

University of Nevada, Reno

**Upstream Effects on Microbial Community Selection in Biological Activated
Carbon Filters**

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of
Philosophy in Civil and Environmental Engineering

by

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THE GRADUATE SCHOOL

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prepared under our supervision by

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Abstract

Limited literature has explored the development of bacterial communities in biofilters used in advanced water reclamation for potable water reuse and their specific role in the biodegradation of contaminants of interest. The study of microbial ecology and the development of bacterial communities in biofilters based on upstream effects in the treatment systems and downstream effects on effluent quality and process stability is imperative. Investigation of the role of upstream effects such as seeding of microorganisms from the conventional water reclamation processes and effects of ozone oxidation on the community before and during biofiltration are the main foci of this research. In addition to microbial ecology assessment for biofiltration processes, this examined the applicability of ozonation - biofiltration as a treatment barrier in water reuse projects and identify future needs to consolidate it as preferred option. This research collected pilot-scale and bench-scale data from experiments with reclaimed water to understand the biofilters microbiology and its capabilities and resilience based on different influent characteristics, either selecting and/or defining microorganisms to enhance biofilter performance and efficiency. Ozonation employed to disinfect pathogens and make trace organics more biodegradable followed by activated carbon based biofiltration (biological activated carbon (BAC) filtration) were studied in combination with Coagulation/Flocculation/Clarification/Granular Media Filtration, Granular Activated Carbon Filtration, and Ultraviolet disinfection. This advanced treatment train combined physical, chemical, and biological processes to successfully produce a high-water quality with low to non-detectable contaminant of emerging concern reducing the risk to potential public health concerns.

In summary, this research highlights how the structure, composition, development, and fate of microbial community in ozone/BAC and other processes such as CFCGMF and GAC/UV/CF in advanced water treatment for water reuse purposes can be a key player to assess and control the overall system performance and ensuring high quality and safe finished water.

¡Terminé! Eso grite.
Para José, mi Niche, mi vida.

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Chapter 1

INTRODUCTION

1.1 Purpose of the Study

Limited literature has explored the development of bacterial communities in biofilters used in advanced water reclamation for potable water reuse and their specific role in the biodegradation of specific organic compounds or contaminants. The study of microbial ecology and the development of bacterial communities in biofilters is needed to make the process stable and highly functional based on the relationship between upstream and downstream effects and contaminants of concern. The role of upstream effects such as seeding microorganisms from the conventional water reclamation processes and the effects of ozone oxidation on the microbial community in feed water and during biofiltration are the main foci of this research project. In addition to microbial ecology assessment for biofiltration processes, this research will examine the applicability of ozonation - biofiltration as a treatment barrier in water reuse projects and identify future needs. The research includes pilot-scale and bench-scale experiments to understand biofilter microbiology and its capabilities and resilience based on different influent characteristics, either to select and/or define microorganisms to enhance performance and efficiency.

1.2 Background

Regions and countries facing water shortages due to infrequent precipitation and higher water demands seek strategies to expand available water resources and improve water resiliency. Water reuse is an attractive strategy to enhance available water resources to address the water deficit and resiliency issue. The highest value option for water reuse would be achieved by making the reclaimed water suitable for potable reuse. In potable water reuse systems, treated wastewater

is further polished to remove pathogens, trace organics (contaminants of emerging concern (CECs)), and bulk organic matter. A promising technology applied in potable reuse projects is the combination of biological activated carbon (BAC) filter and ozone to remove trace and bulk organics. The O₃-BAC method is an alternative to the typical full advanced treatment (FAT), which combines reverse osmosis (RO) and advanced oxidation process (AOP). Compared to FAT, O₃-BAC offers less energy consumption per unit volume of water produced, lower capital costs, reduces/eliminates the need for salinity removal or brine management, diminishes the total organic carbon (TOC) concentration, and removes disinfection byproducts (DBPs) such as trihalomethanes (Sundaram et al., 2010; Gerrity et al., 2014).

In addition to the advantages above, biofiltration using a BAC filter can enhance microorganisms' activity, facilitating the degradation of natural organic matter in water. A biofilter offers the traditional solid-liquid separation in addition to biodegradation in a single process (Mohammed et al., 2011). Several filter media ranging from granular materials such as silica sand, granular activated carbon (GAC), anthracite, and porous ceramics can be used in a biofilter (Basu et al., 2016). Compared to the available options, GAC has been found to have a strong affinity for organic matter attachment and adsorption, a large specific surface area, and shear force-sheltering capability (Xing et al., 2011; Skouteris et al., 2015). BAC biofilter functions as a system that permits the growth of "beneficial" organisms and relies on the properties of contaminant sorption, diffusion, and capacity of biodegradation (Kirisits et al., 2019). The key aspects of biofiltration as a treatment process are assessing its performance and the inherent efficiency of the microorganisms present. Despite the many advantages of O₃-BAC in water reuse projects, limited research has explored the development of microbial communities in biofilters and their specific role in enhancing its performance and treatment efficiency.

1.3 Objectives and Scope

1.3.1 Objectives

The general objective of this research was to investigate the development, structure, and composition of the microbial community involved in the O3/BAC process in advanced water reclamation for water reuse and to understand the impact of the influent water matrix and upstream treatment on microbial communities within a biofilter used as a barrier in water reuse applications.

The following specific objectives are proposed to accomplish that goal:

1. Identify and summarize all relevant research information regarding the combination of ozonation and biofiltration processes as treatment processes in water reuse applications.
2. Analyze biofilter resiliency by assessing and identifying microbial communities developed within a biofilter in water reuse applications.
3. To validate the value of combining ozonation and biofiltration to treat secondary wastewater treatment effluent that will be used for potable reuse purposes.
4. Examine the ozone impact on the robustness, composition, and structure of the microbial community.
5. To study the microbial ecology and development of bacterial communities in biofilters based on the relationship between upstream and downstream processes and treated contaminants.
6. To determine the role of upstream effects such as seeding microorganisms from the conventional water reclamation processes and the effects of ozone oxidation on the microbial community before and during biofiltration.
7. To examine and validate the applicability of biofiltration in the removal of CECs as a treatment alternative in water reuse projects.

1.3.2 Scope

This research is in parallel with the advanced water treatment (AWT) train for indirect potable reuse (IPR) demonstration project by OneWater Nevada, whose primary goal is to validate an advanced water treatment train “to produce category A+ water by utilizing granular media filtration with coagulation, flocculation, and sedimentation pretreatment, ozone-biological activated carbon filtration, granular activated carbon polishing, and UV disinfection.”

Three different source waters were studied in this investigation to understand the microbial community assembly in biofilters and how the microbial community is being selected to enhance its overall performance and assess its impact on the resilience and capabilities of the BAC filters. This research is developed by combining experimental and analytical investigations.

1.4 Chapters and Contents

This dissertation describes the research conducted in the following chapters, and they are briefly described here.

Chapter 1: Introduction

In this chapter, a brief introduction to the entirety of the thesis is presented. This chapter describes the purpose of the study, background, research objectives, and the scope of the research. It briefly presents the subsequent chapters and their contents.

Chapter 2: Microbial Community in Biofilters for Water Reuse Applications: A Critical Review

This chapter gives a detailed literature review about the microbial community diversity in biofilters used in water reclamation applications. This chapter identifies and summarizes all

relevant research information regarding the combination of ozonation and biofiltration processes as treatment processes in water reuse applications.

Chapter 3: Microbial Community Characterization in Advanced Water Reclamation for Potable Water Reuse

This chapter focuses on determining biofilter resiliency by assessing and identifying microbial communities developed within a biofilter used as barrier in water reuse applications. It describes the value of combining ozonation and biofiltration to treat secondary wastewater treatment effluent that will be used for potable purposes. Finally, the ozone impact on the robustness, composition, and structure in the BAC's microbial community are examined and described.

Chapter 4: Microbial Community Characterization and Fate across an Advanced Water Treatment Train Used in Potable Water Reuse: Reno-Stead Water Reclamation Facility Demonstration Study.

This chapter evaluates how AWT processes influence the microbial community across the treatment train based on pilot-scale experiments, how the microbial community changes along with the treatment train, and the potentially pathogenic bacteria in finished water.

Chapter 5: Linking Microbial Community structure and Removal of Contaminants of Emerging Concern across an Advanced Water Treatment Train for Potable Water Reuse

This chapter presents the study of the microbial ecology and development of bacterial communities in biofilters based on the relationship between upstream and downstream processes and the removal of CECs. A 16S metagenomics analysis is presented characterizing the microbial

community structure of the biofilters, and then a function prediction is described to understand biodegradation patterns within the BAC filter.

Chapter 6: Rapid-Small-Scale-Column Test to Assess the Upstream Treatment Impact on Microbial Communities in Biofilters used in Potable Water Reuse.

This chapter will discuss whether upstream treatments are impacting or shaping the microbial community in biofilters by monitoring rapid small-scale column tests (RSSCT) fed with three different source waters: secondary effluent, coagulation/flocculation/clarification/granular media filtration (CFCGMF) effluent, and ozonated effluent. Water quality parameters were monitored, as well as the microbial community developed in each RSSCTs to determine the role of upstream effects such as seeding microorganisms from the conventional water reclamation processes and the effects of ozone oxidation on the microbial community before and during biofiltration.

Chapter 7: Conclusions

This chapter summarizes the research's overall conclusions and identifies future research questions that may benefit the body of knowledge.

Chapter 2

MICROBIAL COMMUNITY IN BIOFILTERS FOR WATER REUSE APPLICATIONS: A CRITICAL REVIEW

This research phase aims to identify and summarize all relevant research information regarding the characterization, structure, and composition of the microbial community on biofilters used in water reuse applications. This chapter was published in Science of the Total Environment, Volume 773, 15 June 2021, 145655.

2.1 Introduction

Regions and countries facing water shortages due to infrequent precipitation and higher water demands seek strategies to expand available water resources and improve water resiliency. Water reuse of reclaimed water from wastewater is an attractive strategy to address the water deficit and resiliency issue. This practice also promotes a circular economy, which is being adopted by many communities and regions. The highest value option for water reuse is achieved by making the reclaimed water suitable for potable reuse or drinking water supply augmentation. In potable water reuse systems, treated wastewater is further polished by advanced treatment to remove pathogens, trace organics [such as contaminants of emerging concern (CECs)], and bulk organic matter. An emerging technology being applied in potable water reuse projects is the combination of a pre-oxidation step coupled with a biofilter. In practice, a commonly applied system comprises a pre-ozonation step and a biologically activated carbon (BAC) filtration step (O₃-BAC). The O₃-BAC

method is an alternative to the typical full advanced treatment (FAT), which combines reverse osmosis (RO) and advanced oxidation process (AOP) (Hooper et al., 2020). Compared to FAT, O₃-BAC offers effective CEC degradation, less energy consumption per unit volume of water produced, lower capital costs, reduces/eliminates the need for salinity removal or brine management, diminishes the total organic carbon (TOC) concentration, and removes disinfection byproducts (DBPs) such as trihalomethanes in the product water (Gerrity et al., 2014; Sundaram et al., 2010).

In addition to the advantages above, biofiltration using a BAC filter can enhance the activity of microorganisms, which degrade natural organic matter that can form DBPs during subsequent disinfection. This is achieved through a single process that combines biodegradation and solid-liquid separation (Mohammed et al., 2011). Several granular filter media such as silica sand, granular activated carbon (GAC), anthracite, and porous ceramics can be used in a biofilter (Basu et al., 2016). Compared to the available options, BAC biofilter with GAC as support media functions as a system that allows the growth of “beneficial” organisms and relies on the properties of contaminant sorption, diffusion, and biodegradation (Kirisits et al., 2019). Hence, the combined mechanisms of oxidation, adsorption, and biological degradation provide interesting capabilities to O₃-BAC treatment as an effective treatment step for trace and bulk organics in water reuse applications. However, the challenge is to fine-tune ozone dose to balance organics reduction, microbial pathogen inactivation, downstream treatment effects (e.g., DBP formation potential), and effects on microbial community seeding to the subsequent biofilter.

The key aspects of biofiltration as a treatment process are assessing its performance and the inherent efficiency of the microorganisms present. Then, a selective enrichment of

microorganisms that biodegrade target pollutants such as CECs in a biofilter would significantly improve the capacity and efficiency of the process. Therefore, O₃-BAC is a viable option in potable reuse water production because of its reliability as a barrier to address regulatory concerns that include pathogen reduction, and removal of regulated and unregulated trace organic compounds (Oliveria et al., 2016; Sundaram et al., 2020; Tchobanoglous et al., 2015). An example of an advanced reclaimed water treatment train that includes O₃-BAC and two other treatment barriers to guarantee pathogens and CEC/bulk organics removal for indirect potable reuse needs is shown in Figure 1. In California (USA), the minimal level of pathogen control required is 12, 10, 10 log reduction of virus, *Giardia*, and *Cryptosporidium*, respectively, from raw wastewater to finished water for groundwater recharge projects with potable reuse quality reclaimed water (Olivieri et al., 2020). Since the two major functions of the train involve microorganisms, the microbial ecology of the influent reclaimed water to the BAC and the effects of ozonation and microbial selection/enrichment within BAC are critical for treatment in this system.

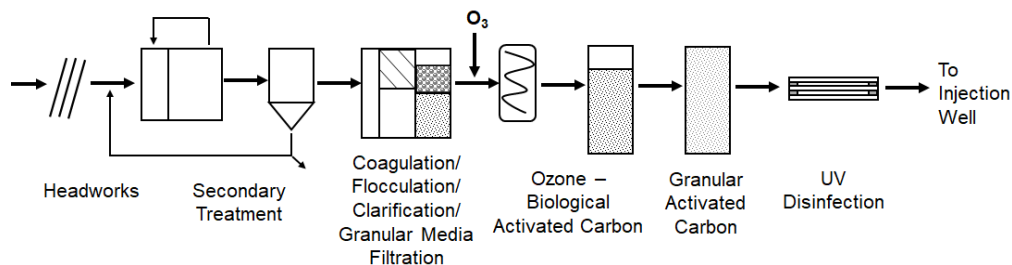


Figure 2-1. Advanced reclaimed water treatment train that includes O₃-BAC to treat CECs in potable reuse applications.

Despite the many advantages of O₃-BAC in water reuse projects, limited research has explored the development of microbial communities in biofilters and their role in the degradation of specific compounds, particularly CECs in water reuse projects. This knowledge gap is addressed in this paper by assessing the state-of-the-art microbial ecology and microbial communities' development in water reuse biofilters. This review paper is divided into six sections. The first

section gives a brief overview of the advantages of combining ozonation and BAC filters and their performance as a barrier for water reuse applications. The second section examines the conditions, characteristics, and tendency of microorganisms to form biofilms in biofilters. In a subsection, the typical phyla identified in secondary wastewater treatment are described as the first attempt to associate taxa classification with phenotypic characteristics. The following section identifies and analyzes the microbial communities that have been reported in biofilters used in water reclamation for water reuse applications at full, pilot, and bench-scale. Then, the fourth section discusses the implication and consequences of pre-ozonation on shaping the microbial communities in the subsequent biofilters. Finally, the fifth and sixth sections describe the research questions and conclusions to assure better implementation and improve the effectiveness of O₃-BAC units for advanced water treatment (AWT) for water reuse purposes.

2.2 Microbial Assembly in Biofilters

The microbial community in the biological treatment process of a water resource recovery facility (WRRF) is strongly associated with the characteristics and composition of the WRRF influent (Fernandes et al., 2013). The selection of biological treatment process type to meet effluent requirements and regulations is the driver that obligates microbial communities' adaptation and enrichment across the treatment train. As a result, the biodegradation process will be controlled by organisms with higher adaptability and resilience. By natural extension, it can be assumed that the microbial community in WRRF effluents would provide the seed microorganisms for biofilters when employed in AWT trains for potable reuse, as shown in Figure 1. Hence, linking the microbial community from WRRFs effluents, pretreatment effects on this microbial community in AWT, and the resulting microbial community in the AWT biofilters is critical to understanding biofiltration performance concerning CECs and bulk organic matter removal. The following subsections provide

insights about why certain microorganisms employed in biological treatment for water reclamation tend to form biofilms in biofilters and describe the specific characteristics of these microorganisms. The reason for identifying these organisms as first step is related to the seeding effect of upstream suspended organisms that will colonize the biofilter media.

2.2.1 Factors Defining the Microbial Community Structure

The concept of ecosystem and species interactions may better explain the performance and efficiency of the microbial community. The microorganisms-contaminant, microorganisms-environment, or microorganisms-community synergies are complex and comprise multiple mechanisms that define their overall role in the microbial community. Therefore, assuming that a particular species is mainly responsible for a specific biodegradation aim is a simplistic approach that denies ecological aspects. Despite the importance of including the vision of the sum of species within a microbial community as a whole, microbiologists and environmental engineers' efforts to include the microbial communities as a whole failed to address it due to its complexity (Braga et al., 2016).

The structure of a microbial community can be defined by multiple and interlinked factors, like nutrients, interactions with other organisms, microorganisms arrangement, presence of disinfectants/inhibitors, among others (Figure 2-2). The availability of nutrients and their concentration may both increase or limit the abundance, composition, and viability of certain organisms (Prest et al., 2016). Competitive relationships between microorganisms will arise based on nutrients availability, and even predation can play a role in shaping microbial community composition. Rittmann (2018) underlines that the available substrate defines microorganisms' metabolic rates in biofilms. For example, slow-growing organisms such as nitrifiers live within the

biofilm between the substratum and outer layers where nutrients are scarce. The opposite is true for fast-growing organisms like heterotrophic bacteria, which live on the outer layer of the biofilm. These communities are arranged either as biofilms, suspended in bulk water, or within sediments add a variable to the prevalence and selectivity in distinguishing microorganisms. The community arrangement is also determined by substrate accessibility, shelter from predators, and toxicity.

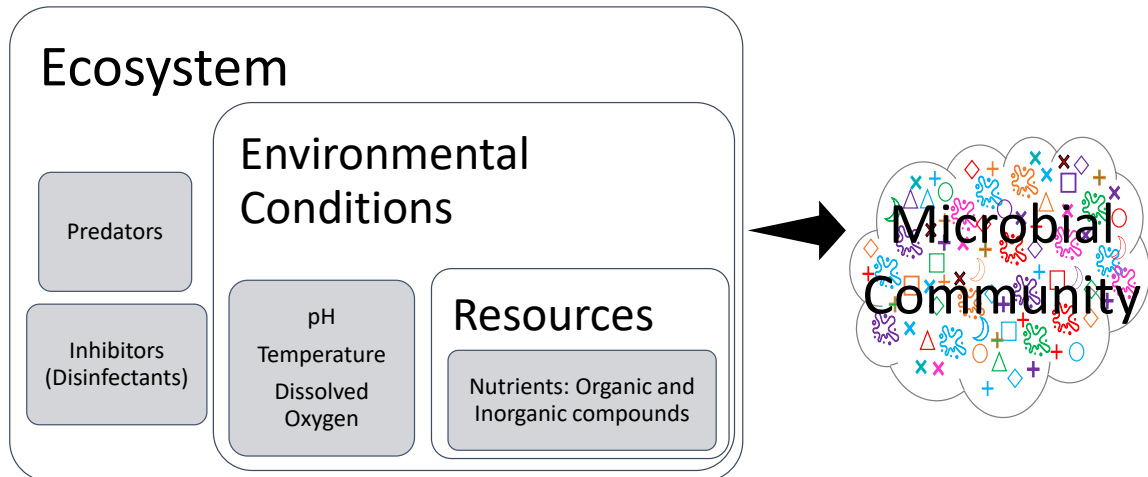


Figure 2-2. Factors defining microbial communities in engineered ecosystems

Likewise, environmental conditions such as dissolved oxygen, pH, and temperature can impact the structure of microbial communities. For instance, dissolved oxygen in BAC filters can cause different redox conditions such as aerobic, anaerobic, or anoxic environments. Here a particular electron acceptor (e.g., oxygen, nitrate, sulfate, bromate, etc.) would be preferred depending upon microorganisms metabolic requirements (Sauter et al., 2021). As for pH, it works as an indicator factor that defines microbial metabolic preferences. Indeed, pH can determine microbial growth and survival, control the chemical activity of protons that define redox reactions, and affect the reactivity of natural organic carbon (Jin and Kirk, 2018).

Similarly, temperature has been identified as a controlling factor of microbial community diversity. Thus low seasonal temperatures may inhibit and slow-down microorganisms metabolism (Li et al., 2021). Combining these factors will impact, define, and shape the microbial community composition that will thrive and actively assume control of the biodegradation process. Nevertheless, biofilters in AWT by nature treat water with low nutrients and organic matter content. Hence, microorganisms that can grow at low substrate concentrations and in biofilm form are likely to be selected in AWT biofilters.

2.2.2 Microbial Community Structure and Attachment Process in Biofilters

Biofilms harbor microorganisms from the surrounding environment by providing constant nutrient availability and metabolic cooperativity and acquiring genetic traits. Watnick and Kolter (2000) stated that microorganisms established in a microbial biofilm are organized based on their needs rather than randomly positioned; in other words, microorganisms distribute themselves around the biofilm depending upon their adaptation to certain environmental factors. According to Donlan (2002), the attachment process could be linked to three characteristics: substratum, bulk fluid, and cell structures properties. Substratum or media properties such as bed texture or roughness, hydrophobicity, and unique conditioning feature can boost the attachment process by conditioning specific cell surface structures to be attracted to. In addition, bulk fluid conditions (nutrient richness, flow velocity, pH, inhibitor presence) can promote cell attachment by attracting microorganisms and colonizing the media due to food availability and protection from the bulk environment. BAC filters with the rough surface texture of GAC grains and intra-particle accumulation and sorption of the substrate could provide ideal conditions for biofilm growth and higher effectiveness in trace organics removal over other media filters. In addition, the effect of

inhibitors in the feed can be mitigated by GAC grains due to the sorption and desorption of the inhibitors. This creates the question of whether certain specialists with specific cell characteristics enriched in the BAC or generalists from the feed water are given an environment for scavenging low substrate concentrations more effectively.

2.2.2.1 Major Phyla Identified in A WRRF's Biological Treatment

As shown in Figure 2-1, the secondary biological treatment is the first barrier in producing reliable and high-quality water and will be followed by further advanced treatment steps in water reuse applications. Thus, understanding the microbial community diversity in secondary treatment is important because the treated effluent seeds microorganisms for subsequent biofiltration. From Midas-database (McIlroy et al., 2017), a total of 115 species were identified as common microorganisms in activated sludge processes in 20 full-scale WWTP over an eight-year study. These species were classified in ten phyla, of which the most abundant (summing up to more than 92%) belonged to Proteobacteria (50.4%), Actinobacteria (17.4%), Chloroflexi (12.2 %), Bacteroidetes (7.0%), and Acidobacteria (5.2%).

The ability of the phyla identified by McIlroy et al. (2017) to form biofilms is not known because only 12 of 115 species were assessed for their potential cell surface hydrophobicity. Surface and cell outer structure interactions strongly define biofilm formation based on hydrophobic and hydrophilic preferences (Krasowska and Sigler, 2014). For instance, microorganisms with highly hydrophobic cell surfaces, such as that found in gram-negative bacteria, have mechanisms to form outer membrane vesicles as a response to stress factors, have been reported as being involved in biofilm formation (Baumgarten et al., 2012). Notably, there is insufficient data to conclude whether a microorganism based on phyla classification can form biofilms. As mentioned in subsection 2.2, microorganism preferences that dictate the biofilm formation rather than suspended growth will depend on environmental factors and genetic markers as well.

However, regardless of the cell structure characteristics, phyla classification differentiation can describe other phenotypic characteristics. Proteobacteria is the most abundant phylum within the bacteria domain (Ettema and Andersson, 2009; Rizzatti et al., 2017). It comprises six classes: *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, *Epsilonproteobacteria*, and *Zetaproteobacteria*. *Proteobacteria* can be chemoorganotrophs, chemolithotrophs, phototrophs, mesophilic or thermophilic, strictly aerobic or anaerobic, or facultative. Some antibiotic-resistant bacteria such as *Pseudomonas*, *Acinetobacter*, *Acidovorax*, and *Sphingomonas* are well-studied *Proteobacteria* in conventional biological treatment (Vaz-Moreira et al., 2017). Meanwhile, *Actinobacteria* can be found in a diversity of habitats such as soil, rhizosphere, marine, or freshwater (Ludwig et al., 2012b, 2012a). In fact, it is the most dominant phylum in lakes and surface water (Newton et al., 2011). A variety of species among *Actinobacteria* are antibiotic producers (*Streptomyces*), biodegraders (*Rhodococcus*), and medically known pathogens (*Mycobacterium leprae*-leprosy and *Mycobacterium tuberculosis*-tuberculosis). *Chloroflexi* is the most reported phyla in anaerobic wastewater treatment systems (Bovio et al., 2019) because its filamentous morphology leads to granule formation and, along with particular fermentative metabolism (Gupta et al., 2013). *Chloroflexi* is a branching and diverse phylum, which can use organohalide compounds as an electron acceptor, could be anoxygenic phototrophs, and under thermophilic conditions act either as aerobic or anaerobic bacteria. Similarly, *Bacteroidetes* is a phenotypic and metabolically diverse phylum, which can be found in soils, aquatic environments, or as symbionts either of humans, animals, or plants (Newton et al., 2011). Examples known are *Flavobacterium* as aerobic free-living organisms and *Bacteroides* as obligate anaerobes. *Bacteroidetes* are gram-negative, non-spore-forming, rod-shaped microorganisms (Gu et al., 2019) considered a primary degrader of polysaccharides making up the largest phylum of gram-negative bacteria inhabiting humans gastrointestinal tract (Thomas et al., 2011). Finally, *Acidobacteria* is a very diverse phylum with species abundant in soil and peat and can endure under polluted and extreme environments (Lee et al., 2008; Quaiser et al., 2003). The occurrence of *Acidobacteria* in acidic and

chemically polluted environments has been linked to extracellular polymeric substances (EPS) production, which might be critical for biofilm formation (Kielak et al., 2017).

Overall, the versatility, robustness, and adaptability of the phyla mentioned above found in secondary biological treatment effluents support the idea that a very rich and complex microbial community composition will actively facilitate and involve the biodegradation processes the advanced treatment biofilters. The richness and diversity of species within the microbial community are strongly related to the WRRF's biological process (Chen et al., 2017; Jaranowska et al., 2013). However, the lack of broad consensus to include and combine multiple factors (standard biomolecular detection techniques, influent characteristics, operational and environmental factors) increased the gap in distinguishing and unique microbial community from the biological process of the WRRF. Hence, understanding the effect of each factor requires further investigation.

2.3 Identification of Microbial Community in Biofilters

Regarding the composition of microbial communities in biofilters, it is better to look more broadly to identify functional members of the microbial community. Hence, this section will focus on studies of different microbial communities through a treatment train and link the development and presence of microorganisms to specific processes in biofiltration (Figure 2-3).



Figure 2-3. The schematic approach is used to study the microbial community in water reuse treatment facilities.

The final aim of any treatment system is to assure chemically and biologically stable water. Indeed, microbial communities in biofilters used in Drinking Water Treatment Facilities (DWTFs) have been well explored due to the needed safety and public health implications of the finished water. Mainly, BAC filters have been used in DWTFs due to their ability to control biological growth downstream, which results in decreased chlorine demand or other disinfectants. Several lessons learned from the drinking water treatment industry may apply to build on and establish BAC filters as a valid and preferred barrier for safeguarding water quality and protecting human health and the environment in AWT trains in water reuse. For instance, some researchers (Lautenschlager et al., 2014; Q. Li et al., 2017; Liu et al., 2019; Pinto et al., 2012; Vignola et al., 2018) focused on the microbial community that colonizes the BAC filters in DWTFs, and how these organisms are being affected by or are affecting upstream and downstream treatments, respectively. Their findings suggest that the microbial community structure within the BAC filter shifts, and it is shaped by environmental factors such as nutrients availability, changing redox, and physicochemical conditions. In addition, when microbial communities from the influent, BAC media and filter effluent are compared, a remarkable statement emerges; BAC filters share more species with the biofilter effluent than the influent water (de Vera and Wert, 2019; Lautenschlager et al., 2014; Q. Li et al., 2017; Pinto et al., 2012). The latter indicates that the seeding microbial community is not shaping the microbial community, and the BAC filter environment assembles and defines the product water microorganisms. This statement thus must be interpreted with caution, as wastewater effluent treated by BAC filters in AWT might reflect similarities with that from DWTFs effluent. However, it cannot be surmised without further comprehensive studies. Hence, the importance of reviewing the microbial community fate and occurrence in AWT for water reuse applications is needed.

2.3.1 Exploring Microbial Communities in Biofilters in Water Resource and Recovery Facilities

To date, there are no available or significant studies on the microbial ecology of BAC biofilters in advanced wastewater reclamation for water reuse. Some studies have investigated and examined changes in the microbial community across the multi-barrier membrane-based FAT train at well-known water reuse facilities, namely, El Paso, Texas (Kantor et al., 2019) and Orange County Water District Advanced Water Purification Facility (Leddy et al., 2017; Stamps et al., 2018). These studies characterize microbial communities and how diversity, abundance, and specificity are shaped and reduced from raw water to the final finished water. Hence, the lack of understanding and knowledge of the same topic applied in membrane-free treatment trains for potable reuse, such as those using the O₃-BAC process, drives the interest and attention of the authors.

Some quantitative and qualitative descriptions have been reported about the microorganisms within biofilters in water reuse (Gerrity et al., 2018). Other studies have focused on the effects of different factors such as pipe material, disinfection, and time on biofilm microbial community development in reclaimed water effluents (Bae et al., 2014; Peng et al., 2018; Zhang et al., 2019). For instance, Zhang et al. (2019) identified a total of 30 phyla, including *Proteobacteria*, *Nitrospirae*, *Bacteroidetes*, *Acidobacteria*, *Planctomycetes*, *Actinobacteria*, and *Verrucomicrobia*, as the most dominant. Compared to the impact of time and pipe material, the upstream disinfection process significantly impacted the downstream microbial community structure and composition. Similarly, Peng et al. (2018) studied the influence of a WRRF effluent in the abundance, distribution, and composition of early biofilm formation communities downstream at the discharge point. *Proteobacteria* (76.8%), *Firmicutes* (12.5%), and *Bacteroidetes* (8.8%) were the dominant phyla in biofilm samples from the downstream discharge point, whereas in the water samples, the

abundance of these last two phyla were lower while Acidobacteria was higher. The main difference between bulk water and biofilm communities was the presence of biofilm-specific species in the latter. Notably, a phylum and a class characterized for being prompt to form biofilms, namely, Proteobacteria and Gammaproteobacteria, respectively (Bae et al., 2014).

The biodegradation of CECs in biological process of the secondary treatment in WRRFs has been studied as well. Table 2-1 presents a compilation of some significant classes of CECs and identified biodegrader microorganisms reported from these WRRF studies. Within the species identified as CEC biodegraders, 52.2% belong to phylum *Proteobacteria*, 30.4% to *Actinobacteria* and 17.4% are *Firmicutes*. Based on observed and identified groups of bacteria present in a WRRF biological process and identified biodegraders, *Proteobacteria* is the most dominant phylum and has been identified as a diverse biodegrader for all CECs' classes shown in Table 2-1. Identifying these phyla and species with the corresponding removal of the CEC classes is needed for water reclamation biofilters used for potable reuse applications. Furthermore, the biofilter operating conditions that enrich the desirable phyla/species that enable the CEC degradation during water reclamation is also required in future research.

Specific studies are needed to produce a clear view of the microbial community within a multi-barrier membrane-free advanced water purification facility incorporating BAC filters. They should be capable of informing not only about bacteria but pathogens, viruses, fungi, parasites, and genes coding antibiotic resistance to ensure public health safety. Further investigations that cover the spatial and temporal state of biofilms and water phase (suspended microorganisms) in the current full and pilot-scale advanced water treatment system with BACs filters in service are needed. The microbial diversity information coupled with monitoring and analysis of environmental parameters (temperature, pH, conductivity, total organic carbon), organic and inorganic nutrients

(nitrate, phosphate, metals) will provide valuable knowledge that eventually will define acceptable ranges for microbial community changes capable of performing and delivering high water quality.

Table 2-1. Microorganisms involved in CECs' biodegradation during water reclamation.

CEC Class	CEC	Biodegrader	Taxonomy (Phylum; Class)	Reference(s)
Pharmaceuticals: Analgesics, anti-inflammatory drugs, antidepressants, stimulants, antibiotics	Acetaminophen	<i>Bacillus pumilus</i> strain NBRC 12092 <i>Arthrobacter ginkgonis</i> strain SYP-A7299	<i>Firmicutes; Bacilli</i> <i>Actinobacteria; Actinobacteria</i>	(Yang et al., 2020)
	Ibuprofen	<i>Sphingomonas</i> spp, <i>Novosphingobium</i> spp.	<i>Proteobacteria; Alphaproteobacteria,</i>	(Navrozidou et al., 2019)
	Caffeine	<i>Burkholderia</i> spp.	<i>Proteobacteria; Betaproteobacteria</i>	(Win et al., 2019)
	Sulfamethoxazole	<i>Achromobacter denitrificans</i> strain PR1, <i>Leucobacter</i> sp. Strain GP	<i>Proteobacteria; Betaproteobacteria</i> <i>Actinobacteria; Actinobacteria</i>	(Ana C Reis et al., 2018)
	Trimethoprim	<i>Bacillus subtilis</i>	<i>Firmicutes; Bacilli</i>	(Liu et al., 2018)
	Carbamazepine	<i>Starkeya</i> sp. C11 and <i>Rhizobium</i> sp. C12	<i>Proteobacteria; Alphaproteobacteria</i>	(Bessa et al., 2017)
Endocrine-disrupting compounds	Estrone (E1)	<i>Sphingomonas</i> sp. Strain AHC-F <i>Sphingobium</i> sp. Strain AX-B	<i>Proteobacteria; Alphaproteobacteria</i>	(Ma et al., 2016)
		<i>Virgibacillus alotolerans</i> , <i>Bacillus flexus</i> , <i>Bacillus licheniformis</i> .	<i>Firmicutes; Bacilli</i>	(Fernández et al., 2017)
	17β-estradiol (E2)	<i>Bacillus</i> sp. <i>Klebsiella</i> sp	<i>Firmicutes; Bacilli</i> <i>Proteobacteria; Gammaproteobacteria</i>	(Khattab et al., 2019)
		<i>Rhodococcus zopfii</i> <i>Pseudomonas putida</i>	<i>Actinobacteria; Order: Actinomycetales</i> <i>Proteobacteria; Gammaproteobacteria</i>	(Menashe et al., 2020)
Herbicides, Pesticides	2,4-D	<i>Rhodococcus</i> sp.	<i>Actinobacteria; Actinobacteria</i>	(Carboneras et al., 2017)
	Atrazine	<i>Klebsiella variicola</i> Strain FH-1 <i>Arthrobacter</i> sp. NJ-1	<i>Proteobacteria; Gammaproteobacteria</i> <i>Actinobacteria; Actinobacteria</i>	(Gao et al., 2020)
Preservatives	Pyridine	<i>Paracoccus</i> sp. Strain NJUST30	<i>Proteobacteria; Alphaproteobacteria</i>	(Wang et al., 2018)
	Bisphenol A	<i>Sphingobium</i> sp. YC-JY1	<i>Proteobacteria; Alphaproteobacteria</i>	(Jia et al., 2020)
PFAS	PFOS/PFOA	<i>Acidimicrobium</i> sp. Strain A6	<i>Actinobacteria; Actinobacteria</i>	(Huang and Jaffé, 2019)
Flame retardants	TCEP	<i>Sphingobium</i> sp. strain TCM1	<i>Proteobacteria; Alphaproteobacteria</i>	(Takahashi et al., 2017)
DBPs	Bromate	<i>Aeromonas</i> sp. <i>Rivibacter</i> sp.	<i>Proteobacteria; Gammaproteobacteria</i> <i>Proteobacteria; Betaproteobacteria</i>	(Wang et al., 2019)
	NDMA	<i>Rhodococcus ruber</i> ENV425	<i>Actinobacteria; Order: Actinomycetales</i>	(Hatzinger et al., 2017)

		<i>Rhodococcus sp. Strain L4</i>		(Na-Phatthalong et al., 2019)
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2.4 Implications of Pre-Ozonation on Microbial Community in O₃-BAC Biofilters

Several studies examined advanced wastewater treatment by ozonation in combination with biofiltration (Gerrity et al., 2014; Gerrity and Snyder, 2011; Hollender et al., 2009; Kalkan et al., 2011; Lee et al., 2012; Reaume et al., 2015; Reungoat et al., 2012, 2010; Sidhu, 2016; Stalter et al., 2010; Yavich et al., 2004). There is general agreement on the positive outcomes of combining both technologies, which give higher water quality, a very effective way of removing trace organic compounds, decreasing non-specific and specific toxicity, sufficient inactivation of bacteria, viruses, protozoa, and parasites. The O₃-biofiltration treatment provides an additional barrier to remove organic contaminants before discharge into receiving water bodies. This barrier made up of the combination of oxidation (ozone) and adsorption and biological biodegradation enhanced both processes.

Ozone can destroy and break carbon-double bonds of organic structural groups and diminish the UV absorbance (Melin et al., 2002; Michael-Kordatou et al., 2018; Tripathi and Tripathi, 2011). UV₂₅₄ absorbance or transmittance is used as an indicator or surrogate of natural organic matter (NOM) concentration (Crittenden et al., 2012), and ozonation causes improvements to UV₂₅₄-indicated water quality by transforming NOM. Ozonation can transform refractory organic matter into smaller and simpler molecular weight compounds, which can be easily removed by biodegradation. The combination of ozone and biological filtration is one of the most promising technologies available to treat secondary effluents (Chen et al., 2018; Wang et al., 2008) to reduce NOM and other TOC.

According to earlier studies (Dempsey and Fu, 1994; Seger, 1996; Yordonov et al., 1996), the exact effect of ozone on the ecology of the bacterial community of the biofilter was neither well

understood nor well researched. However, after developing new tools and methods focused on DNA sequencing of microorganisms and understanding their metabolisms, the identification and characterization of microbial communities became attractive for quantitative and qualitative microbial ecology assessment (Douterelo et al., 2014). Tables 2-2 S1 and 2-3 contain a compilation of research focused on ozone impact on microbial communities in drinking water and wastewater applications. Most of the studies were focused on identifying influences on the microbial community due to influent characteristics, ozone disinfection, and environmental factors. There were more detailed studies concerning the microbial community in drinking water biofilters than those for wastewater (Table 2-2 vs. 2-3). Nevertheless, the current need to develop technologies or combine existing methods capable of producing potable reuse water quality from wastewater effluent emphasizes the need to explore and further investigate the fate of microbial communities in water reuse biofilters.

As for influent characteristics, significant differences have been reported between the biofilm's microbial community and the influent water. Li et al. (2017) reported that sand and BAC biofilms exhibit more common OTUs with their effluents than their influents. The hypothesis to examine here is whether the BAC filter is selecting specific bacteria (i.e., biofilm-promoted-gene carrier) or not. Other studies (Fonseca et al., 2001; Q. Li et al., 2017; Wang et al., 2013) suggest that complex and diverse communities are established after disinfection and filter pretreatments. Notably, influent water quality provides nutrients, electron donors/acceptors, redox conditions, among other variables that define the conditions that microorganisms require to carry out their function. Hence, these factors define the microbial communities that colonize the biofilters.

Table 2-2. Ozone impact on microbial communities in drinking water and potable reuse applications

Ozone Dose	Scale	Method	Microbial identification	Finding	Reference
O ₃ /DOC: 1.3 ±0.52	Pilot-scale	Biological activity rather than microbial community characterization . Phospholipid and fatty acid analyses, PLFA.	Out of scope	Higher activities in filters treated with ozone compared to non-treated. Biomass level from ozonated biofilter was 47% higher than non-ozonated.	(Fonseca et al., 2001)
Pre-ozonation (1.1 mg/L); RSF; Intermediate ozonation (0.5 mg/L)	Full scale	16s rRNA gene based 454 pyrosequencing	Actinobacter was abundant in raw water but not after ozonation. All filters showed similar phyla composition: Proteobacteria, Planctomycetes, Acidobacteria, Bacteroidetes, Nitrospira, and Chloroflexi.	Biomass concentration in RSF was 3x less than that for GAC and SFF. Diversity from high to low RSF (38 bands) > SSF (38 sand+43 schmutz)>GAC (24 bands).	(Lautenschlager et al., 2014)
Pre-ozonation 0.5 mg/L Post-ozonation 1.0 mg/L	Full-scale	PCR Amplification and 16S rRNA Sequencing	Proteobacteria, Actinobacteria, Acidobacteria, and Firmicutes dominant phyla in all samples.	Microbial community was relatively stable through treatment train in water and biofilm samples. Sand and BAC biofilms shared more OTUS with their effluent than their influent. Actinobacteria proportion decreased through treatment train while Proteobacteria increased. Chlorine disinfection decreased the proportion of Proteobacteria but increased Firmicutes and kept the same for Bacteroidetes.	(Q. Li et al., 2017)

Ozone Dose	Scale	Method	Microbial identification	Finding	Reference
Not mentioned	Full-scale	PCR - DGGE, 16S rDNA Sequencing, and community structure analysis.	Alphaproteobacteria and Betaproteobacteria were the dominant bacteria throughout the water treatment process. Bacteroides and Firmicutes were stage-specific and were the dominant bacteria in the early and later treatment stages	Diversity did not continuously change with treatment. Bacterial community structure and composition differ depending on the upstream process. I.e., Samples from preozonation, main ozonation, and active biofilter shifted compared to their influents.	(Tian et al., 2014)
0.9-1.5 mg/L	Full-scale	qPCR, 16S rRNA Illumina MiSeq Sequencing	Most abundant phyla: Proteobacteria (50-75%), Actinobacteria (0-34.1%) , Bacteroidetes (1-50%), Cyanobacteria (1.93-7.93%), Chloroflexi (0-3.07%)	Most changes in the bacterial community occurred before filtration, with ozonation having the greatest impact, especially negatively in Actinobacteria and positively on Bacteroidetes. Filter depth impacted the microbial structure. Total bacterial concentration fluctuation was confirmed the lowest after ozonation and highest at the filter effluent. Treatment process shapes microbial communities within the treatment plant.	(de Vera and Wert, 2019)

Ozone Dose	Scale	Method	Microbial identification	Finding	Reference
0.5-4.0 mg/L, no residual into the BAC filters.	Full-scale DWTP	16S rRNA high-throughput amplicon sequencing	Most abundant phyla: Proteobacteria (71-94%), Bacteroidetes (~3-14%), Acidobacteria (<1%) Nitrospirae (<1-3%)	Environmental factors strongly impact the microbial community richness and evenness. Ozonation enhance the bioavailability of organic substrate promoting a relaxed environment favoring the development of a diverse community and less specialized organisms.	(Li et al., 2021)
0-4.0 mg/L	Bench-Scale	PCR, High-throughput Illumina amplicon sequencing	Most abundant phyla: Proteobacteria (31.7-45.3%), Planctomycetes (29.9-44%), Acidobacteria (5.0-12.4%)	Microbial community not only is defined by influent characteristics but also environment conditions and operational parameters such as media type, EBCT, and ozonation. Because this study focused on CECs removals in BACs. CECs were spiked into the BACs, here microbial communities were impacted and develop unique microbial compositions.	(Zhang et al., 2018a)

Table 2-3. Ozone impact on microbial communities in wastewater applications

Ozone Dose	Scale	Method	Microbial identification	Finding	Reference
0-47.8 kg O ₃ /d / 0-0.15 g O ₃ / kg MLSS-h	Full-scale WWTP	FISH and conventional microscopy	Microbial community composition of filamentous bacteria, nitrifying bacteria, protist and metazoa.	Ozone selectively affected the different morphotypes of filamentous bacteria. No clear correlation between ozone dose and nitrifying community dynamics.	(Barbarroja et al., 2019)
0.15 g O ₃ / g MLSS	Two lab-scale bioreactors Non ozonated and ozonated sludge	PCR-DGGE and RFLP	Bacteria per function was reported.	Protease activity and intracellular ATP concentration increased, hence positive impact of ozonated sludge in diversity of microbial community and metabolic activity.	(Yan et al., 2009b)
0.02-0.14 g O ₃ / g TSS	Two lab-scale bioreactors	PCR-DGGE and RFLP	Quantitative identification rather than qualitative.	Inverse relationship between ozone dose and bacteria concentration.	(Yan et al., 2009a)
148 ±23 mg O ₃ /m ³	Two lab-scale biofilters operated in parallel.	qPCR, Illumina Miseq high- throughput sequencing	Dominated phyla: Proteobacteria, Actinobacteria, and Bacteroidetes (sum > 90%). More Proteobacteria and less Actinobacteria were identified in ozonated biofilter.	Viable dominant species do not change with ozone injection, but some ozone-tolerant genera were enhanced by ozone addition (<i>Rhodanobacter</i> , <i>Dokdonella</i> , and <i>Rhodococcus</i>) while others were sensitive to ozone (<i>Pseudomonas</i>)	(Saingam et al., 2018)
Ozonation process: Time 30 min at 1.2 L/min for reactors A, and 12 L/min for reactors B.	Four batch experiments	Illumina MiSeq sequencing	Reactor 1A: Proteobacteria (45%); Acidobacteria (11%); Chloroflexi (10%); Actinobacteria (8%). Reactor 1B: Proteobacteria (40%), Gemmatimonadetes (12%), Chloroflexi (11%), and Bacteroidetes (9%). Reactor 2A: Proteobacteria (50%), Chloroflexi (10%), Acidobacteria (9%), and Actinobacteria (8%). Reactor 2B: Proteobacteria (63%), Chloroflexi (7%), Firmicutes (5%), and Gemmatimonadetes (5%)	Low residual ozone can change the composition of EPS fractions, improve bioactivity, and adjust the structure of the microbial community.	(Fu et al., 2019)

Ozone Dose	Scale	Method	Microbial identification	Finding	Reference
O ₃ /DOC: 0.80	Both pilot and full-scales	DNA extraction coupled with 16S rRNA gene sequencing	Most abundant phyla: Proteobacteria, Acidobacteria, and Cyanobacteria for DWTP. For WWTP, RP1 Proteobacteria were also detected but lower proportion, highest diversity. RP2 most abundant phyla was Proteobacteria. Microbial community structure Ozone vs Non-ozone at RP2 identified higher proportion of Nitrospirae in the latter, all other phyla were in similar proportion.	Proteobacteria highest relative abundant in DWTP. Backwash frequency can shape the microbial community within the BACs.	(Gerrity et al., 2018)
5-15 mg/L	Pilot scale	DNA extraction coupled with 16S rRNA gene sequencing.	Proteobacteria, Nitrospirae, Planctomycetes, Firmicutes, and Bacteroidetes were the most abundant phyla.	Microbial communities were similar independent of the upstream AOP. Microbial community composition and diversity change across the biofilter bed, showing the highest variations at the top. Biofilter bed depth influenced the microbial population structure more than upstream treatment.	(D. Li et al., 2017)
3.5 mg/l	Pilot-scaled advanced purification facility.	DNA sequencing based analyses and flow cytometry.	Reported at family level. At phylum level mostly Proteobacteria, Actinobacteria, and Bacteroidetes.	Microbial communities at the three GAC filters are similar, but the one with higher EBCT showed more diversity and abundance.	(Kantor et al., 2019)

Concerning ozone effects, biofilters treated with pre-ozonation showed higher microbial activity compared to non-ozonated influents (Table 2-3). This is most likely due to the increased biodegradability of bulk organic matter upon ozonation and hence higher microbial activity in the subsequent biofilter (Abdul Hamid et al., 2019; Hooper et al., 2020; Li et al., 2021; Sundaram and Pagilla, 2020; Zhang et al., 2018b). The fluctuations in total bacteria concentration were the lowest after ozonation and highest at the filter effluent. The listed studies suggest that regardless of low influent microbial community concentration, biofilters with pre-ozonation can be populated and actively biodegrade, assimilate, metabolize NOM and display higher metabolic activities. Returning to the identification of microorganisms up to the phyla level, the most abundant was Proteobacteria. The ozone impact on Actinobacter is higher compared to that on Bacteroidetes.

In summary, three main ideas can be extracted from the combination of ozonation and BAC filters in wastewater, and drinking water treatment process applications are: i) O₃-biofiltration creates a complex microbial ecosystem, ii) ozonation significantly shapes downstream microbial communities in biofilters by making more substrate bioavailable, and iii) the scientific literature mostly addressed microbial communities in drinking water and wastewater, and there is a lack of scientific literature about the microbial community in biofilters of advanced water reclamation for water reuse applications. Also, several research questions arise on how to shape the microbial community by providing conditions to make them specialized for specific tasks in BAC filters for water reclamation (for example, NDMA degradation in the BAC following ozonation).

2.4.1 Pathogens in Biofilter Effluents

Recent studies (Kantor et al., 2019; D. Li et al., 2017) comparing microbial communities upstream and downstream of the O₃-BAC process have shown that they are similar, validating that

ozonation inactivated microbial cells but do not damage the DNA structure. This raises many questions regarding whether a biofilter effluent quality is reliable and safe enough to guarantee potable water free of pathogens and the needed disinfection steps downstream. Some studies (Liu et al., 2019; Nemani et al., 2018; Tian et al., 2014; Zhang et al., 2018b) have detected putative pathogens in the biofilter media and its effluent. Within the identified pathogens were *Legionella pneumophila* and *Plesiomonas shigelloides*, both belonging to Proteobacteria; some Firmicutes such as *Staphylococcus aureus* and *Bacillus cereus*; and Actinobacteria such as *Mycobacterium gordonae*. It must be noted that their relative abundance was very low (<0.09%) in both the biofilter media and effluent and that the most frequently detected were *M. gordonae* and *L. pneumophila* (Lin et al., 2014; Wang et al., 2013; Zhang et al., 2018b). More recent evidence (Liu et al., 2019) highlights that pre-ozonation apparently can reduce gene markers of pathogenic species (*Legionella* spp., *Mycobacterium* spp., *P. aeruginosa*, *Acanthamoeba* spp., and *Aeromonas* spp.). In addition, the ozone acting as an oxidizing agent favored more biodegradable substrate (e.g. BDOC and AOC) triggering a vertical gradient across the BAC filter, limiting both growth and potential regrowth and promoting competition for nutrients and living space between pathogenic and non-pathogenic organisms. Stamps et al. (2018) suggested that filtration treatment units shape microbial community effluent either by sloughing off and reappearing downstream, but more importantly, by diminishing the concentration of nutrients (i.e., TOC, ions) in the treated water resulting in a reduction of microorganism growing rates. However, this area of pathogens in biofilters effluents have been overlooked, and the vast uncertainty about the potential bacteria detachment from filter media should get more attention. The focus should be on factors and mechanisms that allow their occurrence and persistence in finished treated water and potential influence on final discharge or reuse applications.

2.5 Research Questions to Be Addressed

Despite the progress resulting from several investigations like those analyzed in this critical review, key questions still need to be investigated regarding the implementation of O₃-BAC systems in advanced water reclamation. Particular critical needs and gaps that must be addressed to understand the development and behavior of microbial communities in biofiltration in reclaimed water treatment include:

- Is there a particular microbial community (specialists) enriched in the biofiltration process to treat wastewater trace organic constituents, or do generalists develop specialized functions under biofilter process conditions? Current and available studies failed to answer this question. Studies have focused separately on quantifying contaminants removal and microbial community qualitative assessments. The need is to combine both to gain insights about whether the bacterial community populating a biofilter can be engineered to achieve a particular objective or not.
- Is ozonation able to shape the microbial community in the BAC biofilter? Literature suggests it to be true for DWTP applications; however, the question remains unanswered for WRRF O₃-BAC applications.
- What is the final microbial community in the biofilter treated water? Apparently, microorganisms in the finished water will be: i) survivors from the pre-ozonation step before biofiltration and are not able to attach to media bed; ii) microorganisms that prefer a "free-surface" lifestyle; and/or iii) simply microorganisms that detach from media. Solving this question is important because a more biostable and safer finished reclaimed water can

be produced if those organisms and their pathogenic or non-pathogenic traits are identified.

- Besides the external environmental factors, do these microbial communities change due to other pretreatment steps such as chemical treatment (for example, coagulation-flocculation) before biofiltration? In advanced water treatment applications, there are multiple combinations to set up the treatment barriers (Marron et al., 2019).

Understanding the upstream processes on the microbial community in biofilters is crucial to position O₃-BAC as a robust, reliable, and preferred barrier over other options.

- The lack of investigations on the potential leaking of pathogens into the finished water in water reuse applications when O₃-BAC units are implemented was identified. To ensure biologically stable water downstream, a biofilter standardized qualitative and quantitative methods to detect and analyze low microbial community concentrations are necessary and urgent.

Overall, future work should attempt to identify major species in the microbial community consortium and link and associate the microbial community with its inherent capabilities, abilities, properties, and functions. The richness, dynamic, and evenness parameters of a microbial community rather than the biomass content are the governing characteristics and controlling factors to study and comprehend. Besides, the relationships and interaction among the microbial communities and the ecosystem and the environment where they belong are additional characters that should be further investigated to understand the microbial consortium's behavior, performance, and resilience.

2.6 Conclusions

The endless need to provide safe, stable, and reliable water to all consumers has obligated engineers and scientists to study effective and efficient treatment alternatives. The combination of ozonation (oxidation) and biofiltration (adsorption and biological degradation) is a promising technology that can overcome many limitations of conventional water reuse treatment process trains because they reliably remove biodegradable organic matter trace organic pollutants, byproduct precursors, and concerning substances. One key aspect of biofiltration as a treatment process is the success of its performance and the inherent efficiency of the microorganisms involved in biodegradation processes.

A broad understanding and monitoring of the microbial community are vital in any biological system application to ensure sustainable water quality. Though research involving qualitative assessments of microbial communities in biofilter has been developed, supplementary efforts must take place to accept it for water reuse applications fully. Learned experiences and strategies used in drinking water biofilter applications will serve as guidance to propose appropriate methodologies, protocols, and control plans of the microbial process involved in advanced water reclamation biofiltration processes.

Future studies on the microbial ecology through the influent to finished water and the ability, performance, and potential adverse effects of biofilters in advanced water treatment applications are needed. To this purpose, full or pilot-scale should be prioritized to describe those relationships better.

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Chapter 3

MICROBIAL COMMUNITY CHARACTERIZATION IN ADVANCED WATER RECLAMATION FOR POTABLE REUSE

This research phase aims to characterize the microbial community of two conventional barriers for advanced water treatment in potable reuse application: Coagulation/Flocculation/Clarification/ Granular Media Filtration (CFCGMF) and Ozonation/Biological Activated Carbon filtration (O3/BAC). This study was conducted parallel with the Extended pilot-testing of Ozone-BAC treatment processes and the WesTech Trident High Solids filter pilot unit during the 2018-2019 period at Reno, Nevada. Both barriers were monitored and optimized to remove unregulated constituents, trace and bulk organics, and pathogens reduction. In detail, the impact of CFCGMF and O3/BAC treatment processes on the abundance, structure, and composition of the microbial community involved in each process is investigated. This chapter was submitted for publishing to Applied Microbiology and Biotechnology Journal, and it has been under revision (No. AMAB-D-21-00991R1) since July 23, 2021.

3.1 Introduction

Microbial communities have proliferated in every ecosystem known, and their functional role in natural and engineered systems has been explored across multiple disciplines such as agriculture, environmental science, medicine (Huttenhower et al., 2012; Philippot et al., 2013; Wu et al., 2019). For instance, the abundance and richness of microbes in a given human body part

have been linked to diseases (Cho and Blaser, 2012; David et al., 2014). Identification and characterization of microbial communities have become attractive, not only as quantitative studies but also as qualitative assessments of the organisms in their ecosystem and their specific roles in the system that they belong. The development of techniques and analytical methods to understand microbial species' role in biological processes in environmental systems gained popularity in the '90s. Researchers looked to gain a complete picture of the members and the bacterial communities' dynamics (Jenkins, 2008). For instance, analyzing 16S rRNA complemented by metagenomic analyses can provide information regarding the genes responsible for a specific process and the role of the species studied in environmental remediation (Little et al., 2008).

In a water treatment context, biological treatment by microorganisms is a sustainable and cost-effective option (Benner et al., 2013). However, water quality and microbiological safety concerns emerge when microbial quality is not met (Prest et al., 2016). Safety concerns extend further in water reclamation (wastewater treatment) for potable reuse due to potential risks posed by pathogens and constituents that support pathogen persistence in reuse water. In potable reuse projects, the protection of public health is the major driver that defines both treatment requirements and pathogens and chemical control criteria. Several studies have focused on microbial communities' characterization in drinking water and wastewater, but limited work has been conducted in water reuse systems (Gerrity et al., 2018; Kantor et al., 2019; Q. Li et al., 2017; Oh et al., 2018; Stamps et al., 2018; Zhang et al., 2018b). Therefore, the interest in exploring microbial communities' structural and functional characteristics to decipher their composition, richness, and behavioral properties is essential to optimize biological processes and upstream effects on water reclamation systems for potable reuse.

The present study was conducted as a feasibility demonstration to evaluate whether indirect potable reuse (IPR) of highly purified reclaimed water could offer water supply benefits. The treatment system was developed with multiple barriers against pathogens and chemical contaminants referred to as a multi-barrier approach. This multi-barrier approach was selected to investigate advanced water purification at a pilot scale using filtered secondary wastewater effluent as feed water. The pilot project was developed to evaluate the suitability and feasibility of two different treatment steps, namely: Coagulation/Flocculation/Clarification/Granular Media Filtration (CFCGMF) and a combination of ozonation (O_3) and biological activated carbon (BAC) filtration (O_3 /BAC). During this pilot testing, Sundaram et al. (2020) described detailed findings regarding the O_3 /BAC process capabilities concerning trace and bulk organic constituents' removal. This paper focuses on the microbial community characterization and presence of pathogens in water produced by the two treatment steps.

The research involved microbial community assessment, including multiple sampling locations across the treatment processes under steady-state operation. Diversity analyses were conducted based on 16S rRNA sequencing on Illumina MiSeq (RTL Genomics, Lubbock, Texas, USA) to compare microbial communities before and after each treatment process; namely, CFCGMF and O_3 /BAC, in order to i) characterize the abundance and diversity profile of the microbial community inherent to each process, ii) evaluate the effects of each process on the core microbial community structure and composition, iii) identify potential pathogens and resistant species that can feed downstream treatment steps. This information explains microbial communities' dynamics, changes, and fate through these two barriers in advanced water purification of reclaimed water for potable reuse.

3.2 Materials and Methods

3.2.1 Pilot Scale Study System

Water and media samples were collected from the pilot-scale treatment units between November 2018 to January 2019 at the South Truckee Meadows Water Reclamation Facility (STMWRF) (Reno, NV, USA). The STMWRF consisted of a nitrification-denitrification activated sludge process with a mean cell residence time (MCRT) of 10 days and a capacity of 15,520 m³/d, followed by Parkson's DynaSand continuous backwash sand filters and chlorine-based disinfection. A portion of unchlorinated STMWRF filtered effluent (70-109 m³/d) was diverted as influent or fed water into the treatment steps of the pilot-scale system. The first one, CFCGMF, known as Trident HS Pilot unit, was provided by Westech Engineering (Salt Lake City, UT, USA). This unit consisted of chemical coagulation-flocculation-sedimentation followed by up-flow buoyant media and compressible media adsorption clarifier and downflow mixed media filter to produce high-quality water. The second unit was a Xylem Oxelia™ ozone-BAC (O₃/BAC) process provided by Xylem (New York, USA). The unit consisted of two stainless steel tanks (0.53 m³ each), providing an ozone contact time of 20 minutes after injection. Following ozonation, the flow was directed to two BAC (BAC1 and BAC2) filters that were operated in parallel with identical media to perform biological treatment of constituents in reclaimed water. The empty bed contact time (EBCT) of BAC1 and BAC2 were 10 and 20 minutes, respectively. For coagulants and polymers used in CFCGMF, ozone dose in O₃/BAC unit, and other operational details of both pilot units, see Table S1 and S2 (Supplemental Information). The schematics of both treatment steps are shown in Figure 3-1.

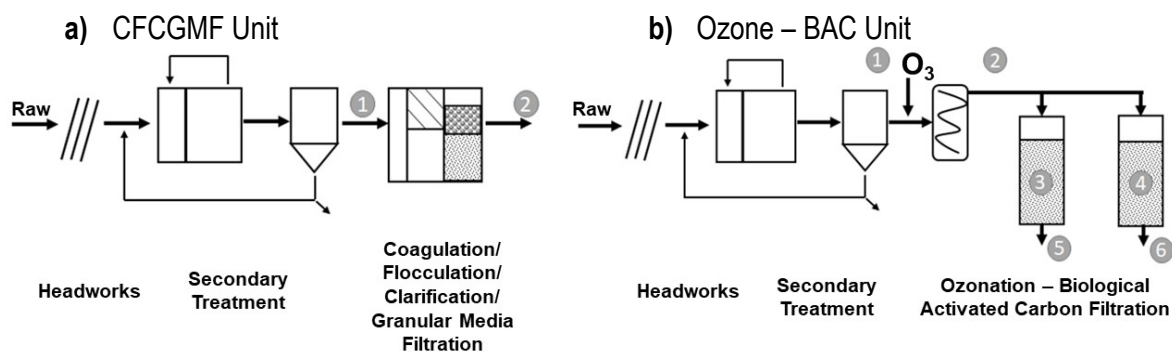


Figure 3-1. Pilot scale treatment unit schemes of a) CFCGMF and b) O₃/BAC filter. Sampling locations are shown with numbers in each scheme: 1) CFCGMF influent (CFCGMF INF), 2) CFCGMF effluent (CFCGMF EFF) and 1) Ozone influent (O₃ INF), 2) Ozone effluent (O₃ EFF), 3) BAC 1 media filter (MBAC1), 4) BAC 2 media filter (MBAC2), 5) BAC 1 effluent (BAC1), and 6) BAC 2 effluent (BAC2).

3.2.2 Data Collection and Sample Processing

Five sampling campaigns were conducted using ozone to total organic carbon (TOC) ratio in water (O₃/TOC ratio) as follows: 0, 0.9, 1.5, and 2.0 (referred to as SE0, SE1, SE2, SE3, and SE4, respectively) in the O₃/BAC unit, whereas two sampling campaigns were held at the CFCGMF unit under steady-state operating conditions. A sample volume of 2 L was collected for water samples at locations as shown in Figure 3-1 using sterile amber bottles. Biomass media samples from both BACs were collected on the same dates as the liquid samples. Samples were transported on ice to the laboratory; then, the liquid samples were filtered through 0.2 μm sterilized membrane filters to recover suspended biomass. DNA from filters and media samples was extracted using DNeasy PowerWater Kit (QIAGEN, USA) and DNeasy PowerSoil Kit (QIAGEN, USA), respectively. Once the DNA was extracted from each sample, the DNA extracts were

sequenced for 16S rRNA (V4 region) on Illumina MiSeq platform (RTL Genomics, Lubbock, Texas, USA). The primers used for DNA amplification were the 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTTC TAAT-3') as recommended by (Caporaso et al., 2010). RTL Genomics analyzed microbial community diversity in both liquid and solid phases following their data analysis pipeline.

3.2.3 Bioinformatics and Statistical Analyses.

The sequencing data of the 16S rRNA amplicons were processed following the analysis pipeline of RTL Genomics. In brief, paired-end reads were trimmed and merged using PEAR Illumina, then denoised, and chimera checks were performed before the downstream process. The number of sequences per sample ranged from 12089 to 39366. Chimera detection and removal were done by using UCHIME in *de novo* mode after denoising the data. To obtain the identity of each remaining sequence, samples were demultiplexed. Then using the UPARSE algorithm (Edgar, 2013), sequences were clustered into operational taxonomic units, OTUs. Taxonomic information is added to each sequence after running each cluster against the USEARCH global alignment program. The data is identified by employing a database of high-quality sequences derived from the NCBI database. Once sequencing data were obtained, all data processing related to microbial community analysis were made following different pipelines (Callahan et al., 2016; Shetty et al., 2018). The *phyloseq* R-package (McMurdie and Holmes, 2013a) was used to manage the sequencing data capable of supporting sequencing importing data format from RTL Genomics. All the analytical techniques used, such as diversity analysis, ordination methods, quality plots, and microbiome detail analyses, were performed using this package. Normalized data were used to determine the observed number of OTUs and to estimate the Shannon Index, Chao1, and Simpson's Diversity Index using R statistical software, version 4.0.3 (R Core Team, 2020) using

packages phyloseq version 1.34.0 (McMurdie and Holmes, 2013a) vegan version 2.5.7 (Oksanen et al., 2020), and microbiome version 1.12.2 (Lahti and Shetty, 2019). All figures were developed using ggplot2, version 3.3.3 (Wickham, 2016).

3.3 Results

3.3.1 Core Microbial Community Description

A core microbial community analysis was performed for the two treatment processes studied. The core microbial community was defined using a threshold for detection with an absence/presence ratio of 0.001 and a prevalence of 0.50. Combining both treatment steps, a total of 3801 OTUs were detected. A total of 38% of these OTUs were detected at the CFCGMF unit process, whereas 98% were detected in O₃/BAC. The identified core microbial members in the CFCGMF and O₃/BAC processes were 386 and 542 OTUs. Comparing both core microbial communities, Figure 3-2 shows the beta diversity or dissimilarities between the 30 samples collected during the sampling campaigns. These dissimilarities between samples are based on their microbial community composition and structure. For further details, the values of diversity indices for each sample are shown in Table S3.

Samples were grouped based on where they were collected (location) and sampling event (SE). The location group assessed the microbial community before and after each treatment process. In contrast, the sampling event checked whether or not time affected or impacted the microbial community in the samples. A beta dissimilarity analysis was performed to characterize the difference between samples. Two different analyses were conducted, one based on the presence/absence of different taxa (Unweighted Unique Fraction (Unweighted UniFrac) in Figure 3-2 left panel) and the other that considered the abundance of the different taxa identified in all the

samples analyzed (Weighted Unique Fraction-Weighted UniFrac, Figure 3-2 right panel). Both included phylogenetic information; hence, the distance between communities was defined as the fraction of branches of the phylogenetic tree that lead to community members being compared but not to all.

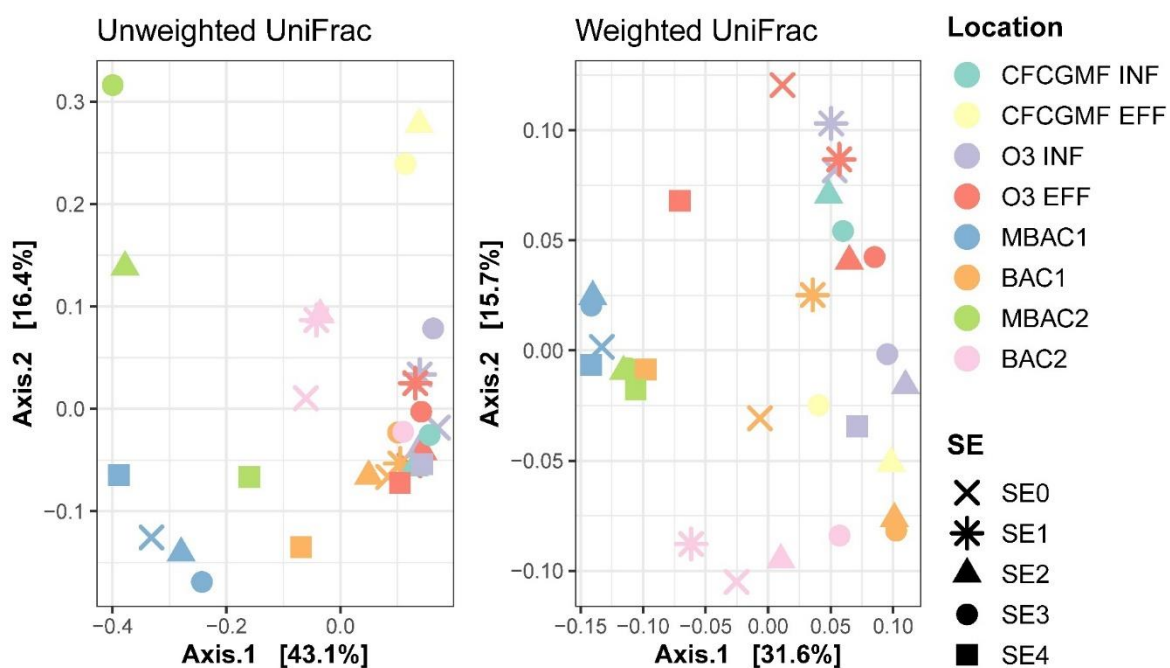


Figure 3-2. Ordination analysis for each microbial community for all the samples by principal coordinate analysis (PCoA) with Unweighted UniFrac and Weighted UniFrac. Point shapes indicate sampling events when samples were collected, and colors indicate the sampling location.

3.3.2 CFCGMF Process

CFCGMF influent and effluent samples were collected during two sampling events (SE2 and SE3), resulting in four samples. CFCGMF effluent samples showed lower diversity (Shannon Index: 3.8) and richness (Chao1: 494) compared with influent samples (Shannon Index = 4.05 and Chao 1 = 873). The identified phyla that covered more than 90% of the total identified OTUs in the

effluent were: *Proteobacteria* (67.5%), *Bacteroidetes* (6.7%), *Actinobacteria* (4.1%), *Firmicutes* (2.4%), and unclassified microorganisms (18.5%). In contrast, the effluent samples showed a shift in the relative abundance of the microbial community detected with an increase of *Proteobacteria* and *Firmicutes* to 72.6% and 4.4%, respectively. *Bacteroidetes* were 3.4%, no *Actinobacteria* was identified, but *Verrucomicrobia* (0.2%) phylum completed the phyla list (unclassified phyla were 18.5%) in the effluent (Figure 3-3).

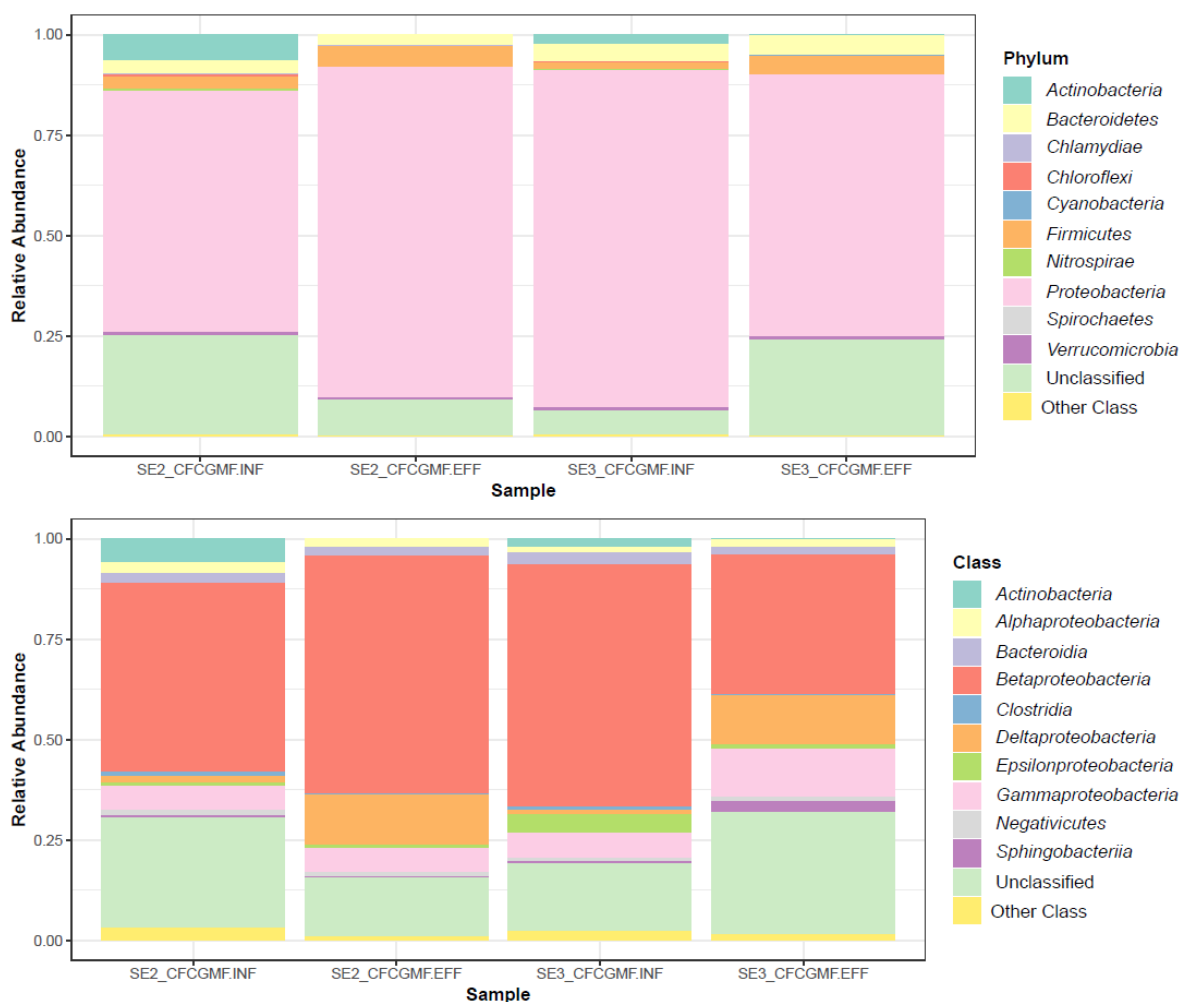


Figure 3-3. Upper panel: Relative abundance of top 10 phyla in the core microbial community across the CFCGMF treatment process. The lower panel: Distribution of all bacteria depending on taxonomic class for each sample analyzed.

3.3.3 O₃/BAC Process

A total of 542 OTUs were identified in the O₃/BAC train as the core microbial community.

Those OTUs detected in more than one location (variable OTUs) corresponded to 52% of the total OTUs. The remaining 48% were the unique OTUs detected at only one of the sampling locations.

The locations that showed the most uniqueness were the BAC and the ozonation effluents (as seen in Table S4). In contrast, the BAC media yielded the lowest diversity of OTUs.

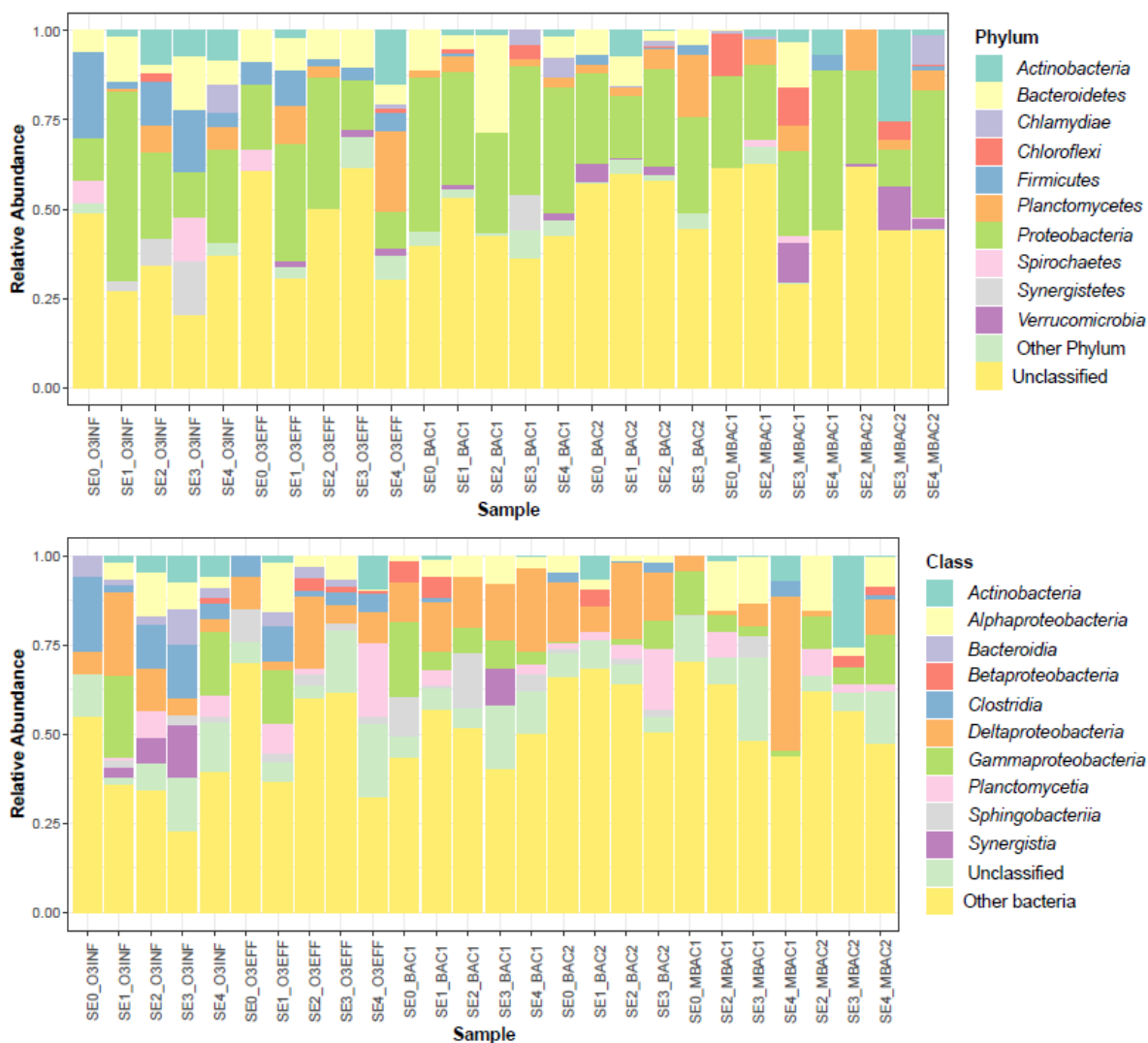


Figure 3-4. Upper panel: Relative abundance of top 10 phyla in the core microbial community across the O₃/BAC treatment process. Lower panel: Distribution of all bacteria depending on taxonomic class for each sample analyzed.

The O₃/BAC process yielded a large microbial diversity. There were 12, 14, 10, 12, 9, and 11 different phyla identified in the O₃ INF, O₃ EFF, MBAC1, BAC1 effluent, MBAC2, and BAC effluent, respectively. Taxonomic assignment showed (Figure 3-4) that the most abundant phylum was *Proteobacteria* at all four sampling locations, followed by *Bacteroidetes*, *Actinobacteria*, *Firmicutes*, and *Chloroflexi*, covering more than 90% of the total relative abundance, including 32.5% for OTUs that were unclassified microorganisms.

3.3.3.1 Ozone Impact

A total of 204 OTUs corresponding to 38% of the core microbial community were present at least once, either before or after ozonation. These were identified as OTUs impacted by ozone. The remaining OTUs were detected at other locations but not at ozone-related locations (O₃ INF and O₃ EFF). Also, those OTUs impacted by ozone were classified in three categories: survivors, eliminated, and occurred OTUs based on ozonation impact: i) O₃ survivors are those that were detected at both influent and effluent of the ozonation step; ii) O₃ eliminated were those OTUs that were not detected after ozonation even though present in influent samples, iii) O₃ occurred correspond to OTUs that were not detected in the influent but were identified after ozonation. The OTUs abundance order based on ozone impact, from highest to lowest, showed no effect, O₃ occurred, O₃ survivors and O₃ eliminated with 62%, 16%, 12%, and 10%, respectively (Figure 3-5).

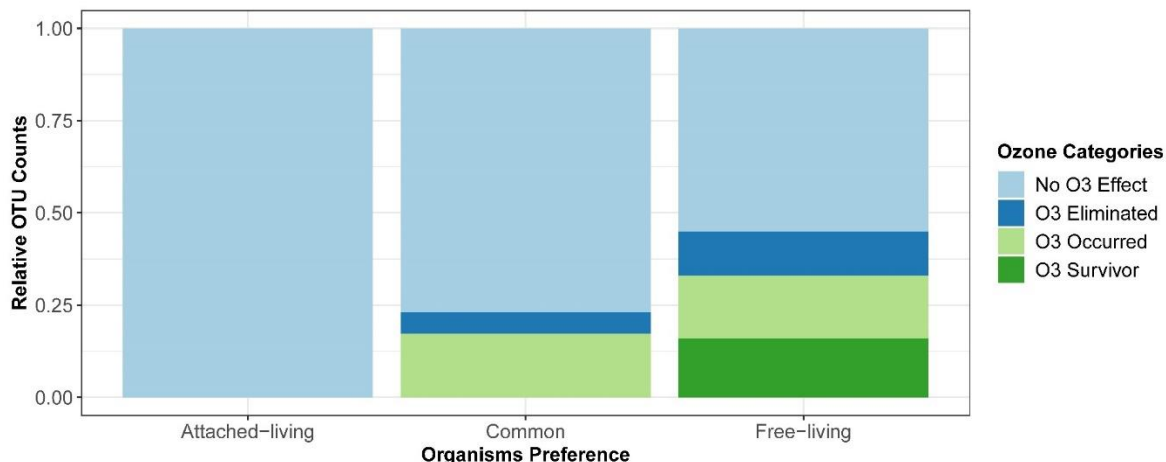


Figure 3-5. Relative abundance of ozone impacted OTU categories for microorganisms based on their life-style preferences.

3.3.3.2 Biological Filtration Impact

A modified approach based on Pinto et al. (2012) was used to understand how the BAC filters shaped the microbial community. All OTUs identified in the core microbial community were classified into eight categories based on their relation or lack thereof with the biological filters. The leaky colonizers (LC) were those OTUs identified in both media filters and BAC effluents. The strict colonizers (SC) were those OTUs detected in the filter media and locations upstream of the filter, but never after the BAC filtration. Because SC can display special features, two categories were added. First, those organisms are exclusively found in both media filters as “both strict colonizers” (SCb). Second, the unique strict colonizer (SCu) those OTUs found at BAC1 or BAC2 but not in any other location.

The fifth category, pass-through (PT) OTUs, is detected before and after the BAC filter but not in the media, suggesting organisms that cannot form biofilms or are not strong competitors compared to those strict and leaky colonizers. The sixth and seventh category groups were those OTUs detected only after BAC filtration, FOu corresponded to those unique OTUs identified at

each BAC filter, and FO as OTUs detected at both BAC filters. The final category was the filter-independent (FI) group, which comprised OTUs never detected on media filters but with some occurrence at other sampling locations. A total of 62 OTUs were classified as strict colonizers corresponding to 1.8%, 4.4%, and 5.2% (from total OTUs) as SCb, SC, and SCu. The next category, LC, comprised 63 OTUs, corresponding to 11.6%. The pass-through OTUs were 68 (12.5%). The filter occurring OTUs included 220, of which 6.6% and 37.1% were classified as filters occurring at both BACs (FO) and unique (FOu) to each BAC, respectively. Finally, the FI OTUs comprised 112, corresponding to 20.7% of the total abundance. Figure 3-6 shows the ozone impacted categories for all detected microorganisms based on their preference to form biofilms.

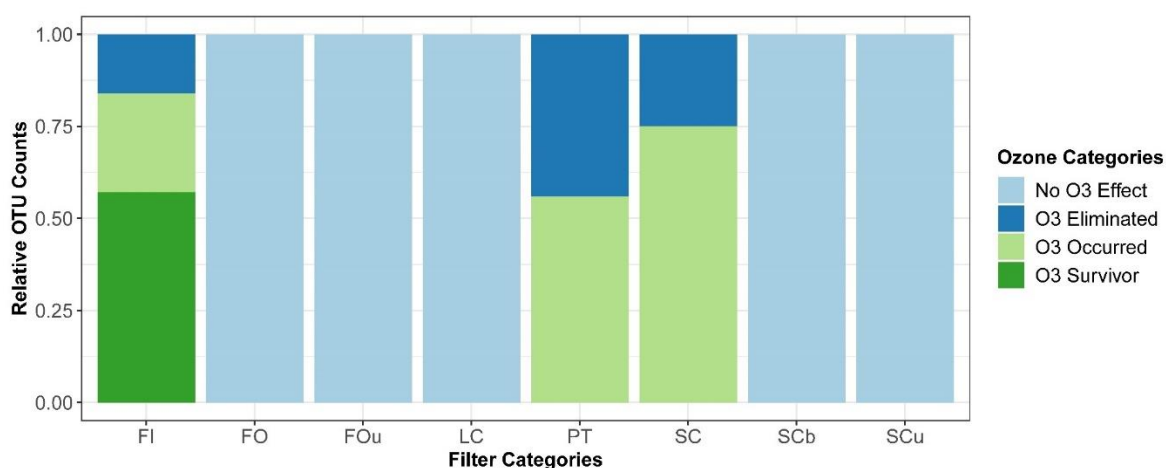


Figure 3-6. Relative OTU abundance of ozone-treated water across the eight filter categories defined (FI: filter independent, FO: filter occurred, FOu: unique filter occurred at either of the BAC filters, LC: leaky colonizer, PT: pass-through, SC: strict colonizer on BAC's media, SCb: strict colonizer and found in both BAC filters, SCu: strict colonizers and found at either of the BAC filters).

3.3.4 Identification of Potentially Pathogenic Bacteria

A total of 19 (4.9%) and 8 (1.5%) OTUs identified as potential pathogenic bacteria were detected at the CFCGMF and O₃/BAC treatment processes, respectively. The percent of pathogenic bacteria relative to core microbial communities identified for each process were about

2.57, 1.57, 0.40, 0.05, 0, 0.17, 0.08, and 0.35% for CFCGMF INF, CFCGMF EFF, O₃ INF, O₃ EFF, MBAC1, BAC1, MBAC2, and BAC2. At the CFCGMF unit, in descending order of relative abundance, the pathogenic bacterial genera were *Mycobacterium* (31.3%), *Legionella* (20.9%), *Aeromonas* (20.8%), *Enterobacter* (7.7%), *Flavobacterium* (6.9%), *Pseudomonas* (6.4%), *Escherichia* (3.8%), and *Clostridium* (3.1%), in both influent and effluent samples. In contrast, only three genera were detected at the O₃/BAC unit. In descending order of counts, these were identified as *Legionella* (47%), *Clostridium* (37.9%), and *Flavobacterium* (15.1%). None of these three genera were detected at the MBAC1, while at MBAC2, only *Clostridium* was found, as seen in Figure 3-7.

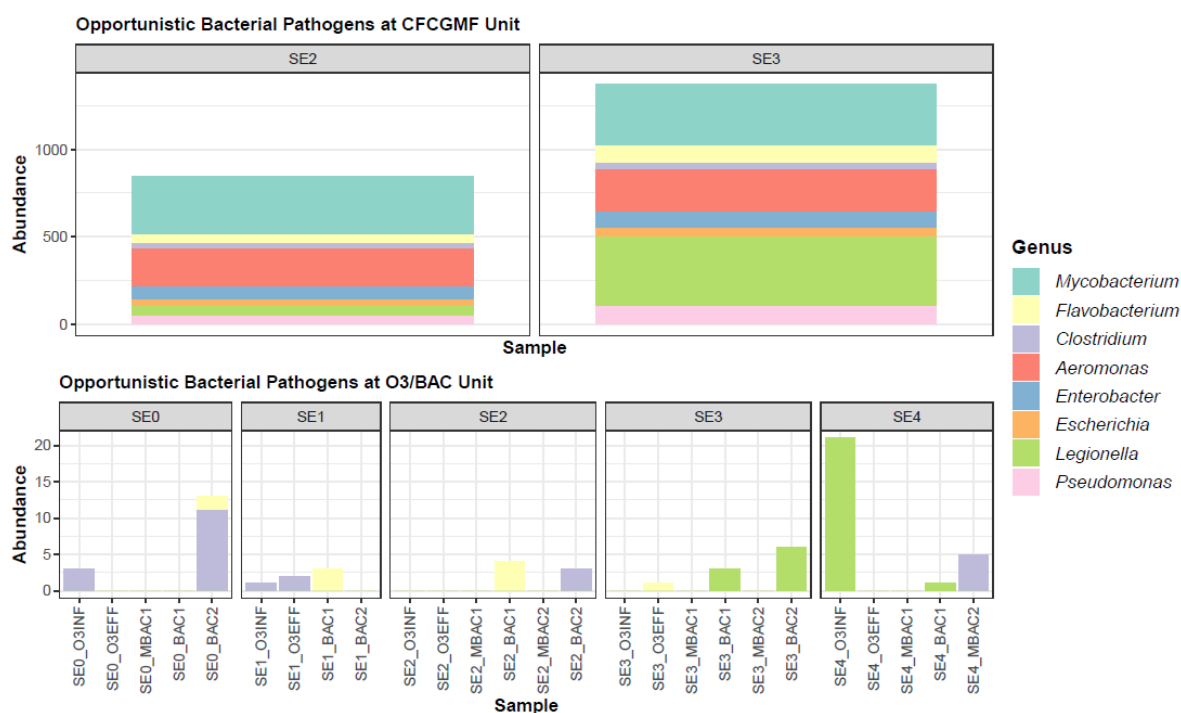


Figure 3-7. Detected opportunistic bacterial pathogens at genus taxonomic levels for CFCGMF process (upper panel) and O₃/BAC process (lower panel).

3.4 Discussion

The microbial community characterization of the two processes studied was emphasized, reviewed, and identified by evaluating the bacterial composition and structure, thus providing insights into the microorganisms involved in each treatment process. Before and after each primary process, the microbial community abundance was measured to change across each treatment process. These changes were examined according to potential impacts from the water quality matrix, environmental factors, and the individual bacteria traits that specify and establish inherent phenotype preferences and behavioral tendencies. When comparing both treatments, including all the 30 samples, Figure 3-2 shows the Unweighted UniFrac approach, which clusters samples based on location: BAC1, CFCGMF EFF, and joined CFCGMF-INF, O₃ INF, O₃ EFF. Samples collected for media of BAC2 (MBAC2) were spread vertically, which reflected that these particular community members did have different taxonomic characteristics with time.

Conversely, Weighted UniFrac analysis accounted for more structural aspects of the microbial community of each sample. The media filter samples (MBAC1 and MBAC2) and one sample from BAC1 collected when the system was under higher ozone dose tested (SE4) was closer, which implied similarity in their microbial structure. Comparing both approaches, Unweighted UniFrac distributed the sample points into more distinguished clusters.

3.4.1 Microbial Community Characterization for Each Process

The relative abundance of the core microbial community in CFCGMF process at the phyla level is shown in Figure 3-3. The most abundant phyla found were *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*. Their relative abundance and respective proportions did not differ significantly ($p=0.67-0.77>0.05$). At the class level, the relative abundance of classes belonging to

the *Proteobacteria* phylum did change. From high to low: *Betaproteobacteria*, *Gammaproteobacteria*, *Epsilonproteobacteria*, *Alphaproteobacteria*, and *Deltaproteobacteria* were detected in the influent. The CFCGMF effluent exhibited a change in composition, whereas the relative abundance of *Betaproteobacteria* decreased (55.2% to 49.1%), and *Deltaproteobacteria* increased (1.3% to 12.3%). Further inspection at the genera level revealed the most significant structural change of CFCGMF effluent microbial community, with *Bdellovibrio* genus being the most abundant. Microorganisms classified under this genus are aerobic obligate predatory bacteria that usually prey on various Gram-negative bacteria (Bratanis et al., 2020; Williams and Chen, 2020). Therefore, their persistence, survival rate, and resilience after coagulation-flocculation-filtration and granular media filtration can be associated with this particular trait.

Characterization of the microbial communities associated with the O₃/BAC process showed that *Proteobacteria* accounted for 19.9-33.7% of the total OTUs in the core microbial community when averaged for all six sampling locations. The changes of phyla profiles for relative abundance across the O₃/BAC process described how the microbial community is affected. For instance, the phylum *Proteobacteria* was impacted by ozonation; its relative abundance was reduced on average by 36.4%, whereas *Planctomycetes* increased 4x its relative abundance. *Actinobacteria* and *Firmicutes* relative abundance decreased along the O₃/BAC treatment process. *Bacteroidetes* decreased after ozonation and in the media samples (it was not detected at MBAC2). However, it was within the two most relatively abundant phyla at the BAC filter effluents, suggesting a low preference for attached-living (biofilm) conditions in contrast to previous observations (Gerrity et al., 2018; Peng et al., 2018; Saingam et al., 2018). *Verrucomicrobia* phylum was not detected in any of the influent samples to the O₃/BAC process. However, it was detected after ozonation, followed by an incremental increase on both media filters and then a decrease in the filter effluents.

This phylum and *Proteobacteria*, *Nitrospirae*, *Bacteroidetes*, *Acidobacteria*, *Planctomycetes*, and *Actinobacteria* were reported as dominant in biofilm samples of water reuse treatment systems (Zhang et al., 2019). Regarding the relative abundance in the media samples, *Proteobacteria*, *Planctomycetes*, and *Actinobacteria* were the most common phyla reported elsewhere (Zhang et al., 2018b).

Despite *Proteobacteria* being the most abundant phylum, the immense diversity and richness of bacterial groups can be noticed by the high proportion of unclassified microorganisms within the samples. Figure 3-8 shows in detail how the *Proteobacteria*'s classes were distributed across each sampling location. Within this phylum, four classes were detected: *Alphaproteobacteria*, *Betaproteobacteria*, *Deltaproteobacteria*, and *Gammaproteobacteria*. *Alphaproteobacteria* increased its proportion by 1.6-1.8 times in the BAC filter effluents compared to the O₃/BAC process influent. *Betaproteobacteria* was the only *Proteobacteria* class that was not detected in all sampling locations. There were no OTUs related to this class at the BAC1 media samples despite being detected before (O₃ EFF) and after (BAC1 EFF) passing through it. Besides, the *Betaproteobacteria* class showed the lowest presence. In contrast, *Deltaproteobacteria* showed the highest abundance. Within the media samples, the abundance proportion was twice as high in the BAC with the lowest EBCT (BAC1) compared to the BAC2. Finally, *Gammaproteobacteria* showed the highest decrease in abundance after ozonation. The relative abundance of OTUs classified under this class decreased 71% compared to samples before ozonation. Once the treated water passed to the BAC filters, the relative abundance of the media samples slightly increased at MBAC1 (1.3x) but highly at MBAC2 (2.7x) compared to the influent samples. This was related to the importance of longer EBCTs in BAC2, allowing the proliferation of slow growers, which is

characteristic of microorganisms belonging to this class, besides the fact that *Gammaproteobacteria* are prompt to form biofilms (Bae et al., 2014).

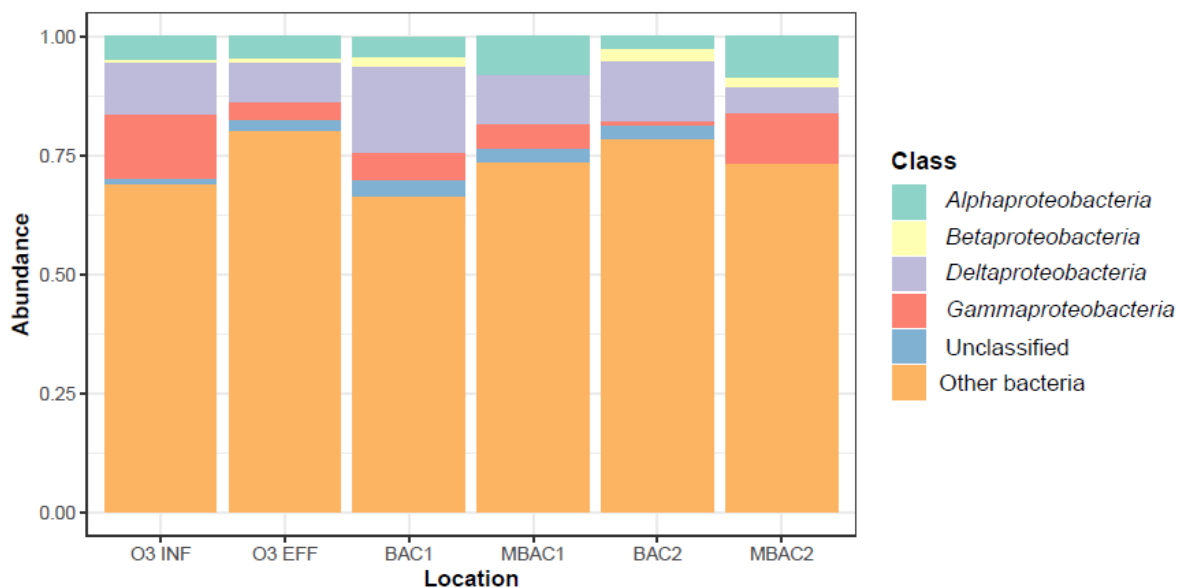


Figure 3-8. Relative abundance of Proteobacteria classes at six sampling locations. The classes included: Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Gammaproteobacteria, unclassified Proteobacteria, and all other bacteria.

Interestingly, among the OTUs present in O3 INF, the majority escaped the ozonation step without any adverse effects. In addition, a classification of OTUs was also proposed based on the microorganisms' preference to form a biofilm: free-living, attached-living, and common (Figure 3-5). Free-living microorganisms (73.8%) included those that were detected at all the liquid samples but not media filter samples, attached-living (7%) were those detected only at the media filter samples, and common (19.2%) were those detected at both liquid and media filter samples. As expected, all the OTUs detected at locations different from media filter samples were also not affected by ozonation. All OTUs classified as O₃ survivors preferred free-living styles. Figure 3-6 shows that those OTUs not affected by ozone were also detected and classified as FO, FOu, LC, SCb, and

SCu, all related to locations at BAC filter effluents and media samples. Ozone survivors were all classified as FI. O₃ eliminated OTUs associated with PT, SC, and FI because these categories excluded OTUs detected downstream of the ozonation step (O₃ EFF particularly).

3.4.2 Identification of Potentially Pathogenic Bacteria

Pathogen control for direct and indirect potable water reuse is essential due to its threat to public health. Current regulations adopted multibarrier approaches that combined different treatment processes, which safeguard the microbial quality of finished water (Mercer, 2020). In the US states where potable reuse projects have been established (e.g., California, Texas, Nevada), guidelines have been developed and implemented (Olivieri et al., 2020; Soller et al., 2019) at the state level with an emphasis on the reduction of viruses and protozoa. Several studies have opted to focus on specific potential opportunistic pathogens to evaluate individual treatment processes or complete treatment trains (Cui and Liang, 2019; Huang et al., 2018; Kulkarni et al., 2018; Liu et al., 2019; Vignola et al., 2018; Wang et al., 2017). Among these studies, the common potential bacterial pathogens up to the genus taxonomic level are *Aeromonas*, *Clostridium*, *Enterobacter*, *Escherichia*, *Flavobacterium*, *Legionella*, *Mycobacterium*, and *Pseudomonas*.

Comparing influent and effluent samples (Figure 3-7) in the CFCGMF process, the total abundance of potential bacterial pathogens did decrease across treatment. The average removal for all pathogenic genera except *Legionella* was $64.9 \pm 17.6\%$. *Legionella* genus increased its abundance across the treatment train by an average of almost ten times. However, the influent's counts were not significantly different from that in the effluent ($p=0.33 > 0.05$). In agreement with our findings, Vignola et al. (2018) reported a higher abundance of *Legionella* at the effluent of a sand filter treating drinking water.

Likewise, *Legionella* was detected in the O₃/BAC process. Its abundance decreased along with the treatment flow, and some counts were detected in BAC filter effluents predominantly during the sampling events with higher ozone doses. We hypothesize that when higher ozone doses are used ($O_3/TOC > 1$), the *Legionella* spp. are inactivated during ozonation and do not physically attach to the filter media, resulting in their abundance increase in BAC effluents. *Legionella* spp. usually accumulates on BAC media and detaches, hence are detected in the effluent (Q. Li et al., 2017; Vignola et al., 2018). *Clostridium* spp. has been identified as a filter colonizer (Vignola et al., 2018) and is resistant to UV disinfection (Kulkarni et al., 2018). This genus has been recognized as organisms that can degrade decaying organic material (Gerardi, 2006). Only one OTU count of *Flavobacterium* genus was detected after ozonation, none in the BAC media, and some were detected in both BAC filter effluents. The latter is consistent with de Vera & Wert (2019), who found this genus after filtration and the most abundant at the top of the filter media.

In summary, eight potential bacterial pathogenic genera were detected in the shared core microbial community. The CFCGMF process compared to O₃/BAC yielded more pathogenic genera, which was supported by the fact that no disinfection agent was added, resulting in a higher survival rate. Pathogen removal during the physicochemical CFCGMF treatment process was attributed to clarification and dual media filtration. O₃/BAC effluents contained some of the studied pathogenic genera. Still, these species accounted for about 1% of the total count of the core microbial community.

In conclusion, the application of 16S metagenomic sequencing methodology combined with bioinformatic analysis tools provided insights into the abundance, composition, and diversity of the

microbial community identified in treatment processes employed in potable reuse purposed advanced water reclamation systems. Microorganism traits such as preference for free-living vs. attached-living style defined the type of organisms that colonized fixed media surfaces. Chemical (e.g., coagulation, ozone addition) and physical (e.g., clarification, filtration) treatments shaped the core microbial community that flows across the treatment train.

Findings show that potential bacterial pathogenic microorganisms are reduced through both barriers (CFCGMF and O₃/BAC). However, their presence and detection (even at low abundance) are serious risks that justify efforts to continue monitoring strategies.

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Chapter 4

MICROBIAL COMMUNITY CHARACTERIZATION AND FATE ACROSS AN ADVANCED WATER TREATMENT TRAIN USED IN POTABLE WATER REUSE: RENO-STEAD WATER RECLAMATION FACILITY DEMONSTRATION STUDY

The goals of this research phase are 1) to study the fate of microbial community across AWT, 2) to identify the microbial community at each sampling location, and 2) to examine potential pathogenic bacteria in the finished water.

4.1 Introduction

Since 2008, a multidisciplinary group comprised of researchers from the University of Nevada Reno, consultants, and regional water agencies have led advanced water treatment pilot testing projects in Reno, NV. These feasibility studies have facilitated the development and design of an advanced water treatment train and polished operating strategies to produce high water quality for potential IPR. This project provided the engineering design criteria, optimization of operational conditions, and maintaining protocols for the AWT demonstration study.

Because the AWT demonstration study evaluated the combination of multiple water treatment technologies, the regulatory framework for finished water was limited by the State of Nevada Category A+ reclaimed water as defined by Nevada Administrative Code (NAC) 45A.27612. In addition, the following rules dictated the performance and efficiency of the study: National Primary

Drinking Water Regulations (NAC 445A.4525), Nevada Secondary Maximum Contaminant levels (NAC 445A.455), specific reductions for pathogens (NAC 445A.27612 and NAC 445A.27618), and a quality monitoring program for unregulated constituents (NAC 445A.27616).

The proposed multibarrier approach (i.e., multiple treatment technologies for each group of contaminants) were operated and monitored to remove chemical, pathogenic, and emerging contaminants. This approach recognizes that a single barrier cannot remove specific contaminants, and multiple barriers guarantee public health protection and safe finished water for potable purposes.

4.2 Materials and Methods

4.2.1 Source Water and Pilot-scale Advanced Water Treatment Description

The system was comprised of CFCGMF, O3/BAC, and Granular Activated Carbon (GAC) filtration, Ultraviolet Disinfection, and Cartridge Filtration (GAC/UV/CF) housed in three separate enclosed units (three barriers). These three barriers were treating secondary effluent from the Reno-Stead Water Reclamation Facility in Reno, NV. The studied AWT train was monitored for full-scale aquifer injection for groundwater augmentation and storage demonstration. This multibarrier system aimed to remove suspended solids, pathogens, bulk organics, and trace organics, such as CECs. For particle removal, the combined effect of CFCGMF with O3/BAC was monitored and assessed, and O3/BAC for pathogens and TOC removal. Other removal objectives per barrier are illustrated in Figure 4-1.

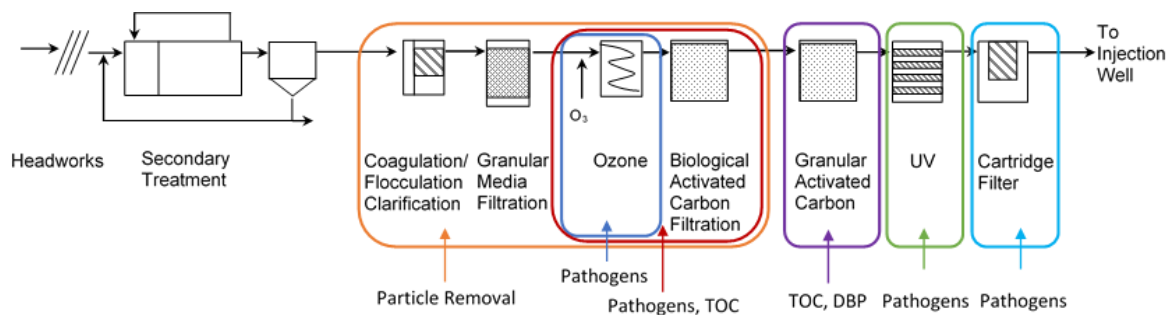


Figure 4-1. Diagram of Control Critical Points in the AWT System

The first barrier against microorganisms was secondary treatment, which was largely outside the demonstration project's control but has provided up to 2-log removal for some pathogens (Teel et al., 2021). The second barrier, CFCGMF, also provided removal of microorganisms and was first in the combined demonstration barriers (CFCGMF-O₃/BAC) to remove particulate contaminants. CFCGMF influent was monitored for biological and particulate indicators. O₃/BAC process performed as a barrier against pathogens focused on virus removal and was also a major barrier for reducing TOC, DBPs precursors, and CECs. GAC was a polishing step to remove further residual CECs (such as perfluorinated contaminants), TOC, and DBPs. Finally, UV disinfection and a cartridge filter provided the final pathogen barriers prior to the conveyance of purified water to the injection well (Figure 4-1). Treatment goals were established for each barrier to ensure and meet regulatory requirements.

The CFCGMF unit (Trident HS by WESTECH Engineering-Salt Lake City, UT) treated an average flow rate of 17 gallons per minute (gpm), providing 15.5 gpm treated effluent for downstream treatment. Coagulants (PAX-XL8 and EC 461) and flocculation aid (VK201) were added, plus frequent automatic backwashing (once every 4-5 hours) was set up to guarantee its performance. A self-contained O₃/BAC unit (Xylem's Leopold – Charlotte, NC) was placed downstream to treat the CFCGMF effluent. The ozone dose was set up in ratio form (O₃/TOC) of

0.9-1.4 (with an influent TOC range of 3.7-7.0 mg C/L) or a fixed dose of 6.4 ppm. Two stainless steel tanks provided an ozone contact time of 18-22 minutes. The ozonated water was stored in a break tank and pumped into two parallel BAC filters to biodegrade organic contaminants (BAC 1 and BAC 2). GAC media was used in the biofilters (4ft² and 60 in bed height) (Filtrisorb F400, Calgon Carbon Corp, Pittsburgh, PA, USA). BAC empty bed contact time (EBCT) was 23 minutes in each biofilter.

Following the BACs, the treated water was sent to the third treatment barrier consisting of GAC filter (Tetrasol Filtration, Jacobi Carbons) with a 60-in bed depth of coconut shell-based GAC. The influent flow to the GAC was 10-12 gpm resulting in 45 minutes EBCT. Then the treated water was sent to the UV system, which included a closed-vessel UV unit (Trojan Technologies, London, Ontario, Canada). The UV system was arranged with four 250 -watt lamps parallel to the direction of the flow. The system maintained a dose of ≥ 300 mJ/cm². Finally, the cartridge filter (CF), a modular 1-micron filter (Harmsco HC/40-0.35) housed in a pressure tank, was the last step for redundancy in pathogen removal. The finished water was stored in two holding tanks. During injection, finished water was pumped into the injection well.

4.2.2 Data Collection and Sampling Campaigns

The pilot study was divided into two phases, namely pre-injection and injection phases. The pre-injection phase started in April/2020, while injection of the finished water into the aquifer started in September/2020. A total of ten sampling events were held for CECs monitoring of the AWT train. The last three events' samples were collected only at the influent and final effluent of the AWT train. For the microbial community samples, a total of 10 sampling events were held for media BAC filters for a total of 28 samples. Liquid samples along the treatment train were 62 in total as follows:

seven from CFCGMF INF, CFCGMF EFF, BAC 1 EFF, BAC 2 EFF, BAC EFF, and UV EFF, six from O3 EFF, five from GAC EFF, and two from CF EFF. From the main seven liquid sampling events, two were taken during the injection phase.

4.2.3 DNA Extraction and High-Throughput Sequencing

A sample volume of 2 L was collected for water samples at locations as shown in Figure 4-1 using sterile amber bottles. Biomass media samples from both BACs were collected on the same dates as the liquid samples. Samples were transported on ice to the laboratory; then, the liquid samples were filtered through 0.2 µm sterile membrane filters to recover suspended biomass. DNA from filters and media samples was extracted using DNeasy PowerWater Kit (QIAGEN, USA) and DNeasy PowerSoil Kit (QIAGEN, USA), respectively. Once the DNA was extracted from each sample, the DNA extracts were sequenced for 16S rRNA (V4 region) on Illumina MiSeq platform (RTL Genomics, Lubbock, Texas, USA). The primers used for DNA amplification were the 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTTC TAAT-3') as recommended by Caporaso et al. (2010). RTL Genomics (Lubbock, Texas, USA) analyzed microbial community diversity in both liquid and solid phases following their data analysis pipeline.

4.2.4 Bioinformatics and Statistical Analyses

The sequencing data of the 16S rRNA amplicons were processed following the analysis pipeline of RTL Genomics. In brief, paired-end reads were trimmed and merged using PEAR Illumina, then denoised, and chimera checks were performed before the downstream process. Chimera detection and removal were done by using UCHIME in *de novo* mode after denoising the data. To obtain the identity of each remaining sequence, samples were demultiplexed. Then using

the UPARSE algorithm (Edgar, 2013), sequences were clustered into operational taxonomic units, OTUs. Taxonomic information is added to each sequence after running each cluster against the USEARCH global alignment program. The data is identified by employing a database of high-quality sequences derived from the NCBI database. Once sequencing data were obtained, all data processing related to microbial community analysis were made following different pipelines (Callahan et al., 2016; Shetty et al., 2018). The *phyloseq* R-package (McMurdie and Holmes, 2013a) was used to manage the sequencing data capable of supporting sequencing importing data format from RTL Genomics. All the analytical techniques used, such as diversity analysis, ordination methods, quality plots, and microbiome detail analyses, were performed using this package. Normalized data were used to determine the observed number of OTUs and to estimate the Shannon Index, Chao1, and Simpson's Diversity Index using R statistical software, version 4.0.3 (R Core Team, 2020) using packages *phyloseq* version 1.34.0 (McMurdie and Holmes, 2013a) *vegan* version 2.5.7 (Oksanen et al., 2020), and *microbiome* version 1.12.2 (Lahti and Shetty, 2019). All figures were developed using *ggplot2*, version 3.3.3 (Wickham, 2016).

4.3 Results & Discussion

Alpha and beta diversity metrics were calculated to describe and characterize the microbial community at each treatment step. For the alpha metrics, the following indices were computed: observed, *chao1*, Shannon, Pielou, coverage, Hill1, and faith's phylogenetic distances. For beta metrics, Weighted (W-UniFrac) and Unweighted Unifrac (UnW-UniFrac) dissimilarity matrix were used to compare the microbial community composition between the sampling locations. The Unifrac distance was chosen to include the effect of phylogenetic information.

4.3.1 Alpha Diversity

Different alpha diversity indices were computed for all the samples at each treatment step. A summary of the diversity indices is shown in Table 4-1 and Figure 4-2. The richness measured by the observed index ranged from 54 to 2442. The average value corresponded to 1231.8 ± 516 . The highest richness was found at the O3 INF, BAC EFF, and GAC EFF with 1569.4 ± 322.5 , 1565.7 ± 178.2 , and 1547.4 ± 635.1 , respectively, while the lowest was at the final two treatment steps (UV and CF EFF). The average was 4.5 ± 1.2 ; lower values were found at CFCGMF EFF, UV EFF, and CF EFF, while the filter effluents (BAC and GAC) showed the highest diversity. In addition, the microbial communities that displayed higher evenness (Pielou's Index 0.71-0.75) were those with higher diversity.

Conversely, the final two treatment steps showed the lowest richness, diversity, and evenness. The number of effective species (Hill1) was calculated by taking the exponent of the Shannon index. The average Hill1 numbers ranged between 58.3 ± 44.2 to 243.3 ± 74.5 . The highest Hill1's numbers were found at the BAC and GAC effluents, while the lowest was at the UV EFF and the CFCGMF EFF. Faith's phylogenetic diversity (PD) is the index to check when measuring biodiversity based on phylogeny. This index defines the phylogenetic diversity of a set of species as "equal to the sum of the lengths of all those branches on the tree that span the members of the set" (Faith, 1992). Therefore, Faith's PD indicates a feature diversity; the lower the number suggests closest are the species in the tree. The lowest Faith's PD was identified at the UV EFF (95.6 ± 32.1) and highest at O3 INF (165.6 ± 21.6), with an average for all sampling locations of 134.6 ± 37.8 .

Table 4-1. Alpha Diversity indices for each sampling location during every sampling event.

Sample Location	Sampling Event	Diversity Index						
		Observed	Chao1	Shannon	Pielou	Coverage	Hill1	Faith's PD
CFCGMF INF	SE1	1482	1703.6	5.3	0.7	21	200.8	161.7
	SE2	1546	1763.3	4.6	0.6	10	103.9	163.9
	SE3	258	323.0	4.4	0.8	20	84.4	48.7
	SE4	1517	1732.9	5.4	0.7	28	221.1	165.0
	SE5	1588	1838.7	5.6	0.8	38	280.2	171.5
	SE6	1622	1887.4	4.6	0.6	11	101.6	167.3
	SE7	1103	1314.5	2.9	0.4	1	17.7	134.8
CFCGMF EFF	SE1	1387	1611.7	5.6	0.8	42	272.9	154.8
	SE2	1667	1925.0	5.3	0.7	32	200.6	173.1
	SE3	1335	1580.5	3.6	0.5	2	38.3	145.9
	SE4	802	992.8	3.5	0.5	4	32.7	103.1
	SE5	54	74.2	2.1	0.5	1	8.0	16.2
	SE6	1060	1267.2	2.6	0.4	1	13.2	130.3
	SE7	784	903.4	1.7	0.3	1	5.5	105.1
O3 INF	SE1	1793	2057.7	6.0	0.8	61	402.6	178.6
	SE2	2061	2530.6	5.6	0.7	35	271.1	196.7
	SE3	1848	2180.0	5.0	0.7	19	148.1	187.7
	SE4	1288	1467.1	4.8	0.7	14	121.4	147.5
	SE5	1391	1662.7	4.7	0.6	10	105.0	153.5
	SE6	1326	1598.9	3.0	0.4	1	20.9	152.0
	SE7	1279	1502.6	2.9	0.4	1	18.1	142.9
O3 EFF	SE1	1227	1353.1	4.4	0.6	9	79.4	137.7
	SE2	1273	1460.0	3.8	0.5	3	43.9	143.1
	SE3	106	126.0	3.5	0.8	9	34.1	24.0
	SE4	1133	1307.8	4.9	0.7	19	133.2	136.1
	SE6	1484	1653.3	5.7	0.8	46	308.9	162.0
	SE7	1388	1476.5	5.5	0.8	37	253.1	152.2
	BAC EFF	SE1	1759	2030.6	6.0	0.8	60	385.3
SE2		1378	1645.2	5.3	0.7	32	202.9	151.7
SE3		1425	1705.3	5.4	0.7	28	217.6	151.4
SE4		1591	1898.0	4.4	0.6	8	80.3	165.5
SE5		1364	1694.1	4.1	0.6	5	57.9	151.0
SE6		1657	1896.6	5.7	0.8	44	309.5	171.3
SE7		1786	1992.1	5.5	0.7	39	248.1	183.9
GAC EFF	SE1	1740	2044.4	5.6	0.7	34	259.9	176.5

Sample Location	Sampling Event	Diversity Index						
		Observed	Chao1	Shannon	Pielou	Coverage	Hill1	Faith's PD
	SE2	1392	1687.7	5.2	0.7	24	182.2	149.9
	SE3	683	772.5	5.3	0.8	42	202.1	91.0
	SE6	1480	1673.3	5.3	0.7	26	205.9	161.0
	SE7	2442	2684.4	5.9	0.8	52	366.2	216.9
UV EFF	SE1	254	296.5	3.1	0.6	4	22.2	47.8
	SE2	922	1003.6	3.8	0.6	5	43.0	116.9
	SE3	1302	1486.4	4.7	0.6	13	105.2	145.3
	SE4	641	718.5	2.2	0.3	2	9.2	85.8
	SE5	408	439.7	3.3	0.5	3	26.6	68.0
	SE6	793	893.7	4.4	0.7	10	79.2	101.4
	SE7	742	785.5	4.8	0.7	14	122.7	103.7
CF EFF	SE6	460	498.6	3.0	0.5	2	20.4	73.1
	SE7	1106	1225.7	5.0	0.7	21	150.5	132.6

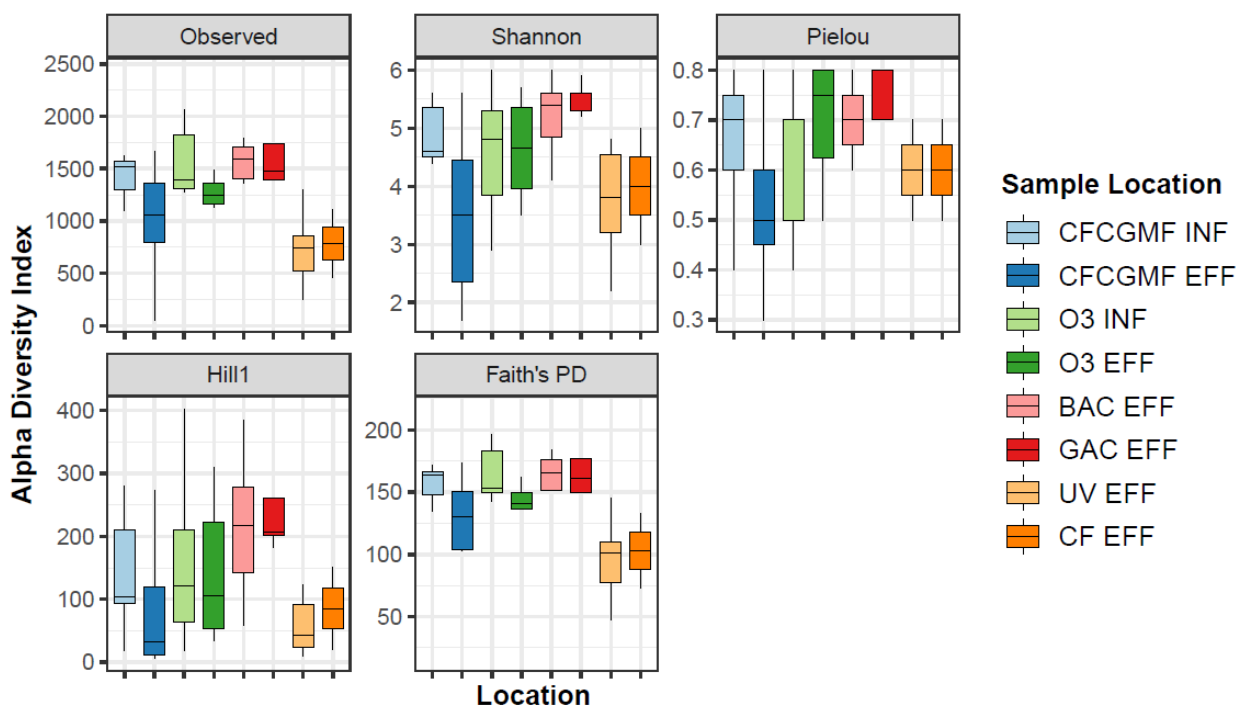


Figure 4-2. Alpha diversity indices Observed (richness), Shannon (diversity), Pielou (evenness), Hill1, and Faith's phylogenetic distance.

✓ **Statistical tests**

After describing the diversity in all sampling locations, it is important to know if these indices are significantly different across all the locations. The first step is to test whether or not the data is normally distributed, then test for significance using a parametric or non-parametric test, and finally test using a post-hoc test to determine which pairwise comparisons are different. The computed steps are summarized in Figure 4-3.

Richness, diversity, Hill1, and PD were tested for normality; only richness data were normally distributed ($p:0.065$). Then, using ANOVA, the richness was found to be significant ($p:0.005$), and with pairwise comparisons using Tukey's HSD (honestly significance difference) post-hoc test only, the diversity between UV-EFF against O3 INF, BAC EFF, and GAC EFF were found significantly different. The non-normally distributed data (diversity, Hill1, and PD) were tested for significance with the non-parametric equivalent of ANOVA, the Kruskal-Wallis test. The corresponding p-values were 0.056, 0.050, and 0.006 for diversity, Hill1, and PD, respectively. The diversity indices represented by the Shannon index and Hill1 number were not significant, meaning that diversity is not significantly different between sampling locations. Conversely, PD was found significant, and UV EFF was found different compared to O3 INF and BAC EFF microbial community samples.

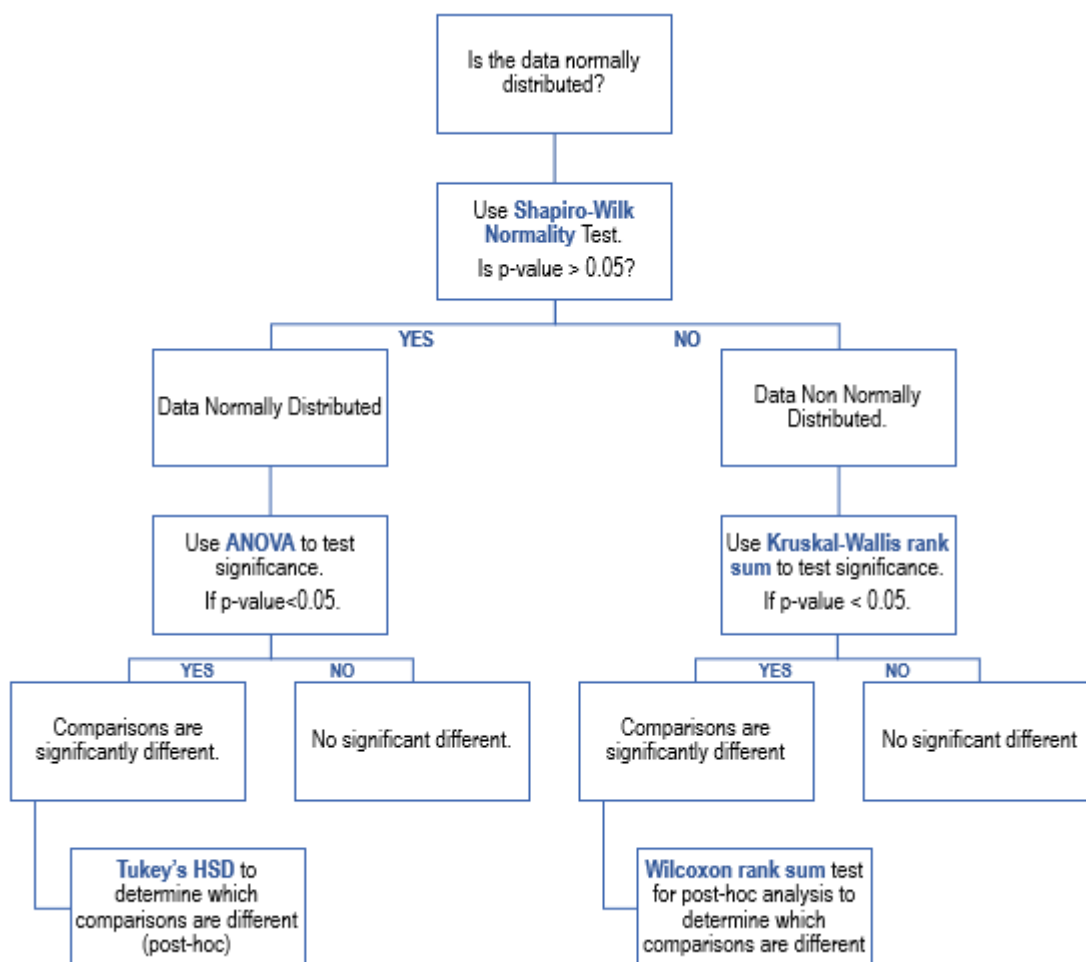


Figure 4-3. Statistical test decision tree for alpha diversity significance.

4.3.2 Beta Diversity

A principal coordinate analysis (PCoA) based on Weighted (W-UniFrac) and Unweighted UniFrac (UnW-UniFrac) dissimilarity matrix was used to compare the microbial community composition between the eight sampling locations. The UniFrac distance was chosen to include the effect of phylogenetic information. Figure 4-4 shows how samples up to O3 EFF clustered together at the bottom right of the plot, and the top left BAC and GAC effluent samples. In contrast, UV and

CF effluents did spread along Axis 1. Axis 1, 2, and 3 explained around 33% of the variation. The main axis of the W-UniFrac (Figure 4-5) explained ~35% of the dissimilarities between the eight sampling locations. Most of the sampling locations, but for CFCGMF INF, EFF, and UV EFF, were clustered together at the top left. Because W-UniFrac takes abundance information into account (Chang et al., 2011), Figure 4-5 suggests that O3 EFF, BAC EFF, GAC EFF, and CF EFF microbial communities abundances are similar. In contrast, CFCGMF INF and CFCGMF EFF may change with time.

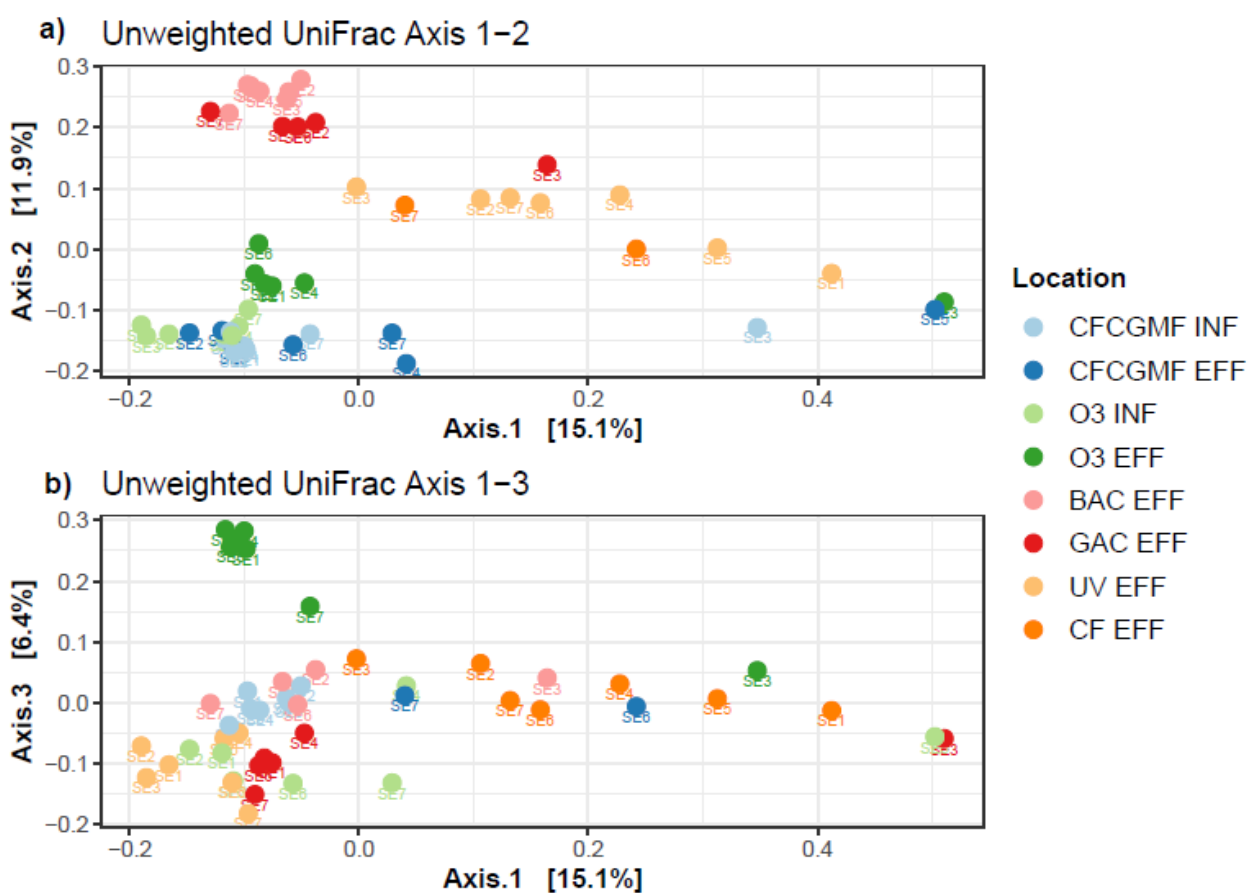


Figure 4-4. Comparison of the microbial community on the different sampling location using Principal Coordinate Analysis (PCoA) of Unweighted Unifrac showing a) Axis 1 and 2 and b) Axis 1 and 3

Statistical tests (PERMANOVA) were performed to test whether microbial communities differ by sampling location. Unweighted Unifrac results detected as significantly different include the following pairs: O3 INF from BAC EFF, GAC EFF and UV EFF, BAC EFF from CFCGMF INF, O3 EFF and UV EFF, and CFCGMF EFF and GAC EFF. These dissimilarities are based on the presence/absence of OTUs. On the other hand, Weighted Unifrac results suggested that the following sampling locations are significantly different (p -values <0.05): UV EFF different from CFCGMF INF and EFF; BAC EFF different from CFCGMF EFF, O3 INF, and O3 EFF, and BAC and GAC effluent. Then, to confirm the equal dispersion assumed by PERMANOVA, the betadisper test (p -value:0.27) confirms the PERMANOVA results.

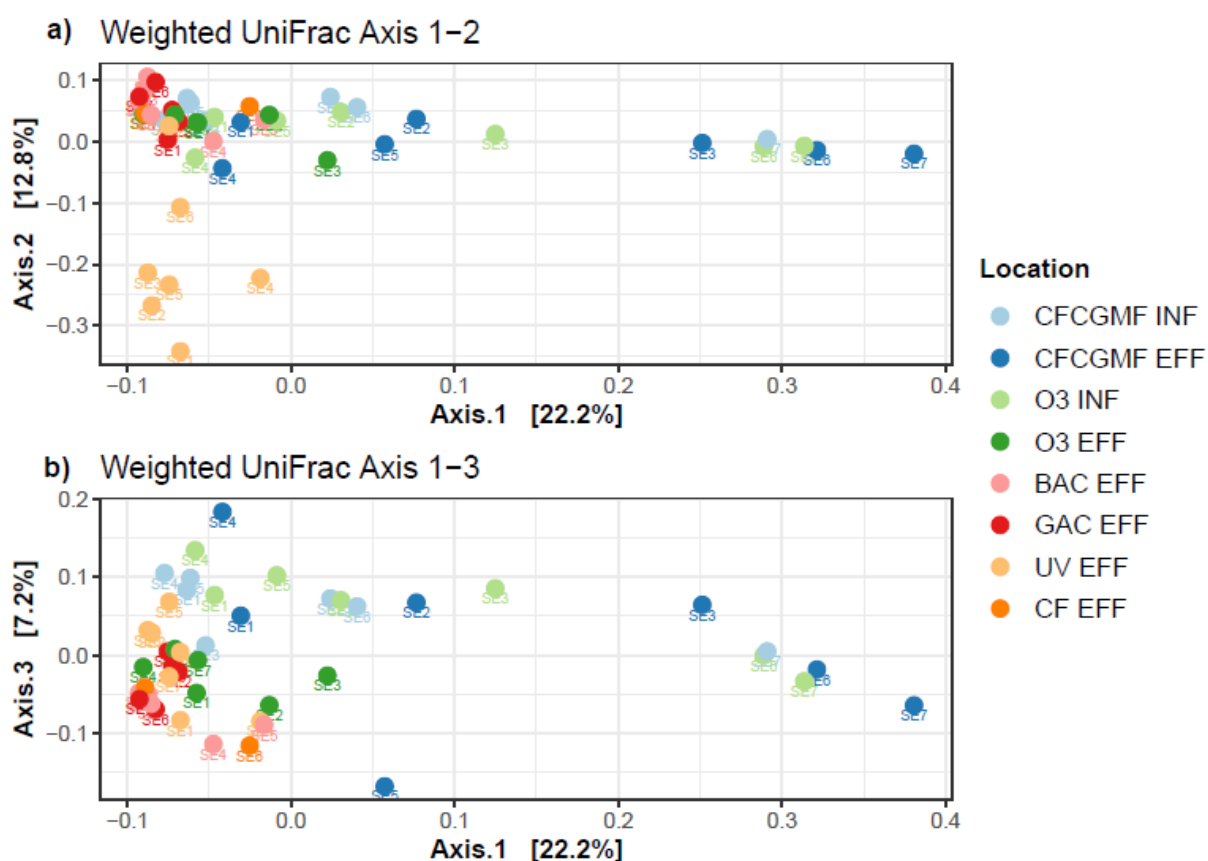


Figure 4-5. Comparison of the microbial community on the different sampling locations using Principal Coordinate Analysis (PCoA) of Unweighted Unifrac showing a) Axis 1 and 2 and b) Axis 1 and 3.

4.3.3 Microbial Community Fate Across the AWT train.

The advanced water treatment train harbored a large diversity of bacterial phyla (22 different phyla). The top 10 phyla (Figure 4-6) were *Proteobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes*, *Verrucomicrobia*, *Actinobacteria*, *Planctomycetes*, *Acidobacteria*, *Nitrospirae*, and *Chloroflexi*. *Proteobacteria*, the most dominant phyla across all locations, range between 37.8% to 73.2% in relative abundance. Ozonation and filter effluents (O3 EFF, BAC EFF, GAC EFF, and CF EFF) showed a lower relative abundance of *Proteobacteria*: 38, 49, 47, and 52% respectively, whereas all locations upstream of ozonation and UV effluent displayed higher *Proteobacteria* dominance, with the average relative abundance of 61%, 63%, 61%, and 73% for CFCGMF influent and effluent, ozone influent, and UV effluent, respectively. *Bacteroidetes* relative abundance ranged between 4.8-26.1% and showed the highest relative abundance at the influent and final effluent to the AWT (CFCGMF INF: 14% and CF EFF: 26%). *Cyanobacteria* relative abundance varied from 0 to 28.2%; it was detected at very low percentages (<0.5%) at all locations but before and after ozonation. O3 EFF showed the highest value with 28%. All the other phyla: *Firmicutes* (0.3-6.4%), *Verrucomicrobia* (0.3-7.0%), *Actinobacteria* (0.2-5.0%), *Planctomycetes* (0.4-3.9%), *Acidobacteria* (0.2-2.1%), *Nitrospirae* (0.1-1.8%), and *Chloroflexi* (0.1-0.9%) were detected at all treatment effluents and kept similar relative abundances throughout. These 10-phylum covered more than 79% of all bacteria identified.

At the class level (Figure 4-7), 46 bacterial classes were detected. The most abundant classes were *Betaproteobacteria* (16-42.9%), *Alphaproteobacteria* (6.1-16.8%), *Gammaproteobacteria* (3.0-27.7%), *Deltaproteobacteria* (2.8-6.9%), *Sphingobacteria* (1.3-10.6%), and *Flavobacteria* (0.4-

22%), which along with unclassified classes (19.9-50.6%) accounted for more than 90% of the total relative abundance for this taxonomic level. *Betaproteobacteria* relative abundance was high upstream of ozonation; then, its relative abundance was reduced by half after ozonation. *Alphaproteobacteria* lowest relative abundance was detected at CFCGMF INF and was highest at UV EFF. *Gammaproteobacteria* relative abundance at UV EFF was the highest, which may indicate that organisms classified under this class may potentially be less susceptible to UV disinfection. The average relative abundance of microorganisms belonging to the *Sphingobacteria* class was $3.6 \pm 1.5\%$, but it was about 11% at CFCGMF INF. *Flavobacteria* class was relatively low ($1.2 \pm 0.7\%$), but at CF EFF increased up to 22%.

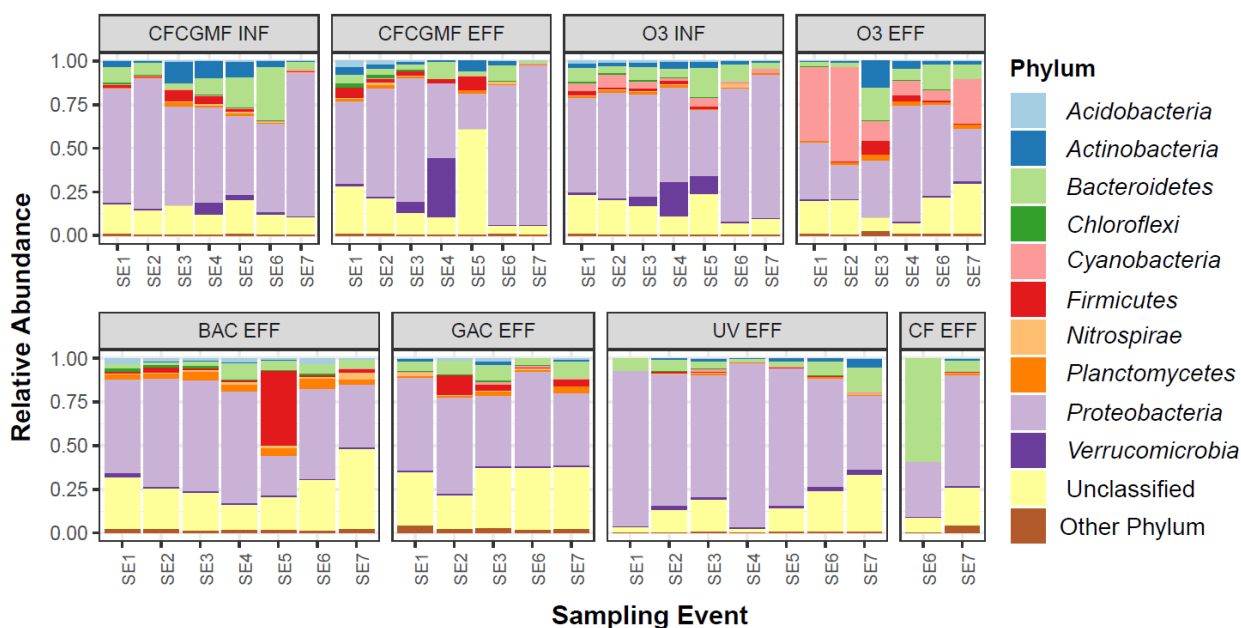


Figure 4-6. Relative abundance of top 10 phyla across each treatment step and sampling event (SE).

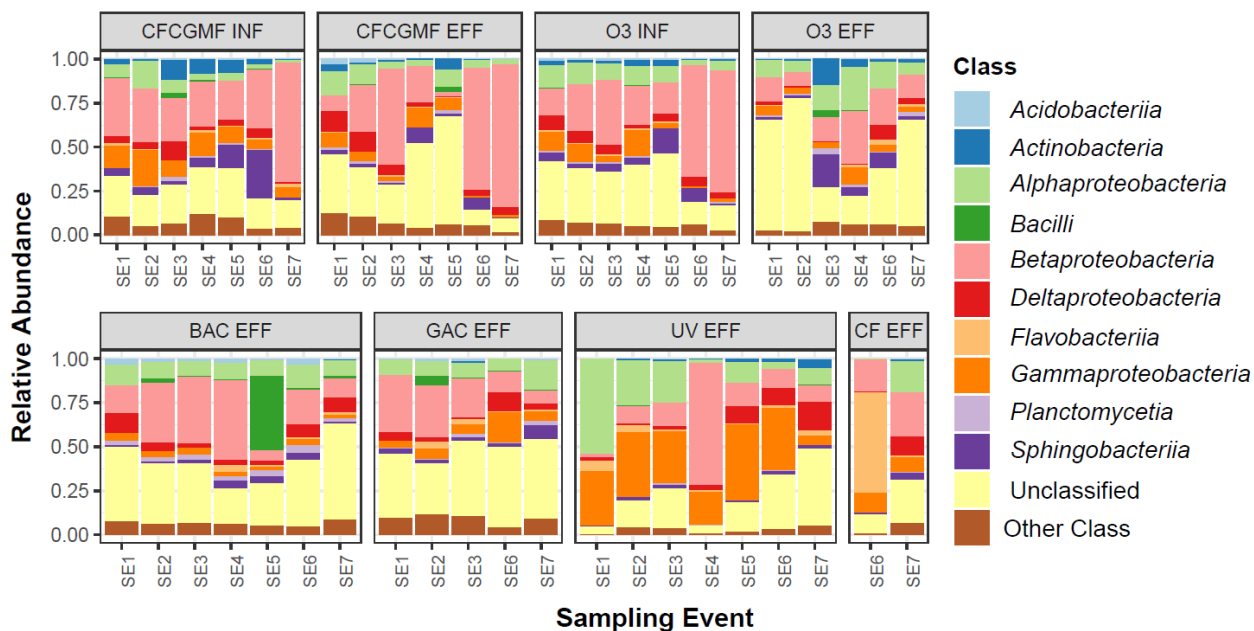


Figure 4-7. Relative abundance of top 10 classes across each treatment step and sampling event (SE).

4.3.4 Archaea Fate Across the AWT Train

Archaea organisms even at low proportion abundance compared to *Bacteria* played key roles in nitrogen and carbon cycles, and methane production. These organisms have been reported as active participants in biological activities such as nitrification and denitrification in aerated bioreactors (Płaza et al., 2021). Particularly, *Archaea* has been reported as ammonia-oxidizing microorganisms (Pan et al., 2018), despite usually being outnumbered by ammonia-oxidizing-bacteria, ammonia-oxidizing archaea (AOA) dominated the ammonia oxidation process during active nitrification.

In this study, a total of 65 out of 10335 OTUs were classified as *Archaea* while 8203 as *Bacteria* at the kingdom level. The primer pairs for 16S rRNA gene amplification were chosen to capture the bacteria domain rather than the archaea. This choice determines quality and

perspective on the community data obtain (Fischer et al., 2016), hence the relative low abundances obtained for archaea organisms ranged between 0-1% along the treatment train were expected. The highest values were detected across the GAC EFF and BAC media filter and effluents, and at very low abundances at the final treatment steps (UV EFF and CF EFF). Archaeal sequences were assigned to 2 phylum, 3 classes, 4 orders, 2 families, 3 genera, and 2 specie. The Archaea phyla detected were *Crenarchaeota* and *Euryarchaeota* (Figure 4-8), and about 97.9% of the detected archaea were not possible to classify under a phylum level. At the class level, *Methanomicrobia*, *Methanobacteria*, and *Halobacteria* were detected all belonging to the *Euryarchaeota* phyla. Methanogenic archaea belong to the phylum Euryarchaeota. Within this phyla, four orders were detected *Halobacteriales*, *Methanobacteriales*, *Methanomicrobiales*, and *Methanosarcinales*. Methanogenic archaea are a relative diverse group that can be found in extreme environments (saline lakes, hydrothermal vents, digesters, biogas plants) and non-extreme environments (rice fields, wetlands, landfills) (Enzmann et al., 2018).

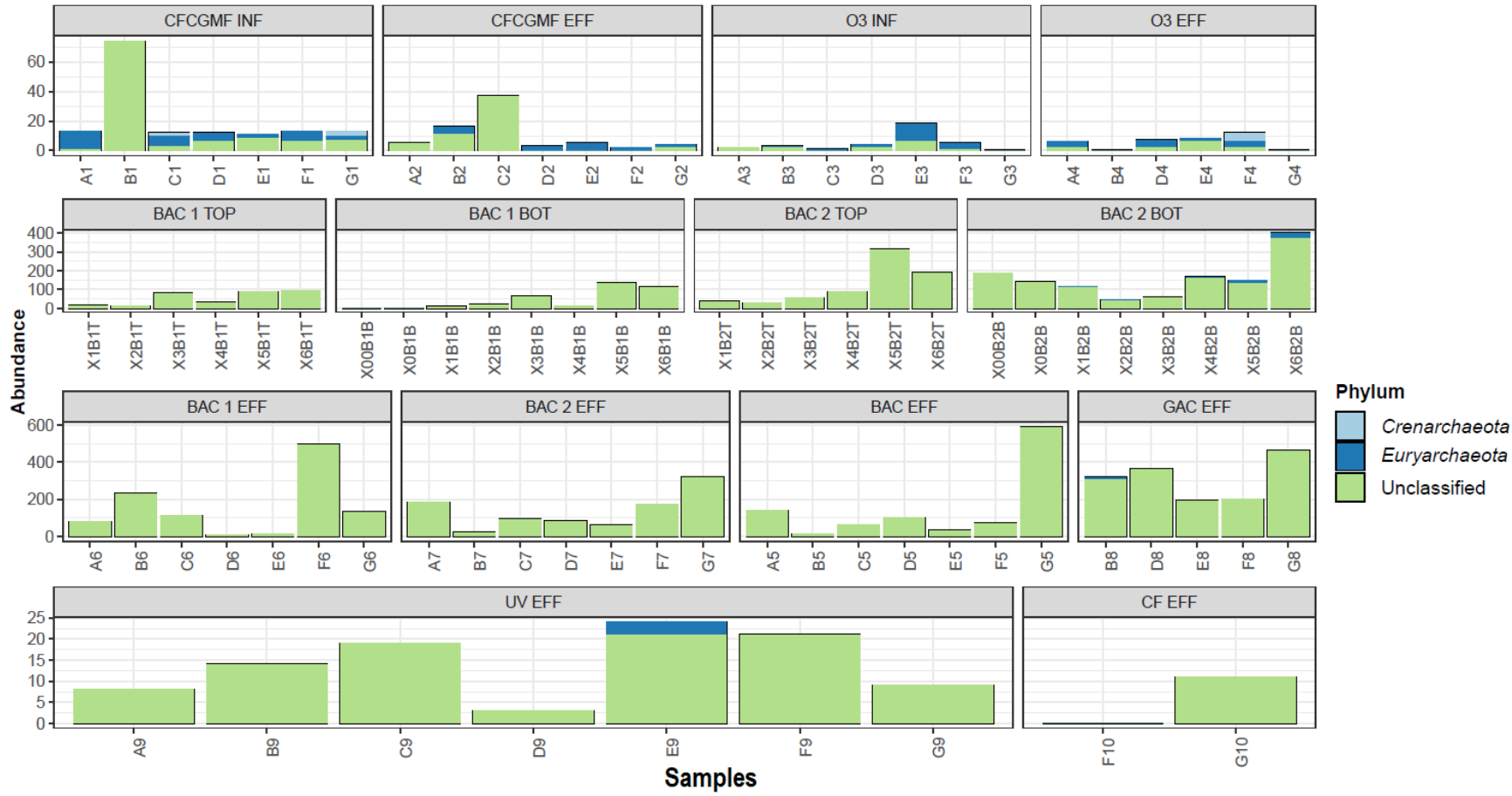


Figure 4-8. Abundance counts for detected phylum belonging to the Archaea domain.

4.3.5 Potential Pathogenic Bacteria Fate Across the AWT Train

The importance of the study and identification of organisms that may resist and pass through a multibarrier system is imperative due to the latent and potential risk to public health. This subsection focuses on identifying those organisms that can be a potential threat to finished water users. Multibarrier approaches are usually adopted to preserve the microbial quality of finished water (Mercer, 2020). Previous studies have focused on specific opportunistic pathogens to assess individual or combined treatment (Cui and Liang, 2019; Huang et al., 2018; Kulkarni et al., 2018; Liu et al., 2019; Vignola et al., 2018; Wang et al., 2017).

The common bacterial pathogens up to the genus taxonomic level are *Aeromonas*, *Clostridium*, *Enterobacter*, *Escherichia*, *Flavobacterium*, *Legionella*, *Mycobacterium*, and *Pseudomonas*. These genera have been observed to regrow in disinfected reclaimed water distribution systems, filter effluents, and filter media. Indigenous species to drinking water treatment systems are *Aeromonas* spp., *Legionella* spp., *Mycobacterium* spp., and *P. aeruginosa* (de Vera et al., 2018; Liu et al., 2019). In addition, a portion of these listed microorganisms are disease vectors. For instance, *L. pneumophila* and another *Legionella* spp. are linked to Legionnaire's disease (Haupt et al., 2012). Likewise, *Mycobacterium* can harbor potential pathogenic species such as *M. avium*, *M. xenopi*, *M. fortuitum*, and nontuberculous mycobacteria (Liu et al., 2019). Indicator bacteria such as coliform bacteria and *Escherichia coli* belong to *Enterobacter* and *Escherichia* genera, respectively. Finally, some *Flavobacterium* spp. has been identified as responsible for meningitis and pneumonia in humans (Crump et al., 2001).

Figure 4-9 and Table 4-2 show the relative abundance and detected counts of the listed opportunistic pathogens at each treatment process effluent. From the 10335 OTUs identified within the 90 studied samples, only 3% of the OTUs were classified as opportunistic pathogens.

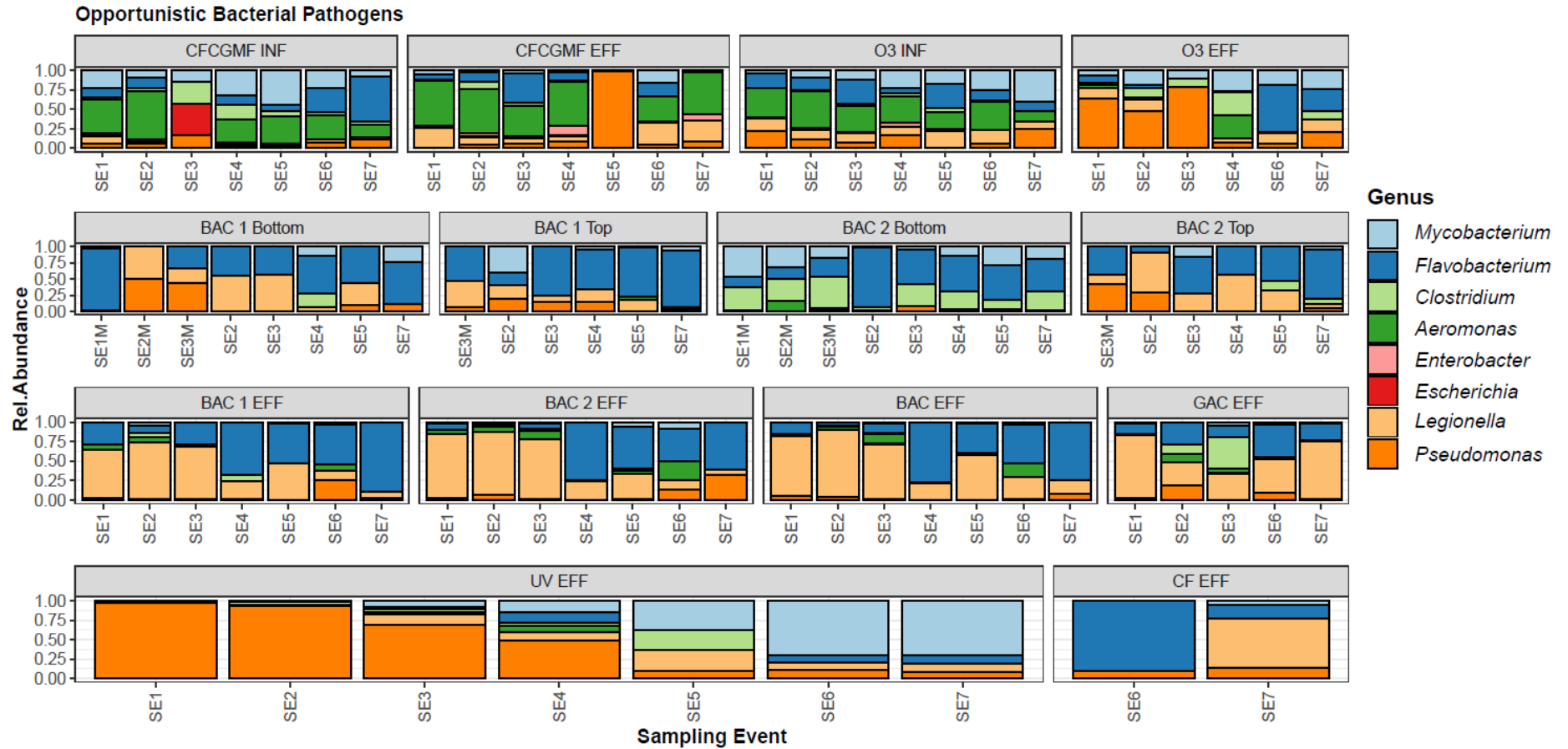


Figure 4-9. Detected opportunistic bacterial pathogens at genus taxonomic level across all effluent processes CFCGMF INF, CFCGMF EFF, O3 INF, O3 EFF, BAC 1 EFF, BAC 2 EFF, BAC EFF, GAC EFF, UV EFF, and CF EFF. Also, samples were taken from solid samples collected from the BAC filters at two depths (BAC 1 Top, BAC 1 Bottom, BAC 2 Top, BAC 2 Bottom).

The average relative abundance at each location was 4.0 ± 6.8 %, and *Flavobacterium* was the most frequently detected pathogenic genus (Table 4-2). The sampling location that yielded the highest number of observed pathogenic bacteria OTUs was the CF unit (27%) and the O3 EFF (4.8%), while the BAC media sample was the lowest (0.1-0.8%). Opportunistic pathogens belonging to the *Legionella* and *Pseudomonas* genera were the most persistent across the treatment train. When comparing the detected counts at the influent to the AWT train and finished water, the effluent counts were higher. *Legionella* genus increased its abundance across the treatment train almost ten times on average downstream the BAC filters; the GAC filter seems to promote its abundance. However, UV disinfection attenuates their concentration by about 86% (455 and 61 observed OTUs in GAC EFF and UV EFF, respectively).

On the other hand, the *Pseudomonas* genus seems resistant to ozonation. There were three times counts increased downstream of ozonation. BAC filter impacted their counts, decreasing the number by about 96% (it went from 374 to 15 observed OTUs). However, after UV disinfection, their OTU counts started with 24 and finished with 654 observed OTUs classified under the *Pseudomonas* genus. *Mycobacterium* genera detection decreased after the BAC and GAC filters by 94-98%, but it was resistant to UV disinfection. It was reported (Bohrerova and Linden, 2006; Lee et al., 2010) that *Mycobacterium* is resistant to not only UV but chlorine disinfection. Microorganisms belonging to this genus tend to aggregate, then disinfection efficacy decreases, as seen in this study.

Table 4-2. Observed OTUs of opportunistic pathogenic microorganisms at genus level detected at each monitored sampling location

Sampling Location	Opportunistic Pathogenic Genus								Subtotal Observed OTUs	Total Observed OTUs
	<i>Aeromonas</i>	<i>Clostridium</i>	<i>Enterobacter</i>	<i>Escherichia</i>	<i>Flavobacterium</i>	<i>Legionella</i>	<i>Mycobacterium</i>	<i>Pseudomonas</i>		
CFCGMF INF	398	178	22	127	215	25	340	112	1417	33022
CFCGMF EFF	202	13	25	4	63	42	12	93	454	26153
O3 INF	207	14	12	2	124	82	88	78	607	25713
O3 EFF	153	187	3	0	208	138	252	374	1315	27249
BAC 1 Top	1	0	0	0	23	6	2	1	33	27053
BAC 1 Bottom	0	0	0	0	19	3	1	1	24	25188
BAC 1 EFF	6	28	0	0	521	278	7	13	853	22821
BAC 2 Top	0	0	0	0	10	4	1	1	16	28833
BAC 2 Bottom	4	48	0	0	124	0	32	4	212	27857
BAC 2 EFF	24	4	1	0	365	232	10	76	712	30939
BAC EFF	27	3	0	0	327	246	6	15	624	28375
GAC EFF	33	132	0	2	171	455	19	23	835	32092
UV EFF	4	18	0	0	55	61	275	653	1066	29239
CF EFF	13	3	6	0	10050	1182	93	1298	12645	46852

To sum up, the highest relative observed OTUs classified as potential pathogenic bacteria was found at the influent to the AWT train (Table 4-3). Before the CF unit, *Aeromonas*, *Enterobacter*, *Escherichia*, *Flavobacterium*, and *Legionella* genera are well diminished along the AWT train; their average relative abundance went from $32.3 \pm 34\%$ to $1 \pm 1.2\%$. *Mycobacterium* and *Pseudomonas* genera showed a high relative abundance after both disinfection steps, ozonation, and UV disinfection. The uniqueness of *Pseudomonas* in tap water and ozone effluents have been reported before, also their antibiotic and toxic metal resistance (Vaz-Moreira et al., 2017),

suggesting their resilience and robustness to environmental stress. In another study, where pathogenic nontuberculous mycobacteria in humans were investigated (Hilborn et al., 2006), it was reported that after adding filtration and ozonation units to a drinking water treatment facility, the detection of *Mycobacterium* organisms persisted. Hence, the persistence of potentially pathogenic bacteria belonging to *Mycobacterium* and *Pseudomonas* is a risk that deserves further attention in advanced reuse treatment.

Table 4-3. Relative abundance of observed OTUs related to pathogenic bacteria at genus level along with the AWT trains.

Sampling Location	Aeromonas	Clostridium	Enterobacter	Escherichia	Flavobacterium	Legionella	Mycobacterium	Pseudomonas
CFCGMF INF	37.1%	28.3%	31.9%	94.1%	1.8%	0.9%	29.9%	4.1%
CFCGMF EFF	18.8%	2.1%	36.2%	3.0%	0.5%	1.5%	1.1%	3.4%
O3 INF	19.3%	2.2%	17.4%	1.5%	1.0%	3.0%	7.7%	2.8%
O3 EFF	14.3%	29.8%	4.3%	0.0%	1.7%	5.0%	22.1%	13.6%
BAC 1 Top	0.1%	0.0%	0.0%	0.0%	0.2%	0.2%	0.2%	0.0%
BAC 1 Bottom	0.0%	0.0%	0.0%	0.0%	0.2%	0.1%	0.1%	0.0%
BAC 1 EFF	0.6%	4.5%	0.0%	0.0%	4.2%	10.1%	0.6%	0.5%
BAC 2 Top	0.0%	0.0%	0.0%	0.0%	0.1%	0.1%	0.1%	0.0%
BAC 2 Bottom	0.4%	7.6%	0.0%	0.0%	1.0%	0.0%	2.8%	0.1%
BAC 2 EFF	2.2%	0.6%	1.4%	0.0%	3.0%	8.4%	0.9%	2.8%
BAC EFF	2.5%	0.5%	0.0%	0.0%	2.7%	8.9%	0.5%	0.5%
GAC EFF	3.1%	21.0%	0.0%	1.5%	1.4%	16.5%	1.7%	0.8%
UV EFF	0.4%	2.9%	0.0%	0.0%	0.4%	2.2%	24.2%	23.8%
CF EFF	1.2%	0.5%	8.7%	0.0%	81.9%	42.9%	8.2%	47.3%

The CF unit yielded the highest relative abundance of *Flavobacterium* (81.9%), followed by *Pseudomonas* and *Legionella*. As noted from Table 4-3, the highest yields of *Flavobacterium* and *Legionella* were detected after the filtration steps (BAC 1 EFF, BAC 2 EFF, BAC EFF, GAC EFF,

and CF EFF), while *Pseudomonas* highest detection was after disinfection steps (O3 EFF, and UV EFF). *Flavobacterium* genus is characterized by its ability to attach to surfaces forming biofilms (Jo et al., 2016; Levipan et al., 2018; Ríos-Castillo et al., 2018). A study (Stewart et al., 2012) reported that *Legionella* persists when *Flavobacterium*, *Pseudomonas*, and *Klebsiella* form a biofilm, hence due to the filter's membrane properties of the CF process step the potential development of a biofilm and subsequent sloughing of microorganisms that colonized the upstream filter. Interestingly, when *Flavobacterium* and *Pseudomonas* genera coexisted in biofilms, the former overgrew the latter (Zhang et al., 2013). *Flavobacterium* is usually established at the top layers of the biofilm, whereas *Pseudomonas* preferred deeper biofilm regions. Therefore, the nutrients availability combined with a faster-specific growth rate favors the abundance of *Flavobacterium*, explaining the relative abundances detected in this study.

As a note, not all organisms classified under these eight genera are pathogenic, pathogenicity cannot be extrapolated of sole amplicon data, complementary analysis such as targeting specific organisms with qPCR analysis is strongly suggested. In addition, some of the detected observed OTUs were removed through the train, survived, or grew downstream treatment processes. To explain higher abundances downstream the AWT system, (Kantor et al., 2019) added Illumina barcode bleed, sample cross-contamination to persistence along with treatments.

4.4 Conclusions

In this chapter, the fate of the microbial community across the AWT was investigated at each sampling location, and potential bacterial pathogenic bacteria occurrence was examined. The top 10 phyla were *Proteobacteria* (38-63%), *Bacteroidetes* (38-63%), *Cyanobacteria* (0-28%), *Firmicutes* (0.3-6.4%), *Verrucomicrobia* (0.3-7.0%), *Actinobacteria* (0.2-5.0%), *Planctomycetes*

(0.4-3.9%), *Acidobacteria* (0.2-2.1%), *Nitrospirae* (0.1-1.8%), and *Chloroflexi* (0.1-0.9%) were detected at all treatment effluents and kept similar relative abundances throughout. This 10-phylum covered more than 79% of all organisms identified. Richness and diversity measurements were calculated and indicated that the highest richness was found at the O3 INF, BAC EFF, and GAC EFF with 1569.4 ± 322.5 , 1565.7 ± 178.2 , and 1547.4 ± 635.1 , respectively, while the lowest was yielded at the final two treatment steps (UV and CF EFF). The average diversity value was 4.5 ± 1.2 . The lower values were found at CFCGMF EFF, UV EFF, and CF EFF, while the filter effluents (BAC and GAC) showed the highest diversity. When testing whether the microbial communities differ by sampling location, the BAC EFF microbial community was significantly different from that at CFCGMF EFF, INF, O3 INF, O3 EFF, BAC, and GAC EFF.

Even though the detected pathogenic bacteria genera accounted for about 3% of the total counts, the need for optimizing current treatment barriers, establishing monitoring and risk assessment is a never-ending endeavor to ensure public health protection. Findings show that eight potential bacterial pathogenic genera were detected along the AWT train. The ozonation step yielded more pathogenic bacteria genera than any other sample location, then the GAC and BAC filters. However, the percentage removal was around 75-100% for *Aeromonas*, *Clostridium*, *Enterobacter*, *Escherichia*, and *Flavobacterium*. The persistence of some genera (*Flavobacterium*, *Legionella*, and *Pseudomonas*) at finished water (even at low relative abundances) are serious risks that justify further efforts to continue validating monitor strategies and unit process optimizations.

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Chapter 5

LINKING MICROBIAL COMMUNITY STRUCTURE AND REMOVAL OF CECs ACROSS AN ADVANCED WATER TREATMENT TRAIN FOR WATER REUSE

The goals of this research phase are 1) to characterize the microbial community within a BAC filter under operational conditions, 2) to investigate the removal efficiency of CECs across an AWT system, 3) to identify and link specific bacteria capable of CEC removal. Samples were taken from the AWT pilot demonstration project mentioned in Chapter 4.

5.1 Background

CECs are present in municipal wastewater primarily due to human excretion of unabsorbed pharmaceuticals, formed products during water treatment (DBPs), and consumer and industrial chemical products. Ternes et al. (2005) investigated fate, occurrence, and treatment of human acidic drugs (diclofenac, ibuprofen, bezafibrate); neutral drugs (diazepam, carbamazepine); personal care products (tonalide, galaxolide; both musk fragrances); antibiotics (sulfamethoxazole, roxithromycin); iodinated contrast media (iopromide); and estrogens (17 α -ethinylestradiol, 17 β -estradiol, estrone). Based on this research, biological degradation and sorption were defined as promising techniques to evaluate the removal of CECs during wastewater treatment. Besides, ozonation as an advanced oxidation treatment was found to potentially diminish CECs concentration in water as well (Ternes et al., 2005). The authors reported that ozonation could reduce the estrogenic effect of CECs in the environment because ozonation generates biodegradable byproducts from CECs. The additional biofilter step to degrade these byproducts

was recommended for those cases when the treated water is to be reused for potable purposes. According to Douterelo et al. (2014), the main idea of environmental microbial research is to look for the activity and function of the microbial communities in degrading the target pollutants. Hence, understanding how microbial community shifts depending upon water being treated and relates this to treatment performance and efficiency is needed to ensure water quality and biofiltration treatment process success. With this, a closed picture of the microbial ecosystems is obtained by linking the presence of microorganisms to a particular biochemical activity such as dehalogenation, oxidation, and reduction that leads to organic contaminant degradation and mineralization. Based on observed and identified groups of bacteria present in a WRRF biological process, some key insights can be developed on the selected species or strains that can degrade the CECs in advanced treatment water reuse systems. To do so, it is critical to identify the bacterium types capable of CEC biodegradation and then determine if they are present in the biological process and effluent of the WRRF.

5.2 Materials And Methods

5.2.1 Source Water and Pilot-scale Advanced Water Treatment description

As described in subsection 4.2.1

5.2.2 Data Collection and Sample campaigns

As described in section 4.2.2.

5.2.3 DNA Extraction and High-throughput Sequencing

As described in section 4.2.3

5.2.4 Bioinformatic and Statistical Analyses

As described in section 4.2.4.

5.2.5 Contaminants of Emerging Concern Studied

The reduction in CEC concentrations through the system was evaluated during the pre-injection phase by collecting and analyzing samples for unregulated contaminants at the CFCGMF influent, as well as before and after each CEC treatment barrier (CFCGMF EFF, O3 EFF, BAC EFF, GAC EFF, UV EFF, and CF EFF). The CECs list included 1,4 Dioxane, Estradiol, Atenolol, Caffeine, Carbamazepine, Cotinine, N, N-diethyl-metatoluamide (DEET), Estrone, Ethinyl estradiol, Gemfibrozil, Iohexol, Meprobamate, Phenytoin, Sucralose, Sulfamethoxazole, Tris (2-chloroethyl) phosphate -TCEP, and Triclosan. For CEC analysis, samples were shipped to Eurofins Eaton Analytical (Monrovia, CA) laboratory, which used a fully automated online solid-phase extraction, high-performance liquid chromatography (HPLC), mass spectrometry-mass spectrometry (SPE-LC/MS/MS) system. The laboratory provided bottles and sample preservatives. Samples were shipped within 24 hours of collection. The samples were packed with wet ice and contained in coolers to preserve temperatures below 5°C. A strict protocol was followed for sample collection to guarantee successful field sampling by avoiding external contamination, errors, and controlling process conditions.

5.3 Results and Discussion

5.3.1 Unique OTUs at Each Treatment Step.

A total of 8904 OTUs were identified in all three main barriers of the AWT train. 21.5% of the total OTUs were detected in all three main barriers, and the unique OTUs presence was 10.5%, 17.3%, and 12.3 % for the CFCGMF, O3-BAC, and UV barriers, respectively. Figure 5-1a shows details of shared and unique OTUs for each treatment barrier. Only 239 OTUs (Figure 5-1b) were commonly detected at all locations. The location that showed the highest percentage of unique OTUs was CFCGMF INF (7.8%), followed by the GAC (5.9%) and BAC (5.7%) effluents.

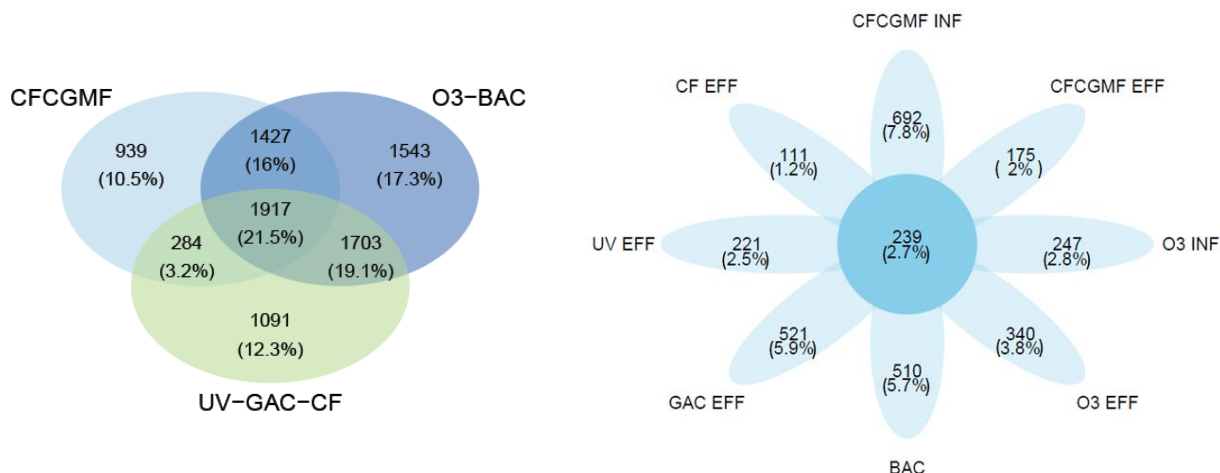


Figure 5-1. Venn Diagrams showing unique and shared OTUs detected at a) main barrier level and b) each treatment barrier effluent.

When exploring the detected OTUs at the phylum level (Table 5-1) and order level (Figure 5-2), CFCGMF EFF showed a relative low abundance of *Proteobacteria* (~2% compared to at least 17% detected at all other locations), *Actinobacteria* and *Firmicutes* were the dominant phylum, at order level *Micrococcales* and *Selenomonadales* were the most representative. O3 EFF, GAC EFF, and CF EFF most abundant unique OTUs belonged to *Proteobacteria* and *Bacteroidetes*, at order level *Sphingobacteriales* and *Bdellovibrionales* were found at O3 EFF, *Sphingobacteriales* and *Legionellales* at GAC EFF, and later *Rhizobiales* at CF EFF. BAC EFF unique observable OTUs detected at order level are *Myxococcales*, *Planctomycetales*, and *Rhizobiales*. UV EFF had *Proteobacteria* and *Cyanobacteria* in the order: *Enterobacteriales*, *Rhizobiales*, and *Prochlorales*.

Table 5-1. Unique OTUs observed at phylum taxonomic level in CFCGMF EFF, O3 EFF, BAC EFF, GAC EFF, UV EFF, and CF EFF.

Phylum	CFCGMF EFF	O3 EFF	BAC EFF	GAC EFF	UV EFF	CF EFF
<i>Acidobacteria</i>	4	2	24	14	4	1
<i>Actinobacteria</i>	504	33	11	21	200	30
<i>Bacteroidetes</i>	151	894	108	331	176	152
<i>Chlamydiae</i>	14	127	37	138		31

Phylum	CFCGMF EFF	O3 EFF	BAC EFF	GAC EFF	UV EFF	CF EFF
<i>Chloroflexi</i>	2	8	143	142	16	
<i>Cyanobacteria</i>	4	34	6	29	324	
<i>Deferribacteres</i>				2		
<i>Deinococcus-Thermus</i>		2				
<i>Elusimicrobia</i>	2		11			
<i>Euryarchaeota</i>				14		
<i>Firmicutes</i>	773	322	56	5	18	83
<i>Fusobacteria</i>			6	22		
<i>Lentisphaerae</i>		2				
<i>Nitrospirae</i>	5		31	2		
<i>Planctomycetes</i>	3	108	239	116	55	8
<i>Proteobacteria</i>	202	1041	1019	1188	8408	601
<i>Spirochaetes</i>	17	19	17		15	
<i>Tenericutes</i>	3					
<i>Verrucomicrobia</i>	8	39	23	11	496	5
<i>Unclassified</i>	7868	2123	1035	4795	2306	586
<i>Abundance Subtotal</i>	9560	4754	2766	6830	12018	1497
<i>OTUs observed</i>	175	340	510	521	221	111

Table 5-1 shows that the abundance of *Proteobacteria*, *Bacteroidetes*, and *Planctomycetes* increased after ozonation, while *Actinobacteria* and *Firmicutes* decreased their relative abundance of unique observed OTUs. After the BAC filters, the unique phylum detected were *Proteobacteria*, *Planctomycetes*, and *Chloroflexi*. At the GAC EFF, *Bacteroidetes* significantly showed a higher relative abundance. Downstream UV disinfection, the unique OTUs were classified as *Proteobacteria*, *Cyanobacteria*, *Actinobacteria*, and *Bacteroidetes*. At the final treatment step (CF filter), the number of observed unique OTUs diminished by 78% compared to the BAC EFF samples.

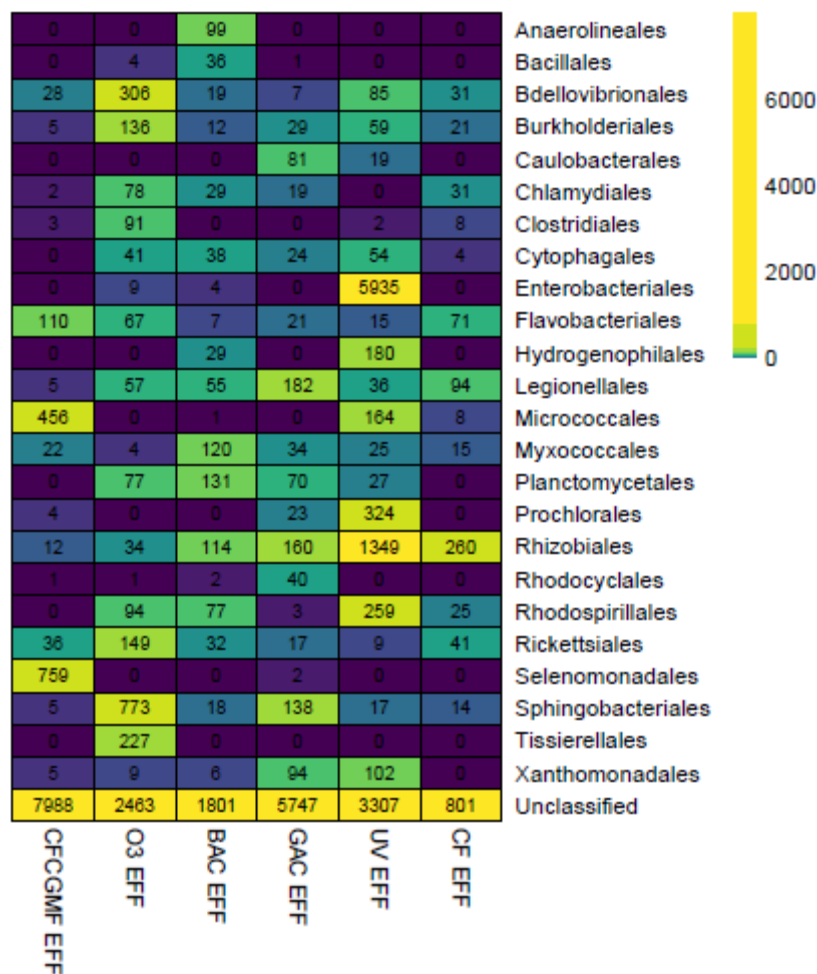


Figure 5-2. Heatmap for unique OTUs abundance at order taxonomic level at CFCGMF EFF, O3 EFF, BAC EFF, GAC EFF, UV EFF, and CF EFF.

5.3.2 Persistent Bacteria Through the Treatment Train

The persistence of taxonomical groups at genus and species level are shown in Figures 5-3 and 5-4, respectively. The percentage reduction of unique OTUs after CFCGMF EFF, O3 EFF, BAC EFF, GAC EFF, UV EFF, and CF EFF were 10, 52, -63, 41, -133, and 56%, respectively. The negative percentage suggests that the counts increased at the BAC and UV effluents.

Microorganisms belonging to *Acidovorax* and *Pedobacter* genera were the most dominant across

the treatment train. The relative abundance of the former ranged between 2 to 30% across the treatment train, it decreased after CFCGMF, but it built up to 29% at BAC EFF, then a 2/3 decrease is achieved after GAC filter, but it goes back to 30% at UV EFF. *Pedobacter* relative abundance decreased along with the treatment steps (14.5% to 0.1%), the biggest relative abundance reduction was detected after the BAC filter. After UV disinfection, the genus *Lysobacter* was detected with 38% of relative abundance.

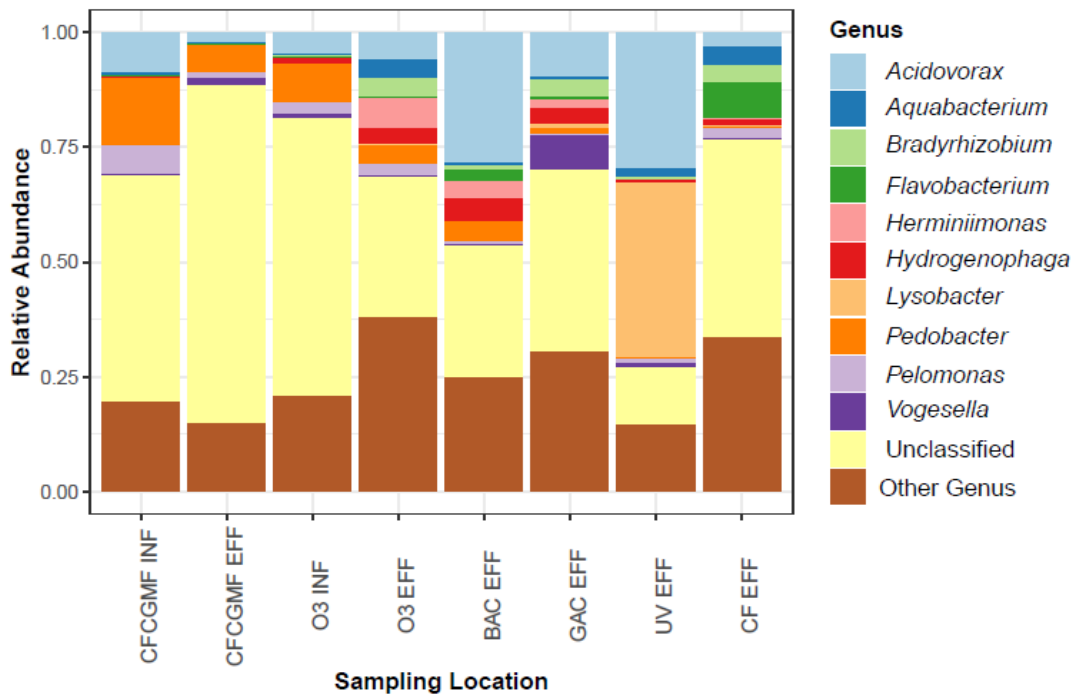


Figure 5-3. Relative abundance of ten persistent OTUs at genus level across the treatment train.

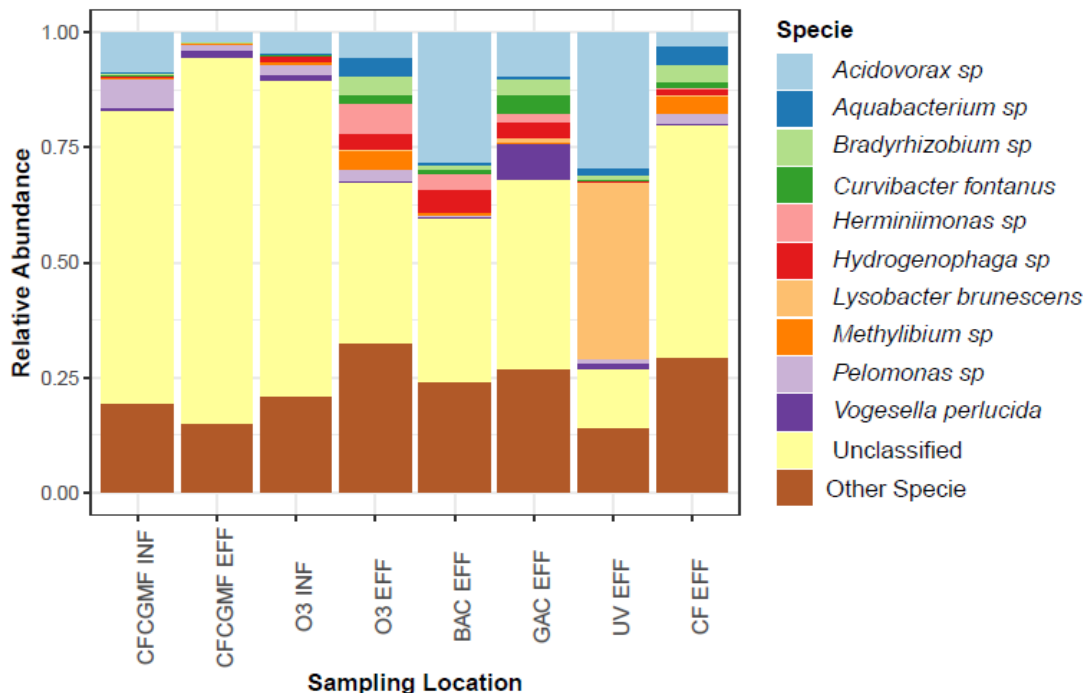


Figure 5-4. Relative abundance of ten persistent OTUs at species level across the treatment train.

At species level, *Acidovorax sp*, *Bradyrhizobium sp*, *Hydrogenophaga sp*, *Lysobacter brunescens*, and *Pelomonas sp* were the most dominant across the treatment train. The *Acidovorax sp* was dominant in both BAC (28%) and UV (30%) effluents, while the average relative abundance at other sampling locations was $6 \pm 3\%$. These microorganisms are aerobic and commonly found in soil, water, and plants. *Acidovorax sp* are divided into two groups: phytopathogens and environmental species found in soil and water habitats. The environmental species have been reported as pollutants degraders. Among the pollutants that these species can degrade are biphenyl, 2-nitrotoluene, PHBs, etc. (Boycheva et al., 2015).

Bradyrhizobium sp are aerobic microorganisms and have been reported to participate in diverse processes such as nitrification, sulfur oxidation, and aromatic degradation (Nguyen et al., 2018). *Hydrogenophaga sp* has been isolated from water, activated sludge, soil, and compost.

They can be aerobic or facultative anaerobic (Choi et al., 2020). *Lysobacter brunescens* was detected with a relative abundance of 38% at UV EFF, suggesting resistance to UV light.

Lysobacter genus is known to be a gliding and bioactive antibiotic; it has been reported as capable of producing exoenzymes that lyse and degrade other organisms (Gökçen et al., 2014; Ling et al., 2019). *Pelomonas sp.* relative abundance was reduced along with the treatment train; the lowest abundances were detected in filter effluents BAC and GAC.

5.3.3 Identification of Microbial Communities on BAC Filters

The most abundant phyla (Figure 5-5) identified were *Proteobacteria* (38-61%), *Firmicutes* (1-6%), *Cyanobacteria* (0-28%), and unclassified (15-38%). Media samples showed distinct features from liquid samples (O3 INF, O3 EFF, BAC 1 EFF, BAC 2 EFF, BAC EFF). The relative abundance of *Nitrospirae* and *Acidobacteria* were more dominant in media BAC samples (12-35% and 5-15%, respectively) than they were in liquid samples (0-2% and 0-5%, respectively). In addition, *Planctomycetes*, *Chloroflexi*, and *Verrucomicrobia* completed the most detected phyla on solid media samples as found elsewhere (Gerrity et al., 2018; Lautenschlager et al., 2014). On the order level, few groups were dominant (Figure 5-6). The order was *Burkholderiales* (Liquid:8-17%; Media: 3-4%), *Rhizobiales* (Liquid:4%; Media:5-6%), *Sphingobacteriales* (Liquid:2-6%; Media:2-3%), and *Acidobacteriales* (Liquid:0-1%; Media:3-4%) across the O3/BAC barrier. Additionally, in media BAC samples, *Acidobacteriales* (L:0-1%; Media:3-4%), *Nitrospirales* (Liquid:0-1%; Media:2-3%), and *Rhodospirillales* (Liquid:0-2%; Media:1-3%) were detected. In general, samples taken from liquid phase (O3 INF, O3 EFF, BAC 1 EFF, BAC 2 EFF, and BAC EFF) showed similar microbial community composition but they were distinct from media samples. *Acidovorax* (Liquid:2-10%; Media:0.1-0.3%), *Pedobacter* (Liquid:2-5%; Media:0-0.1%), and *Bdellovibrio* (Liquid:1-3%;

Media:0.1-0.2) were the most prevalent genera in the liquid samples, while *Nitrospira* (Liquid:0-1%; Media:2-3.0%) was the most common genus on solid media samples.

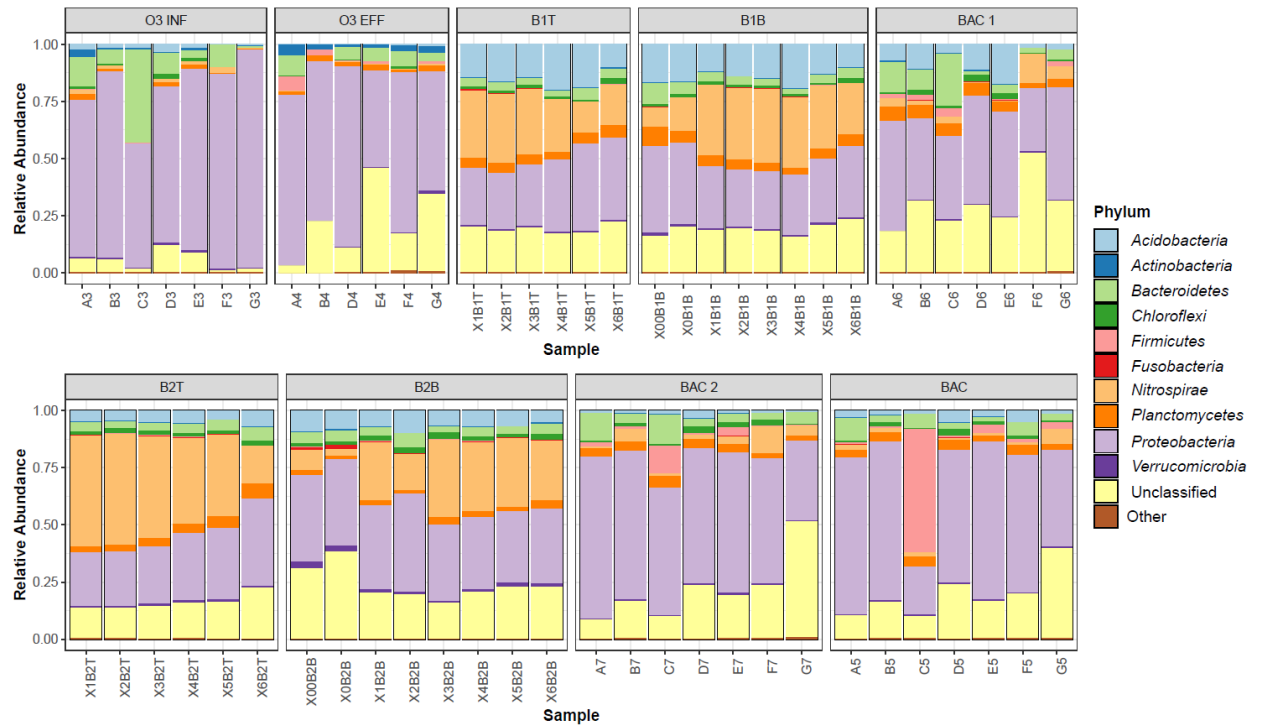


Figure 5-5. Relative abundance of top 10 phyla before, within, and after the BAC filter. Liquid samples were taken at O3 INF, O3 EFF, BAC 1, BAC 2, and BAC effluents. Solid samples were taken at two depths (top: B1T, B2T, and bottom: B1B, B2B).

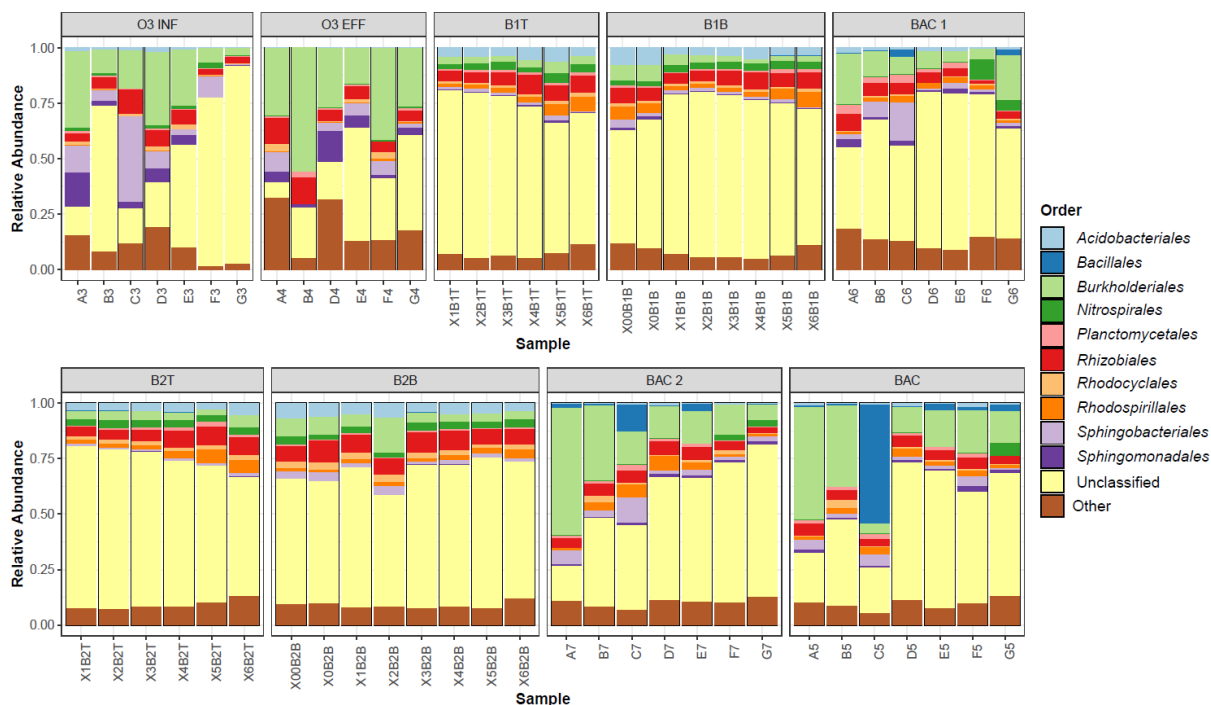


Figure 5-6. Relative abundance of top 10 order before, within, and after the BAC filter. Liquid samples were taken at O3 INF, O3 EFF, BAC 1, BAC 2, and BAC effluents. Solid samples were taken at two depths (top: B1T, B2T, and bottom: B1B, B2B).

A trend of increasing diversity and richness after ozonation was detected in other studies (de Vera and Wert, 2019). The Shannon diversity index did increase downstream of the ozonation step. These samples (media BAC samples at top and bottom, BAC effluents, and combined BACs effluents) ranged from 4.6-5.8, with an average evenness value of 0.76, and 1156-1905 observed OTUs. The high evenness indicates an even distribution within these communities suggesting higher diversities as detected. BAC filters effluent communities may reflect microbial media communities that slough off, microorganisms with high ozone tolerance, and those who prefer a planktonic lifestyle. When testing the microbial community differences across sample locations, non-parametric statistical tests suggested that the microbial community diversity in the deeper media samples at the BAC 2 filter were significantly different from all other locations, including its influent and treated effluent. Results from an analysis of similarity (ANOSIM) were significant, indicating that sample location impacts microbial community composition.

These results concur well with previous findings (Lautenschlager et al., 2014; Q. Li et al., 2017; Zhang et al., 2018b). Microbial communities on BAC media filters differ from upstream and downstream process units. Factors such as nutrients, interactions with other organisms, the presence of disinfectants/inhibitors, and their arrangement will define the microbial community structure and composition (Guarin et al., 2021; Prest et al., 2016; Rittmann, 2018).

5.3.4 Fate of Contaminants of Emerging Concern across the AWT

The occurrence of 18 CECs monitored was analyzed (Figure 5-7) across the AWT system. A total of ten sampling events occurred during the monitoring phase. The percent reductions observed from the influent through the finished water across the three barriers are presented in Table 5-2. The removal of CECs across the three main barriers was gradual. The CFCGMF step did not provide a strong barrier against CECs removal, the highest percent reduction of detected CECs was for Meprobamate, TCEP, and caffeine with 35-58%, 7-43%, and 0-39%, respectively. No reduction was observed for 1,4-Dioxane, Carbamazepine, Cotinine, DEET, Gemfibrozil, Phenytoin, and Primidone.

Downstream, Carbamazepine, Estrone, Gemfibrozil, and Phenytoin were not detected further along the treatment train due to the oxidative ozone effect provided by the ozonation step. These four compounds shared common aromatic ring structures coupled with active groups such as amino and hydroxyls, making them susceptible to ozonation. The average percentage reduction after ozonation was 44 - 60%. The compounds that showed the lowest removals were TCEP, meprobamate, and sucralose, with an average of 27%. Following ozonation, the BAC filtration step achieved non-detectable levels for Atenolol and Sulfamethoxazole. In addition, the average percent removal was between 58-75%. The three compounds with the lowest removals were 1-4 Dioxane,

Meprobamate, and lohexol, with an average of 47% removal. The third barrier, GAC/UV/CF, further decreased six CECs (Caffeine, Cotinine, DEET, Meprobamate, Primidone, and TCEP) to non-detect levels. Only three compounds were detectable before passing through UV disinfection: 1,4-Dioxane, Sucralose, and lohexol. The latter was not detected after UV irradiation even though it is not susceptible to UV oxidation, while the percentage removal for 1,4-Dioxane and Sucralose were 7% and 97%, respectively.

Summarizing the removal across the three barriers, three out of 18 compounds were never detected in the influent during the ten sampling events. These were: Estradiol, Ethinyl estradiol, and Triclosan. Only three compounds were detected 25% of the time in the finished water and were: DEET, lohexol, and Sucralose. In addition, the only compound that was detected in both influent and finished water in all ten sampling events was 1,4-Dioxane. The remaining eleven compounds were removed up to the detection level, as seen in Figure 5-7. The lowest percent reduction was between 5-65% for Caffeine and Phenytoin. However, the concentration in the finished water was always below detection limits. None of the compounds showed higher concentration in the effluent; a specific percent removal was achieved for all the listed compounds (Table 5-2). Even though the percent reductions across the treatment barrier were not 100%, all the compounds except two (1,4-Dioxane and Sucralose) achieved concentrations below detection levels. The percent reductions when concentrations achieved non-detect levels were computed using the minimum report level (MRL) as the final concentration. Regarding two remaining compounds, 1,4-Dioxane was detected below notification level (California 1000 ng/L) and an average percent removal of 60-73%, while sucralose was 99 - 100%.

1,4-Dioxane is a recalcitrant groundwater compound used primarily as a solvent in industry and personal care products. Its inherent physical and chemical properties make it a challenge for removal through biological, physical, or chemical treatment (Chitra et al., 2012; DiGiuseppi et al., 2016; Simonich et al., 2013). It is highly mobile and not very susceptible to biodegradation. It has a very low Henry's law constant and is very miscible, making its extraction or removal from water complicated during treatment. In addition, it has a very low organic carbon partitioning coefficient ($\log K_{oc}$ of 0.54). Hence it can display very low sorption (Horst et al., 2019). 1,4-Dioxane ring structure adds resistance to chemical oxidation and requires an oxidation potential of at least 2V for effective oxidation (DiGiuseppi et al., 2016). UV light addition does not result in sufficient oxidation because 1,4-Dioxane is a weak absorber of UV. As a potential remediation tool, advanced oxidation processes such as combination of UV with hydrogen peroxide (UV/ H₂O₂) may react and oxidize it.

Sucralose is an artificial sweetener with very high solubility (2.75×10^4 mg/L at 25°C). Therefore, its hydrophilic characteristics make it pass through wastewater treatment processes, mainly due to its high stability, persistence, and low removal efficiency (Henderson et al., 2020; Subedi and Kannan, 2014). Because of its ubiquitous occurrence, it has been used as a tracer of wastewater contamination. In fact, sucralose is even found at detectable concentrations in drinking water systems. For example, a study (Mawhinney et al., 2011) monitored the amount of sucralose in nineteen drinking water systems across the US; they found that 15 showed sucralose in their influents, of which the concentrations were still detectable in finished water at 13 plants and in 8 distribution systems. Sucralose concentrations in these systems ranged between 48-2400 ng/L, demonstrating its persistence. Biodegradation and advanced oxidation processes have been studied as processes to remove sucralose (Sharma et al., 2014). No particular microorganism has

been identified as sucralose-degrader, but a microbial consortium may be capable of biodegrading it (Buerge et al., 2010; Mead et al., 2009). In addition, it has low reactivity with ozone and hydroxyl radicals (Sundaram and Pagilla, 2020), explaining its low removal percentage after ozonation.

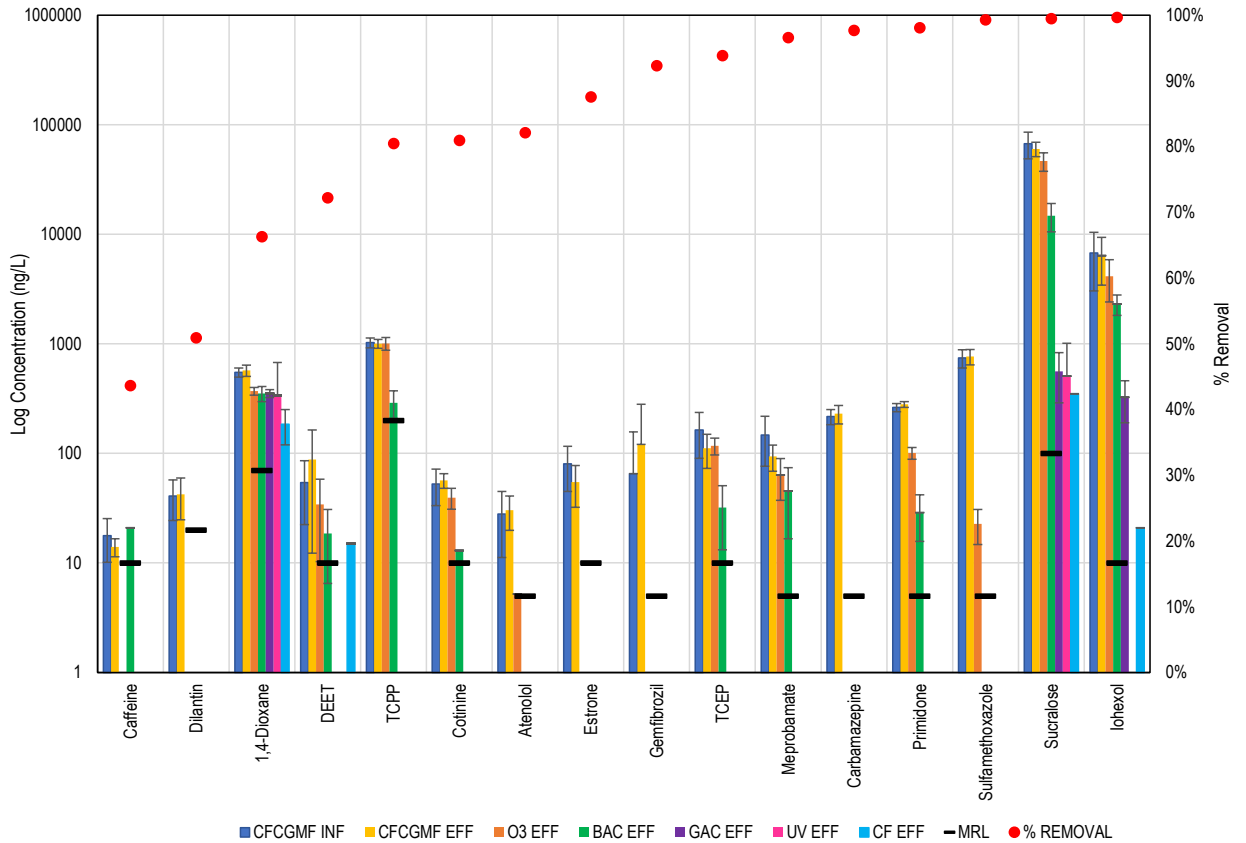


Figure 5-7. Log Concentration of listed CECs across the AWT train and corresponding cumulative removal percentage at each treatment barrier (colored bar plots): CFCGMF influent and effluent, ozone effluent (O3 EFF), BAC filter effluent (BAC EFF), GAC filter effluent (GAC EFF), UV irradiation effluent (UV EFF), and cartridge filtration effluent (CF EFF), and percentage removal up to the indicated barrier (red dots)

Table 5-2. Percent Reduction of Detected CECs After Main Treatment Barriers

CEC	Percent Reduction of Detected CECs (%)					
	CFCGMF EFF	O3 EFF	BAC EFF	GAC EFF	UV EFF	CF EFF
1,4-Dioxane	-	35 - 38 %	37 - 39 %	0 - 9 %	0 - 14 %	40 - 54 %
Atenolol	0-25 %	74 - 88 %	75 - 88 %	**		
Caffeine	0 - 39%	17 - 41 %	0 - 100 %	0 - 52%	0 - 52%	0 - 52%
Carbamazepine	-	97 - 98 %	97 - 98 %	*		
Cotinine	-	31 - 41 %	74 - 81 %	0 - 23 %	0 - 23 %	0 - 23 %
N,N-diethyl-metatoluamide (DEET)	-	56 - 66 %	68 - 80 %	9 -75 %	9 -75 %	0 - 63%
Estrone	24 - 31 %	66 - 88 %	66 - 88 %	*		
Gemfibrozil	-	76 - 99 %	76 - 99 %	*		
Iohexol	8 - 31 %	24 - 38 %	49 - 75 %	83 - 91 %	99%	99%
Meprobamate	35 - 58 %	15 - 18 %	32 - 53 %	82 - 94 %	82 - 94 %	82 - 94 %
Phenytoin (Dilantin)	-	9 - 68 %	9 - 68 %	*		
Primidone	-	61 - 68 %	84 - 93 %	72 - 90 %	72 - 90 %	72 - 90 %
Sucralose	0 - 33 %	27 - 35 %	70 - 78 %	96 -97 %	96 -97 %	97 - 98 %
Sulfamethoxazole	0 - 9%	98 - 97 %	99 - 100 %	**		
Tris (2-chloroethyl) phosphate (TCEP)	7 -43 %	0 - 18 %	62 - 77 %	38 - 84 %	38 - 84 %	38 - 84 %

Note:

* CECs removed to not detectable levels after ozonation and were not further detectable.

** CECs entirely removed to not detectable levels after BAC filtration and were not further detectable.

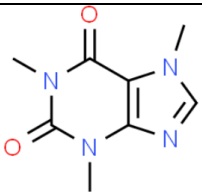
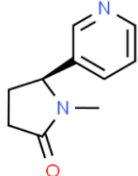
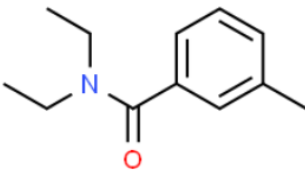
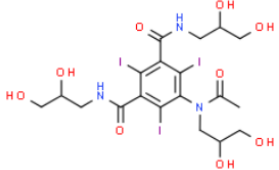
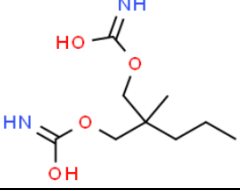
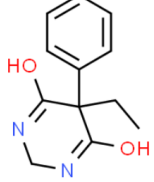
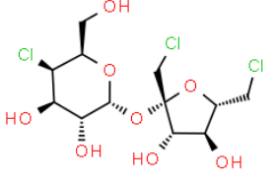
5.3.5 CECs Descriptors and Molecular Structure Details

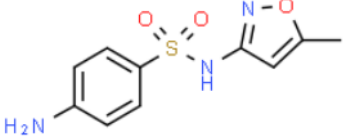
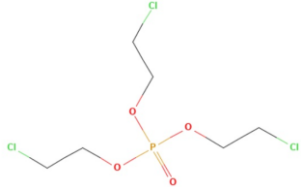
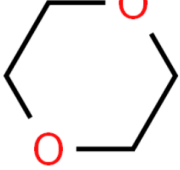
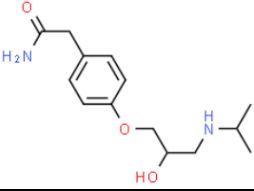
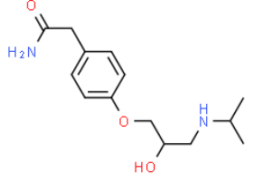
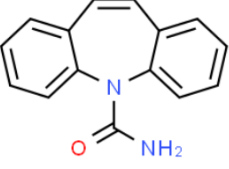
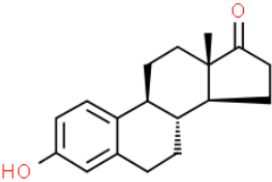
A compound response to physical, chemical, and biological processes depends on their molecular structure, which includes their molar mass, availability of functional molecules, molecular weight, structure, functionality, and shape (Tadkaew et al., 2011).

Table 5-3 listed eleven molecular descriptors: molecular formula, chemical molecular weight (MW; g/mol), chemical structure, polar surface area (\AA^2), the distribution coefficient at pH=7.4 (LogD), Octanol-Water partition coefficients (LogKow and LogP), the biodegradability indicator represented as its probability of rapid aerobic biodegradation (BIOWIN6), number of hydrogen bond donors and acceptors, and rotatable bond count. Descriptors were taken from ChemSpider database¹. These descriptors provide information about the size of the molecules (MW), their ability to engage in hydrogen bonding, the molecule flexibility, the lipophilicity of the molecule, and polarity as the sum of surface contributions of the electronegative atoms (oxygen, nitrogen, and attached hydrogens) in a molecule (Ivančev-Tumbas et al., 2021). The most hydrophilic compound based on their logKow (<3) and LogD (<0) value would be the least adsorbable due to its affinity for water. In addition, those with electron donor functional groups are amenable to ozonation. Within the highly reactive compounds include activated aromatic structures, such as amine functionalities.

¹ <https://www.chemspider.com/Default.aspx>, properties predicted by ACD/ Labs (2021) online, last accessed December 1st, 2021

Table 5-3. CECs descriptors of adsorption, biodegradability, and ozonation amenability.

CEC	Chemical Structure	Polar Surface Area, Å ²	Log K _{ow}	LogP	LogD	BIOWIN6	#H bonds	
							Acceptors	Donors
Caffeine C ₈ H ₁₀ N ₄ O ₂ MW: 194.19		58	0.16	-0.13	0.28	0.0521	6	0
Cotinine C ₁₀ H ₁₂ N ₂ O MW: 176.21		33	0.34	-0.23	0.1 0.17	0.2890	3	0
DEET C ₁₂ H ₁₇ NO MW: 191.27		20	2.26	1.96	2.24 2.24	0.3954	2	0
Iohexol C ₁₉ H ₂₆ I ₃ N ₃ O ₉ MW: 821.138		200	-	-4.16	-3.17	-	12	8
Meprobamate C ₉ H ₁₈ N ₂ O ₄ MW: 218.25		107	0.98	1.14	-1.82	0.3140	6	4
Primidone C ₁₂ H ₁₄ N ₂ O ₂ MW: 218.25		65	0.73	0.03	-0.86	0.4211	4	2
Sucralose C ₁₂ H ₁₉ Cl ₃ O ₈ MW: 397.63		129	-1	0.68	-0.17	0.004	8	5

CEC	Chemical Structure	Polar Surface Area, Å ²	Log K _{ow}	LogP	LogD	BIOWIN6	#H bonds	
							Acceptors	Donors
Sulfamethoxazole C ₁₀ H ₁₁ N ₃ O ₃ S MW: 253.278		107	0.48	0.89	0.56 -0.56	0.006	6	3
TCEP C ₆ H ₁₂ Cl ₃ O ₄ P MW: 285.49		55	1.63	0.48	1.42	0.0193	4	0
1,4-Dioxane C ₄ H ₈ O ₂ MW: 88.105		18	0.32	-0.27	-0.29	0.6713	2	0
Estradiol C ₁₈ H ₂₄ O ₂ MW: 272.382		40	3.94	4.13	3.62	0.1058	2	2
Atenolol C ₁₄ H ₂₂ N ₂ O ₃ MW: 266.336		85	-0.03	0.1	-2.82 -1.85	0.2349	5	4
Carbamazepine C ₁₅ H ₁₂ N ₂ O MW: 236.269		46	2.25	2.67	2.28	0.0364	3	2
Estrone C ₁₈ H ₂₂ O ₂ MW: 270.366		37	3.43	3.69	3.38	0.1074	2	1

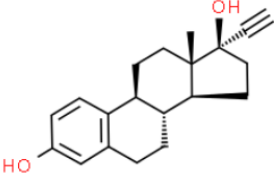
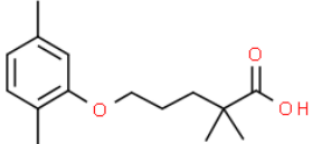
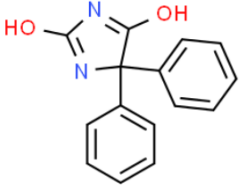
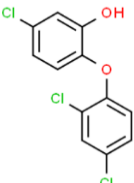
CEC	Chemical Structure	Polar Surface Area, Å ²	Log K _{ow}	LogP	LogD	BIOWIN6	#H bonds	
							Acceptors	Donors
Ethinyl estradiol C ₂₀ H ₂₄ O ₂ MW: 296.403		40	4.12	4.52	3.87	0.0354	2	2
Gemfibrozil C ₁₅ H ₂₂ O ₃ MW: 250.33		47	4.77	4.39	3.37 1.58	0.6123	3	1
Phenytoin C ₁₅ H ₁₂ N ₂ O ₂ MW: 252.26		65	2.16	2.29	0.79 -0.98	0.0408	4	2
Triclosan C ₁₂ H ₇ Cl ₃ O ₂ MW: 289.54		29	4.66	5.17	5.27	0.0187	2	1

Table 5-4 contains a description of all the eighteen compounds under study with the main functional chemical groups of their molecular structure.

Table 5-4. Molecular structure description of CECs under study.

CECs	Molecular Structure Description
Caffeine	Purine base (xanthine ring), alkyl group, and c=c double bond.
Cotinine	Ketone and pyridine ring
DEET	Amide in their aromatic rings.
Iohexol	Benzene, carbonyl groups, iodo substitutes, secondary and tertiary amides, primary and secondary alcohols
Meprobamate	Double bonds, carbamates (amide)
Primidone	Aromatic bonds, six membered rings, secondary amides.
Sucralose	Trichlorinated disaccharide (two monosaccharide joined by glycosidic linkage)
Sulfamethoxazole	Sulfanilamide: aromatic bonds, five membered rings, six membered ring, primary amide, sulfonamide, isoxazole.

TCEP	Aliphatic structure with chlorine functional groups.
1,4-Dioxane	Heterocyclic organic compound: six membered rings, ethers
Estradiol	Phenol and aliphatic rings
Atenolol	Aromatic ring, carboxylic acid, ether, amine.
Carbamazepine	Aromatic rings, amide.
Estrone	Phenol and aliphatic rings
Ethinyl estradiol	Phenol and aliphatic rings
Gemfibrozil	Aromatic ring, carboxyl group, ether.
Phenytoin	Five membered ring, six membered rings, ester, urea derivative, imide
Triclosan	Aromatic bonds, six membered ring, aromatic hydroxyls, ether.

5.3.6 Functional and Metabolic Analyses

Using abundance and phylogeny information available in the 16S data, functional and metabolic predictions can be extrapolated using bioinformatic tools. Hence, the tool Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 -PICRUSt2 version 2.4.1 (Douglas et al., 2020) was used to predict the functional potential of the bacterial communities based on marker gene sequencing profiles. Also, the public pipeline and tutorial (Douglas, 2021) was followed for the analysis. Because the CECs concentration was being attenuated across the AWT, the metabolic capacity of the microorganisms that were detected along with the treatment train was explored. Thus, PICRUSt2 was used to predict KEGG pathway abundances in all the collected samples.

PICRUSt2 (Douglas et al., 2020) was developed "to predict the functional potential of a bacterial community based on marker gene sequencing profiles." However, there are limitations, such as the biased function predictions towards existing reference genomes, suggesting that rare environment-specific functions are harder to identify. Also, the resolution to differentiate strain-

specific functionality because PICRUST2 can only distinguish taxa to the point they differ at the amplified marker gene sequence.

Nevertheless, PICRUST2 is a tool that provides flexibility for marker gene metagenome inferences. A total of 180 predicted KOs were grouped up to level 1 of categorization (Figure 5-8). The KO analysis showed that the dominant metabolism categories were the metabolism of cofactors and vitamins, amino acids, carbohydrates, terpenoids and polyketides, and other amino acids. Out of the 180 predictions, 17 were related to xenobiotic biodegradation and metabolism. Figure 5-9 shows the average number of predictions of this category along the treatment train. The highest number of prediction pathways were found after the ozonation step (O3 EFF), followed by the BAC filtration process. Under section 3.2.1, ozonation was the treatment process that achieved the highest removal of CECs, and BAC filtration provided a polishing removal stage. Within the pathways detected, the degradation of bisphenol, polycyclic aromatic hydrocarbons, ethylbenzene, chloroalkane, and chloroalkene compounds showed higher activity after ozone addition.

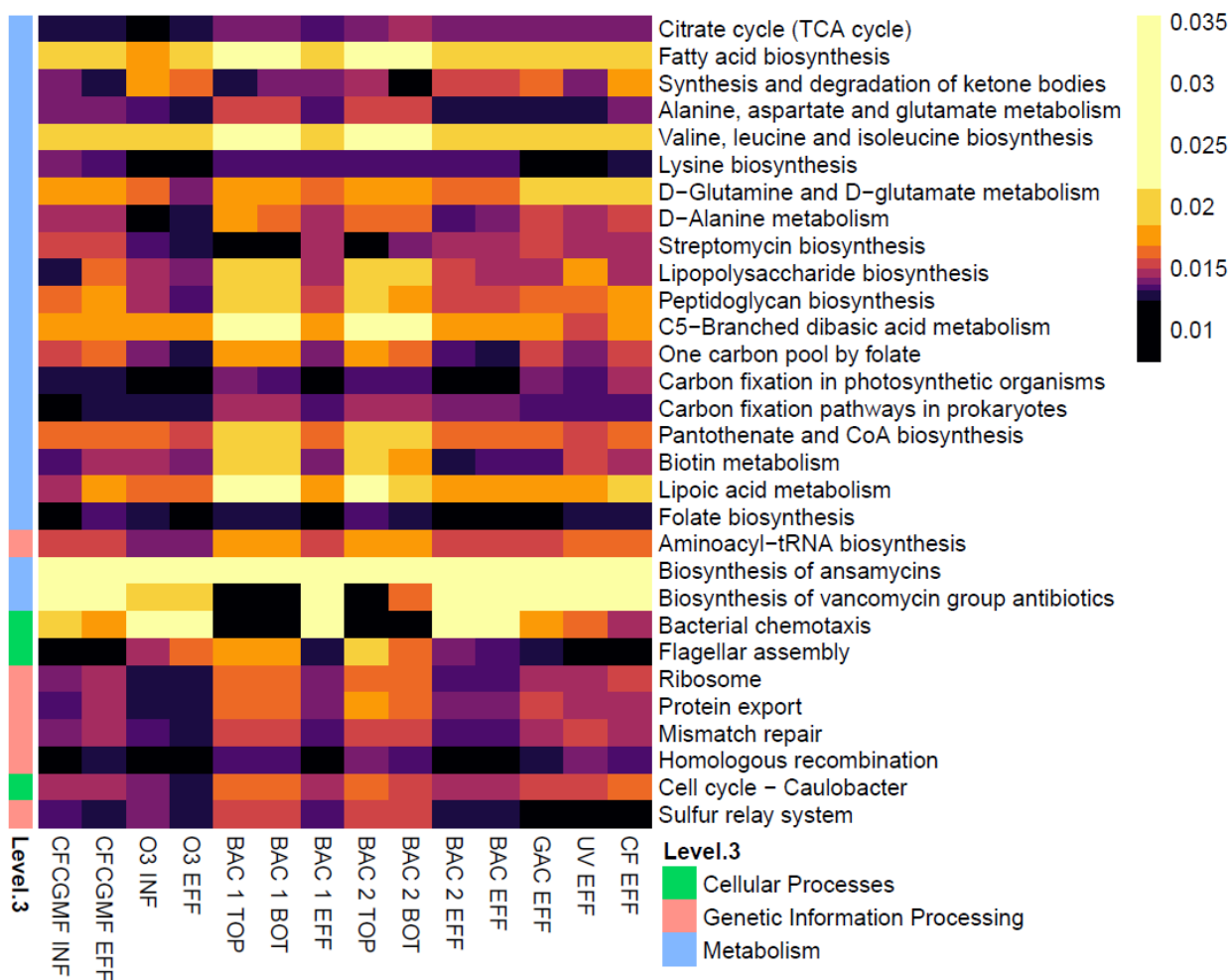


Figure 5-8. Heatmap of top 25 most abundant KEGG pathways identified in each media sample using PICRUST2 output, predicted pathways to Level 1. The color represents pathway abundance; the closer it is to black, the lower the abundance, and closer to light yellow, the higher the abundance.

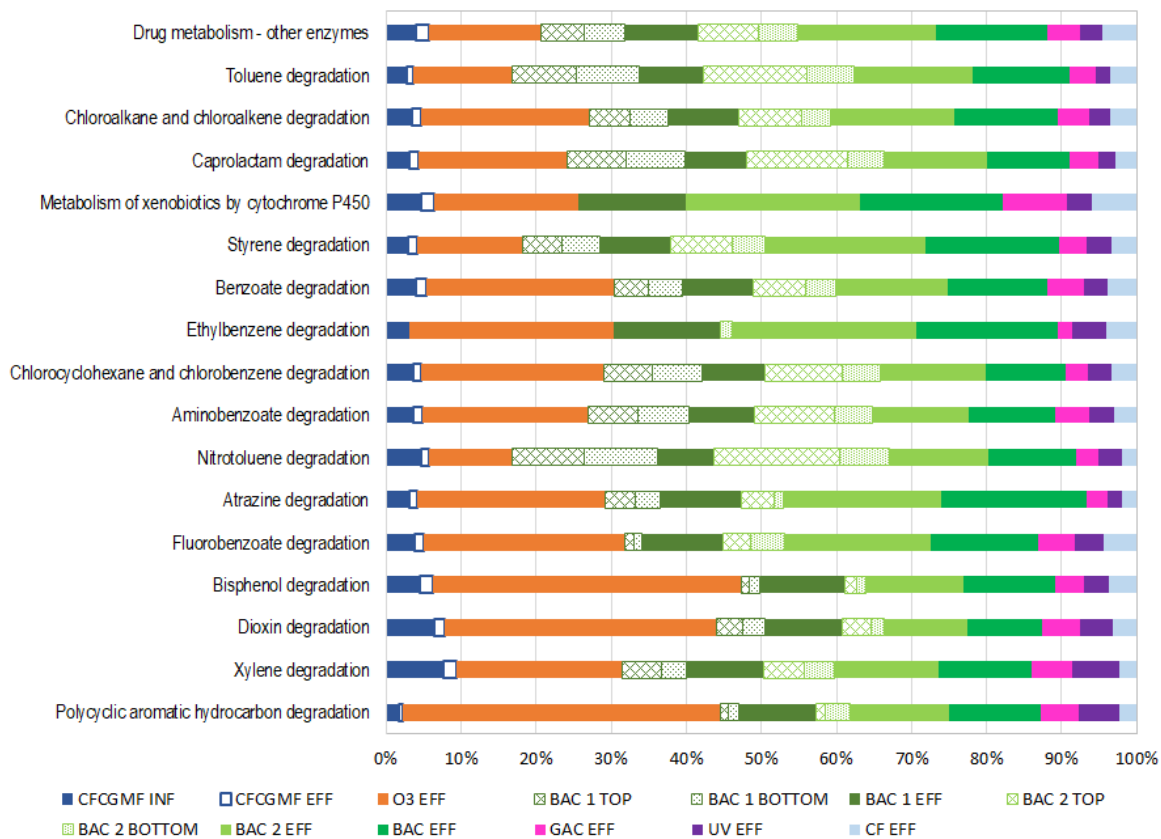


Figure 5-9. Xenobiotic biodegradation and metabolism pathways predicted at each treatment step.

Past studies (Escolà Casas et al., 2017; Falås et al., 2018; Liang et al., 2021) have reported that the transformation of CECs is conducted by individual specialized organisms (less than 0.1% of the microbial community). The top 30 most abundant genera included *Acidovorax*, *Caulobacter*, *Flavobacterium*, *Limnothrix*, *Lysobacter*, *Methylibium*, *Nitrospira*, *Pedobacter*, *Pelomonas*, *Phormidium*, and *Spirobacillus* (Figure 5-10).

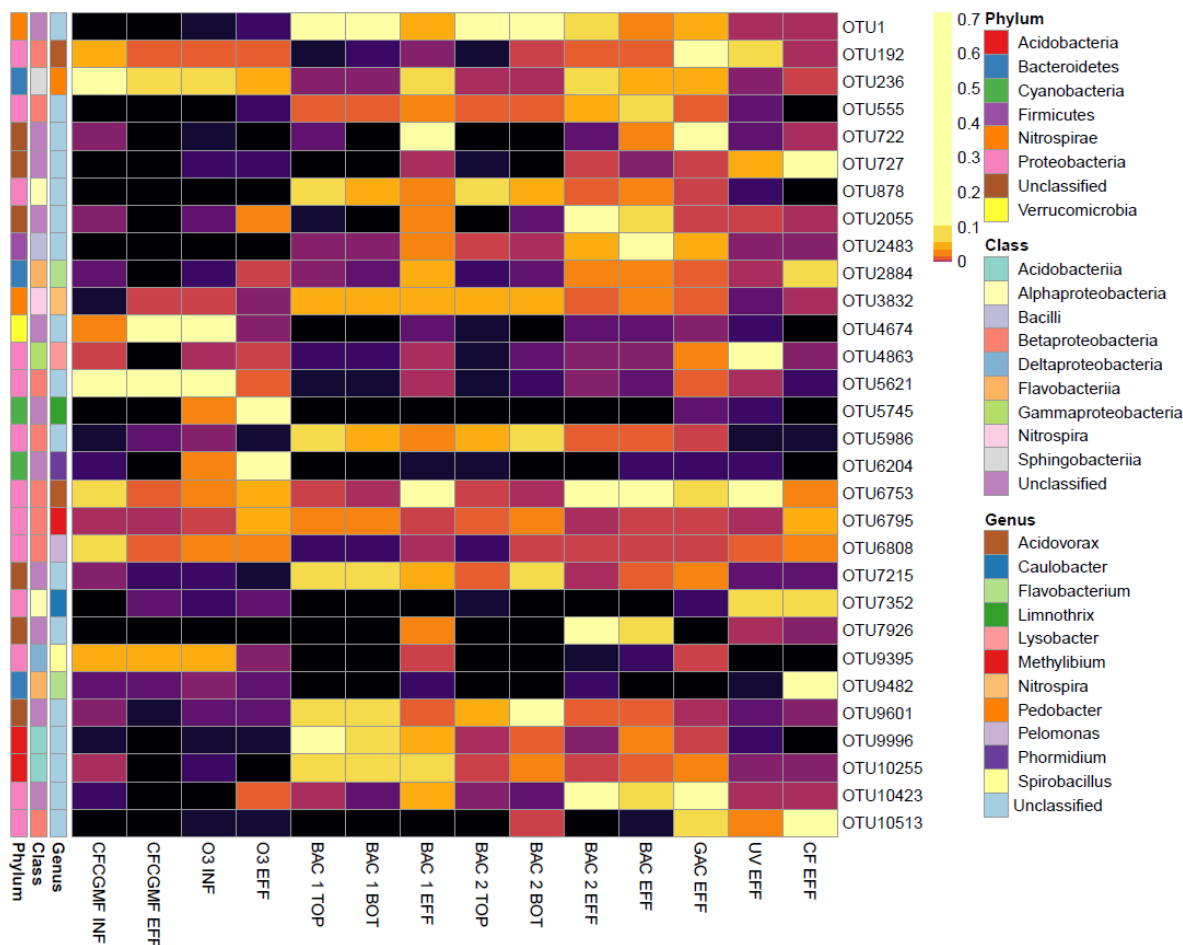


Figure 5-10. Heatmap depicts the mean relative abundance of the top 30 bacterial genera by locations. Color-scaling has been optimized for distinguishing differences between taxa with smaller mean relative abundance. A cell-colored black can be considered a value close to zero, meaning there was very low to non-detection of that genus.

For example, *Acidovorax* species have been reported as polychlorinated, biphenyl, phenanthrene-degrading organisms (Ohtsubo et al., 2012; Singleton et al., 2018). *Flavobacterium* as a cellulose-degrading bacterium (Kim and Yu, 2020). *Methylibium* are methylotrophs with the ability to metabolize the fuel MTBE, degrade aromatic compounds (benzene, toluene, and xylene), and straight-chain (C5-C12) hydrocarbons found in petroleum products, it was isolated from a biofilter treating discharges from oil refineries (Kane et al., 2007). *Nitrospira* is a nitrite-oxidizing bacteria key player on nitrite oxidation, and it cleaves urea to ammonia and CO₂ (Koch et al., 2015;

Lücker et al., 2010). The versatility and high genus diversity detected along with the AWT highlight the complex ecology involved at the microbiological level, revealing the crucial need to deepen understanding of these micro-ecosystems interactions.

5.3.7 Linking Microbial Community Composition and CEC Removal Across the AWT Train

Based on the AWT train employed in this research, biological degradation and sorption are the possible mechanisms to evaluate the removal of CECs during treatment. Besides, ozonation as an advanced oxidation treatment was found to have the potential to diminish PPCPs concentration in water (Ternes et al., 2005). The authors reported that ozonation could reduce the estrogenic effect of PPCPs in the environment because ozonation generates biodegradable byproducts from PPCPs. An additional biofilter step to degrade these byproducts was recommended for those cases when the treated water is to be reused for potable purposes. Recently, Reungoat et al. (2012, 2011) found that BAC filtration can remove PPCPs due to the media filter capacity to allow for larger biomass, enhancing the combination of biodegradation and adsorption mechanisms. In addition, a positive effect on the degradation of CECs due to the oxidation of natural organic matter can be achieved by adding a pre-ozonation step.

Supporting these past studies, this study validates their findings. The combination of O3/BAC did improve the removal percentages of the monitored CECs. However, there is no statement regarding the microbial community present on the BAC media that potentially mediates the degradation processes of some of these compounds. In this study, half of the CECs monitored in detail exhibit some removal after passing through the BAC filter, suggesting that microbial activity may be involved in the removal activity.

These compounds were caffeine, cotinine, DEET, lohexol, meprobamate, primidone, sucralose sulfamethoxazole, and TCEP (Table 5-2). Some of these compounds (meprobamate, primidone, TCEP) have reported adsorption as the main mechanisms of removal (Ma et al., 2018; Sundaram and Pagilla, 2020; Vaidya et al., 2020; Zhang et al., 2021), whereas caffeine, cotinine, DEET, sulfamethoxazole have been removed by biodegradation to some extent (Table 5-5). The remaining contaminant, lohexol, with a complex chemical structure (benzene rings with iodine substituents) was not significantly removed across the O3/BAC process. Due to its low log Kow (-3.05) and high polarity, lohexol is weakly removed by adsorption (Vaidya et al., 2020). However, advanced oxidation can potentially be removed as seen in Figure 5-7 and reported elsewhere (Hu et al., 2019).

Table 5-5. Monitored CECs reported being removed by biodegradation.

CECs	Microorganisms	Chemical Structure	Reference
Caffeine	<i>Pseudomonas putidas</i> , <i>Serratia marcescens</i> , <i>Klebsiella pneumonia</i> , <i>Rhodococcus</i> , <i>Piscinibacter</i> , <i>Hydrogenophaga</i> , and <i>Rubrivivax</i>	Heterocyclic aromatic with two-ringed structures composed of nitrogen and carbon atoms.	(Korekar et al., 2020; Zhou et al., 2022)
Cotinine	<i>Nocardioides sp.</i>	Alkaloid with a pyrrolidine ring with an oxo group and the pyridine nitrogen converted into the N-oxide.	(Qiu et al., 2018)
DEET	<i>Pseudomonas putida DTB</i>	N,N-disubstituted aromatic carbonamide	(Rivera-Cancel et al., 2007)
Sucralose	<i>Bacteroides-Flavobacterium family</i> , <i>Sphingomonas</i> , <i>Xanthomonas</i>	Chlorinated carbohydrate	(Iwai et al., 2009)
Sulfamethoxazole	<i>Arthrobacter</i> , <i>Pimelobacter</i> , <i>Achromobacter denitrificans</i> , <i>Leucobacter</i> , <i>Microbacterium</i> .	Sulfonamide (sulfur atom with two sets of double bonds to two oxygen atoms, a carbon-based side	(Deng et al., 2018, 2016; Ana C. Reis et al., 2018; Ricken et al., 2017)

CECs	Microorganisms	Chemical Structure	Reference
		group, and a nitrogen bonded to the sulfur.	

Table 5-5 compiled some microorganisms identified as CEC-degraders. Compared to those detected in this research, at least one microorganism for each CECs listed was detected. However, their relative abundances were very low (average about 0.32%). The complexity behind assigning particular metabolic degradation to a specific organism is vast and additional studies must be carried out to understand further the kinetics and pathways involved.

5.4 Conclusions

In this study the fate and identification of microbial community across an advanced water treatment train and its relationship with the removal efficiency of CEC were investigated. The results indicated that the multibarrier approach proposed CFCGMF, O3/BAC, GAC/UV/CF combined physical, chemical, and biological processes that successfully produced a high-water quality with low to non-detectable contaminant of emerging concern reducing the risk to potential public health concerns. The AWT train, included traditional treatment processes such as coagulation/flocculation/ sedimentation, BAC and GAC filtration, and disinfection steps (ozonation and UV) can reduce the total bacterial count, but had an impact on the microbial community composition downstream each unit process.

The CFCGMF barrier only provided 7-58% average percentage removal to Estrone, Iohexol, Meprobamate, sucralose, and TCEP, the destabilization of suspended particles and colloids, followed by formation of aggregates was the main mechanism. The second barrier combined ozonation and BAC filtration did achieve an average percentage reduction of 44-60% after ozonation and 58-75% downstream the BAC filters. Once the treated water passed throughout the

third barrier, GAC/UV/CF only 1,4 Dioxane, iohexol, and sucralose remained detectable but well below guidance levels.

The identification of the bacterial community structure and diversity coupled with the prediction of potential functional activity provide insights into the metabolic activity of the microorganisms involved along the treatment train. The functional activity predicted showed a direct relationship with the CEC degradation activity and detected specialized CEC-degraders actively involved in biodegradation.

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Chapter 6

RAPID SMALL-SCALE COLUMN TESTS TO ASSESS THE UPSTREAM TREATMENT IMPACT ON MICROBIAL COMMUNITIES IN BIOFILTERS USED IN WATER REUSE

The goals of this research phase are 1) to develop a rapid small-scale column test experiment based on existing literature and 2) to investigate the impact of three different upstream treatments on microbial communities in biofilters used in water reuse applications. The RSSCT setup was operated over 187 days of continuous operation at the Reno-Stead Water Reclamation Facility in parallel with the AWT pilot demonstration project. This experimental setup was designed to take advantage of the AWT pilot demonstration feasibility study and as an attempt to relate the microbial community that populated the media and the one that passes throughout the biofilter and its impact on overall treatment performance in advanced water treatment applications.

6.1 Introduction

Smaller, scaled-down fixed bed (RSSCT) that utilizes the actual raw water can predict the performance of full-scale adsorbers if the transport processes scale according to the dimensionless groups that appear in the fixed bed models. The proportional diffusivity approach was used to successfully simulate full-scale DOM breakthrough to design the RSSCT filters (Crittenden et al., 1991; Summers et al., 2014, 1995; USEPA, 1996). When the Reynolds numbers for the small and large columns are equal, the spreading in the mass transfer zones concerning column lengths are equal (Equation 2). However, when proportional diffusivity exists, Equation 4 should be used to reduce the pressure drop and the RSSCT column length as proposed by Crittenden et al. (1987).

Constant diffusivity (CD) is assumed when the intraparticle diffusivities do not change with the particle size; hence similarity is maintained. The proportional diffusivity (PD) approach has been used for organic adsorption on GAC systems when the internal mass transfer is limiting. PD-RSSCT is applied to simulate DOM adsorption and micropollutant removal in the presence of DOM (Corwin and Summers, 2010). The equations used for downscaling are listed in Table 6-1. The empty bed contact time (EBCT) and hydraulic loading rate of the small scale (SC) columns are related based on the ratios of corresponding particle diameters (d_p). In this experiment, GAC for the small-scale columns was obtained by grinding, sieving, and washing the media from the AWT GAC.

Table 6-1. Rapid Small-Scale Column Test (RSSCT) Scaling Equations (Adapted from (Westerhoff et al., 2005))

Scaling Assumption	Constant or Proportional diffusivity-specific relationships	General Relationships
Constant Diffusivity (CD) X=0	Eq. (1): $\frac{EBCT_{SC}}{EBCT_{LC}} = \left[\frac{d_{p,SC}}{d_{p,LC}}\right]^{2-X} = \frac{t_{SC}}{t_{LC}}$	Eq. (4): $\frac{V_{SC}}{V_{LC}} = \left[\frac{d_{p,LC}}{d_{p,SC}}\right] \cdot \frac{Re_{SC} \cdot Sc}{Re_{LC} \cdot Sc}$
Proportional Diffusivity (PD) X=1	Eq. (2): $\frac{V_{SC}}{V_{LC}} = \left[\frac{d_{p,LC}}{d_{p,SC}}\right]$ Eq. (3): $\frac{D_{SC}}{D_{LC}} = \left[\frac{d_{p,SC}}{d_{p,LC}}\right]^X$	Eq. (5): $Re = \frac{V \cdot \rho_L \cdot d_p}{\mu \varepsilon}$ Eq. (6): $Sc = \frac{\mu}{D_L \cdot \rho_L}$

Note: EBCT=Empty Bed Contact Time; SC: Small Scale; LC: Large Scale; d_p =media diameter; t = run time; V = loading rate; ε =absorber bed void fraction; D = effective surface diffusivity; Re =Reynolds number; Sc =Schmidt number; ρ_L : liquid density; μ =viscosity; D_L =liquid diffusivity of interest compound.

The RSSCT design is summarized in Table 6-2.

Table 6-2. RSSCT design parameters

Design Parameter	Pilot -scale	RSSCT (PD)
Particle radius (cm)	0.046	0.0115
Column diameter (cm)		1.39
Column area (cm ²)	3716.12	1.51
Bulk density (g/ml)	0.49	0.49
EBCT (min)	22.09	5.53
Loading rate (m/h)	4.28	4.04
Loading rate (cm/min)	7.13	6.73
BV to be processed	30000	30000
Flow rate (gpm)	7.00	
Re*sc (200,000-200)		2000
μ (pa s)	1.14E-03	1.14E-03
ρ (kg/m ³)	999.1	999.1
ν , m ² /s	1.14E-06	1.14E-06
D _L (free liquid diffusivity) (m ² /s)	5.16E-10	5.16E-10
Diameter, d (m)	9.20E-04	2.30E-04
V (m/s)	0.0012	0.0011
ϵ (porosity)	0.25	0.25
Re	3.84	0.91
Sc	2207.74	2207.74
Re*Sc	8468.43	2000.00
Design data		
Parameters	Pilot-Scale	RSSCT (PD)
Flow rate (ml/min)	26495.0	10.2
Flow rate (gal/d)	10080.0	3.87
Volume of media (ml)	585214.8	56.2
Bed Length (cm)	157.5	37.2
Mass of the media (gm)	286755.3	27.5
Water required (L)	17556444.9	1685.5
Water required (gals)	4638426.7	445.3
Duration of experiment (days)	460.2	115.2

The minimum Reynolds number was calculated by dividing the Schmidt number (for the smallest micropollutant of interest 1,4-Dioxane with a molar volume of ~235.5 cm³/mol) by 500 to ensure dispersion and kept it less in the RSSCT column compared to the full-scale adsorber. The hydraulic loading rate was calculated by defining a Peclet number of 2000 (recommended range $R_e \cdot S_c = 200000 - 200$) and following the recommendation of a $Re_{sc} > Re_{sc,min}$.

The influent water to treat using the RSSCTs was taken from the advanced water treatment (AWT) train (shown in Figure 6-1). The system consists of secondary effluent flowing into CFCGMF, Ozone-BAC, GAC, and UV treatment processes as multiple barriers to producing category A+ water. The proposed multi-barrier approach and the specific purpose of each treatment step are summarized in Table 6-3.

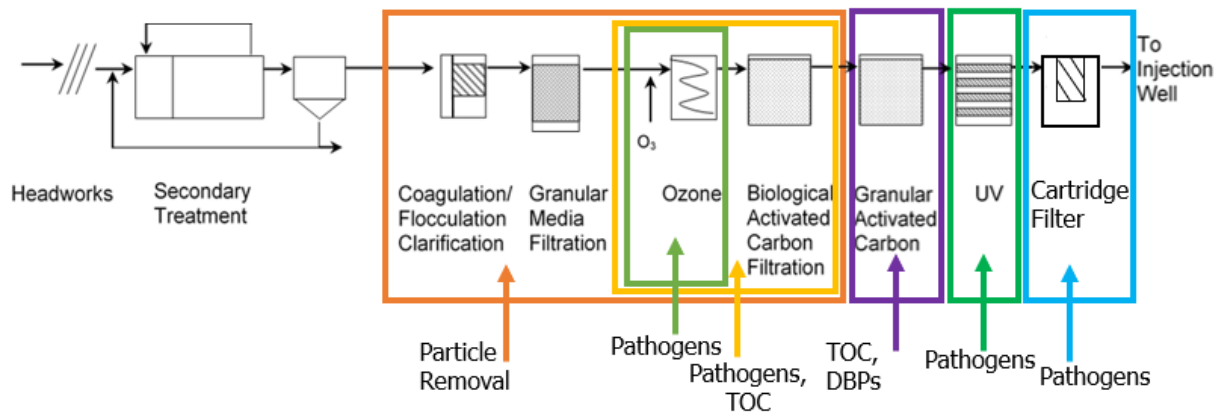


Figure 6-1. IPR Advanced Water Treatment Train at RSWRF with critical points control.

Table 6-3. Treatment Technologies and Purpose

Treatment Technology	Purpose
Secondary Treatment	Removes organics and nutrients. Provides some refractory organics removal and pathogen inactivation
Granular media filtration (GMF) with coagulation/flocculation/clarification pretreatment	Removes suspended solids and turbidity. Provides considerable log removal of <i>Cryptosporidium</i> and <i>Giardia</i> cysts
Ozonation	Removes CECs and provides pathogen inactivation
Biofiltration	Removes CECs and ozonation byproducts
Granular Activated Carbon (GAC) Filtration	Removes refractory organics and provides polishing treatment for a wide range of bulk and trace organics
UV Disinfection	Provides pathogen inactivation

6.2 Materials and Methods

6.2.1 Experimental Plan

To understand the microbial community development in biofilters and how upstream treatments can select specific microorganisms, three different water influents with different characteristics were passed through RSSCT biofilters (Figure 6-2). The experimental plan was divided into two phases: a biological acclimation period followed by an upstream treatment impact assessment on the microbial community established on BAC filters.

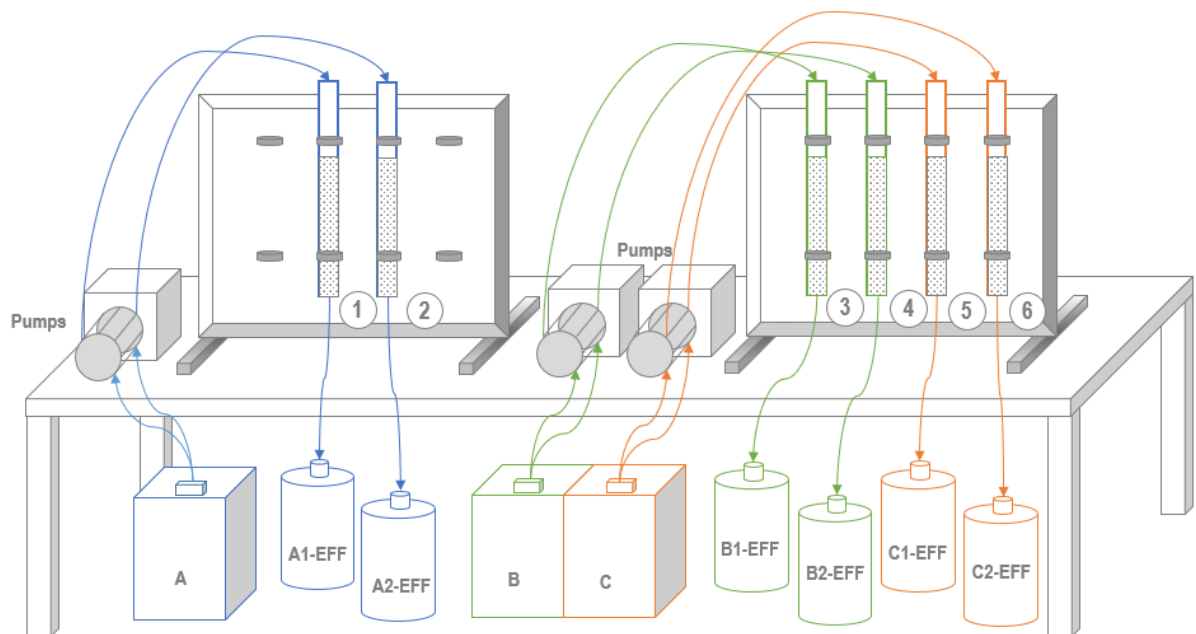


Figure 6-2. RSSCT BAC scheme setup where A, B, and C correspond to three different water matrix influents fed to the BACs: secondary effluent, CFCGMF, and ozonated effluent, respectively.

Phase I: Biological acclimation

This phase aims to establish microbial populations in the GAC media using organisms from three different influent characteristics. Three different influents: secondary effluent (SE EFF),

CFCGMF EFF, and ozonated effluent (O₃ EFF), were used as an upstream treatment to each RSSCT BAC under study. A flow rate of 10.2 ml/min was used with an EBCT of 5.5 min and a hydraulic rate of 4.04 m/h. The RSSCT columns were monitored following the sampling plan described in Table 6-4.

Table 6-4. Water Quality Monitoring Plan for RSSCT BACs

Category	Parameter	Mon	Tue	Wed	Thu	Fri	Location
General	O ₃ dose	X			X		O ₃ contactor
	pH	X	X	X	X	X	Influent and Effluent
	Temperature	X	X	X	X	X	Influent and Effluent
Organic	TOC	X	X		X	X	Influent and Effluent
	DO	X	X		X	X	Influent and Effluent
Microorganisms	ATP Measurement	X	X		X	X	Influent and Effluent

DO will indicate (indirectly) when microbial colonization within biofilters reached a steady state. Once, steady state is achieved to evaluate the microbial community population of the BAC filters, DNA was extracted from the column media for microbial characterization.

6.2.2 RSSCT Materials and Media Preparation

Six ½ diameter PVC tubes were used for the RSSCTs studies. The absorbent bed was supported by glass wool and glass beads to help distribute flow and avoid channeling. For the adsorbent preparation and characterization, the quantities were sieved after virgin media (GAC) was crushed with a mortar and pestle. The resulting d₁₀, d₃₀, d₆₀, coefficient of uniformity (C_u), and curvature (C_c) are 0.158 mm, 0.218 mm, 0.286 mm, 1.81, and 1.05, respectively. Once the media was at optimal size, it was baked to ensure no microorganisms presence in the media before starting the experiment with feed water.

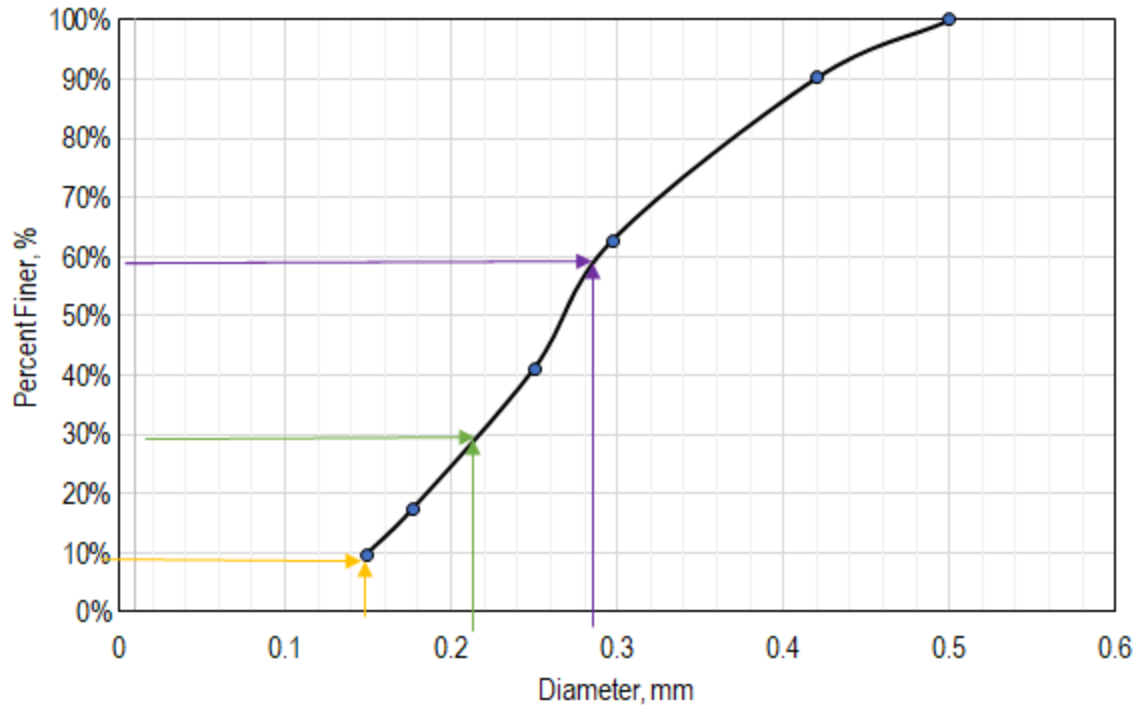


Figure 6-3. Sieve Curve of GAC used as bed material for the RSSCTs.

6.2.3 Water Quality Parameters Collection

Water quality parameters were collected and recorded daily during the first 60 days of the experiment at the RSSCT influents and effluents. During each media sampling event, data was also collected. The parameters collected were temperature, pH, dissolved oxygen, total organic carbon (TOC), nutrients such as nitrate, nitrite, and ammonia. Water quality parameters such as dissolved oxygen (DO), temperature, and pH were measured immediately onsite during sampling, using HACH portable meter and probes (HQ40D) (Hach, Loveland, CO, USA). Other parameters were measured in the laboratory using standard methods.

6.2.4 Sample Processing

Water quality parameters were measured daily during the first stages of the RSSCT experiment to capture the microbial community's development and time. A total of 30 media samples were collected, as seen in Table 6-5. However, three samples (two from CFCGMF INF collected on 06/05/2020 and one from CFCGMF EFF collected on 03/17/2020) did not pass the first quality check to guarantee a successful 16S sequencing (very low to no amplification). Therefore, samples were withdrawn for downstream analysis. Then, a total of 27 samples are included in this analysis.

Table 6-5. Sampling events for RSSCT media collection

Sampling Date	Experiment Time (days)	CFCGMF INFLUENT		CFCGMF EFF		O3 EFF	
		A	B	A	B	A	B
3/17/2020	29	X	X	X	X ¹	X	X
6/5/2020	113	X ¹	X ¹	X	X	X	X
7/10/2020	148	X	X	X	X	X	X
8/3/2020	172	X	X	X	X	X	X
11/5/2020	266	X	X	X	X	X	X

¹ Samples during the amplification process showed very low to no amplification; then, samples were labeled as failed, and no sequencing was performed.

The experiment started on 02/13/2020, and then because of lockdowns due to COVID-19 pandemics, the experiment was shut down 34 days later (03/17/20). The experiment was resumed on 06/05/2020 (It was off for 80 days). During this shutdown period, the RSSCTs columns were kept wet. Media sampling collection was held five times (Table 6-5) on days 29, 113 (when the experiment was resumed), 148, 172, and 266.

6.2.5 DNA Extraction and High-Throughput Sequencing

DNA from media samples was extracted using DNeasy PowerSoil Kit (QIAGEN, USA). Once the DNA was extracted from each sample, the DNA extracts were sequenced for 16S rRNA (V4 region) on Illumina MiSeq platform (RTL Genomics, Lubbock, Texas, USA). The primers used for DNA amplification were the 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTTC TAAT-3') as recommended by Caporaso et al., (2010). RTL Genomics analyzed microbial community diversity following their data analysis pipeline.

6.2.6 Bioinformatic Analyses

The sequencing data of the 16S rRNA amplicons were processed following the analysis pipeline of RTL Genomics. In brief, paired-end reads were trimmed and merged using PEAR Illumina, then denoised, and chimera checks were performed before the downstream process. Chimera detection and removal were done by using UCHIME in de novo mode after denoising the data. To obtain the identity of each remaining sequence, samples were demultiplexed. Then using the UPARSE algorithm (Edgar, 2013), sequences were clustered into operational taxonomic units, OTUs. Taxonomic information is added to each sequence after running each cluster against the USEARCH global alignment program. The data is identified by employing a database of high-quality sequences derived from the NCBI database. Once sequencing data were obtained, all data processing related to microbial community analysis were made following different pipelines (Callahan et al., 2016; Shetty et al., 2018). The phyloseq R-package (McMurdie and Holmes, 2013b) was used to manage the sequencing data capable of supporting sequencing importing data format from RTL Genomics. All the analytical techniques used, such as diversity analysis, ordination methods, quality plots, and microbiome detail analyses, were performed using this package. Normalized data were used to determine the observed number of OTUs and to estimate

the Shannon Index, Chao1, and Simpson's Diversity Index using R statistical software, version 4.0.3 (R Core Team, 2020) using packages phyloseq version 1.34.0 (McMurdie and Holmes, 2013b), vegan version 2.5.7 (Oksanen et al., 2020), and microbiome version 1.12.2 (Lahti and Shetty, 2019). All figures were developed using ggplot2, version 3.3.3 (Wickham, 2016). The tool Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 -PICRUST2 version 2.4.1 (Douglas et al., 2020) was used to predict the functional potential of the bacterial communities based on marker gene sequencing profiles. A public pipeline and tutorial (Douglas, 2021) was followed for the analysis.

6.2.7 Statistical Analyses

Statistical comparison of bacterial diversity levels between samples groups was calculated with parametric pair-wise t-tests. Beta diversity for microbial community analysis and function abundance analysis of KEGG pathways estimations were calculated with Principal Coordinate Analysis (PCoA) using unweighted and weighted Unifrac distances. PERMANOVA tests were used to assess the statistical differences between the samples. Because for each influent, there are two RSSCT (duplicate) that correspond to a technical replicate. The sample and respective duplicate would be merged because the consistency or precision of the assay was not the priority but a more extensive dataset.

6.3 Results and Discussion

6.3.1 Chemical Water Quality Monitoring

✓ Total Organic Carbon

The average influent TOC concentrations (Figure 6-4) were 5.2 ± 0.6 , 4.5 ± 0.6 , and 4.7 ± 0.4 mg/L for SE EFF, CFCGMF EFF, and O3 EFF RSSCTs experiments, respectively. The RSSCTs

BAC average TOC effluents were: 1.5 ± 0.7 , 1.3 ± 0.6 , and 1.6 ± 0.7 mg/L. The TOC removal achieved was 71, 72, and 66% for the RSSCT BACs treating SE EFF, CFCGMF EFF, and O3 EFF, respectively. Approximately after 80 days, the TOC effluents achieved a steady removal. The main removal mechanism for organic carbon in BAC filters are adsorption and biodegradation, but in fresh granular activated carbon, adsorption is the main mechanism (Hess and Morgenroth, 2021). The RSSCTs columns showed carbon exhaustion on day 80, when the media available empty pores may have clogged and saturated, and the biodegradation pathway started.

✓ [Nitrate Concentration](#)

The average influent nitrate concentrations (Figure 6-5) were 4.6 ± 0.8 , 4.6 ± 0.9 , and 4.5 ± 0.8 mg/L for each RSSCT treating SE EFF, CFCGMF EFF, and O3 EFF, respectively. The average effluents were 4.0 ± 1.2 , 4.2 ± 0.8 , and 4.3 ± 0.9 mg/L achieving a nitrate removal of 12, 9, and 4%. After 100 days of operation, the RSSCT treating SE EFF and CFCGMF EFF started showing some nitrate reduction. In addition, the RSSCT treating the O3 EFF started denitrification after 80 days.

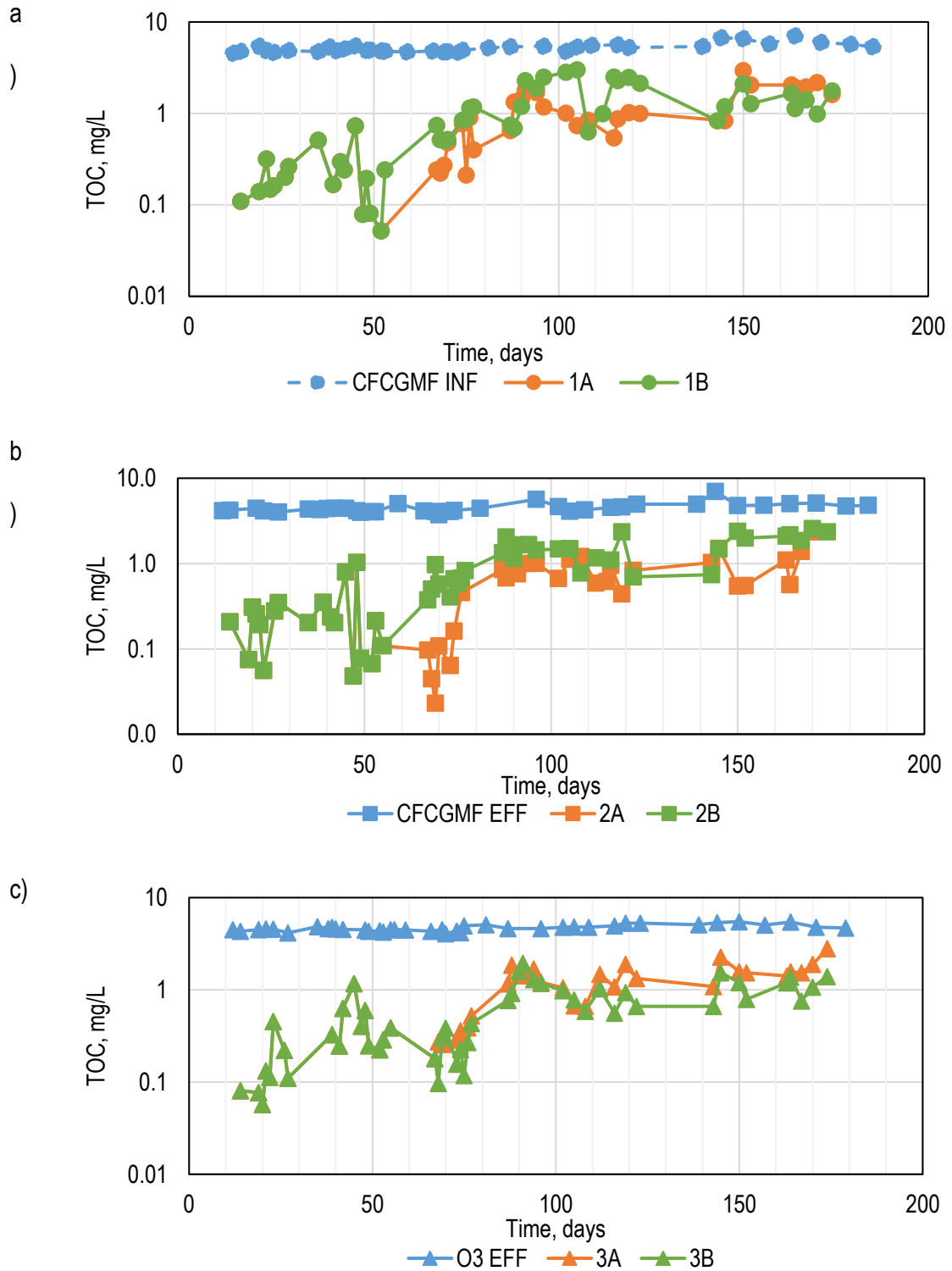


Figure 6-4. Total Organic Carbon at the RSSSCT influent and effluents (A and B BACs) for each experimental condition using influent as a) SE EFF, b) CFCGMF EFF, and c) O3 EFF.

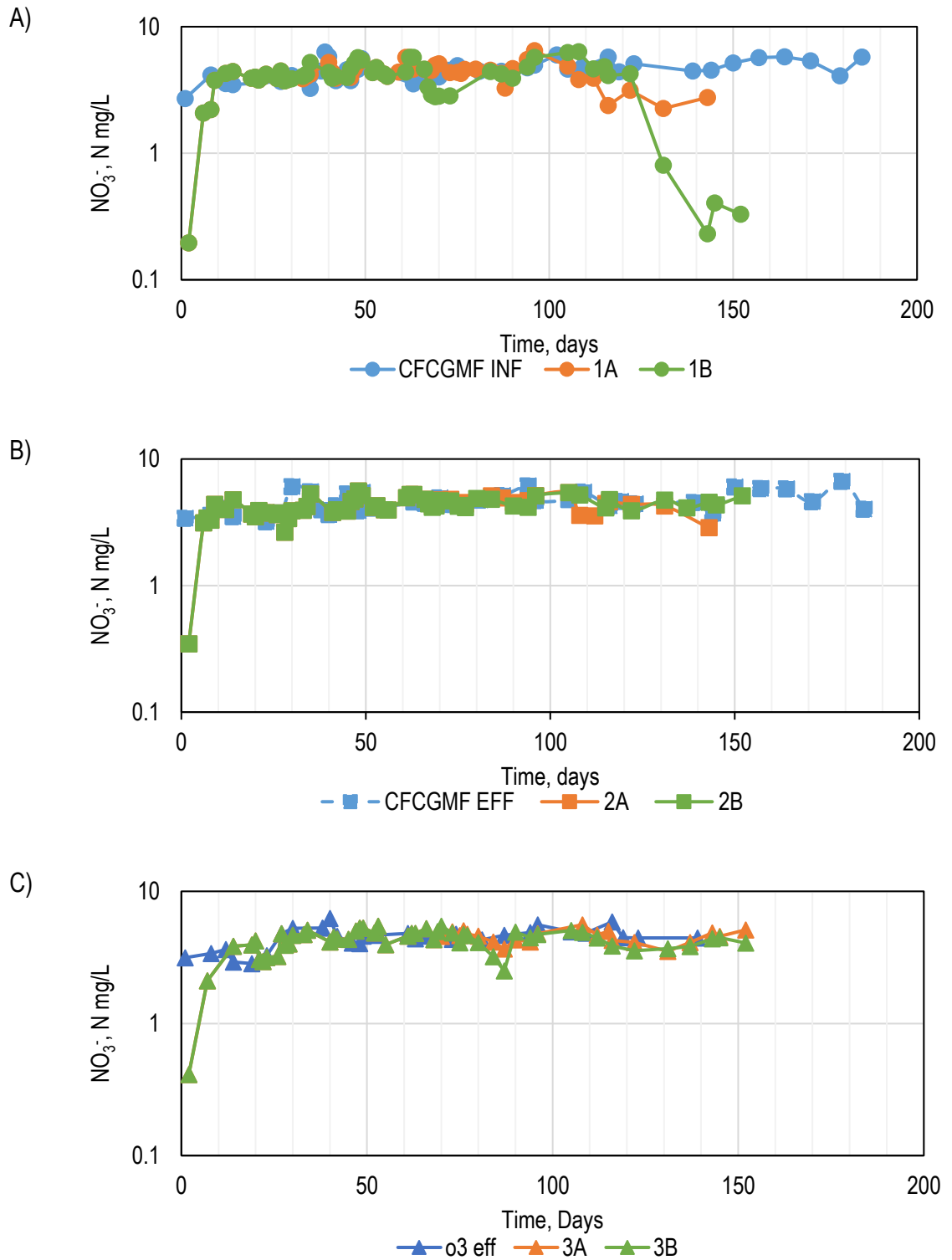


Figure 6-5. Nitrate concentration at the RSSSCT influent and effluents (A and B BACs) for each experimental condition using influent as a) SE EFF, b) CFCGMF EFF, and c) O3 EFF.

✓ Ammonia and Nitrite Concentration

The average influent nitrite concentrations (Figure 6-6) were 0.07 ± 0.04 , 0.03 ± 0.03 , and 0.02 ± 0.03 N-mg/L for each RSSCT treating SE EFF, CFCGMF EFF, and O3 EFF, respectively. The average effluents were 0.06 ± 0.05 , 0.05 ± 0.05 , and 0.15 ± 0.24 N-mg/L achieving a nitrite removal of 19% when SE EFF is treated. Inspecting Figure 6-7a for ammonia removal, the RSSCT treating SE EFF after day 60 achieved a high removal due to its oxidation leading to a nitrite concentration spike, as seen in Figure 6-6a. From Figure 6-7b, some ammonia removal started after day 70, and it matches the increase of nitrite at the RSSCT treating CFCGMF EFF (Figure 6-6b). Similarly, earlier on day 30, the RSSCT treating O3 EFF (Figure 6-6c) shows higher nitrite effluent concentrations than its influent, and the ammonia concentration decreased (Figure 6-7c). Ammonia removal is achieved during the duration of the experiment after day 30. The potential mechanisms involved in removing ammonia and higher nitrite effluent concentrations can be linked to ammonia oxidation to nitrite by ammonia-oxidizing bacteria that can biodegrade it, so ammonia is converted into nitrite.

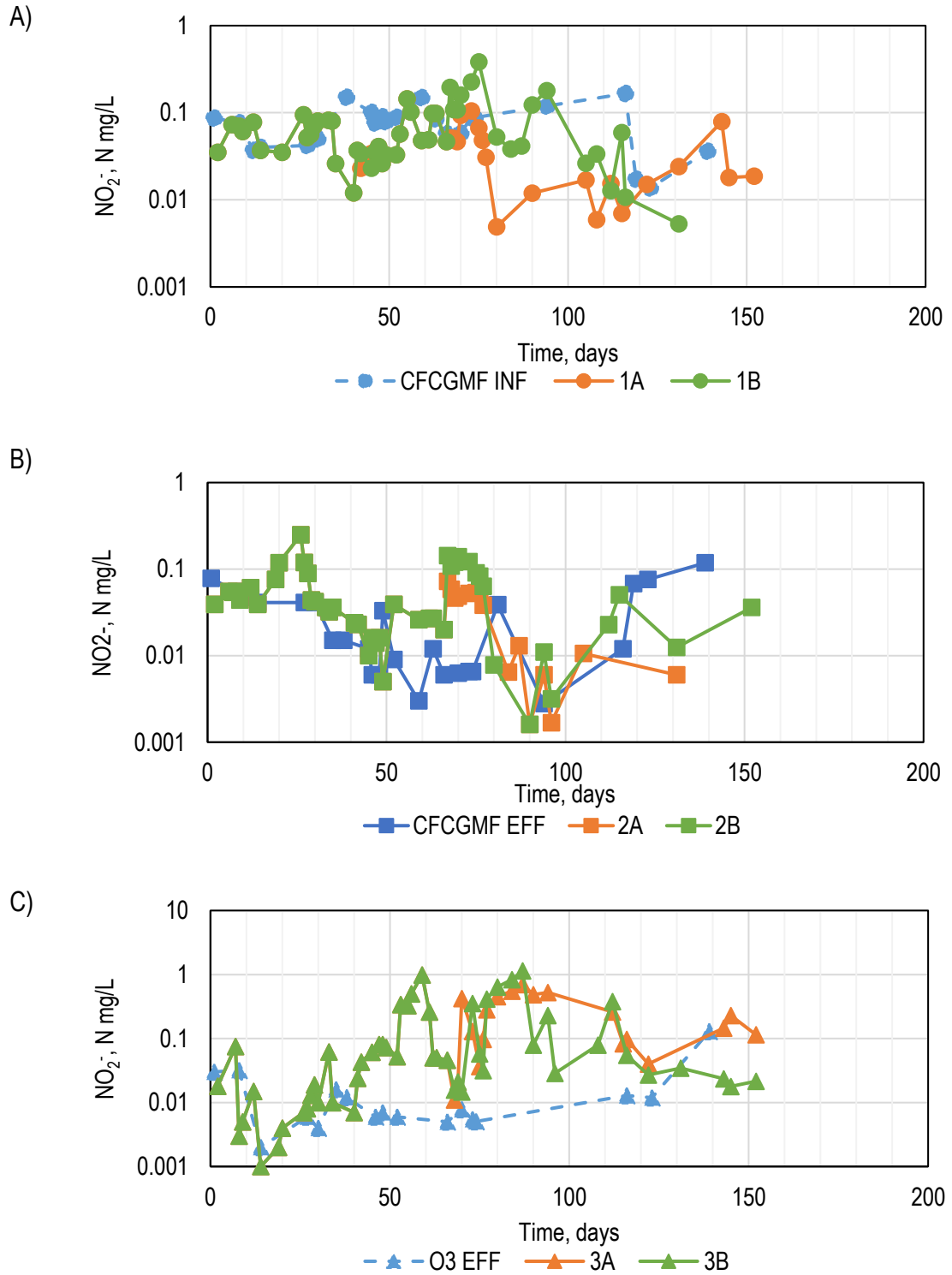


Figure 6-6. Nitrite concentration at the RSSCT influent and effluents (A and B BACs) for each experimental condition using influent as a) SE EFF, b) CFCGMF EFF, and c) O3 EFF.

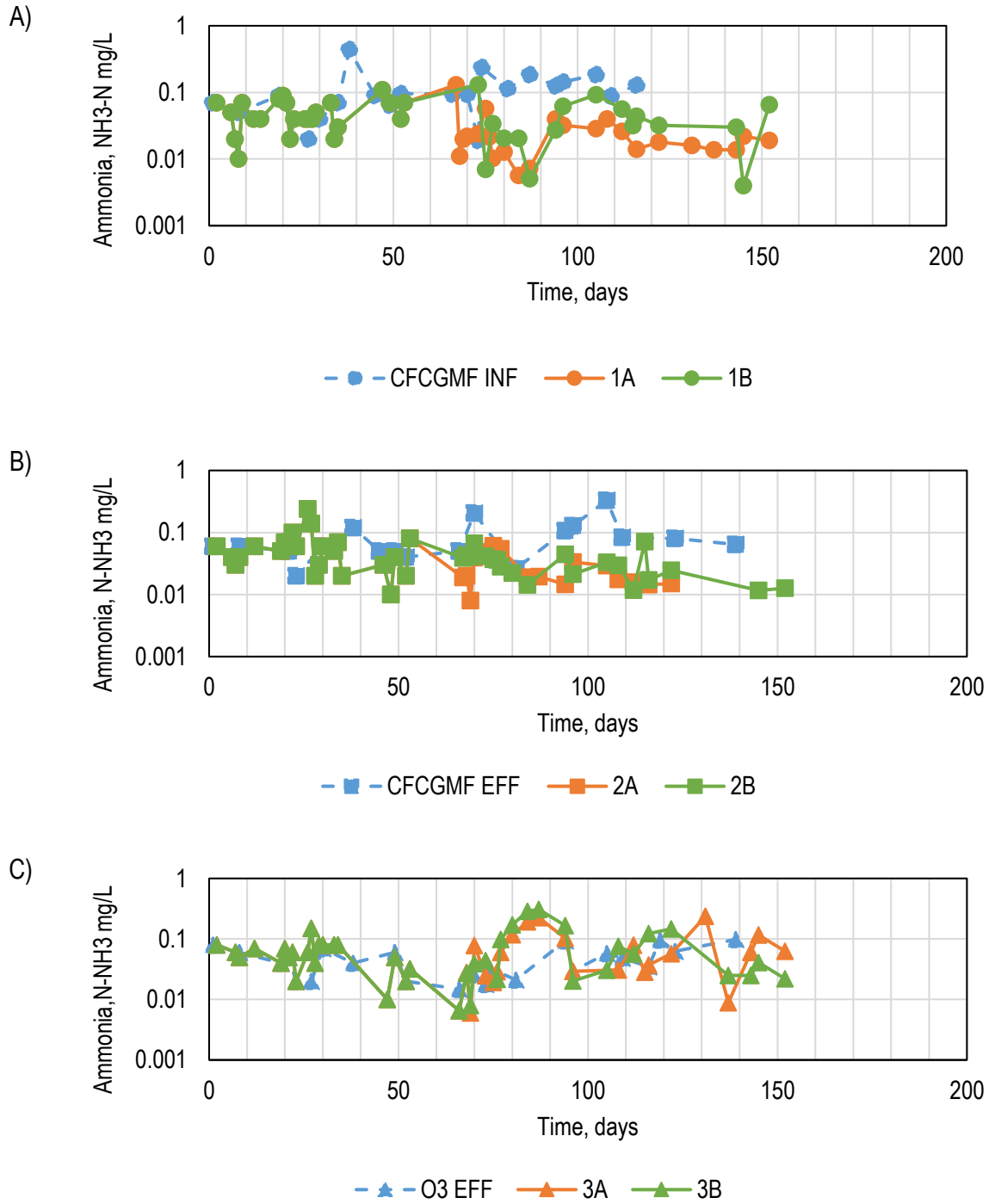


Figure 6-7. Ammonia at the RSSSCT influent and effluents (A and B BACs) for each experimental condition using influent as a) SE EFF, b) CFCGMF EFF, and c) O3 EFF.

✓ pH and Temperature:

The influent and effluent pH values (Figure 6-8) at the influents were 7.3 ± 0.2 , 7.3 ± 0.3 , and 7.6 ± 0.2 and effluents 8.0 ± 0.3 , 8.1 ± 0.3 , and 8.1 ± 0.3 °C for RSSCT treating SE EFF, CFCGMF EFF, and O3 EFF. In addition, average influent temperatures (Figure 6-9) for each RSSCT were values of 21.5 ± 3.8 , 21.8 ± 3.4 , and 21.5 ± 3.7 °C. and at effluents 19.1 ± 5.3 , 18.8 ± 5.5 , and 19.3 ± 5.5 °C. Both temperature and pH are within the optimal ranges to ensure microbial growth and activity.

✓ Dissolved Oxygen (DO):

The dissolved oxygen (DO) along the three RSSCTs kept aerobic conditions (Figure 6-10), influent and effluents DO concentrations were above 3 mg/L. The average influent DO concentrations were 4.4 ± 0.5 , 4.6 ± 1.0 , and 15.4 ± 2.1 mg/L, the effluents were 6.4 ± 2.0 , 6.9 ± 1.6 , and 6.5 ± 2.2 mg/L for the RSSCTs treating SE EFF, CFCGMF EFF, and O3 EFF, respectively.

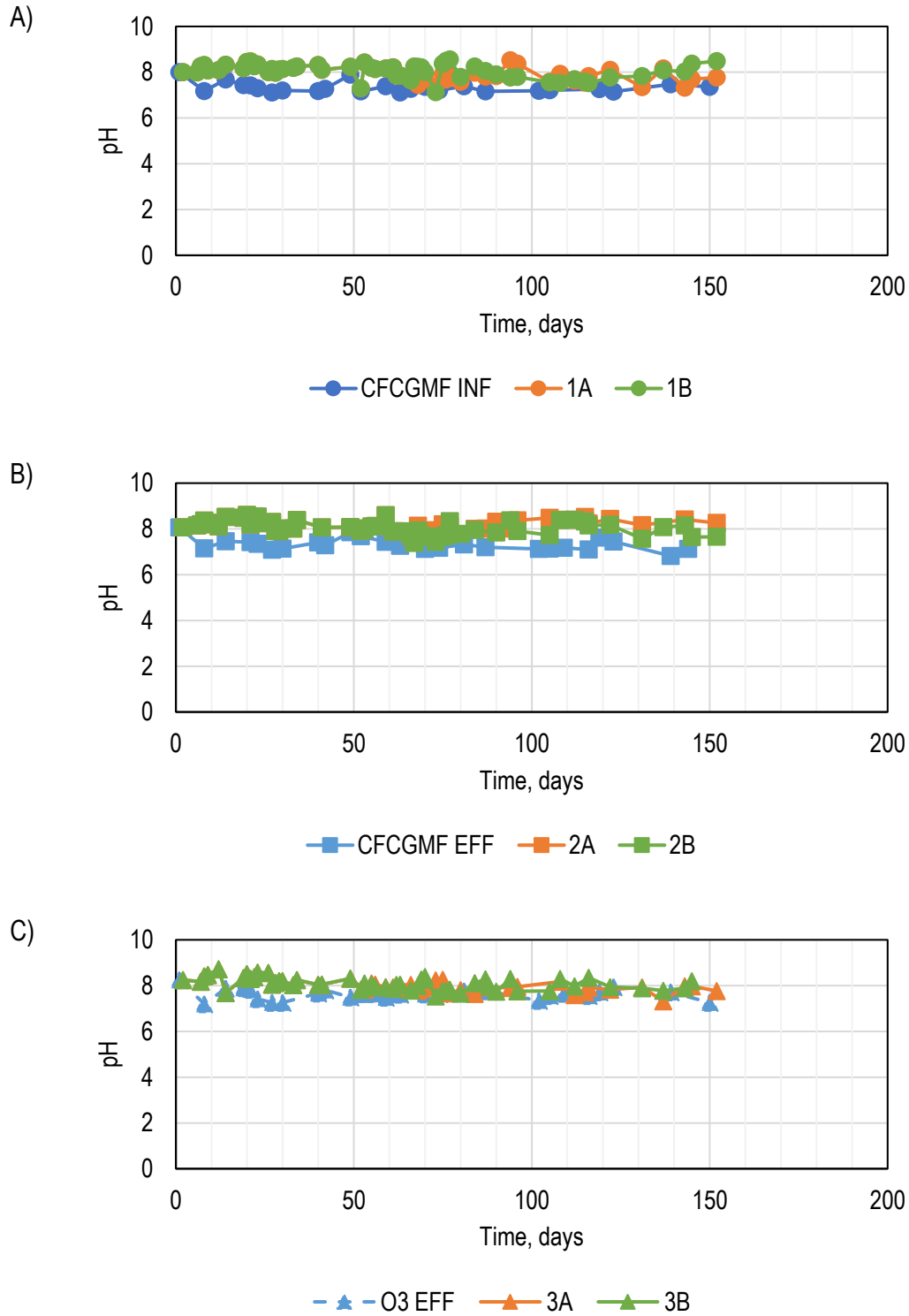


Figure 6-8. pH at the RSSSCT influent and effluents (A and B BACs) for each experimental condition using influent as a) SE EFF, b) CFCGMF EFF, and c) O3 EFF.

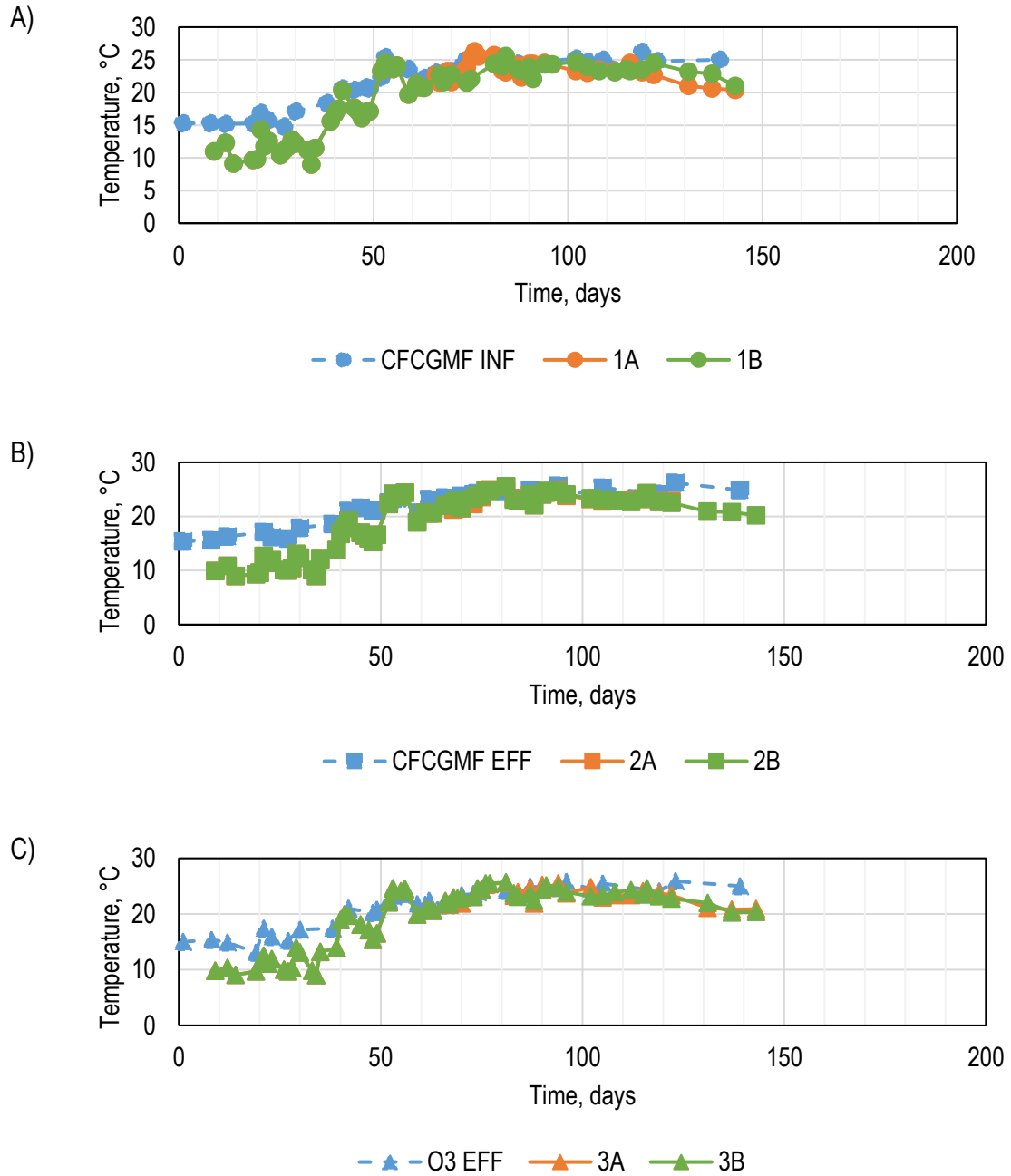


Figure 6-9. Temperature at the RSSSCT influent and effluents (A and B BACs) for each experimental condition using influent as a) SE EFF, b) CFCGMF EFF, and c) O3 EFF.

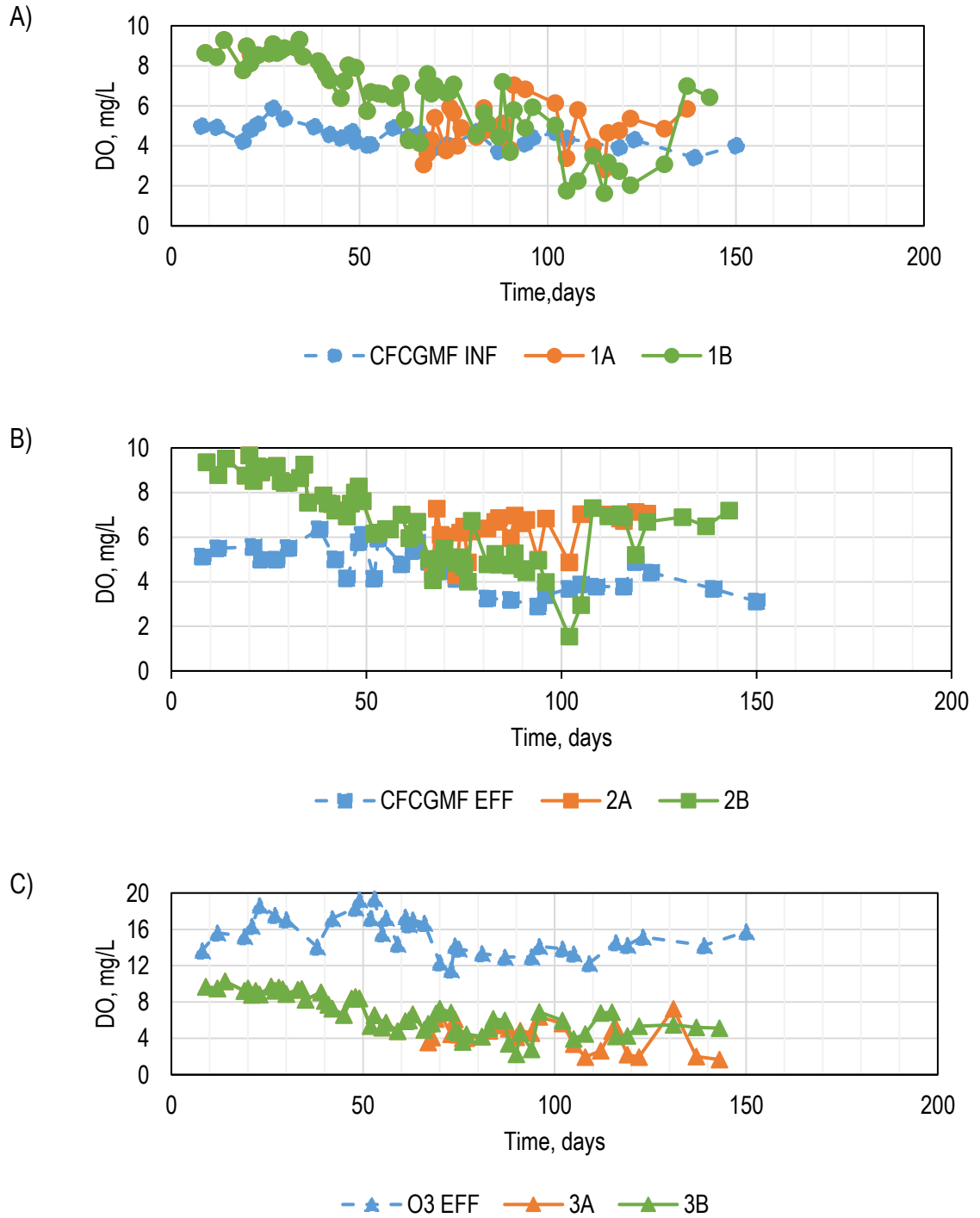


Figure 6-10. Dissolved oxygen concentration at the RSSSCT influent and effluents (A and B BACs) for each experimental condition using influent as a) SE EFF, b) CFCGMF EFF, and c) O3 EFF.

6.3.2 Microbial Community Analysis for Each RSSCT BAC

6.3.2.1 Taxonomic Abundance Analyses

After sequencing, data denoising, normalization, and 97% similarity cutoff, a total of 5728 OTUs and 14 grouped samples were analyzed. Alpha diversity indices are plotted in Figure 6-11. Each boxplot has a middle darker line representing the medians of each group; when this line is entirely outside the other boxes, then there is likely to be a difference between respective groups. Following this logic, the RSSCT BAC fed with SE EFF is different from the other two influents.

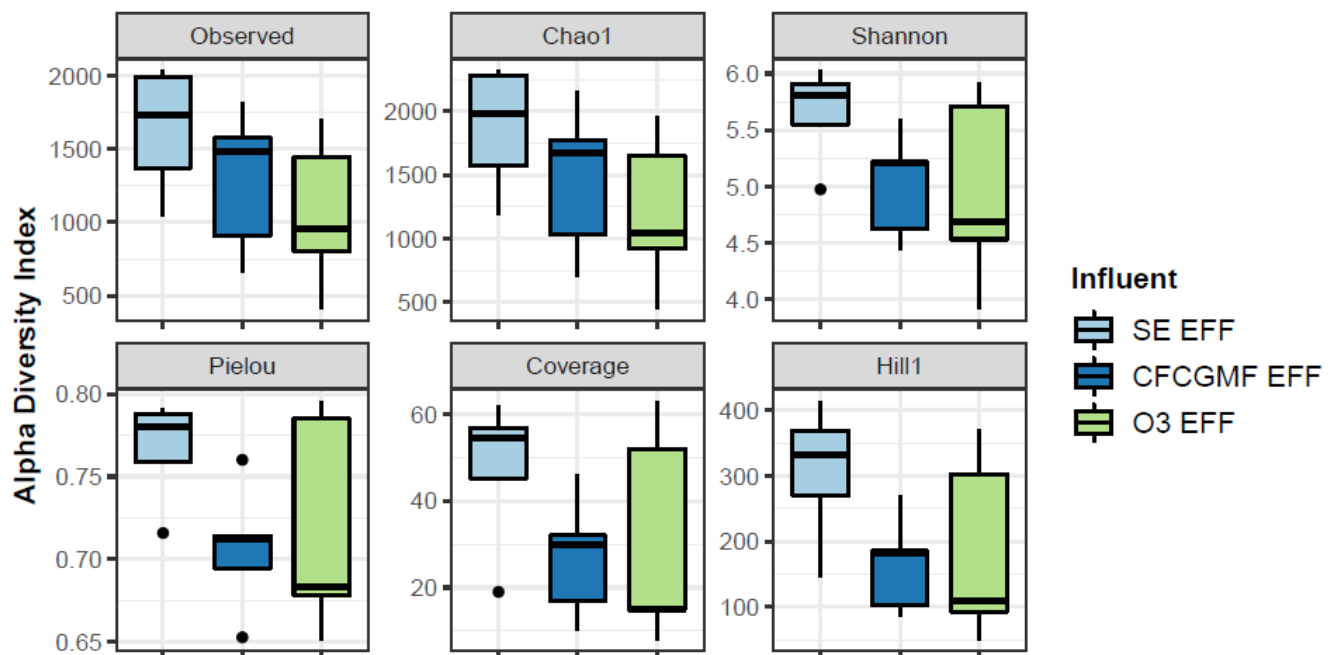


Figure 6-11. Alpha Diversity Indices for all three RSSCTs experimental setups

Observed and Chao1 index account for richness, showing that the RSSCT richness decreased depending upon the type of influent treated. The highest richness was found in the RSSCT fed with SE EFF, followed by CFCGMF EFF and O3 EFF. The microbial community diversity kept the same trend as the one from richness. The microbial community evenness in species abundance is represented by the Pielou evenness number with median values of

0.78, 0.71, and 0.68 for SE EFF, CFCGMF EFF, and O3 EFF influents. The coverage diversity indicates the number of species needed to cover at least half of the total abundance suggesting that the O3 EFF column has the lowest coverage number compared to the other two. The Hill biodiversity index (Hill1) accounts for species' relative abundance was computed by taking the Shannon diversity index's exponent; the average values were 302.9, 164.6, and 184.8 for SE EFF, CFCGMF EFF, and O3 EFF. The diversity indices did not have sufficient evidence to indicate that their means values across each type of influent treated are different ($p > 0.05$ in order 0.275, 0.246, 0.254, 0.227, 0.333, and 0.205 for observed, chao1, Shannon, Pielou, coverage, and Hill1 index, respectively).

6.3.3 Effects of Upstream Treatment on Beta Diversity

The microbial community characterization of all the samples collected from each RSSCT column were compared using UniFrac distances. Multivariate differences among the influent were evaluated with Permutational Multivariate Analysis of Variance Using Distance Matrices, function ADONIS. For ADONIS, the distances among samples were calculated using unweighted (presence and absence of OTUs), followed by weighted (abundance of OTUs) UniFrac. Then an ANOVA-like simulation was conducted to test for differences based on influent treated in the BACs. UniFrac distance measures the evolutionary separation between communities by dividing the length of the ancestral branches by the total branch length of the evolutionary tree. In addition, UniFrac distances can be used to assess if communities are significantly different, compare several communities simultaneously using ordination and clustering techniques, and compute the relative contributions of environmental factors to similarities between samples (Lozupone and Knight, 2005). Unweighted UniFrac shows the distance between communities as the proportion of

evolution unique to one community or the other. In contrast, Weighted UniFrac accounts for the abundance of OTUs in the community.

Figure 6-12 shows three different layers depending upon the influent treated; the lowest to highest are O3 EFF to SE EFF. In addition, when the experiment was during the first stage (less than 115 days), the samples were not similar or forming clusters. However, the end of the experiment (more than 170 days – triangles and circle shapes) did get closer. The Unweighted Unifrac PERMANOVA test (Adonis) comparing the effect of influent type and experiment duration were significant (p -values < 0.05 , $1.00E-4$ for both), suggesting that there is a significant difference between the microbial community structures when both influent treated and experiment duration are assessed. A pair-wise permutation test for homogeneity of multivariate dispersion using `permutest` (`vegan` package) validates that the communities are different by the influent treated and when experiment duration was evaluated. To validate the Adonis result, a test for homogeneity of dispersion among groups was performed (p -value: $0.29 > 0.05$), suggesting that the groups analyzed (influent treated and experiment duration) have homogeneous dispersion, then meeting Adonis assumption.

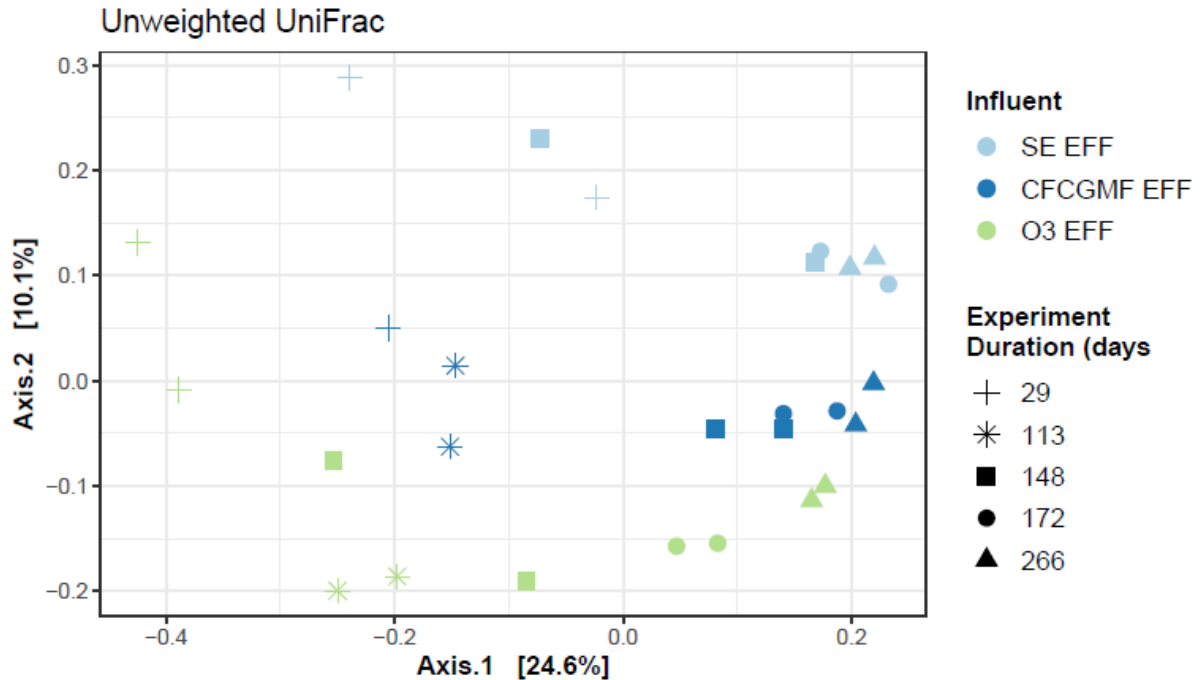


Figure 6-12. Principal Coordinate Analysis (PCoA) with Unweighted UniFrac distance to assess the difference of treated water and experiment duration.

In addition, based on Weighted UniFrac (Figure 6-13), samples are clustered together.

Those were collected at the latter experiment stages (Experiment Day > 150 days) and from the RSSCTs fed by SE EFF and CFCGMF EFF. Samples treating O3 EFF are dispersed in the plot space. The microbial community composition was found to be different depending on the influent treated and experiment running time. However, when a betadisper test was performed (p -value: $0.0053 < 0.05$) to evaluate the homogeneous dispersion assumption, the Adonis result cannot be accepted, and the differences between samples are possibly due to differences in group dispersions.

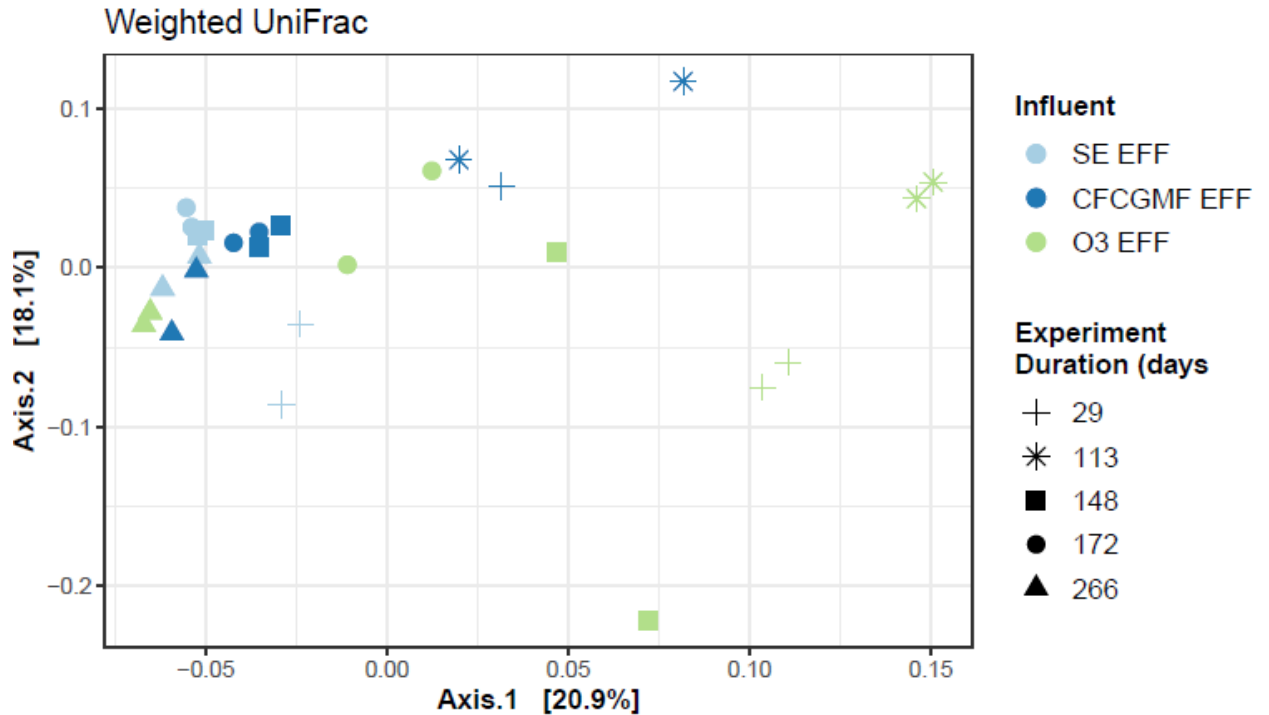


Figure 6-13. Principal Coordinate Analysis (PCoA) with Weighted UniFrac distance to assess the difference of treated water and experiment duration time.

Finally, an Analysis of Similarity (ANOSIM) to test whether overall microbial communities differ depending on the influent treated was performed. The ANOSIM results are significant (p -value=0.001<0.05), meaning the upstream treatment or influent treated influences the microbial community composition developed on the studied biofilters (RSSCTs).

6.3.4 Microbial Community Taxa at Phylum and Class level

A total of 5728 OTUs were identified across all the media samples. The ten most abundant phyla are shown in Figure 6-14. *Proteobacteria* was the most abundant phyla identified with a relative abundance of 48.8, 50.9, and 56.7 % for the RSSCTs fed with SE EFF, CFCGMF EFF, and O3 EFF, respectively. *Bacteroidetes* relative abundance range 11.6% to 16.8%, followed by *Actinobacteria* (4.8-6.2%), *Planctomycetes* (3.0-3.7%), and *Chloroflexi* (1.7-3.4%). These five phyla

sum up, on average, 92.1% of the relative abundance for each RSSCTs, when unclassified phyla are included (14.5-15.8%). Richness is significantly different depending upon the influent treated (p-value: 0.0027) and the experiment duration time (p-value: 2.14×10^{-5}). However, it is not different when comparing samples taken during the same sampling event (p-value: 0.127). On the other hand, the Shannon diversity index is not significantly different when the influent treated is changed (p-value: 0.066). However, it is different along with the experiment duration (p-value: 0.0038), as seen in Figures 6-12 and 6-13.

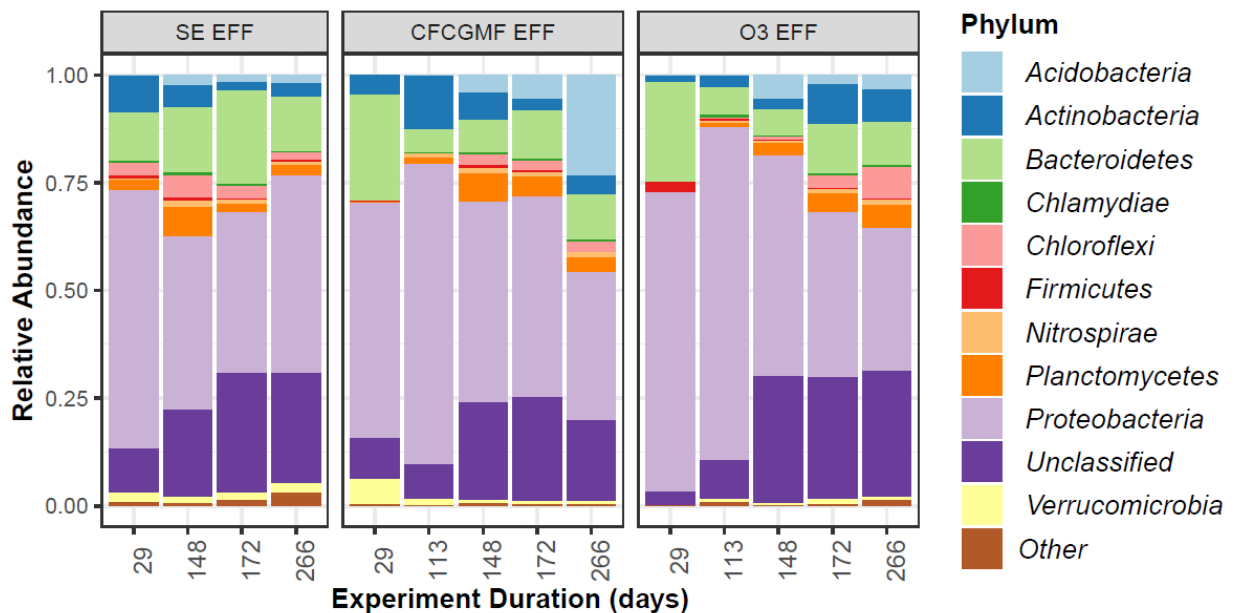


Figure 6-14. Microbial community composition and distribution at Phylum taxonomic level on relative abundance

A total of 32 classes were identified; within the most abundant were *Betaproteobacteria* (14.3% 21.7%), *Gammaproteobacteria* (12.0-20.8%), *Alphaproteobacteria* (10.7-17.5%), and *Deltaproteobacteria* (1.9-3.0%), which all belong to the *Proteobacteria* phylum. The most abundant class was *Sphingobacteriia* (5.2%-12%), belonging to the *Bacteroidetes* phyla. Due to microbial

richness, 21-26.3% of the OTUs were unclassified classes. *Actinobacteria* class showed a relative abundance of 4.4-5.8%.

Comparing classes shifts between RSSCTs (Figure 6-15), the most significant changes were identified for the following classes: *Gammaproteobacteria*, *Betaproteobacteria*, *Alphaproteobacteria*, *Sphingobacteriia*, and *Acidobacteriia*. In Chapter 3, two of the RSSCT influents were characterized: the CFCGMF and O3 effluents. *Gammaproteobacteria* has been found to form biofilms (Bae et al., 2014), then its identification in all the media samples was expected. The relative abundance of *Betaproteobacteria* increased when ozonation was the upstream treatment (Liao et al., 2015, 2013, 2012) as seen in Chapter 3 and Figure 6-15 but decreased when CFCGMF was upstream of RSSCT BAC.

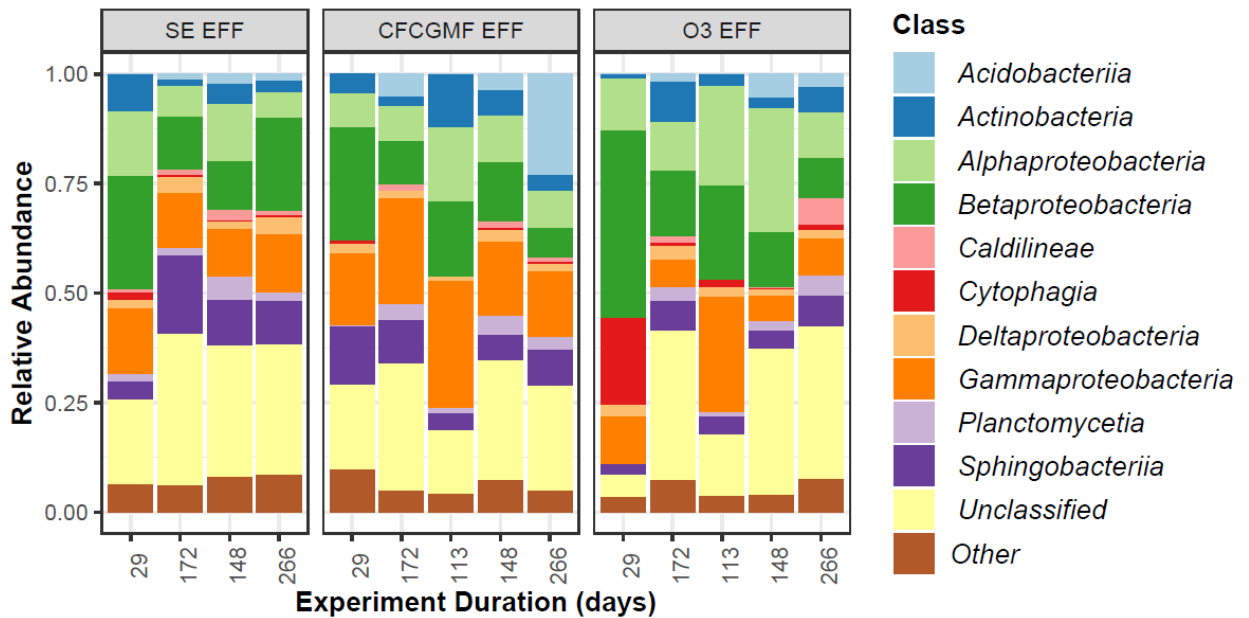


Figure 6-15. Microbial community composition and distribution at the Class taxonomic level on relative abundance.

The higher relative abundance of *Alphaproteobacteria*, *Gammaproteobacteria*, and *Sphingobacteriia* has been reported in filters and membranes, as major media colonizers (Nagaraj

et al., 2017). However, to assess the microbial community difference between RSSCTs, Figure 6-16 shows the microbial structure at the order taxonomic level.

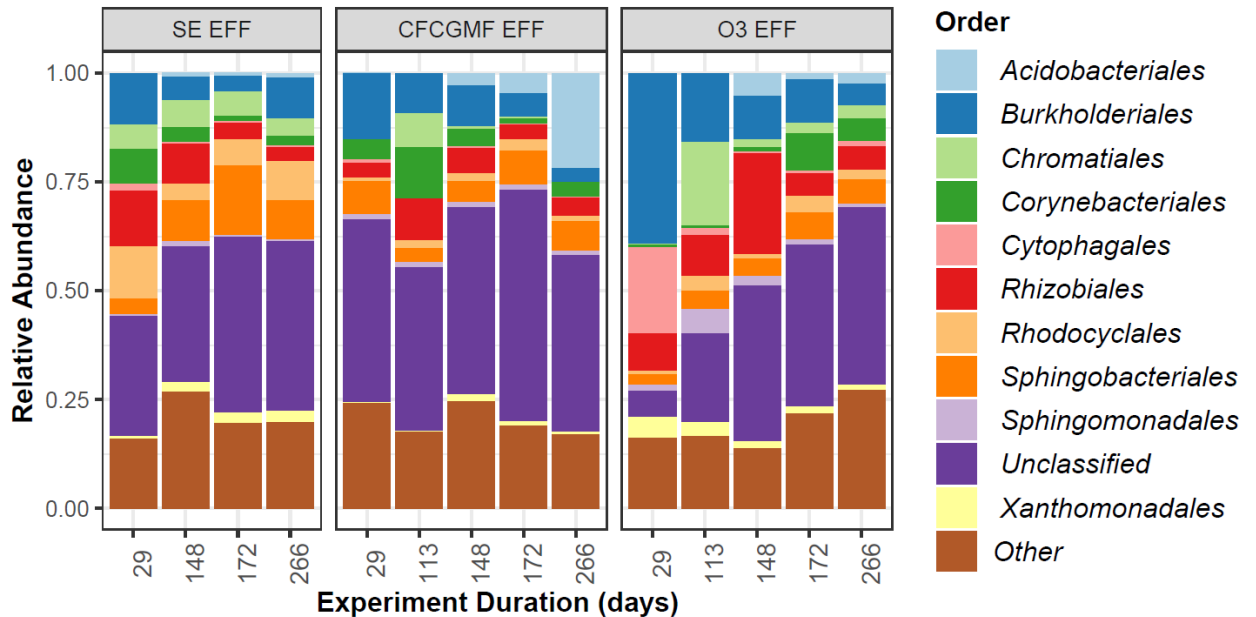


Figure 6-16. Microbial community composition at Order taxonomic level

From the ten most abundant orders identified, the ones that showed the highest variability depending on the influent treated (SE EFF, CFCGMF EFF, and O3 EFF) were: *Acidobacteriales* (0.7, 6.9, and 2%), *Burkholderiales* (7.7, 8.3, and 17.2%), *Rhodocyclales* (8.1, 1.6, and 2.3%), *Sphingobacteriales* (10.7, 6.2, 4.7%), and unclassified (39.7, 50, and 28.5 %). Organisms belonging to the *Burkholderiales* order are associated with the mineralization of dissolved organic matter in BAC filters (Liu et al., 2012), and so is *Rhizobiales*. Both order taxa have been identified as major colonizers in BAC filters (Niemi et al., 2009). The highest relative abundance of *Burkholderiales* in the RSSCT fed with ozonated water can be explained by the tolerance of these taxa organisms to ozone (Islam et al., 2015). *Sphingobacteriales* order has been identified as predominant abundant at activated sludge plants in the US (Zhang et al., 2011).

6.3.5 Differential Abundance Taxa Between Influent and Media Microbial Communities.

Previous subsections have been focused on comparing the microbial community between RSSCTs under study; this section will include a description and analysis of composition between each RSSCT and its respective influent. The objective is to detect differentially abundant taxa between them. For this analysis, the Analysis of Composition of Microbiomes – ANCOM- was used (Lin and Peddada, 2020). Briefly, the ANCOM-BC methodology tests the differential absolute abundance hypothesis of an individual taxon and delivers confidence intervals, includes the compositionality of the OTU table, and does not rely on strong parametric assumptions.

Twenty-five phyla were identified in influent and media samples of the RSSCT BACs. At the phylum level (Figure 6-17), a total of 22 were identified to be differentially abundant. The three genera that were not different were *Proteobacteria*, *Actinobacteria*, and *Spirochaetes*. The differences are notable when exploring the ten most abundant phyla for influent and media samples (Figure 6-18). The most abundant were *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Firmicutes* at influent and media samples. The *Proteobacteria* average relative abundance (37-63%) decreased $17 \pm 1.6\%$, compared to influent and media microbial communities. However, ANCOM results established that these shifts are not significant. *Bacteroidetes* average relative abundance (5%-15%) decreased 19% and 53% when comparing RSSCT1 and RSSCT2 with their influents, but RSSCT3 increased 115%. Comparing three RSSCT media relative abundance of *Bacteroidetes*, the prevalence in the post ozonation column (RSSCT3) has been reported elsewhere (Gerrity et al., 2018). Microorganisms belonging to this phylum are active players in the human digestive system (Gibiino et al., 2018). Hence its high relative abundance prevalence in wastewater is expected. *Actinobacteria* relative abundance (5-15%) increased on RSSCT1 and RSSCT3 media filters compared to its influents, while RSSCT2 decreased. *Cyanobacteria* did not

show high relative abundance, but RSSCT2 took part in the *Proteobacteria* typical abundance and accounted for almost a third of phyla abundance (28%). When genus taxa level was assessed (Figure 6-19), a total of 438 genera were identified, and 90% were found to be differentially abundant; only 30 are shown in Figure 6-20. Figure 6-20 shows that cell colors vary vertically, indicating different influent and media samples.

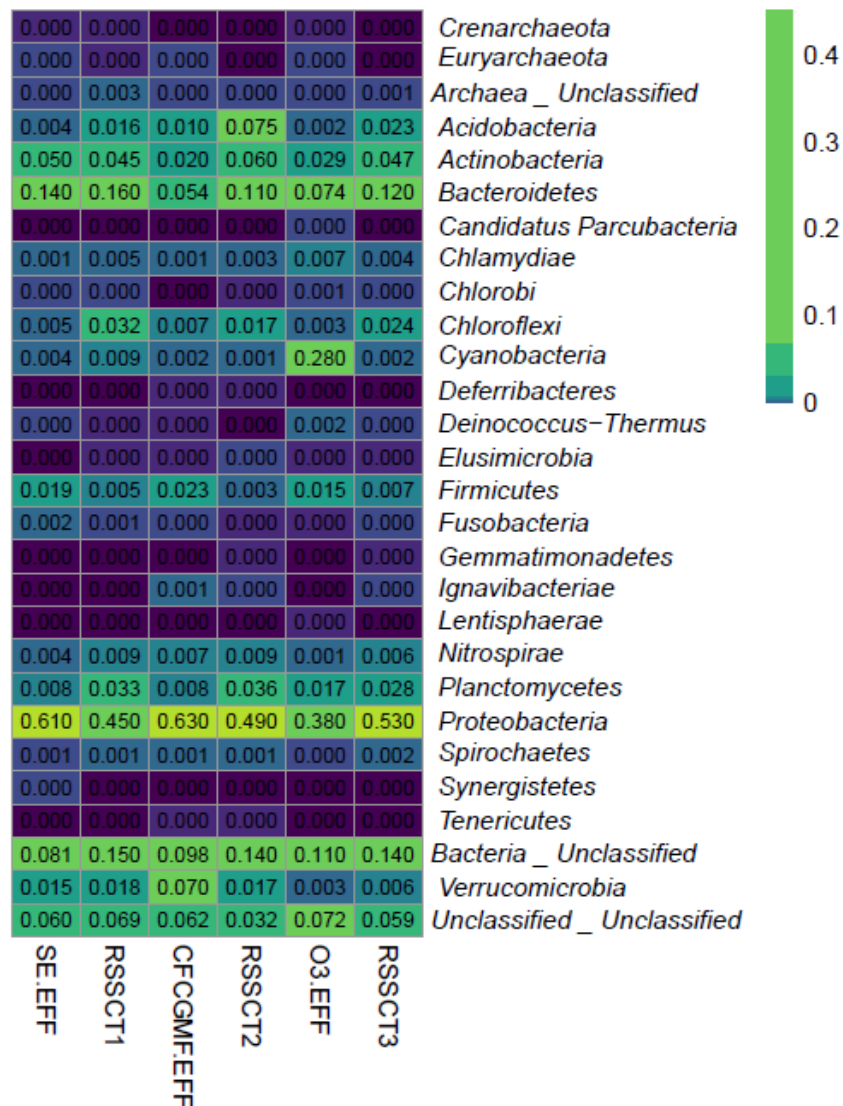


Figure 6-17. Phyla - Heatmap depicts mean relative abundances of microorganisms detected to be differentially abundant by ANCOM procedure. Color-scaling distinguishing differences between phyla with smaller mean relative abundance. A cell-colored dark purple can be considered zero, whereas there were no detections of microorganisms for that given cell.

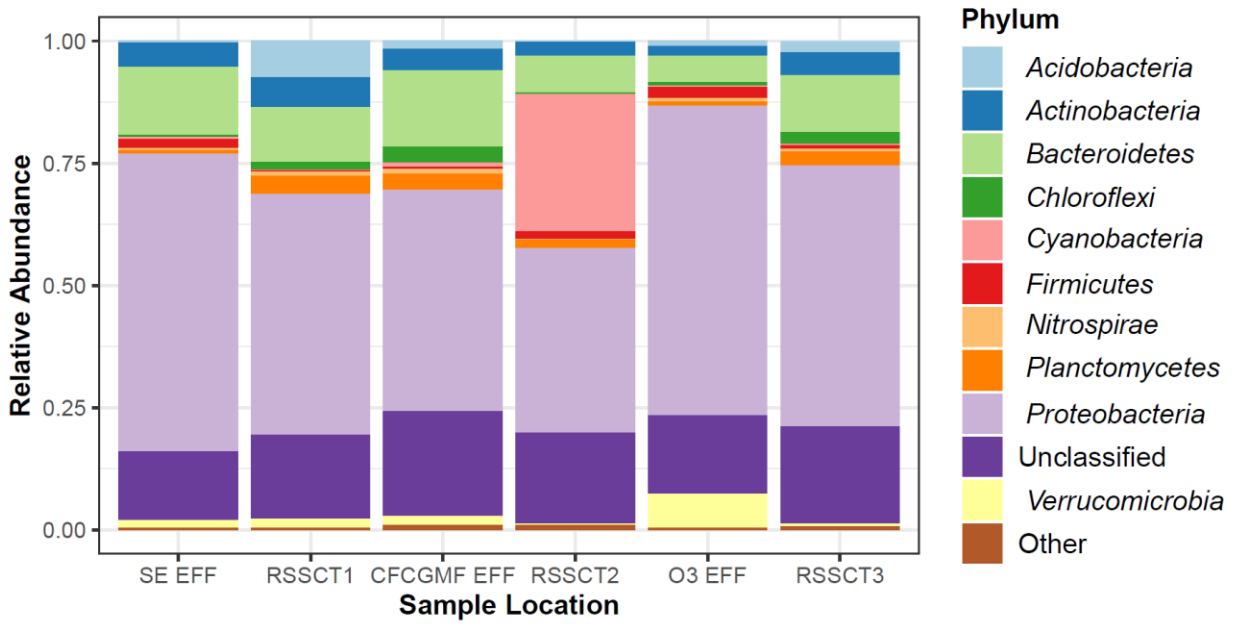


Figure 6-18. Ten most abundant phyla and respective average relative abundance for influent and media samples under study.

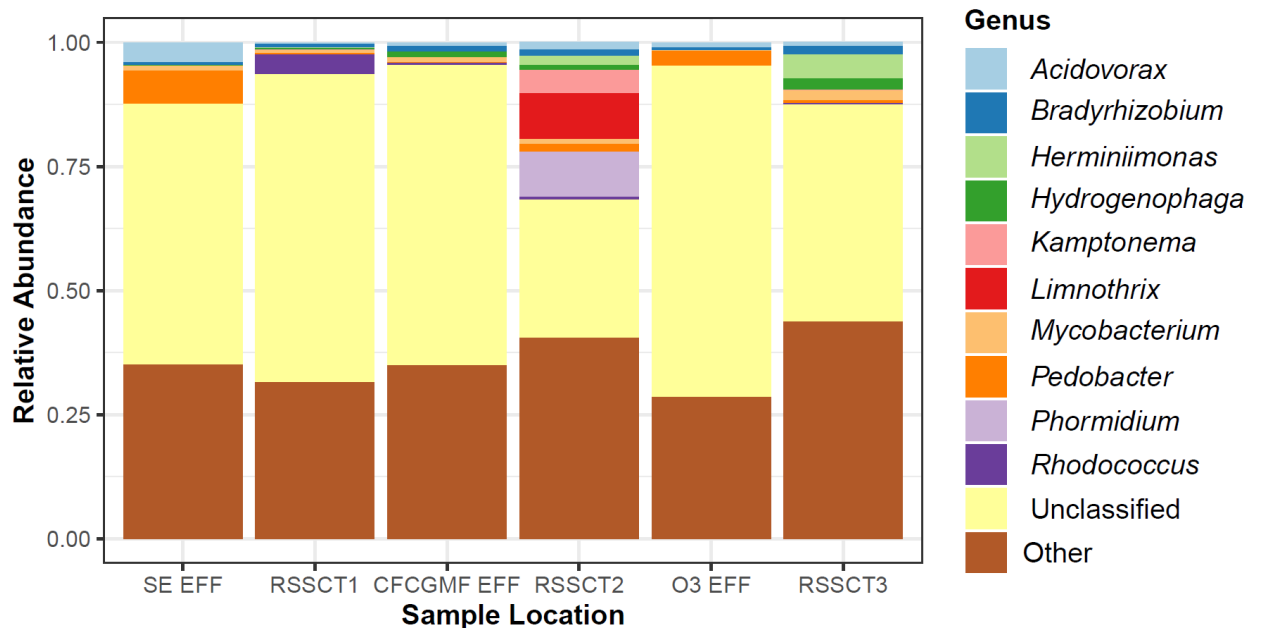


Figure 6-19. Ten most abundant genus and respective average relative abundance for influent and media samples under study.

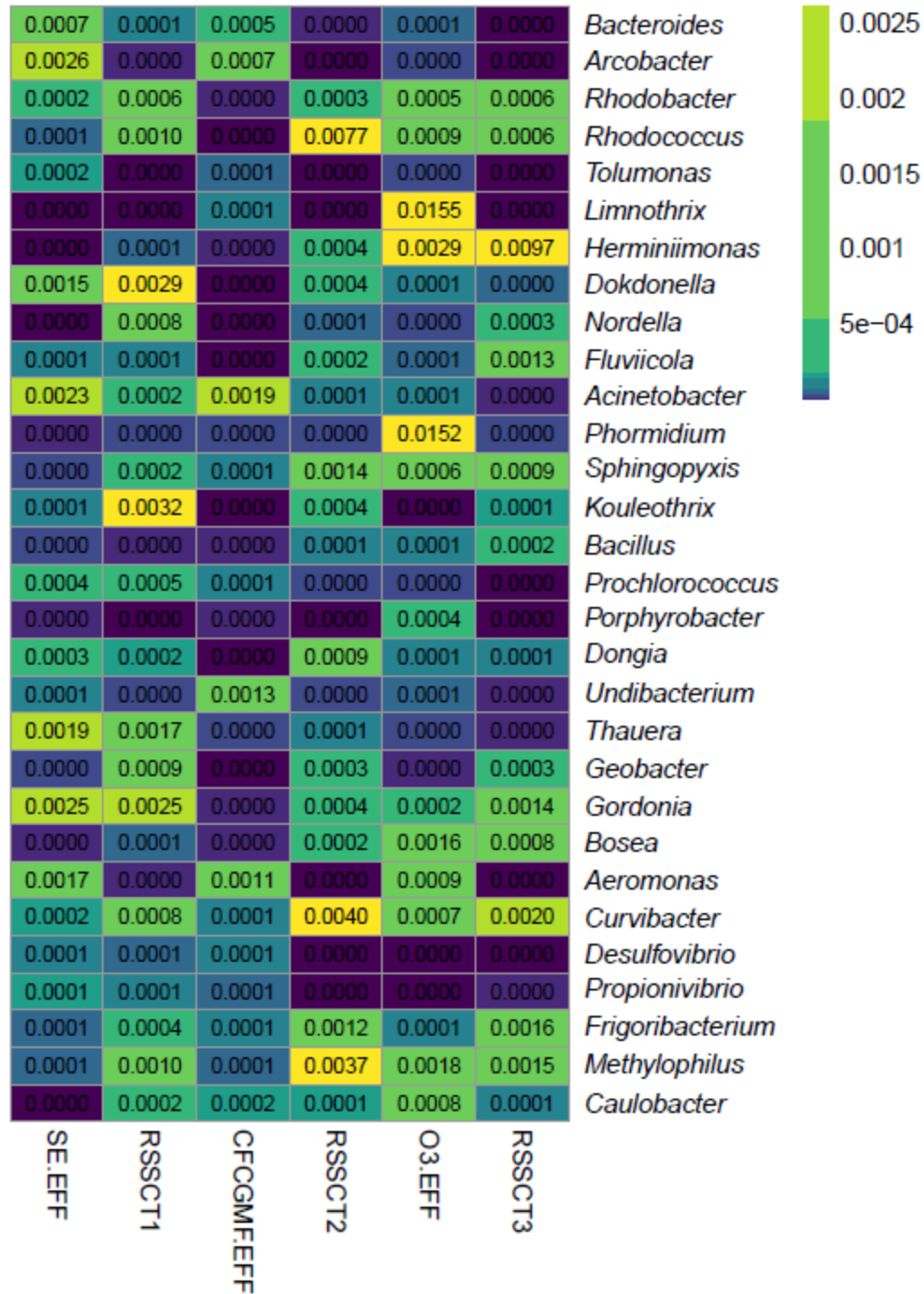


Figure 6-20. Genera - Heatmap depicts mean relative abundances of microorganisms detected to be differentially abundant by ANCOM procedure. Color-scaling distinguishing differences between Genera with smaller mean relative abundance. A cell-colored dark purple can be considered zero, whereas there were no detections of a particular microorganisms for that given cell.

6.3.6 Functional and Metabolism Analyses

Figure 6-21 shows the variation of functional analysis of KEGG metagenome information crossed with the upstream treatment to each RSSCT and the sampling date. Samples collected between July and November are closed independently of the influent treated by the RSSCT, suggesting that the microbial communities' capabilities are similar once the system is mature. In contrast, the microbial communities are different at the initial stages of the RSSCT experiment 30-60 days (3/17/20-6/05/20), particularly the community developed on the RSSCT treating CFCGMF INF or SE EFF.

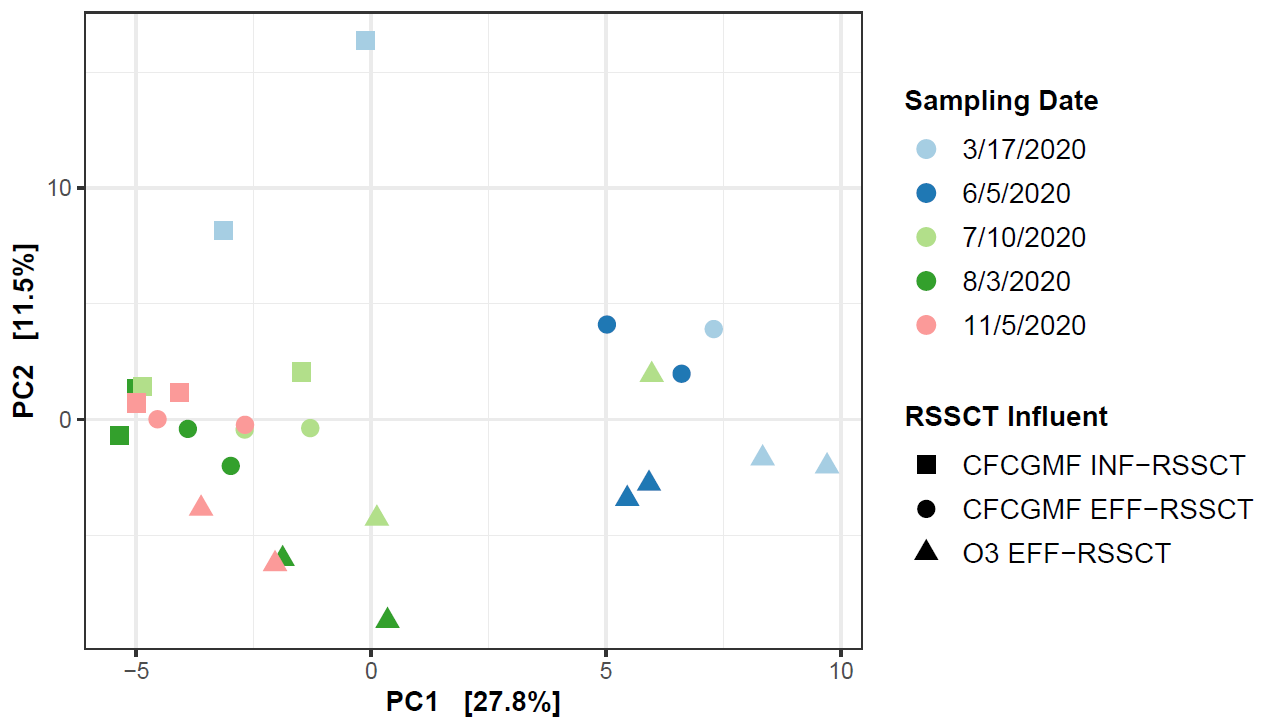


Figure 6-21. Canonical Ordination - Redundancy Analysis (RDA) for the functional analysis of KEGG metagenome as dependent matrix against the RSSCT influent and sampling date variables.

When KEGG pathways are analyzed (Figure 6-22), the samples are scattered distributed. However, the ordination space is separated into three levels depending upon the influent treated by

each RSSCT. The levels at the top, the O3 EFF (square-shaped points), the CFCGMF EFF (triangle-shaped points), and the CFCGMF INF (circle-shaped points), suggesting that the KEGG pathways are distinct based on influent treated by the RSSCT. If a sampling event is included in the analysis, the RSSCTs show dissimilarities of KEGG pathways.

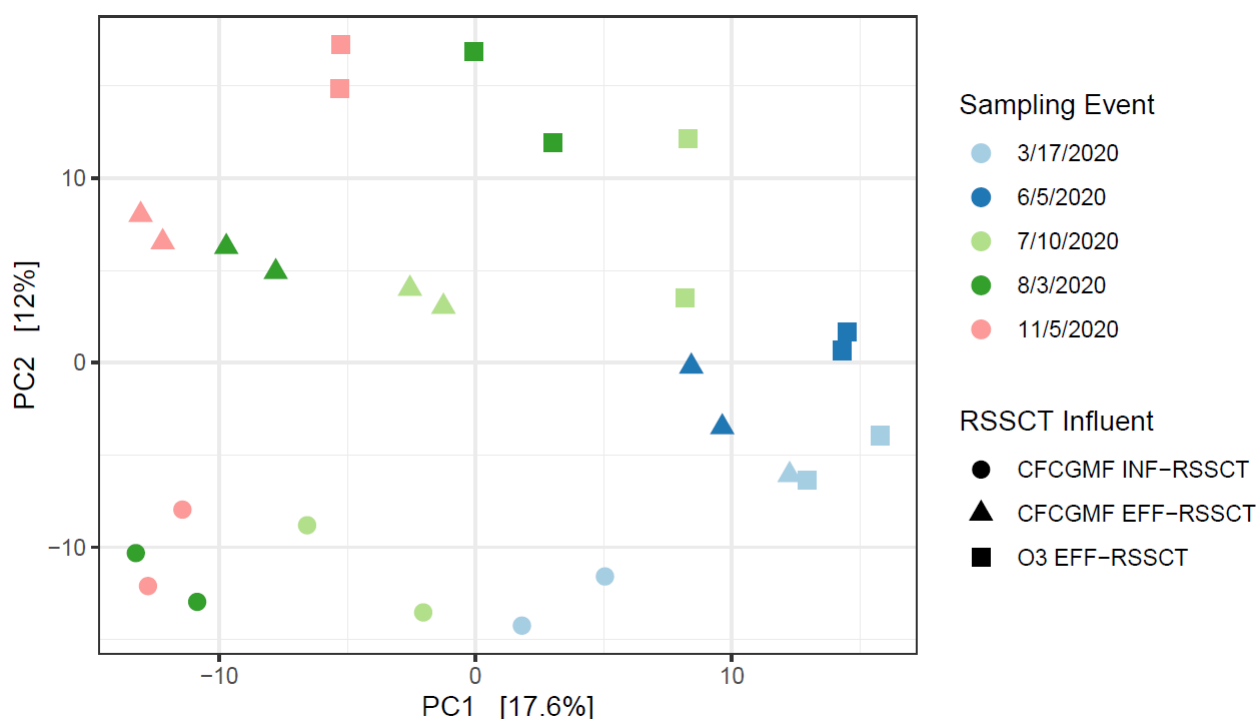


Figure 6-22. Canonical Ordination - Redundancy Analysis (RDA) for the METACYC pathway prediction as dependent matrix against the RSSCT influent and sampling date variables.

To compare the functional potential of microorganisms identified, abundance was predicted based on the OTUs using PICRUST2. The pipeline followed to run the functional analysis was reported earlier (Douglas, 2021). The predicted pathways were significantly enriched in the metabolism class at Level 1, followed by Genetic Information Processing, and Cellular processes (Figure 6-23. a). Figure 6-23. b shows the top 25 most abundant KEGG pathways identified within the samples analyzed. At level 1, a total of 167 pathways were identified. The most abundant

KEGG pathways identified were biosynthesis of ansamycins, biosynthesis of vancomycin group antibiotics, Fatty acid biosynthesis, Valine, leucine, and isoleucine biosynthesis, C5-Branched dibasic acid metabolism, and Biotin metabolism. The level 1 identified pathways mentioned are classified in level 2 as the metabolism of terpenoids and polyketides (ansamycins and vancomycin), lipid metabolism, amino acid metabolism, carbohydrate metabolism, and metabolism of cofactors and vitamins. The samples that showed the highest abundances were those receiving the CFCGMF EFF and the O3 EFF. Exploring Level 3 for Cell Processing and Environmental Information Processing (Figure 6-24), the relative abundance combined accounted for less than 7% of the total predicted function pathways. Nevertheless, predicted functions such as phosphotransferase, bacterial secretion, meiosis-yeast, bacterial chemotaxis, biofilm formation-*Vibrio cholerae*, flagellar assembly, and apoptosis were identified. These functions are predicted to provide a scanned capability of the microbial communities developed in each RSSCT under study.

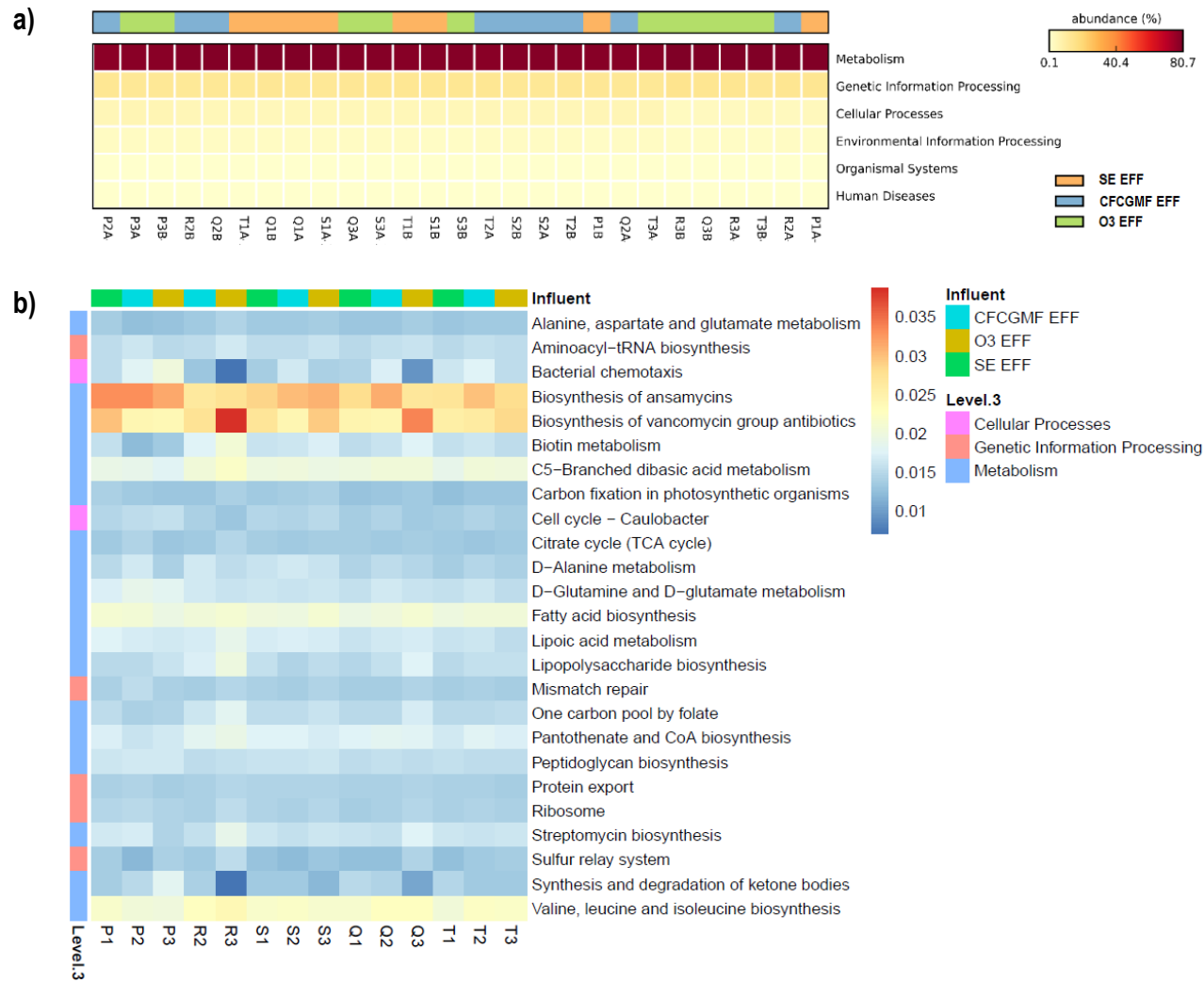


Figure 6-23. Heatmap of Top25 most abundant KEGG pathways identified in each media sample using PICRUST2 output, panel a) Predicted pathways to Level 3 and b) Level 1. The color represents pathway abundance, the closer is to dark blue the lower the abundance and closer to red the higher.

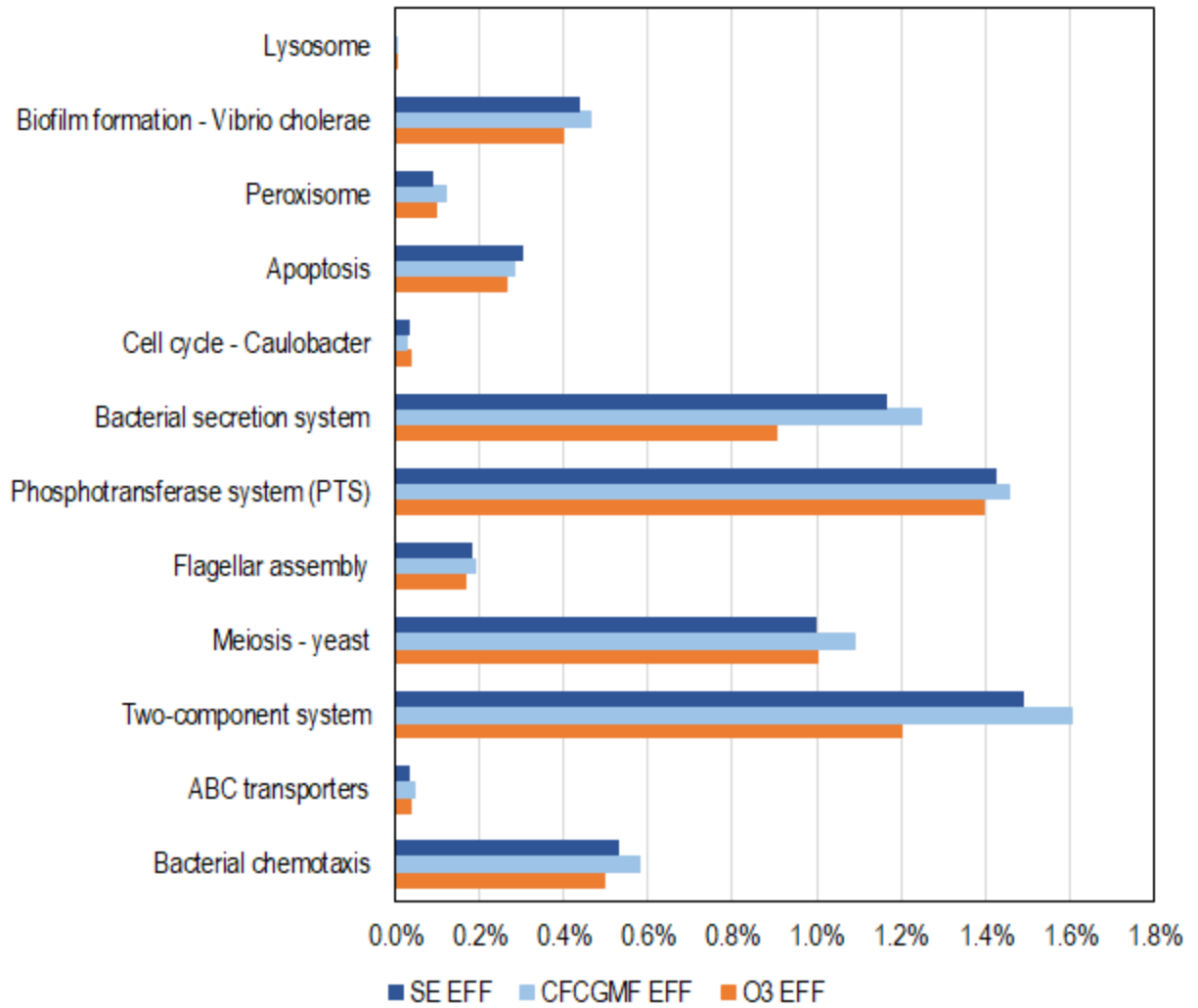


Figure 6-24. Level 3: Cellular processes and Environmental Information Processing predicted the listed Level 1 function for the samples analyzed

6.4 Conclusions

This study assessed the upstream water treatment impact on the microbial community structure and composition developed in RSSCT biofilters. *Proteobacteria* is the most dominant in all the samples on the phylum level, followed by *Bacteroidetes*, *Actinobacteria*, *Planctomycetes*, and *Chloroflexi*. The microbial community richness, diversity, and evenness assessed by different alpha diversity indices decreased depending on upstream treatment treated by each RSSCT, from higher to lower SE EFF, CFCGMF EFF, and O3 EFF. However, no significant difference (p -values >0.05) was observed in the richness, diversity, and evenness of the microbial communities developed in each RSSCT treating different influents. Two metrics were computed when comparing the microbial community diversity between the three RSSCTs treating different influent water. Weighted Unifrac takes abundance into account, and Unweighted Unifrac is based on OTUs presence-absence. UniFrac metrics were selected because it includes the phylogenetic information. The beta diversity analysis suggests that the microbial communities at each RSSCT are different based on the influent that were treated. Finally, an Analysis of Similarity (ANOSIM) to test whether overall microbial communities differ depending on the influent treated was performed. The ANOSIM results were significant (p -value=0.001 <0.05), meaning the upstream treatment or influent treated influences the microbial community composition developed on the studied biofilters (RSSCTs).

In addition, when influent and RSSCT media microbial communities were compared, the differential abundance analysis showed a significant difference to the phylum level and genus level. This finding corroborates what was discussed in Chapter 2 for drinking water and wastewater biofilters. Upstream treatment or influent microbial community structure and composition differ from downstream biofilters when the latter is used in water reuse applications.

Functional profiling was predicted for each RSSCT using PICRUSt2. The most abundant KEGG pathways predicted that the metabolism of amino acids, carbohydrates, cofactors and vitamins, terpenoids, polyketides, and lipids were abundant, particularly the biosynthesis of ansamycin and vancomycin. The former (ansamycin) is a bacterial secondary metabolite that shows antimicrobial activity (antibiotic). The latter, an antibiotic, suggests the bacterial community's maturity developed in the columns. This is the first research to focus on the impact of upstream treatment processes on the microbial community in biofilters treating advanced water for potable reuse. Further studies are needed to decode and confirm these findings.

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Chapter 7

CONCLUSIONS

The studies conducted in this research together demonstrate how the structure, composition, development, and fate of microbial community in O₃/BAC and other processes such as Coagulation/Flocculation/Clarification /Granular Media Filtration, Granular Activated Carbon Filtration, and Ultraviolet Disinfection in advanced water treatment for water reuse purposes can be key considerations to assess and control the overall system performance and ensuring water quality in finished water. The impact of influent water characteristics, available nutrients, environmental conditions, and optimal operational conditions will define the microbial community that will be developed and established after each treatment process.

The following conclusions can be drawn from the experimental and analytical studies presented in this dissertation:

- ✓ The combination of ozonation (oxidation) and biofiltration (adsorption and biological degradation) can reliably remove biodegradable organic matter, trace organic pollutants, byproduct precursors, and contaminants of emerging concern. Biofiltration is the key aspect for trace organics removal due to the active microorganisms' role in biodegradation processes.
- ✓ Tools such as 16S metagenomic sequencing and bioinformatic analytic tools provide insights into the abundance, composition, and diversity of the microbial community identified in treatment processes used in advanced water treatment systems.

- ✓ Microorganisms preference for free-living vs. attached-living style leads to the type of organism that will colonize fixed media surfaces in biofilters.
- ✓ The core microbial community that flows across an AWT is shaped by chemical (e.g., coagulation, ozone addition, UV disinfection) and physical (e.g., clarification, filtration) treatment processes along the system train.
- ✓ Potential bacterial pathogenic microorganisms are eliminated throughout the multibarrier treatment system. However, their ubiquitous presence and detection (even at low abundances) are latent risks that require continuous monitoring strategies.
- ✓ The microbial community detected across an AWT train at phylum level were: *Proteobacteria* (38-63%), *Bacteroidetes* (38-63%), *Cyanobacteria* (0-28%), *Firmicutes* (0.3-6.4%), *Verrucomicrobia* (0.3-7.0%), *Actinobacteria* (0.2-5.0%), *Planctomycetes* (0.4-3.9%), *Acidobacteria* (0.2-2.1%), *Nitrospirae* (0.1-1.8%), and *Chloroflexi* (0.1-0.9%). The highest richness was found at the O3 INF, BAC EFF, and GAC EFF with 1569.4 ± 322.5 , 1565.7 ± 178.2 , and 1547.4 ± 635.1 , respectively, while the lowest was at the final two treatment steps (UV and CF EFF). Diversity measurement indicated by the Shannon index resulted in an average value of 4.5 ± 1.2 ; lower values were found at CFCGMF EFF, UV EFF, and CF EFF while the filter effluents (BAC and GAC) showed the highest diversity. When testing whether the microbial communities differ by sampling location, the BAC EFF microbial community was significantly different from that at CFCGMF EFF, INF, O3 INF, O3 EFF, BAC, and GAC EFF.
- ✓ *Aeromonas*, *Clostridium*, *Enterobacter*, *Escherichia*, *Flavobacterium*, *Legionella*, *Mycobacterium*, and *Pseudomonas* are pathogenic bacteria genera detected along the AWT train. The ozonation step yielded more pathogenic bacteria genera than any other

sample location, followed by the GAC and BAC filters. On average, the percentage removal was around 75-100%, but *Mycobacterium* and *Pseudomonas* were the genera with the highest counts in the finished water. However, the detected pathogenic bacteria genera accounted for less than 3% of the total bacterial counts.

- ✓ The RSSCT BAC experiments assessed the upstream treatment impacts on biofilters' microbial community structure and composition. *Proteobacteria* was the most dominant on the phylum level, followed by *Bacteroidetes*, *Actinobacteria*, *Planctomycetes*, and *Chloroflexi*. The microbial community richness, diversity, and evenness assessed by different alpha diversity indices decreased along the treatment train. In addition, the beta diversity analysis pointed out that the developed microbial communities at each RSSCT were different based on the influent water that was being treated. Hence, the upstream treatment or influent treated characteristics influences the microbial community composition developed on biofilters (RSSCTs). However, when influent and RSSCT media microbial communities were compared within each BAC, the differential abundance analysis indicates a significant difference at phylum and genus levels. This finding supports several previous studies which stated that upstream or influent microbial community structure and composition are not like the community structure downstream of biofilters in water reuse applications.