biofilters

Given the scarcity of data on the long-term intermittent operation of biofilters, particularly regarding Mn and Fe removal, this study's findings are contextualized alongside other research focusing on both short and long-term shutdowns of biofilters. These studies extend to various metals, including arsenic, hexavalent chromium, organics, and Mn and Fe. Our results demonstrate that, upon acclimation, intermittently operated biofilters (12 h/d and 6 h/d) align closely with continuously operated biofilters in removing Mn and Fe to levels below the aesthetic objectives of 20 µg/L. Although the actual microbial processes would be different this observation is similar to that by McKie et al. (2019), who reported similar removal efficiencies for organics and trihalomethanes in cyclically operated biofilters (8-16 h/d), though continuous operation showed enhanced haloacetic acids (HAA) removal. However, cyclically operated filters consistently removed more DOC than continuously operated filters (Nemani et al., 2018). Upadhyaya et al. (2019) further corroborate the biofilters' adaptability to intermittent operation (7 days off followed by 7 days on), observing no significant change in hexavalent chromium effluent levels post-restart.

After a 10-day shutdown, a temporary rise in Mn and Fe concentrations was noted, yet levels normalized within 6 and 2 h, respectively. This pattern aligns with literature indicating that shutdown duration variably affects different contaminant removal efficiencies. For instance, a ~36-h shutdown did not impact nitrate removal, while a 35-day break led to effluent nitrate concentrations nearing influent levels (Upadhyaya et al., 2022). Similar dynamics were observed for arsenic, with recovery within 12 h and a slight increase in hexavalent chromium levels after a 3-day break, which also returned to baseline within 3 h, underscoring the biofilter's resilience (Upadhyaya et al., 2019). The observed increase in effluent concentrations for different contaminants post-restart may stem from several interconnected factors, including the potential leaching of metals triggered by shifts in oxidation-reduction reactions under anaerobic conditions, disruption of the biofilm, changes in the activity of reducing bacteria, or a combination thereof. In the present study, we identified (Fig 6.6) the presence of bacteria capable of reducing Fe, such as *Ferribacterium* (Cummings et al., 1999) and Mn, such as *Bacillus* and *Pseudomonas* (Cerrato et al., 2010; Swain et al., 2018), which could contribute to the observed changes in metal concentrations. The observed increase in concentrations post-restart can be mitigated by backwashing the biofilters before placing them online (Niquette et al., 1998). This approach slightly improved Fe concentration following restart in the present study. However, as the influent Mn and Fe levels were lower in the present study, the effect may be more pronounced in raw

water with higher Mn and Fe concentrations. Additionally, the effect of pre-backwashing the biofilters prior to shutdown should also be investigated.

The post-restart biofilter performance can notably be influenced by the shutdown condition—drained or submerged. Drying of the media can diminish biological activity and decrease substrate availability, whereas submersion may lead to substrate depletion, consequently impacting microbial community dynamics. Although our study maintained submerged conditions without exploring the other options, literature findings highlight potential challenges and conflicting results (Hess et al., 2020; Niquette et al., 1998). Factors such as filter media type, shutdown duration, and water quality differences contribute to these variations. Zeng et al. (2019) noted that submerged conditions adversely affect Mn removal, attributed to diminished DO levels inhibiting MnOB. This suggests careful management of shutdown conditions is crucial for maintaining optimal biofilter performance. Despite the divergent contaminant removal mechanisms, parallels drawn from previous studies and this study offer a comprehensive view of biofilter efficacy under intermittent operation and the consequential effects of shutdown periods on biofilter performance.

6.5 Conclusions

- Intermittently operated biofilters (12 h/d and 6 h/d) achieved performance comparable to continuously operated biofilter (24 h/d) post-acclimation, effectively maintaining Mn and Fe levels below 20 µg/L.
- A temporary increase in Mn and Fe effluent concentrations post a 10-day shutdown was observed, with levels returning to normal within 6 hours and 2 hours of restarting biofilter operations, respectively. This rapid recovery underscores the resilience of the biofilter systems post-shutdown.
- Pre-restart backwashing slightly enhances biofilter efficiency following intermittent operation shutdowns.
- Continuously operated biofilters exhibited higher ATP and EPS levels in comparison to intermittently operated biofilters.
- Shutdowns may affect biofilm stability, as observed by changes in ATP and EPS levels.
- The presence of multiple known MnOB genera was confirmed within the biofilters. Moreover, the overall microbial community composition showed no significant differences between biofilters operated continuously and those operated intermittently.

7 Conclusions and Recommendations

This thesis provides an in-depth analysis of Mn removal from groundwater via biofiltration, exploring a range of media types, operational strategies, and stages of biofilter maturity. The research combined both pilot-scale and bench-scale batch studies to examine the relationships between biofilter media characteristics, microbial dynamics, operational conditions, and Mn removal mechanisms. Pilot-scale biofilters were established at a water treatment plant in Southern Ontario, Canada and operated for approximately 400 days using raw groundwater containing Mn and Fe under various configurations. Concurrently, bench-scale batch experiments were conducted at the University of Waterloo further to elucidate the Mn removal processes within the pilot-scale biofilters.

7.1 Overall conclusions

The research presented in this thesis demonstrates that under tested conditions with low influent concentrations of Mn and Fe, biofilters effectively reduced Mn levels to below the Health Canada aesthetic objective of 20 µg/L, achieving greater than 90% removal efficiency. This high level of Mn removal was consistently maintained at lower water temperatures (<15°C). Although the influent water exhibited relatively low Mn concentrations, biofiltration can effectively remove higher levels of Mn with adjustments to operational conditions, such as increased bed depth or EBCT. Additionally, Mn removal effectiveness in biofilters is influenced by the presence of co-contaminants like Fe. Spatial and temporal patterns in Mn and Fe removal were observed within the biofilters. Initially, Fe was predominantly removed in the top layer, whereas Mn removal was more evenly distributed throughout the biofilter. As biofiltration progressed, the primary zone of Mn removal shifted upward, with over 80% of Mn and Fe being removed in the top 40 cm of the filter bed. This stratification, demonstrating effective Fe and Mn removal predominantly in the upper layers of the biofilter media, highlights key considerations for biofilter design. These findings suggest that adjusting bed depths can be an effective strategy to optimize Fe and Mn removal for different influent water conditions.

The startup period of Mn removal is influenced by filter media characteristics (GAC, anthracite, sand). In GAC biofilters, Mn removal initially occurs through adsorption, facilitated by the high porosity of GAC which provides a large surface area to adsorb Mn(II) from the water. Once the active sites on the GAC are saturated with Mn(II), the efficiency of the adsorption process decreases. However, the adsorbed Mn is oxidized through homogeneous or biological oxidation, leading to the formation of MnO₂, which continues to facilitate Mn removal in biofilters because of its high adsorptive, cation exchange and autocatalytic properties. In contrast, biological processes predominate in sand and anthracite biofilters which do not have any adsorptive properties. These findings were supported by

batch tests using media collected from various bed depths of the pilot biofilters. Sand and anthracite media showed biological dominance for the top layer where the majority of Mn removal takes place, while deeper layers of sand and anthracite biofilters showed greater physicochemical removal. In contrast, GAC media exhibited physicochemical dominance throughout.

The PSO model provided a more accurate depiction of Mn removal kinetics in the batch tests of biofilter media samples compared to the PFO model. This highlights the importance of filter media characteristics and the role of MnO_x in Mn removal. Microbial community analysis revealed a diverse range of MnOB across the biofilters, indicating a relationship between media type and microbial composition. Cultural plating techniques further confirmed the capability of MnOB to oxidize Mn(II) to MnO_x . Over time, as the biofilter matured, the abundance of MnOB decreased on Mn Supp and R2A+Mn culture media, suggesting a decrease in microbial Mn oxidation. Biological activity quantified using ATP, correlated moderately with Mn removal efficiency during start-up, highlighting ATP as a potential indicator for monitoring biofilter acclimation.

Over time, a shift from biological to physicochemical Mn removal mechanisms was observed in a pilot-scale biofilter using sand as the media. This can be explained as follows: 1) The rate of microbial Mn oxidation is faster than physicochemical oxidation, leading to the formation of Bio- MnO_x . 2) Competitive dynamics between MnO_x and MnOB likely led to a reduction in microbial oxidation, favoring physicochemical removal over time. 3) As the biofilter matures, a shift in the nutrient consumption patterns of MnOB may occur. The dynamic environment within the biofilter likely favors MnOB that adapt and utilize a broader spectrum of available nutrients beyond Mn. This adaptation might lead to a wider utilization of nutrients by MnOB, resulting in decreased microbial Mn oxidation despite an overall increase in their relative abundance. 4) Further, autocatalytic oxidation of adsorbed Mn by MnO_x enhances physicochemical removal mechanisms. MnOB genera such as *Sphingomonas*, *Hyphomicrobium*, *Caulobacter*, and others were prevalent from start-up to maturity. Genes associated with direct and indirect Mn oxidation pathways were also predicted, highlighting Mn oxidation's complex, multi-pathway nature involving reactive oxygen species, fatty acid metabolism, and electron transport. Understanding these dynamics is essential for optimizing biofilter designs and operation and to enhance Mn removal, specifically tailoring to the microbial communities most effective in given operational conditions.

This research further validated the effectiveness of intermittently operated biofilters for treating groundwater in small-scale and remote communities, and in utilities where operations are not

continuous. The performance of these intermittently operated biofilters (12 h/d and 6 h/d), following an acclimation period, was found to be on par with that of continuously operated biofilters, consistently maintaining Mn and Fe concentrations below 20 µg/L. Pre-restart backwashing slightly improved Fe removal performance, although it did not affect Mn removal. These biofilters were also able to recover quickly from a 10-day shutdown, although shutdown may impact biofilm stability.

7.2 Summary of findings

Chapter 3

In **Chapter 3**, the impact of filter media characteristics such as pore size, surface area, and media size on the performance of biofilters removing Mn during startup at low water temperatures in the presence of Fe was investigated. The pilot-scale setup, which ran for 133 days, utilized three different types of GACs, two types of anthracite, and one type of sand.

The biofilters successfully achieved simultaneous removal of Fe and Mn under low-temperature conditions. The filter media characteristics notably influenced the start-up period of the biofilter. With their higher surface area and pore volume, GAC media exhibited significant Mn adsorption, leading to an early onset of Mn removal. For the two-anthracite media, the smaller effective media size resulted in a shorter start-up time owing to its higher external surface area. This study also underscored the importance of the macropore volume and BET surface area of the media in enhancing the start-up efficiency of biofilters for Mn removal. Macropores were particularly important, likely due to their suitability for microbial colonization and MnO_x accumulation. However, the contribution of micropores to overall adsorption efficiency appeared limited, likely due to the blocking of micropores by MnO_x accumulation on the biofilter media, suggesting the need for further investigation into the dynamics of Mn adsorption in biofilters.

The physical characteristics of MnO_x accumulated on the biofilter media were evaluated using SEM-EDX and Raman spectroscopy. Birnessite was identified as one of the MnO_x in the biofilters, and variations in the morphology of MnO_x were observed. The presence of heterotrophic bacteria and MnOB was confirmed using HPC and LBB analysis. Over time, the HPC decreased, and MnOB was detected in all biofilters. Biofilm activity on the biofilter media, quantified using ATP analysis, ranged between 100 and 140 ng/cm³ dry weight of media. GAC media exhibited ATP levels comparable to anthracite and sand media ($p > 0.05$). A moderate correlation between ATP levels and Mn removal

efficiency during the start-up of the biofilter was observed, suggesting that ATP may serve as a potential indicator for monitoring biofilter acclimation.

Chapter 4

In **Chapter 4**, the impact of filter media characteristics on the mechanisms of Mn removal and microbial communities in the biofilters was further investigated through a combination of pilot-scale biofilters and bench-scale batch studies. The kinetics of Mn removal in the batch tests using media from matured pilot-scale biofilters at day 126 were studied using non-linear PFO and PSO kinetic models. The contributions of biological and physicochemical processes to Mn removal were quantified in batch tests across different biofilter bed depths. NaN_3 was used to inhibit biological activity to ascertain the extent of physicochemical removal. Biological contributions were determined by subtracting physicochemical removals from total Mn removals. Additionally, potential functional genes associated with Mn oxidation pathways were predicted.

The PSO model provided a more accurate depiction of Mn removal kinetics in the batch tests of biofilter media samples compared to the PFO model. This highlights the importance of filter media characteristics and the role of MnO_x in Mn removal. An examination of biological versus physicochemical Mn removal mechanisms indicated that while sand and anthracite media predominantly engaged biological processes at the top layer, GAC media primarily facilitated physicochemical removal. The observed differences in B/P ratios across sand, anthracite, and GAC media highlight the unique removal mechanisms, which are likely influenced by the characteristics of the filter media. A significant inverse correlation ($p < 0.05$) between B/P ratios and media properties such as BET surface area, and the volume of micro, macro, and mesopores was identified at the top of the biofilter media. This suggests that media with larger pore volumes and greater surface areas tend to support physicochemical removal mechanisms more. In contrast, at a bed depth of 100 cm, a positive correlation ($p < 0.05$) was found between B/P ratios and media properties, indicating that these media characteristics are conducive to biological processes at this depth.

The presence of MnOB genera varied across biofilter media types and depths, highlighting the complex interplay between biofilter media characteristics and microbial colonization patterns. Predictive functional analysis detected genes linked to two types of Mn oxidation pathways: directly through *cueO* and *cotA* genes and indirectly via *katE*, *poxB*, and *cmmE* genes, among others. This analysis underscores the complexity of Mn oxidation, which involves multiple biological pathways.

Chapter 5

In **Chapter 5**, the mechanism of Mn removal in a sand biofilter and its evolution from startup to maturity was studied using a combination of a pilot-scale biofilter and bench-scale batch studies. The pilot-scale biofilter operated for 240 days, and bench-scale batch studies were conducted using biofilter media from the top of the pilot biofilter collected on days 70, 150, and 240 to assess shifts in the contributions of different removal mechanisms as the biofilter matured. Changes in MnO_x morphology were observed using SEM-EDX, and Mn accumulation on the biofilter media was quantified. Additionally, changes in the microbial community composition were monitored over time.

Mn accumulation in the biofilter media was marked by an initial rapid increase due to biological Mn oxidation, leading to the formation of Bio- MnO_x , which is known for its high adsorption capacity. This phase was succeeded by a slower, continuous increase in MnO_x , indicative of a transition towards a physicochemical morphology of MnO_x . As the biofilter media aged, there was a noticeable decline in the B/P ratio, starting at a high of 0.95 on day 70, which suggests that biological processes were nearly as influential as physicochemical processes in Mn removal at this stage. Over time, as the filter matured, the dominance shifted towards physicochemical processes, with B/P ratios dropping to 0.24 and 0.14 by days 150 and 240, respectively. This shift may be due to the competitive dynamics between MnO_x and MnOB, where the strong adsorptive and autocatalytic properties of MnO_x could outcompete MnOB, resulting in reduced biological Mn oxidation and Bio- MnO_x formation.

Several MnOB genera known for Mn oxidation, such as *Sphingomonas*, *Hyphomicrobium*, *Caulobacter*, *Pseudomonas*, *Flavobacterium*, *Reyranella*, *Nocardia*, *Sphingobium*, *Rhizobacter*, and *Sphingopyxis* were identified in the biofilter. As the biofilter aged, there was a decrease in the relative abundance of bacteria like *Pseudomonas*, *Leptothrix*, and *Bacillus*, which are recognized for producing Bio- MnO_x . Despite these shifts in the relative abundance of these MnOBs, the overall composition of the microbial community remained largely unchanged as the biofilter matured ($p > 0.05$).

Chapter 6

Chapter 6 evaluated the effectiveness of intermittently operated biofilters for removing Mn and Fe. Three pilot-scale biofilters were operated over 240 days under different operating conditions: 24 h/d, 12 h/d, and 6 h/d, and different methodologies for restarting biofilters, such as backwashing and without backwashing before restart, were assessed for their impact on the performance of Mn and Fe removal. The effect of intermittent operation on biological activity (ATP), biofilm development (EPS),

and microbial community composition was also explored. Furthermore, the impact of a long-term shutdown (10 days) on biofilter performance was also examined.

The biofilters operated intermittently achieved performance levels comparable to those operated continuously after the acclimation period, successfully keeping Mn and Fe concentrations below 20 µg/L. Following the acclimation phase, there were no significant differences in Mn and Fe removal efficiencies among the biofilters ($p > 0.05$), indicating that biofilters can be effectively used for intermittent operation. It was also observed that pre-restart backwashing slightly improved the efficiency of biofilters after periods of intermittent operation. However, a temporary spike in Mn and Fe concentrations was noted following a 10-day shutdown, but these levels normalized within 6 hours and 2 hours of resuming operations, respectively.

Biofilters operating continuously exhibited higher ATP levels relative to those operating intermittently. Following a 10-d shutdown, a decline in ATP concentrations was observed across continuously and intermittently operated biofilters, with levels returning to their pre-shutdown values within two weeks of resuming operations. As was the case with ATP, continuous biofilter operation was linked to higher EPS protein and polysaccharide concentrations compared to intermittent operation, and a 10-d shutdown led to a decline in both EPS proteins and polysaccharides across all biofilters, with levels not returning to pre-shutdown concentrations even after three weeks once operations were resumed. MnOB genera such as *Sphingomonas*, *Hyphomicrobium*, *Caulobacter*, *Pseudomonas*, *Flavobacterium*, *Reyranella*, and *Sphingopyxis*, which are known for Mn oxidation, and IOB genera linked to Fe oxidation such as *Gallionella*, *Crenothrix*, and *Pseudomonas* were detected across all biofilters. Despite differences in the operational mode of the biofilter, the overall microbial community composition was not significantly different between continuously (24 h/d) and intermittently (12 h/d and 6 h/d) operated biofilters ($p > 0.05$).

7.3 Key contributions and implications for the drinking water industry

- This study conducted a comparative analysis of non- adsorptive (sand and anthracite) and adsorptive media (GAC) on the performance of biofilters for removing Mn and Fe during the start-up and examined the impact of filter media properties (BET surface area, pore size distribution, and media size) on Mn removal. The findings of this research will assist in optimizing the selection of biofilter media.

- The characteristics of the filter media also influenced the Mn removal mechanisms in biofilters. During the startup phase, adsorption was primarily responsible for Mn removal in GAC biofilters, whereas biological processes dominated anthracite and sand biofilters. This distinction underscores the suitability of GAC biofilters at low temperatures. The early onset of Mn removal in GAC biofilters may mitigate the adverse effects of reduced temperatures on biological Mn removal mechanisms.
- This study provides a comprehensive understanding of how the Mn removal process evolves in a biofilter by documenting changes in MnO_x morphology, microbial community composition, and the contributions of biological and physicochemical processes to Mn removal.
- This study predicted potential functional genes associated with Mn oxidation pathways in pilot-scale biofilters based on 16S rRNA gene sequences. To the best of the author's knowledge, no previous study has predicted such genes in pilot-scale biofilters that remove Mn employing different biofilter media. The prediction of genes associated with direct and indirect Mn oxidation pathways highlights the complexity of biological Mn oxidation in biofilters.
- This research also includes a comparative analysis of biofilters operated continuously and intermittently over 240 days, from startup to maturity. The results successfully demonstrated the viability of intermittently operated biofilters for removing Mn and Fe. The performance of these biofilters in treating groundwater containing Mn and Fe was not impacted by intermittent operations, making them particularly suitable for small-scale and remote communities or utilities where operations at drinking water treatment plants are not continuous. Additionally, the findings indicate that biofilters can endure long-term shutdowns (up to 10 days) and rapidly recover their Mn and Fe removal performance within hours, underlining their robustness in varying operational conditions.

7.4 Recommendations

- This study focused on the total Mn and Fe removal primarily for two reasons: the use of groundwater where Mn and Fe predominantly exist in dissolved form, and Health Canada's more stringent guidelines for total Mn and Fe concentrations in drinking water. However, it is recommended that future research should include the speciation of both Mn and Fe into dissolved, particulate, and colloidal forms. This would help optimize biofiltration processes

and enhance understanding of the physicochemical processes involved in biofiltration for Mn and Fe removal.

- The influent Mn and Fe concentrations were relatively low; further studies involving higher Mn and Fe concentrations are recommended to thoroughly test the limits of the biofiltration process for Mn removal.
- The role of filter media characteristics, such as surface roughness, chemistry, and granular morphology, in the formation of MnO_x coatings and Mn accumulation on the biofilter media should be studied in detail. Additionally, the use of analytical techniques such as X-ray Diffraction (XRD), X-ray Photoelectron Spectroscopy (XPS), and Electron Paramagnetic Resonance (EPR) should be employed to determine the crystallinity of oxides, surface chemistry, and the origins of birnessite formation, respectively.
- This study utilized coal-based GAC, future studies should explore different types of GAC, such as those derived from wood, coconut shell, and other biomass sources, to evaluate their efficacy and characteristics in biofiltration. Comparative analysis across various GAC types may reveal differences in adsorption capacities, surface functionalities, and overall performance in removing Mn from groundwater.
- Continuous flow column studies, rather than batch studies, are recommended to elucidate biological and physicochemical contributions in Mn removal within biofilters with continuous addition of inhibitors such as NaN_3 . However, NaN_3 does not completely inhibit microbial activity.
- Although numerous studies have demonstrated the ability of the PICRUSt2 method to reveal functional profiles of microbial communities accurately and effectively, it has limitations. Therefore, employing more comprehensive metagenomics, proteomics, and other approaches to study microorganisms is necessary.
- Initial findings suggest pre-restart backwashing marginally enhances biofilter efficiency for Fe removal following an intermittent operation. However, the full impact of pre-restart backwashing requires further investigation, particularly with feedwater containing higher concentrations of Mn and Fe, where the effects may be more pronounced. Further, the impact of backwashing on a long-term shutdown should be evaluated. It is also important to analyze the effect of pre-shutdown backwashing on immediate post-restart performance.

- During periods of shutdown or intermittent operation, the optimal storage condition for biofilters, whether drained or submerged, remains unclear due to conflicting reports in the literature. Consequently, further research is required to establish the most effective biofilter status during these non-operational periods.
- Following a 10-day shutdown period, an increase in the concentrations of Mn and Fe was noted upon restarting the biofilters. The factors contributing to the release of Mn and Fe post-restart require further investigation to understand their dynamics fully. Specifically, the role of accumulated Mn within the biofilters in influencing the likelihood of Mn(II) release requires additional research.

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Appendices

Appendix A1

Table A1.1: Summary of Mn removal options from drinking water (Adopted from Tobiasson et al., 2016; Brandhuber et al., 2013 & Health Canada, 2019)

Technique	Description	Advantages	Disadvantages
Chemical Oxidation/ followed by Physical Separation e.g., Filtration	Permanganate: $3\text{Mn}^{2+} + \text{KMnO}_4 + 4\text{H}_2\text{O} \rightarrow 4\text{MnO}_2 + 4\text{H}^+ + \text{K}^+$ Requires 1.92 mg KMnO_4 /mg Mn Second-order reaction (k_2) = $1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ (pH 7)	Possible to achieve more than 80% removal, achieving an average Mn concentration of less than 0.045 mg/L and less under appropriate conditions.	Increased dosage in the presence of NOM. Ineffective at a lower temperature (5°C) and pH (5.5)
	Chlorine Dioxide: $5\text{Mn}^{2+} + 2\text{ClO}_2 + 6\text{H}_2\text{O} \rightarrow 5\text{MnO}_2 + 12\text{H}^+ + 2\text{Cl}^-$ Required 2.45 mg ClO_2 /mg Mn Second-order reaction (k_2) = $1 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ (pH 7)	Works over a wide range of pH values with low DOC levels ($< 2.5 \text{ mg/L}$).	Formation of by-products chlorite and chlorate. Higher demand in the presence of co-occurring contaminants
	Ozone: $\text{O}_3 + \text{Mn}^{2+} + \text{H}_2\text{O} \rightarrow \text{O}_2 + \text{MnO}_2 + 2\text{H}^+$ Required 0.87-mg O_3 /mg Mn (II) Second-order reaction (k_2) = $2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ (pH 7)	Similar to ClO_2 , ozone can work over a wide range of pH values.	Higher actual dosage depending on alkalinity and DOC levels. Challenging to achieve $<0.02 \text{ mg/L}$ of Mn in the effluent.
Adsorption Combined with Catalytic Oxidation	Manganese greensand: Granular filter media with a coating of MnO_x that provides higher adsorption capacity	Mn removal in the range of 86-100 % (Average concentration $< 0.02 \text{ mg/L}$). Smaller effective size than silica, hence good at capturing smaller particles.	The use of oxidants to regenerate the adsorptive capacity of the media at regular intervals or continuous dosing of oxidants is required.
	MnOx coating on filter media (using chlorine): The presence of Mn(II) and free chlorine can develop a $\text{MnO}_x(\text{s})$ on the filter media, which can adsorb	The process takes place under appropriate conditions, such as the presence of a free chlorine	Formation of DBPs. Release of accumulated Mn from the MnO_x

	Mn(II) and serve as a catalyst promoting the oxidation of adsorbed Mn(II) to MnO _x	concentration of 0.5–1.0 mg/L in the effluent (to ensure regeneration of MnO _x (s) coating)	coating. Ineffective at lower pH levels (<6)
Softening	Raising the pH of the water (> 9.5) by using lime or soda ash can achieve manganese removal by increasing the rate of Mn (II) oxidation.	Suitable for situations where softening is already taking place or required.	Raising the pH only for Mn removal is associated with high costs.
Ion Exchange	Cation exchange processes like zeolite softening can also remove Mn (II).	Concurrent removal of other contaminants such as Fe and calcium.	Decrease in the overall cation exchange capacity of the resin over time. Also, there is the problem of regenerant disposal.
Sequestration	Sequestering agents such as polyphosphates can bind dissolved manganese, which is unavailable for oxidation or precipitation.	It can only be used to prevent water discoloration and should only be used by water utilities with Mn levels below the MAC in the source water.	Temporary measure: sequestrant's effect is time limited. It requires continuous dosing and cannot prevent the adverse health effects associated with Mn.
Membranes	Membranes such as microfiltration and ultrafiltration (MF/UF) can remove particulate Mn from direct oxidation with KMnO ₄ , ClO ₂ , etc. Destabilization of particles may be required if the size of the Mn particles is larger than the size exclusion of the membrane pores.	Suitable for groundwater that has only dissolved Mn (no other contaminants)	Fouling concerns due to the deposition of Mn on the membrane, a higher cost than other methods if used explicitly for Mn removal.

Appendix A2

Table A2.1: MnOB identified in drinking water biofilters

Scale	Filter material	Species/Genera identified	Comment	Reference
Bench	Quartz sand + Inoculum: Mature filter media from the top 10 cm of a GWTP	<i>Leptothrix</i> , <i>Pseudomonas</i> , <i>Hyphomicrobium</i> , <i>Arthrobacter</i>	Samples were collected from different depths after 180 days of operation.	Li et al., 2013
Bench	Different sets of filter media: Quartz sand, virgin sand, polystyrene, calcium carbonate, and MnO _x	<i>Pedomicrobium</i> *, <i>Hyphomicrobium</i> , <i>Ralstonia</i> *, <i>Pseudomonas</i> *, <i>Hydrogenophaga</i> , and <i>Leptothrix</i>	Samples were collected from the filter bed after 75 days of operation	Breda et al., 2017
Bench	Silica sand + Inoculum: Nitrifying sludge and backwashing sludge	<i>Crenothrix</i>	Samples were collected from the filter bed at a steady-state	Cai et al., 2015
Bench	Sand + Inoculum: MnOB-181 (a <i>Pseudomonas sagittaria</i> strain)	<u>Winter conditions</u> (8-18 ⁰ C) <i>Pseudomonas</i> , <i>Planctomyces</i> , <i>Sphingopyxis</i> , <i>Hydrogenophaga</i> , <i>Sphingomonas</i> , <i>Microbacterium</i> , and <i>Halomonas</i> <u>Summer condition</u> (19-30 ⁰ C) <i>Pseudomonas</i> , <i>Sphingopyxis</i> and <i>Sphingomonas</i>	Samples were collected from the filter bed at a steady-state	Ciancio Casalini et al., 2020
Pilot	Manganese sand	<i>Crenothrix</i> , <i>Leptothrix</i> , and <i>Hyphomicrobium</i>	Samples were collected from the filter bed at a steady-state	Cai et al., 2016
Pilot	Anthracite and sand + Inoculum: Backwashed solids	<i>Azospirillum</i> sp., <i>Solimonas soli</i> , <i>Paenibacillus</i> sp. , and <i>Paenibacillus</i> sp.	Samples were collected from water, backwash sludge at day	Dangeti et al., 2020

			110, and filter media after 183 days.	
Pilot	Quartz Sand	<i>Crenothrix</i> , <i>Bacillus</i> , <i>Gallionella</i> , <i>Arthrobacter</i> , <i>Pseudomonas</i> , and <i>Hyphomicrobium</i>	Samples were collected from different depths at 31, 93, and 158 days	Cheng et al., 2019
Pilot	Sand	<i>Bacillus</i> , <i>Arthrobacter</i> , <i>Crenothrix</i> , <i>Pseudomonas</i> , <i>Gallionella</i> , and <i>Hyphomicrobium</i>	Samples were collected from different depths at 25, 69, and 97 days.	Cheng et al., 2017
Full	Sand	<i>Leptothrix</i>	Samples were collected from the filter bed at steady-state	Burger et al., 2008a
Full	Sand and anthracite	<i>Pseudomonas</i> spp, <i>P. putida</i> , and <i>Leptothrix</i> sp.	Samples were collected from backwash water during the ripening process.	Bruins et al., 2017
Full	Sand	<i>Pseudomonas</i> , <i>Crenothrix</i> , <i>Hydrogenophaga</i> , <i>Leptothrix</i> , <i>Erythrobacter</i> , <i>Hyphomicrobium</i> , <i>Variovorax</i> , <i>Rhodobacter</i> , <i>Sphingomonas</i> , <i>Flavobacterium</i> and <i>Acidovorax</i>	Samples were collected from the supernatant and the filter bed at a steady-state	Piazza et al., 2019
Full	Quartz sand with and without Mn Oxide coating	<i>Leptothrix</i>	Samples were collected from different depths after 120 days	Breda et al., 2019
Pilot	Two different media: Magnetite sand and Mn sand	<u>Magnetite sand filter:</u> <i>Acidiferrobacter thiooxydans</i> and <i>Sulfurifustis variabilis</i> <u>Manganese sand:</u> <i>Hyphomicrobium zavarzinii</i> and <i>Hyphomicrobium nitrativorans</i>	Samples were collected from different depths after 40, 80, and 120 days	Zhao et al., 2021

Table A2.2: Strategies to accelerate the start-up period of biofilters

Scale	Inoculum/Modification	Filter Media	Impact: Reduction in start-up time /increased removal efficiency	Reference
Bench	Ferromanganese oxidizing bacteria	Sand	No significant difference was observed in the start-up of a Biofilter	Zeng et al., 2019
Bench	Nitrifying sludge and backwashing sludge containing MnOB	Sand	Separate inoculation (30 days) Simultaneous inoculation (38 days)	Cai et al., 2015
Pilot	Matured filter sand was inoculated at the top and middle of the filter bed with a thickness of 10 cm each.	Virgin Sand	Non-inoculated filter: 41 days Inoculated filter: 25 days	Breda et al.2019
Full Scale	Biomass obtained by backwashing the biofilters of a well-established plant	Quartz Sand	Two weeks	Štembal et al., 2005
Full scale	Additional layer of manganese oxide-coated sand (MOCS) or manganese oxide-coated anthracite (MOCA)	Sand and Anthracite	Virgin sand: 55 days Virgin anthracite/sand: 16 days MOCA: Complete elimination of the ripening time MOCS: No difference	Bruins et al., 2015a
Bench	Use of MOCS as a filter media	MOCS	High removal efficiency after 2 h start-up	Jia et al., 2014
Pilot	Iron – manganese co-oxide film	Virgin Quartz Sand	Reduction in start-up period from 26 to 17 days	Guo et al., 2019

Bench	<i>Pseudomonas sagittaria</i> strain	Sand	Summer condition: 95% removal efficiency reached within 23 days as compared to the control filter(without strain) 29 days Winter Conditions: 95% removal efficiency reached within 39 days as compared to control filter 549 days	Ciancio Casalini et al., 2020
Pilot	Use of oxidants: Addition of potassium permanganate & sodium hypochlorite in influent to the filters	Virgin sand	Reduction of start-up time to 36 days with potassium permanganate as compared to 90 days for sodium hypochlorite	Yang et al., 2021
Pilot	Iron-manganese co-oxide film (using different Fe/Mn molar ratios)		90 % Mn removal in 15 days, higher Fe/Mn > 2.2 was not conducive to improving catalytic activity.	Cheng et al, 2022
Pilot	Addition of (i)NaOH (ii) both H ₂ O ₂ and NaOH	Anthracite and GAC	GAC: With the addition of NaOH, both H ₂ O ₂ and NaOH acclimation time (Mn <20 µg/L) reduced to 59 and 63 days, respectively, while the ambient filter took 168 days Anthracite: With the addition of NaOH, both H ₂ O ₂ and NaOH acclimation time was 226 and 251 days, respectively	McCormick et al., 2021

Appendix A3

Text

A3.1: Description of pilot scale biofilters and their operation

A3.1.1 Pilot plant location

The pilot plant was set up at one of the treatment plants of a drinking water utility in Southern Ontario (Fig. S1). The source water used in the experiment was from groundwater wells, which were collected in two 1000 L tanks and aerated before being pumped into the top of the biofilters.



Fig S1: Pilot-scale biofilter

A3.1.2 Biofilter design and operation

The column filters used for the study were made from transparent PVC (PVC SCH 80, Fabco plastics) to visually determine the percent of bed expansion during the backwash. The pilot-scale setup consisted of six PVC columns, each with an inner diameter of ~ 0.1 m and a total height of 3 m (Fig. 3.1). The columns were set up using two support structures, each with three columns. The columns' bottom and middle parts rested on the support structure. For additional support, columns were hinged to the wall on the upper part of the columns. The biofilters were covered with aluminum foil to avoid algae growth in the biofilter due to light. Columns were packed with a depth of 1.2 m of media and 20 cm of gravel to support the bed. The columns were gravity-fed and fitted with an

overflow weir to ensure a constant head of 1.5 m above the media. The columns were equipped with seven sampling ports at 20 cm along the length of the filter bed (0, 20, 40, 60, 80, 100, and 120 cm) to collect the media and water samples. The underdrain of the columns was constructed using a 1-inch PVC pipe. The bottom part of the filter includes a perforated PVC plate to uniformly distribute the flow over the biofilter cross-section during the backwash procedures. The top and bottom of the perforated PVC plate were connected to flanges with a screen at the top to avoid passing biofilter media into underdrains (Fig. S2).

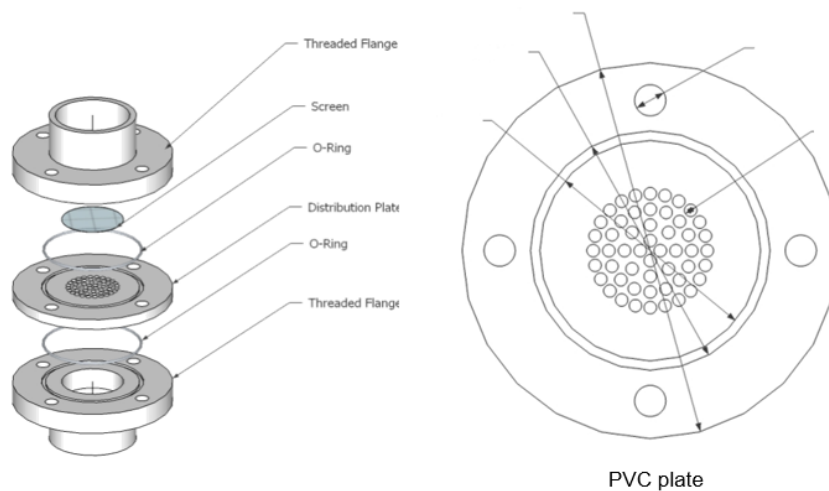


Fig S2: Design of the bottom part of the biofilter

The effluent flow rate in each column was maintained at 5 m/h, resulting in an empty bed contact time of 14.4 min or less. Flow meters were connected to the effluent line of the biofilters, and the flow rate was continuously recorded on a computer. The flow rate was adjusted through an outlet valve if required. The effluent of the biofilters was automated via solenoid valves that controlled start/shutdown times and logged data. The effluent from the biofilters was stored in a tank that was used for backwashing.

A3.1.3 Biofilter media

Three different types of commercially sourced GAC (GAC-1, GAC-2 & GAC-3), two types of anthracite (A-1, A-2), and one type of sand (S-1) were used (Table 3.2). Before the experiments commenced, all the filter media were sieved, quartered, and washed with tap water to remove fines. Sieving was performed to ensure the effective media size was 0.85-1.00 mm, except for A1 (1.10-1.25 mm). The suppliers of the biofilter media are as follows:

- GAC1: F300, Calgon Carbon Corp., Pittsburgh, PA
- GAC2, GAC3 and A1: Greenbird Canada Inc.
- S1, A2: Continental Carbon Group, Canada

A3.1.4 Backwashing procedure

Backwashing was done once per week with a flow rate of 10-20 m/h using the effluent from the biofilters, resulting in bed expansion of 20-30%, and was performed for 5-10 minutes until clear water was observed in the supernatant and backwash effluent.

A3.2: Description of filter media characterization techniques

Filter media characterization techniques were performed by National Research Institute, Poland, for MIP, and BET analysis.

Nitrogen subcritical gas adsorption (SGA) isotherms at 77 K were obtained using a Tristar II 3020 apparatus (Micromeritics). 1 g of media was heated to 105°C using a SmartPrep apparatus operating in a pre-programmed 15 h cycle with a ramp of 10°C/min. Later, the sample was cooled to ambient temperature by a dry gas-blowing (Nitrogen) process. After drying, the samples were placed inside the measuring tube, flushed with helium, and evacuated. Full adsorption and desorption cycles were performed over the entire partial pressure range. Specific surface area (SSA) was determined using a modified BET analysis procedure proposed by Rouquerol et al. (2007).

Micropores volume was estimated using the t-plot technique using the Harkins–Jura thickness equation. The t-plot method involves plotting the volume of gas adsorbed against the statistical thickness of the adsorbed layer (t). The statistical thickness is calculated based on standard isotherms for non-porous reference materials. Obtain adsorption isotherm data using nitrogen or another inert gas at liquid nitrogen temperature (77 K). The data should include the volume of gas adsorbed at a series of relative pressures. The statistical thickness of the adsorbed nitrogen layer at different relative pressures we used the Boer, Harkins & Jura equation

$$t = \frac{0.354 (V_L P_o)}{V(P_o - P)}$$

X-axis: Plot the statistical thickness of the adsorbate layer (t).

Y-axis: Plot the corresponding volume of gas adsorbed.

This plot should ideally show a linear relationship at lower thicknesses (indicative of micropore filling) and then deviate as multilayer adsorption begins on external surfaces. The intercept of the linear regression line with the Y-axis at $t=0$ gives an estimate of the total micropore volume. This intercept represents the volume adsorbed exclusively by micropores.

The mesopore volumes were derived from the desorption branches of isotherms using the Barrett–Joyner–Halenda (BJH) model based on the adsorption data (Lu et al., 2020).

The MIP measurements were performed using an AutoPore IV 9500 Series Mercury Porosimeter (Micromeritics) with pressure steps from 0 to 60,000 psi, covering the pore throat diameter range from approximately 300 μm to 0.004 μm (4 nm) to determine the macropore volume (Dymaczewski et al., 2020). The conformance and compaction corrections were applied to all MIP curves using the approach of Brown (2015). The corrected pressure data were converted to the pore throat size distribution (PTD) using the Washburn equation (Washburn, 1921).

The total pore volume was calculated as the sum of micropore, mesopore, and macropore volume.

A3.3: Description of microbiological analysis

A3.3.1 Media biofilm ATP

2-3 g of the filter media were collected from the top of the biofilter using a sterilized spatula in a sterile container. The collected media was gently rinsed with effluent 2-3 times to remove loose debris. Samples were stored at 4° C, and analysis was performed within 2 h of collecting the samples.

A deposit and surface analysis kit (DSA™, LuminUltra Technologies, Canada) was used for ATP measurements. According to the manufacturer's instructions, 1 g of filter medium was transferred to 5 mL of Ultralize™7 and homogenized. After 5 min incubation at room temperature, 1 mL of the solution was diluted in a 9 mL UltraLute™ (dilution) tube. Finally, using 100 μL of the sample and 100 μL of Luminance™ enzyme, the reading was taken on a luminometer (PhotonMaster™) as relative light units (RLUs). The RLU value was converted to ATP values, which were reported as the ng/cm^3 dry weight of the media. The ATP analysis was conducted in duplicate and represented as average with standard deviation.

A3.3.2 HPC & MnOB

~1.5 g of filter media from the top of the filter bed and at a depth /of 40 cm and suspended in 10 mL of 0.85% NaCl solution. The samples were placed in a stomacher for around 4 min to allow for

sufficient biofilm detachment. The resulting supernatant appeared black, suggesting that biofilm detachment had succeeded, at least to some degree. A small aliquot (1 mL) of the detached biofilm supernatant was used in conjunction with 0.85% NaCl solution to prepare dilution tubes to allow for the plating of the detached biofilm (Burger et al., 2008b).

Dilutions of logs 1–4 were prepared and plated on three culture media using Standard Methods, 9215. The first media was R2A agar + 17 mg/L MnSO_4 (R2A+Mn), the second media was 17 mg/L MnSO_4 + 15g/L agar (Mn min), and the third was supplemented media using 1 g/L beef extract, 75 mg/L yeast extract, 150 mg/L ferrous ammonium sulfate, 150 mg/L sodium citrate, and 12 g/L agar with 17 mg/L MnSO_4 (Mn Supp). The spread plating technique was used to plate 0.1 mL of the sample. Plates were left in the dark at room temperature for approximately four weeks. Plate counts were performed at the end of the incubation time (APHA, 2017).

For MnOB counts, the LBB method was employed. After an incubation period of four weeks, two drops of the LBB reagent (0.04% w/v dissolved in 45 mM acetic acid) were added to the colonies (Araya-Obando et al., 2021). This qualitative method is based on the LBB oxidation reaction with Mn^{3+} or Mn^{4+} producing a blue color. The blue-color colony counts correspond to MnOB.

Figures

A3.1

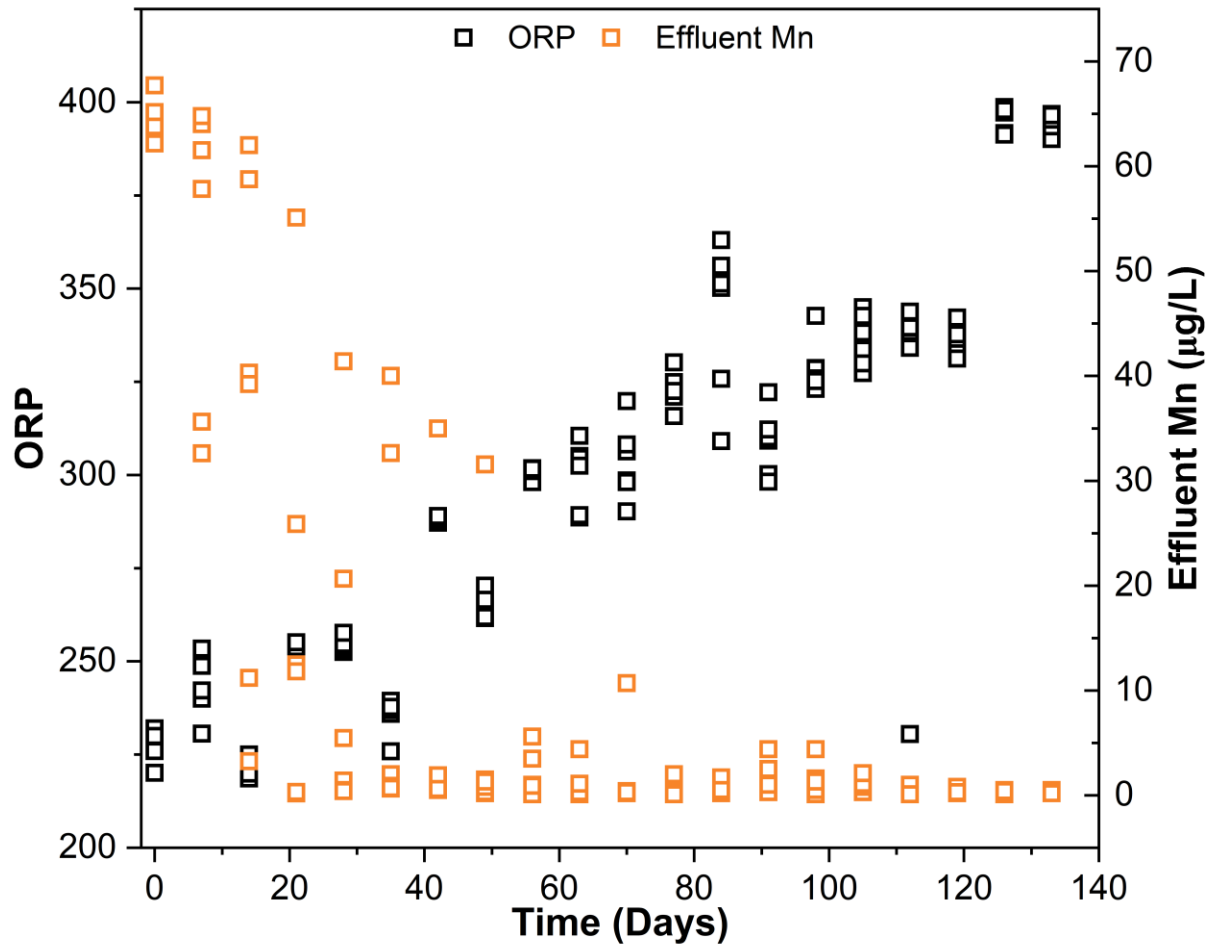


Figure A3.1: Scatter plot of effluent ORP and effluent Mn in biofilters

A3.2

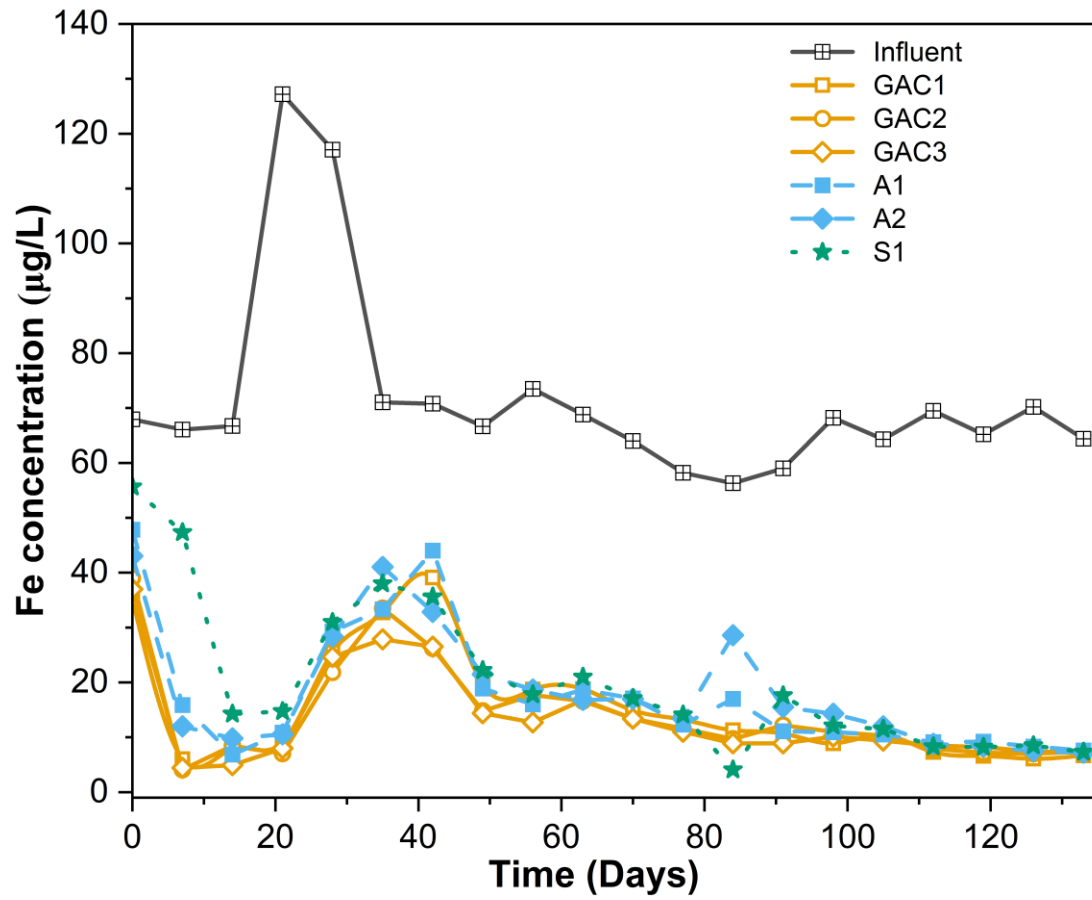


Figure A3.2: Biofilter influent and effluent Fe concentrations

A3.3

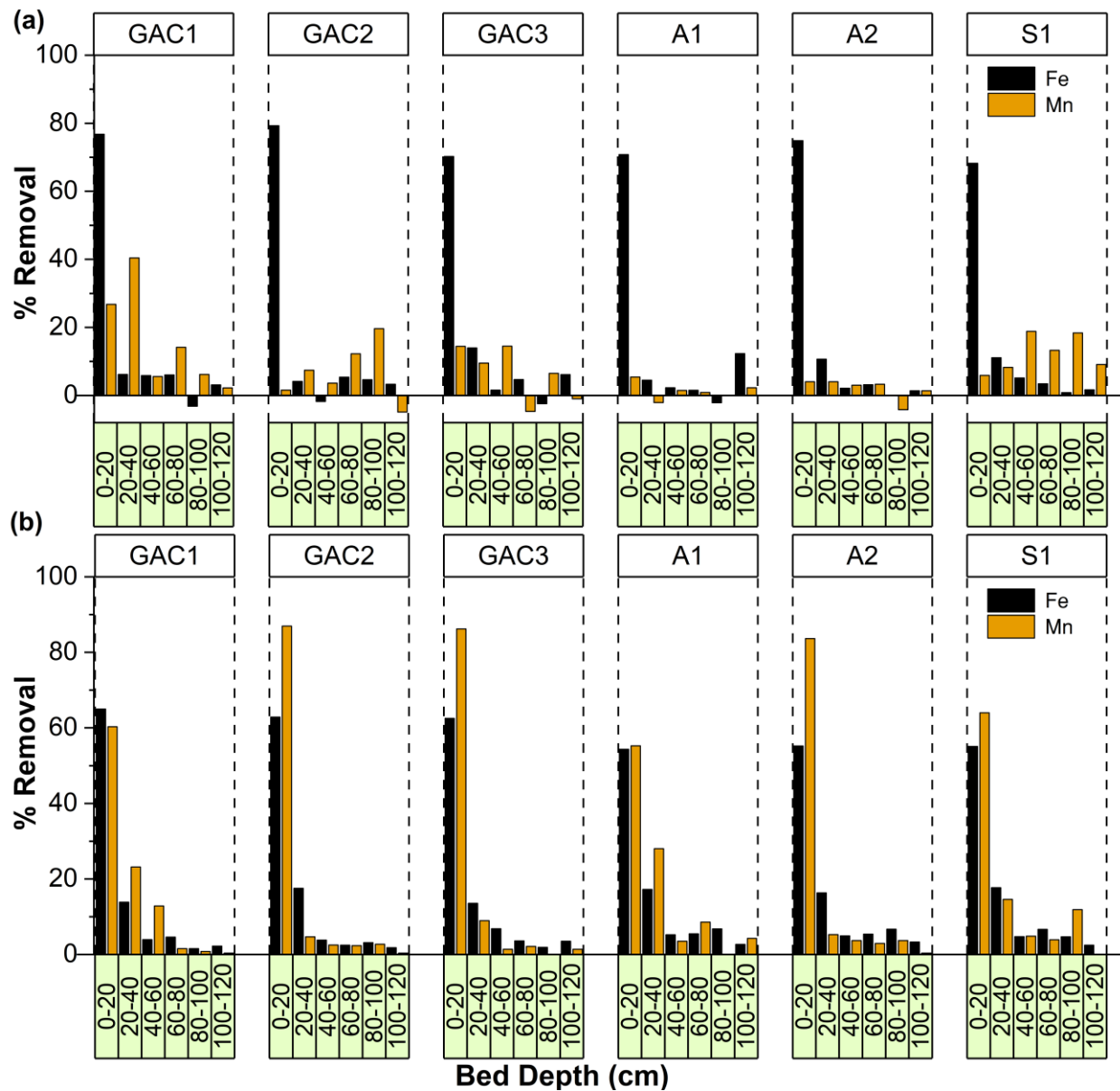


Figure A3.3: Extent of Mn & Fe removal at different bed depths in the biofilters at (a) day 18 and (b) day 133

Table

Table A3.1: Kinetics of Mn removal by pilot scale biofilters (k & R^2_{adj} values for biofilters at day 18, 56, 84, 126, and 133)

Biofilter	Day 28		Day 56		Day 84		Day 126		Day 133	
	R^2_{adj}	k (min^{-1})	R^2_{adj}	k (min^{-1})	R^2_{adj}	k (min^{-1})	R^2_{adj}	k (min^{-1})	R^2_{adj}	k (min^{-1})
GAC1	0.946	1.026	0.900	1.100	0.912	0.684	0.953	0.766	0.804	0.731
GAC2	0.941	0.904	0.877	0.975	0.928	0.682	0.946	0.808	0.974	0.880
GAC3	0.919	0.186	0.921	1.033	0.904	0.686	0.935	0.901	0.946	0.87
A1	0.769	0.010	0.919	0.504	0.898	0.452	0.888	0.606	0.980	0.601
A2	0.992	0.228	0.978	0.770	0.917	0.686	0.928	0.697	0.979	0.688
S1	0.967	0.213	0.974	0.553	0.979	0.686	0.934	0.697	0.949	0.688
SD**	0.072	0.388	0.037	0.231	0.026	0.103	0.020	0.101	0.015	0.099

Appendix A4

Figure

A4.1

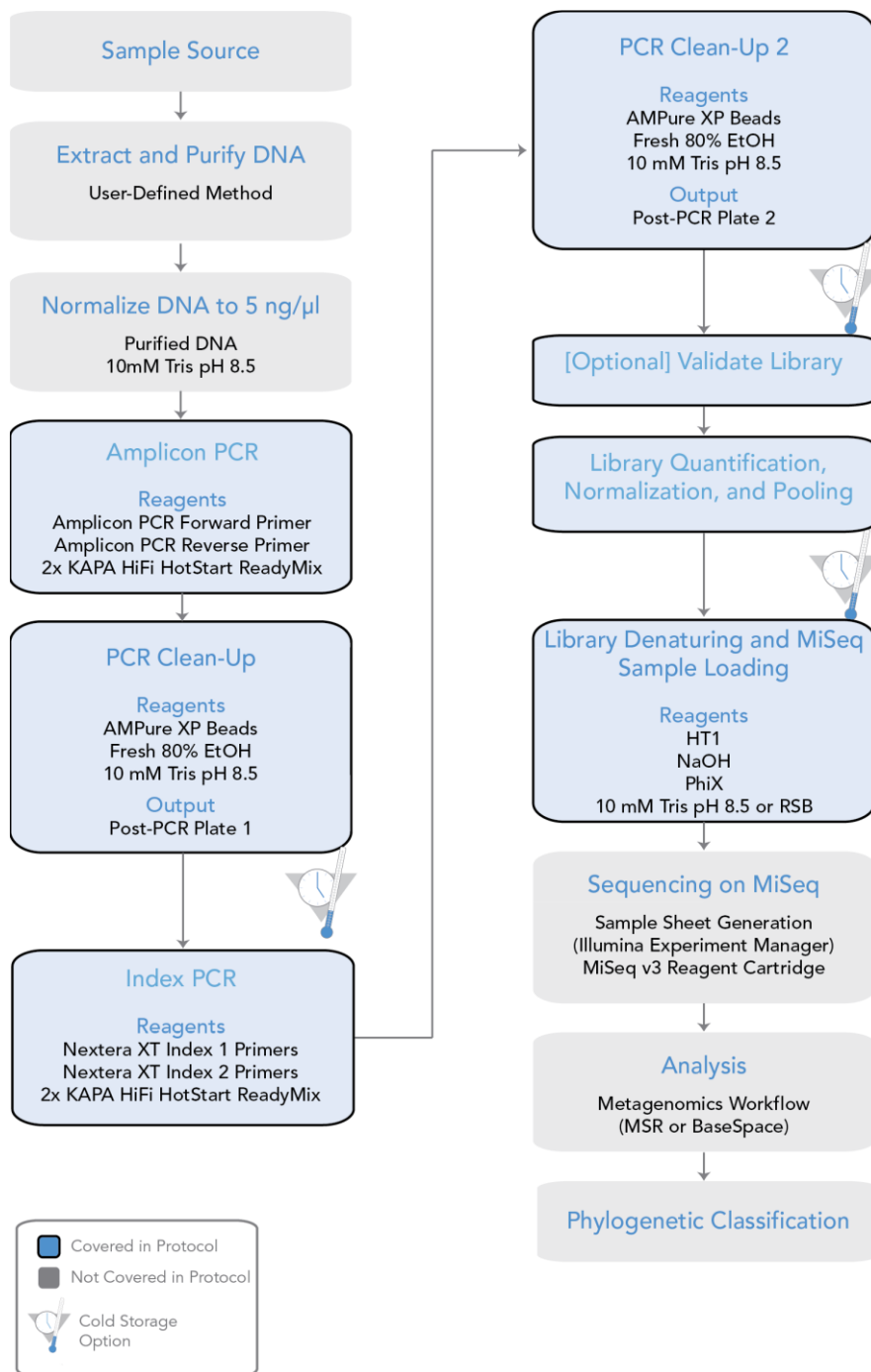


Figure A4.1: 16S Library Preparation Workflow

A4.2

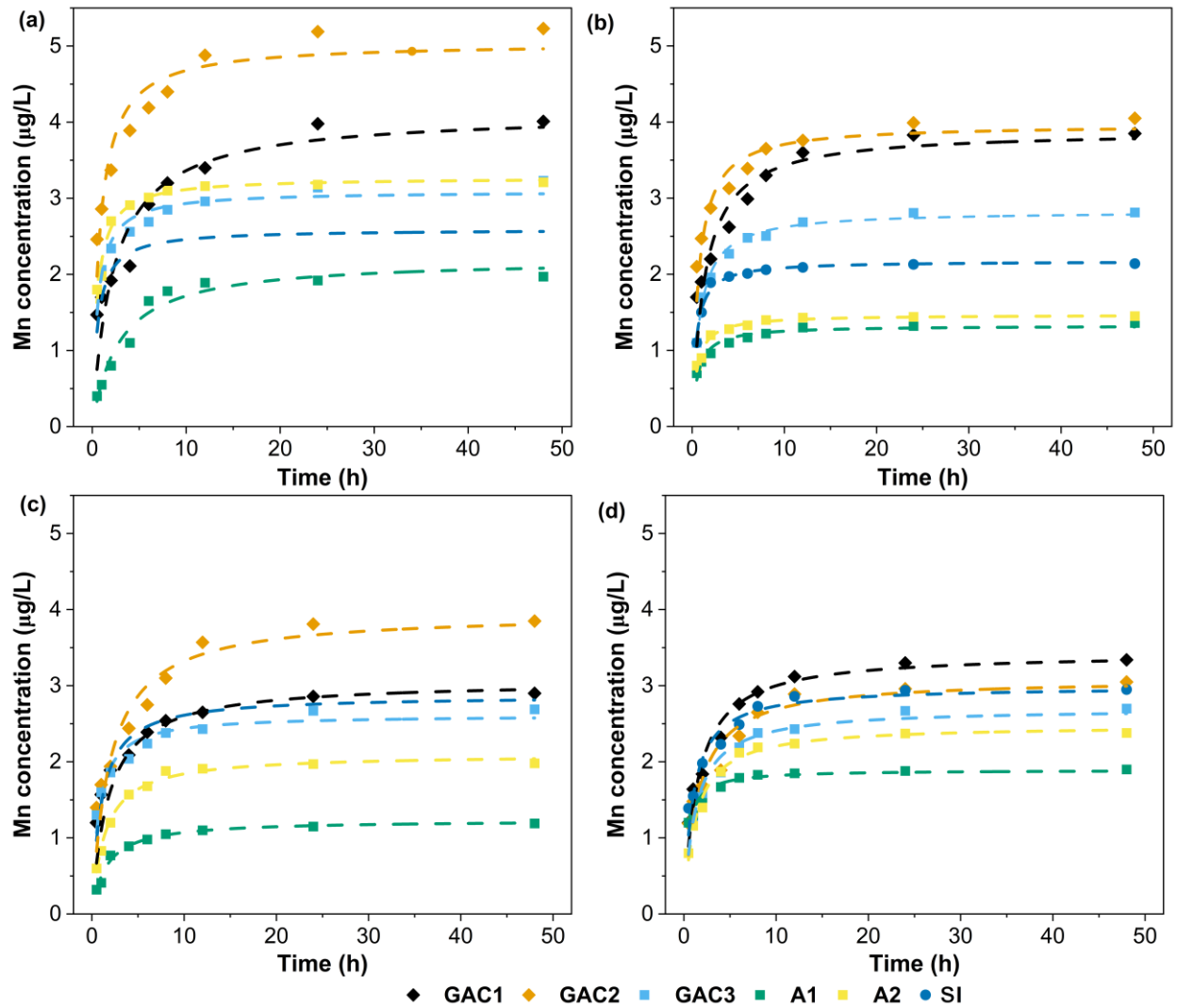


Figure A4.2: Desorption batch tests conducted on inhibited media sampled from pilot-scale biofilters on day 126 at various depths: (a) top, (b) 20 cm, (c) 40 cm, and (d) 100 cm

A4.3

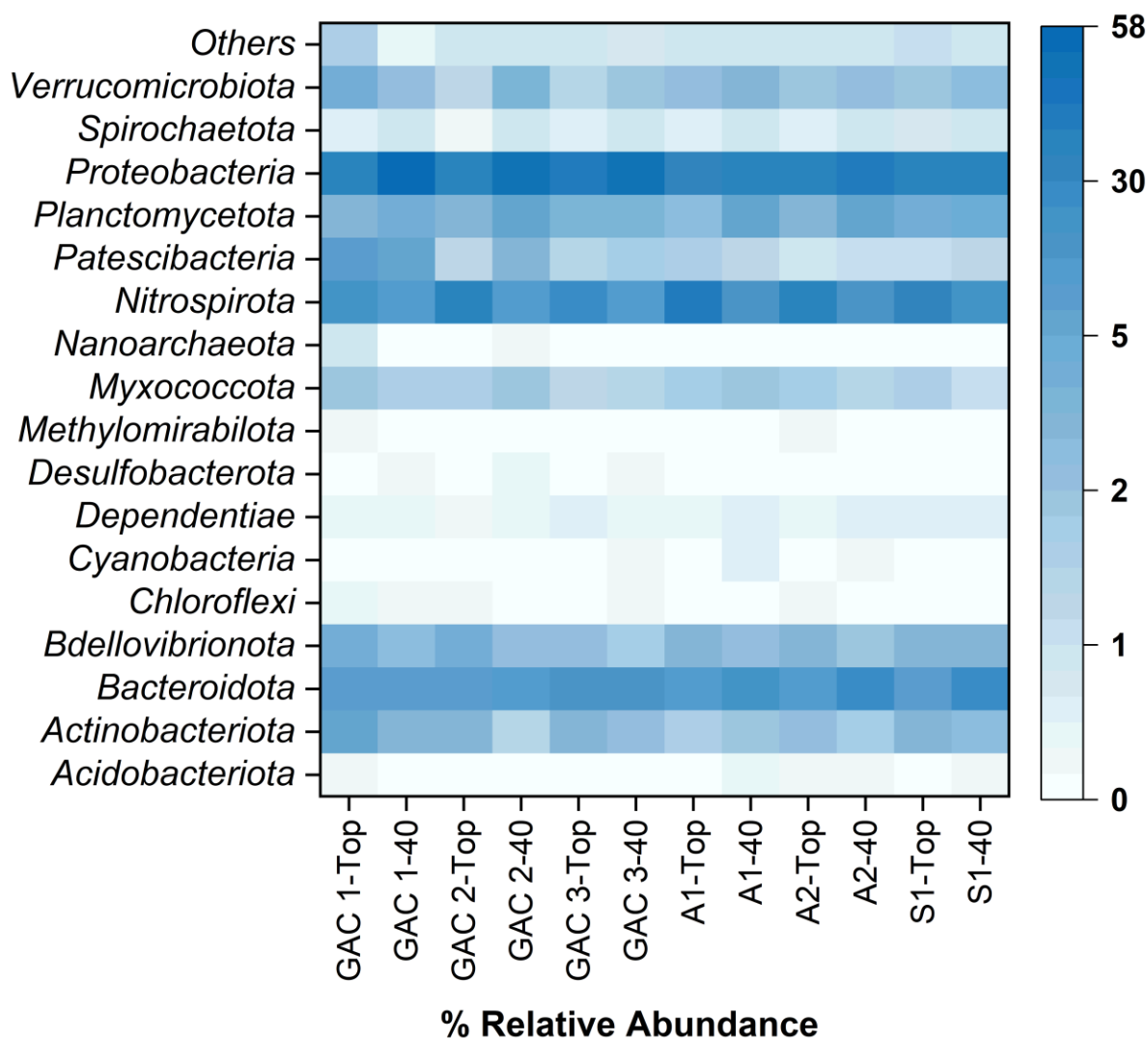


Figure A4.2: Heatmap of the relative abundances of microorganisms at the phyla level on pilot-scale biofilter media samples from day 126

Tables

Table A4.1: Shannon and Simpson Diversity of biofilter media collected on day 126

Media	Shannon Index		Simpson Diversity	
	Top	40 cm	Top	40 cm
GAC1	4.40	4.65	0.97	0.98
GAC2	4.42	4.62	0.97	0.98
GAC2	4.46	4.60	0.98	0.98
A1	4.35	4.47	0.97	0.98
A2	4.42	4.51	0.97	0.98
S1	4.51	4.54	0.98	0.98

Table A4.2: Pathway numbers associated with genes involved in Mn oxidation identified on biofilter media sample from day 126 in this study

Pathway number	Genes	Function
K03781	<i>katE</i>	ROS
K03782	<i>katG</i>	ROS
K06324	<i>cotA</i>	MCO
K00156	<i>poxB</i>	Pyruvate metabolism
K01716	<i>fabA</i>	Fatty acid metabolism
K00645	<i>fabD</i>	Fatty acid metabolism
K02372	<i>fabZ</i>	Fatty acid metabolism
K04564	<i>sodA</i>	ROS, superoxide dismutase, Fe-Mn family
K02197	<i>ccmE</i>	Direct Mn oxidation
K04016	<i>ccmF</i>	Direct Mn oxidation
K02193	<i>ccmA</i>	Direct Mn oxidation
K00426	<i>cydB</i>	Two-component system- electron transfer reaction
K04755	<i>fdx</i>	Two-component system- electron transfer reaction
K14588	<i>yacK</i>	MCO
K02199	<i>ccmG</i>	Direct Mn oxidation
K14588	<i>CueO</i>	MCO
K01637	<i>aceA</i>	Metabolic pathway
K02456	<i>xCPT</i>	Direct Mn oxidation
K01657	<i>trpE</i>	Metabolic pathway

ROS: Reactive oxygen species; MCO: Multicopper oxidase enzymes

Appendix A5

Text

A5.1 EPA Method 3050B

Accumulated Mn was extracted from the media by acid digestion and hydrogen peroxide addition according to EPA Method 3050B. A representative 0.5 g dw (oven-dried) sample was digested at 95°C with 1:1 trace metal grade nitric acid (ARISTAR® PLUS, VWR Chemicals BDH®) for 10 to 15 minutes, cooled, and digested at 95°C for 30 minutes. The sample was then cooled and reacted with sequential additions of 30 percent hydrogen peroxide (30% ACS, VWR Chemicals BDH®) while heated. Particles in the digestate were removed by decanting, centrifugation (3600 revolutions per minute (rpm) for 10 min), and filtration (0.45 µm). The digestate was diluted as required for analysis by QQQ-ICP-MS (section 3.3.1). The mass of metal extracted per mass of media was calculated by multiplying the concentration in the digestate by the sample volume and then dividing by the mass of dried media processed. Samples were analyzed in duplicates.

Figures

A5.1

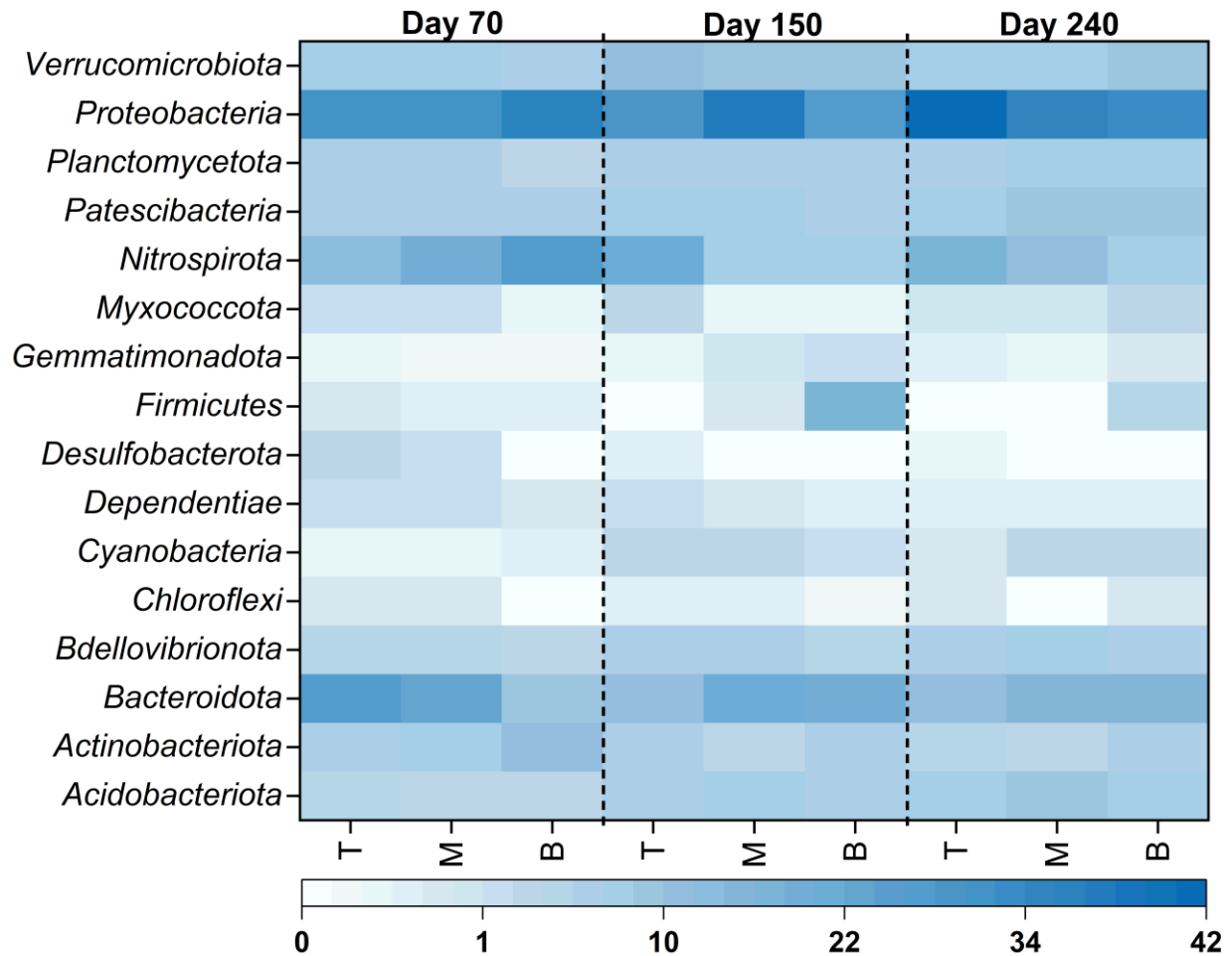


Fig. A5.1: Heatmap of relative microbial abundance at the phyla level (> 0.25%) on days 70, 150, and 240 on biofilter media (T: Top, M: Middle, B: Bottom)

A5.2

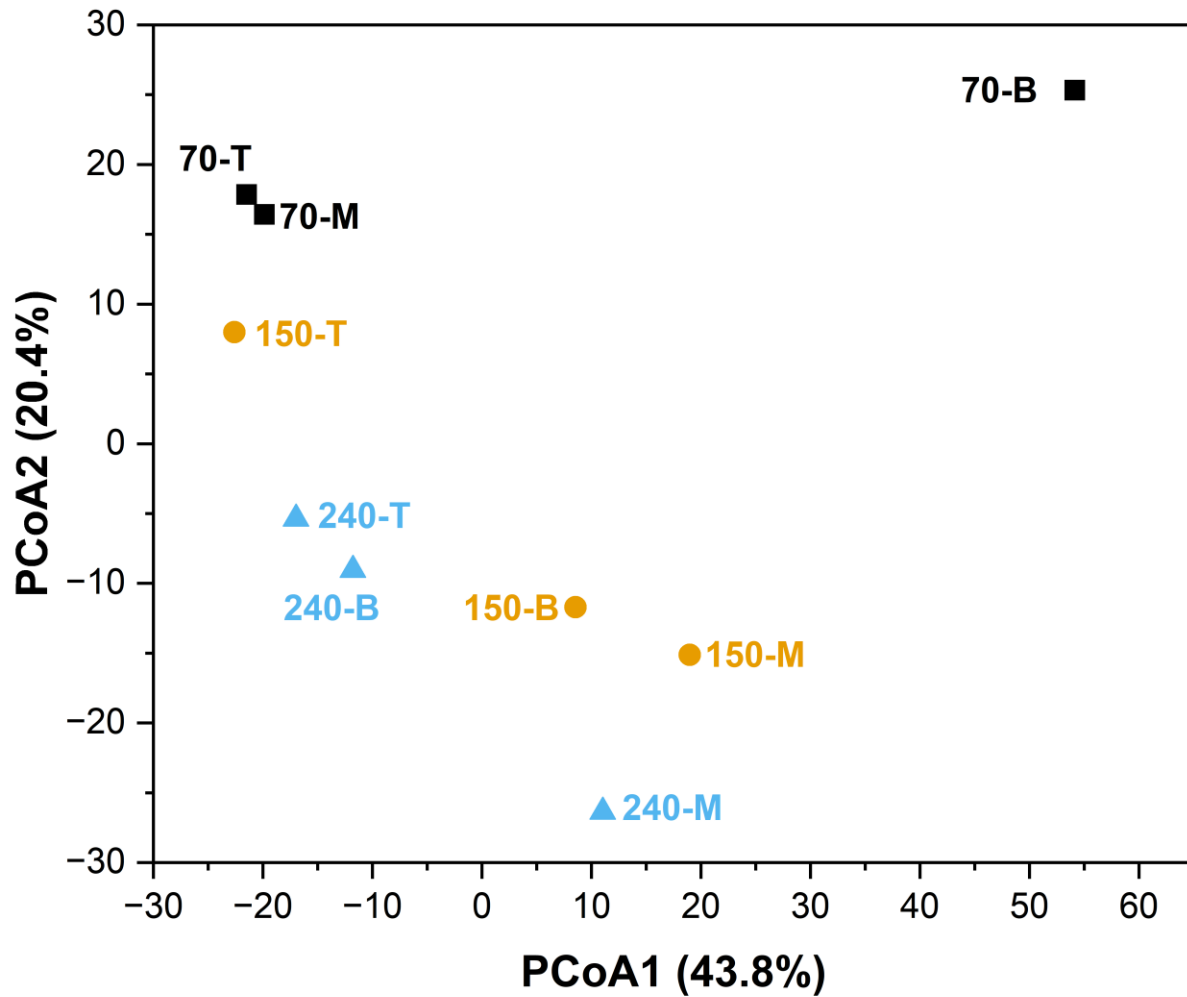


Fig. A5.2: PCoA generated using Bray Curtis dissimilarity for the biofilter media on days 70, 150, and 240 (T: Top, M: Middle, Bottom)

A5.3

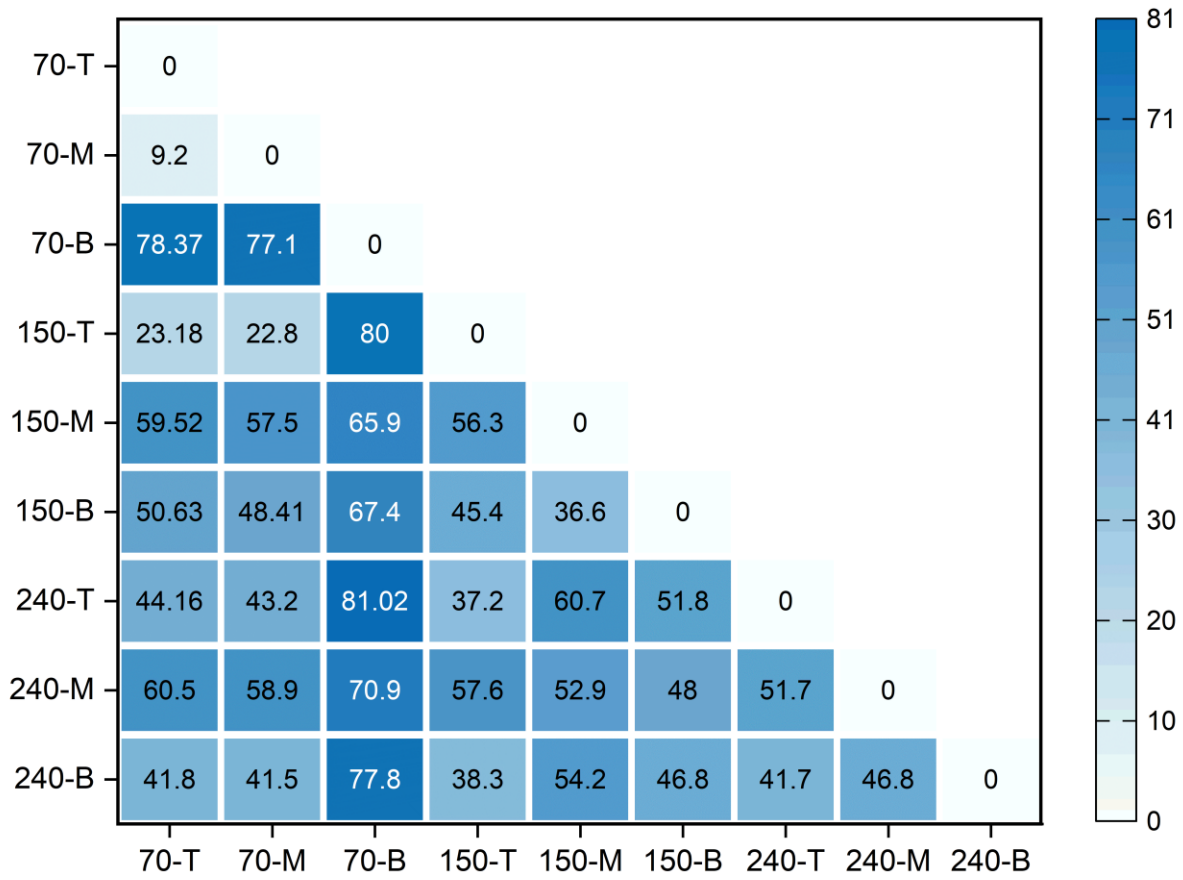


Fig A5.3 Bray-Curtis dissimilarity matrix of biofilter media collected on days 70, 150, and 240 (higher value represents higher diversity between two samples. T: Top, M: Middle, B: Bottom)

Table

A5.1 Shannon and Simpson Diversity of biofilter media collected on days 70, 150, and 240

	Shannon Index			Simpson Diversity		
	Top	Middle	Bottom	Top	Middle	Bottom
Day 70	3.95	3.84	3.16	0.95	0.94	0.90
Day 150	3.99	3.87	3.96	0.94	0.96	0.96
Day 240	5.15	4.97	4.67	0.99	0.98	0.98

Appendix A6

A6.1 EPS Analysis

EPS extractions were performed following the procedure outlined in Keithley & Kirisits (2018). Briefly, ~2 g of biofilter media were added to a 15-mL conical tube, followed by 10 mL of extraction buffer (10 mM Tris, 10 mM EDTA, 2.5% NaCl, pH), which was vortexed for 1 min, and incubated at 35°C with shaking at 200 rpm for 4 h. After that conical tube was centrifuged at 10,000 x g and 25° C for 10 min. The EPS supernatant was filtered through a 0.45-µm nylon filter (Chromospec, Canada), and the pellet was retained for total solids (TS) analysis. EPS was quantified by measuring polysaccharides and proteins in the extractant as follows:

EPS Carbohydrates: The total carbohydrate content of the extracted EPS was determined using the phenol sulfuric acid method described by Dubois et al. (1956). In short, 0.5 mL of the EPS extract, 0.5 mL of 5% (in water) phenol solution (Sigma Aldrich, USA), and 2.5 mL of 98-99% sulfuric acid (Sigma Aldrich, USA) were added to acid-washed (7% HNO₃) glass test tubes. The tubes were gently shaken, capped, and heated to 30° C in a water bath for 15 minutes. The samples were left to cool to room temperature and then analyzed on a Cary 100 UV/VIS spectrophotometer (Agilent Technologies, USA) at 490 nm using a 1 cm glass cuvette. The measured absorbance values were calibrated against D-glucose standard solutions (0, 1, 2.5, 5, 7.5, 10 mg/L as D-glucose). All samples and standards were analyzed in duplicates.

EPS Proteins: The total protein content of the extracted EPS was determined using the bichronic acid method (Pierce micro-BCA protein kit, Thermo Scientific). A 2 mL sample of the EPS extract was added with 2 mL of the prepared reagent in an acid-washed glass test tube. The solutions in the tubes were mixed gently and heated at 60° C in a water bath for 1 h. The samples were left to cool to room temperature, then measured at 562 nm using a Cary 100 UV/VIS spectrophotometer (Agilent Technologies, USA) in a 1 cm glass cuvette. The recorded absorbance values were calibrated using bovine serum albumin (BSA) standards (0, 1, 2.5, 5, 7.5, and 10 mg/L BSA). All samples and standards were analyzed in duplicates.

EPS polysaccharide and protein concentrations were normalized to the mass of filter media used in the extraction.

Table A6.1

A6.1 Shannon and Simpson Diversity of biofilters media collected on day 240

	Shannon Index			Simpson Diversity		
	Top	Middle	Bottom	Top	Middle	Bottom
Biofilter A	4.72	4.78	4.81	0.98	0.98	0.99
Biofilter B	5.15	4.77	4.84	0.99	0.99	0.99
Biofilter C	5.15	4.97	4.67	0.99	0.98	0.98