MICROBIAL COMMUNITY STRUCTURAL AND FUNCTIONAL RESPONSE TO OPTIMIZATION OF A WATER RESOURCE RECOVERY FACILITY FOR BIOLOGICAL NUTRIENT REMOVAL

A Dissertation

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by

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Doctor of Philosophy

Civil and Environmental Engineering

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AN ABSTRACT OF A DISSERTATION

MICROBIAL COMMUNITY STRUCTURAL AND FUNCTIONAL RESPONSE TO OPTIMIZATION OF A WATER RESOURCE RECOVERY FACILITY FOR BIOLOGICAL NUTRIENT REMOVAL

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As regulatory limits on nutrient discharge from water resource recovery facilities (WRRFs) become more stringent, understanding the microbial communities in biological nutrient removal (BNR) systems can become beneficial for process optimizations. Traditional BNR occurs in more than one reactor to accommodate nitrification, denitrification and enhanced biological phosphorus removal (EBPR) processes. However, unconventional BNR includes optimizing a single reactor, such as an oxidation ditch, to have a dissolved oxygen gradient to accommodate these processes simultaneously. As an emerging practice, process optimization often encounters instabilities that can be averted by understanding the factors contributing to the structure, function, and stability of the BNR community, especially with regards to polyphosphate accumulating organisms (PAOs). Therefore, to contribute to the body of knowledge of unconventional BNR microbiology and process performance, this study evaluated how microbial communities adapt to unconventional operations of oxidation ditches and assessed whether the community and process stabilize over time after an optimization. Specifically, this research addressed whether the community structure and potential function change in response to optimization and attempted to further characterize the PAO community. Full and laboratory-scale studies were conducted to address the research goals. The full-scale optimized WRRF of this study modified the aeration patterns of their oxidation ditches to accommodate BNR. Two additional facilities were investigated to establish how a microbial community might change over time without the influence of optimization. One of these facilities operated a conventional oxidation ditch, while the other was designed and operated for BNR. The optimized WRRF was able to accomplish simultaneous nitrification and denitrification, while fluctuating ortho-P concentrations were observed in the oxidation ditch. Supporting evidence provided by measured P-release and uptake rates suggested that the facility has the potential to accommodate EBPR. Furthermore, the community of this facility changed differently compared to the other WRRFs, implicating that the changes were in response to optimization and were not completely temporal. Diversity and the core community structure of the optimized facility were more similar to the BNR-designed facility. Furthermore, dissolved oxygen correlated with the diversity of the optimized facility, whereas temperature was correlated with the reference facilities. PAOs were detected as active in the optimized oxidation ditch when the facility did not exhibit characteristics that would support PAOs. During the laboratory-scale study, *Dechloromonas* and a glycogen accumulating organism were detected as active and possibly more important to the denitrifying EBPR process than the well-known PAO, *Candidatus* Accumulibacter phosphatis. The outcomes of this study suggest optimization of oxidation ditches for BNR has the potential to be a successful and reliable practice.

CERTIFICATE OF APPROVAL OF DISSERTATION

MICROBIAL COMMUNITY STRUCTURAL AND FUNCTIONAL RESPONSE TO OPTIMIZATION OF A WATER RESOURCE RECOVERY FACILITY FOR NUTRIENT REMOVAL

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DEDICATION

This dissertation is dedicated to my inspiring, trail-blazing Grandmother and Great Aunt Ayn. Thank you for starting the path…I hope to continue it and pass it on.

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based on the DNA and cDNA dataset. Nitrate represents the concentration of nitrate in the reactor due to feed input. DO is the concentration measured at the end of the aerobic or anoxic/microaerobic stage.

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CHAPTER 1: INTRODUCTION

1.1 Background and Problem Statement

Eutrophication is the alteration of ecosystem structure and function by unnaturally high nutrient pollution (Dodds & Whiles, 2010). The ecological and economic impact of eutrophication in fresh and coastal water bodies has been a long-standing cause for environmental concern (Dodds & Smith, 2016; Nixon, 1995; Sharpley et al., 2014; Smith et al., 2014; USEPA, 2015b). It is well-established that the excessive discharge of nitrogen (N) and phosphorus (P) from both point and nonpoint sources is the primary reason for this problem. Eutrophication has resulted in the growth of harmful algal blooms, hypoxia, fish kills, and loss of biodiversity in surface water bodies, including major water systems such as the Mississippi River Delta, the Gulf of Mexico, and the Chesapeake Bay (Carpenter et al., 1998; Chislock, Doster, Zitomer, & Wilson, 2013; Smil, 2000; USEPA, 2015b). It has also significantly impacted the surrounding economy. In the United States, various researchers have estimated that nutrient pollution in fresh and coastal waters has resulted in annual economic losses of $2.2 billion (Dodds et al., 2009) and $82 million (Hoagland & Scatasta, 2006), respectively. Therefore, to protect aquatic ecosystems and the surrounding economy, state and federal regulatory agencies are establishing numeric criteria to limit the discharge of N and P, especially from point sources, such as municipal water resource recovery facilities (WRRFs) (USEPA, 2011, 2015a). Since reducing nutrients through upgrading wastewater treatment infrastructure can be expensive, a cost-effective alternative for WRRFs that is gaining popularity is to optimize the existing processes in order to attain the lowest possible effluent nutrient concentrations, before opting for capital expenditure for new processes (Collivignarelli & Bertanza, 1999;

USEPA, 2015a). In 2015, USEPA published a report highlighting twelve different WRRFs that were successful in implementing various optimization strategies for nutrient removal. Biological nutrient removal (BNR), a set of microbial processes employed to reduce the concentrations of ammonia, nitrite/nitrate, and phosphorus in wastewater, is commonly the primary focus of such optimizations (Cherchi, Onnis-Hayden, El-Shawabkeh, & Gu, 2009; Daigger & Littleton, 2000; Grote, 2010; Onnis-Hayden, Majed, Schramm, & Gu, 2011).

Conventional BNR, such as the anaerobic-anoxic-oxic (A_2O) design shown in Figure 1-1, incorporates the nitrification, denitrification and enhanced biological phosphorus removal (EBPR) processes. Nitrification is the two-step biochemical oxidation of ammonia to nitrate; denitrification is the biochemical reduction of nitrate to nitrogen gas; and EBPR is the luxury uptake of soluble phosphorus (or orthophosphorus) present in wastewater by certain microorganisms followed by removing them from the facility. Traditionally, these processes require multiple reactors to be successful. However, to optimize an existing process for BNR, a WRRF ideally will want to avoid building new reactors, and therefore may undergo modifications such as changing dissolved oxygen (DO) concentrations in existing aeration tanks (Collivignarelli & Bertanza, 1999; Zhou et al., 2012), adding alkalinity to stabilize pH for nitrification (JJ Environmental, 2015), enriching the BNR microbial community by altering the solids retention time (SRT) (Daigger & Littleton, 2014) or by increasing readily biodegradable chemical oxygen demand (rbCOD)that is fermented to volatile fatty acids in an anaerobic environment through the addition of an external carbon source (Cherchi et al., 2009). While there are several ways to optimize a WRRF based on the type of reactor configurations being implemented for the biological process, investigating WRRFs operating oxidation ditches

(Fig. 1-2) utilizing the extended aeration variant of the activated sludge process is the primary focus of this research.

There are approximately 10,000 oxidation ditches in the United States, most of which are operated utilizing the extended aeration variant of the activated sludge process (Water Environment Federation, 2010). Oxidation ditches incorporate a ring or racetrack configuration, utilize characteristics of complete mix and plug flow ideal reactors, and can be operated with different types of aeration systems, which can affect the macroenvironment (Metcalf & Eddy Inc, 2014). Such processes have a typical hydraulic retention time (HRT) of 18 to 24 hours and an SRT ranging from 20 to 30 days (Metcalf & Eddy Inc, 2014; USEPA, 2000; Water Environment Federation, 2010, 2015). Oxidation ditches are widely used for biological wastewater treatment (Grady, Daigger, Love, & Filipe, 2011), especially in small to medium-scale facilities treating flows ranging from 0.5 to 5.0 MGD and serving communities 5,000 to 50,000 in population (Water Environment Federation, 2010). This is noteworthy because smaller communities typically do not have the resources for expensive upgrades and complex operations (Bartlett et al., 2017; Hughes et al., 2005; USEPA, 2016), leaving process optimization a more favorable option. Even though oxidation ditches are common and their optimization to incorporate BNR is often recommended, very little knowledge exists on their long-term performance and process reliability following modifications in operating procedures. In order to address this gap, over a period of three years, we investigated the performance and related microbial community of a full-scale oxidation ditch undergoing optimization to assess whether the microbial community structure and function, and thereby the BNR process, stabilize over time. Additionally, we attempted to characterize the microbial community members

involved with BNR through a laboratory-scale study. To our knowledge, this is the first research study to investigate how the microbial community responsible for BNR responds to process optimization of an oxidation ditch.

1.2 Current State of Knowledge

Oxidation ditches, even though traditionally operated for biochemical oxygen demand (BOD_5) removal and nitrification (Metcalf & Eddy Inc, 2014), are among the wastewater treatment reactor types commonly optimized for simultaneous biological nutrient removal (SBNR), which includes the simultaneous nitrification and denitrification (SND) process and the simultaneous nitrification, denitrification and enhanced biological phosphorus removal (SNDP) process (Daigger & Littleton, 2014; USEPA, 2015a; Zhou et al., 2012). In order to accommodate SBNR in an oxidation ditch, aeration modifications have been widely investigated since altering aeration patterns allow for a more complex environment within a single reactor (Chen et al., 2010; Daigger & Littleton, 2000; Datta & Goel, 2010; C. Guo, Fu, Chen, Peng, & Jin, 2013; Jin, Wang, Wang, Ngo, & Jin, 2015; Zhou et al., 2012; Zhou, Han, & Guo, 2015). According to Daigger and Littleton (2000), successful SBNR can be achieved in a single reactor, such as an oxidation ditch, due to any combination of the following:

(1) creating microenvironments within bioflocs, which are aggregates of biomass within the activated sludge that contain anaerobic, anoxic, and aerobic components (Fig. 1-3);

(2) the bioreactor macroenvironment (Fig. 1-4), in which a DO gradient is maintained to create anaerobic, anoxic, and aerobic zones in a single reactor; and

(3) the presence of microorganisms such as comammox, anammox, and denitrifying polyphosphate accumulating organisms (DPAOs) with novel metabolic pathways, which

are each capable of performing more than one function for SBNR and for which the aforementioned unconventional environments may provide an ecological niche.

The micro and macroenvironments generated in oxidation ditches are essential for successful SBNR as they can allow for the enrichment of functionally diverse microbial communities and thereby encourage a more stable nutrient removal process (Flowers, Cadkin, & McMahon, 2013; Mccann, 2000). Few studies have investigated the operational and chemical components of the SBNR environment in oxidation ditches and even fewer have attempted to connect their studies to the microbial communities involved. For example, Daigger and Littleton investigated the mechanisms that allow SBNR by studying seven WRRFs operating the Orbal® oxidation ditch process. The Orbal® oxidation ditch layout is slightly different from a traditional racetrack design, in that it uses concentric channels within the same reactor and utilizes disk aerators (Fig. 1-5) (Metcalf & Eddy Inc, 2014). They observed N removal and EBPR in facilities that did not have defined anaerobic, anoxic, or aerobic zones (Daigger & Littleton, 2000), thus setting the groundwork for the possibility of successful SBNR in unconventional settings. It should be noted that even though the Orbal® process is not typically referred to as a configuration with defined aerobic, anoxic, or anaerobic zones, it does have separate channels that can allow for a DO gradient to occur and be manipulated (Fig. 1-5). To further explain SND performance, Littleton et al. 2003 extended this study by attempting to identify potential novel microorganisms, specifically the autotrophic denitrifiers and heterotrophic nitrifiers, and concluded that these microorganisms did not significantly contribute to SND. However, this study only utilized batch testing to investigate these organisms and lacked molecular methods, therefore this approach may not have allowed for enough analytical

depth to justify such a broad conclusion. Zhou et al. 2012 went a step further by testing a variety of aeration patterns in an Orbal® oxidation ditch to generate various DO gradients throughout the channels and were able to achieve SND in the outer channel of the ditch with a low DO concentration (0.20 mg/L) environment. While testing these aeration patterns, they observed changes in microbial diversity even though they were only able to look into a portion of the community. Later, Zhou et al. (2015), continuing with this study, found that smaller bioflocs consisted of more nitrifying bacteria than the larger flocs and therefore allowed for better nitrification when the DO concentration was low (0.20 mg/L). By simulating an oxidation ditch environment with a laboratory-scale sequencing batch reactor, Guo et al. 2013 investigated the different impacts on N removal in an oxidation ditch resulting from injecting air at a single, specific location, or point aeration, and from injecting air at multiple locations, or step aeration. They found that step aeration increased the nitrifying population while also improving denitrification. Jin et al. (2015) added to Guo's work by implementing step and point aeration into a full-scale oxidation ditch. They found that under step aeration, the oxidation ditch exhibited better N removal and an increase in diversity of the denitrifying microbial community. One of the very few studies that investigated the biological P removal microbial community in an oxidation ditch observed PAOs performing EBPR in an Orbal® oxidation ditch (Zilles, Peccia, & Noguera, 2002). They found that the outer channel provided an anoxic zone for P-release and the aerated inner channels allowed for P-uptake. They also determined that PAOs made up around 22% of the total microbial community in the activated sludge. Another study on P removal in an oxidation ditch was able to identify microbial community members, specifically the family *Rhodocyclaceae* and genus *Dechloromonas*, but the study did not

demonstrate the microbial community's function in that environment (Terashima et al., 2016).

Ammonia-oxidizing bacteria and archaea (AOB, AOA), nitrite-oxidizing bacteria (NOB), denitrifiers, and polyphosphate-accumulating organisms (PAO) are well-known classifications of microorganisms that remove either ammonia, nitrate/nitrite, or phosphorus in SBNR. Their structure and function have been studied extensively in many different WRRFs, with the exception of PAOs. The PAOs are primarily studied in either laboratory-scale or conventional EBPR facilities and are still not completely understood because unlike the other microorganisms mentioned, PAOs have not been culturable. Apart from the previously identified microorganisms, there are unique microorganisms being identified that contribute to SBNR in WRRFs by processing multiple nutrients (as opposed to a single nutrient). Due to their more recent discoveries, these microorganisms are still not completely understood. The presence of these unique microorganisms in SBNR environments may be due to the unusual ecological niches the wastewater process provides, as well as the microorganisms' unique metabolic pathways. The extent to which these microorganisms contribute to SBNR in an oxidation ditch is yet to be determined, however what is known about their multifunctionality makes them potentially very useful for a WRRF required to remove N and P. Recently, in regards to SND, has been the discovery of complete ammonia oxidation (comammox) bacteria, a class of microorganisms that are individually capable of completely oxidizing ammonia into nitrate (Daims et al., 2015; van Kessel et al., 2015). Previously, it was understood that the two-step nitrification process could only be carried out by two separate groups of microorganisms (AOB/AOA and NOB). The genus *Nitrospira,* conventionally classified as NOB, contains three bacteria,

Candidatus Nitrospira inopinata, nitrosa, and nitrificans that have recently been observed performing complete nitrification and therefore are now classified as comammox bacteria (Camejo, Domingo, McMahon, & Noguera, 2017; Daims et al., 2015; Kits et al., 2017; van Kessel et al., 2015). Other microorganisms that contributes to SND, even though less common in oxidation ditches, are the anaerobic ammonium oxidation (anammox) bacteria. Anammox bacteria are able to convert ammonium to nitrogen gas by using nitrite or nitrate as an electron acceptor (Kuenen, 2008). Their activity in a WRRF can reduce aeration costs and since they are autotrophic, as opposed to the conventional heterotrophic denitrifiers, they can function in carbon-deprived environments (Zeng, Li, Wang, Bai, & Peng, 2014). Denitrifying PAOs (DPAO) are also among the microorganisms that cannot be classified into a single functional group and have yet to be fully understood. They have been investigated as key players in SNDP as they can contribute to both N and P removal from a system (Freitas, Temudo, & Reis, 2005; Guerrero, Guisasola, & Baeza, 2011; Lopez-Vazquez, Hooijmans, Brdjanovic, Gijzen, & Van Loosdrecht, 2008; W. Zeng, Zhang, Wang, & Peng, 2016). One study found that a micro-aerobic environment (DO<0.2mg/L) can allow for DPAOs to perform denitrification and P removal because the low DO encourages them to use nitrate as an electron acceptor (Camejo et al., 2016). DPAOs studied so far have been classified in the family *Rhodocyclaceae*, specifically the genera *Candidatus* Accumulibacter phosphatis (CAP) and *Dechloromonas* (Camejo et al., 2016; Lv, Shao, Li, & Li, 2015). When considering their functional diversity on the clade level in the CAP community, it is still unclear as to which CAP clades are DPAOs and which are just PAOs (Camejo et al., 2016; Mao, Graham, Tamaki, & Zhang, 2015; Saad et al., 2016; Stokholm-Bjerregaard et al., 2017a).

Microbial communities can vary among WRRFs depending upon the operational configurations as well as environmental factors such as climate and influent characteristics (Griffin & Wells, 2017; Lopez-Vazquez et al., 2008; Xia et al., 2016; Xu, Liu, Chen, & Ni, 2017). In order to correlate microbial community structure and function to these factors, particularly those that support BNR process stability, a variety of molecular techniques must be employed. Traditionally, nutrient removal activity of activated sludge has been measured through batch-scale kinetic studies (Littleton, Daigger, Strom, & Cowan, 2003; R. J. Zeng, Lemaire, Yuan, & Keller, 2003) and members of SBNR microbial communities have been investigated through a variety of culture-independent methods (Ferrera & Sánchez, 2016; Terashima et al., 2016), such as fluorescent *in situ* hybridization (FISH) and community fingerprinting. These methods have been informative by providing a way to seek out a particular microbe or take a quick measure of diversity. However, recent technology, such as high-throughput sequencing (HTS), has allowed for a deeper look into the identity and functionality of the microbial community members of activated sludge (Xu et al. 2017; Keene et al. 2017; Guo et al. 2017). For example, Xu et al. (2017) used high-throughput amplicon sequencing to compare the microbial communities performing SND in six different oxidation ditch facilities and were able to determine correlations between community members and environmental factors. For the genera *Nitrospira, Nitrosomonadaceae* (uncultured), and *Denitratisoma*, they found strong positive correlations with the geographical location of a facility and with influent/effluent COD and ammonia concentrations. For the genera *Dechloromonas, Thauera, Rhodocyclaceae,* and *Comamonadaceae,* they found strong positive correlations with flow rate and with temperature.

As stated above, several studies have investigated various operational configurations to improve SBNR in oxidation ditches and some have examined the microbial communities involved. However, there is a lack of cohesive understanding of the relationship between the findings of these studies and long-term BNR process reliability, particularly in an unconventional system such as an optimized oxidation ditch. The following section provides a more in-depth discussion of such gaps.

1.3 Knowledge Gaps and Research Justification

As discussed previously, optimization for SBNR can be beneficial for WRRFs required to remove N and P. This optimization can provide for nutrient removal without chemicals and, with aeration modifications for lower DO concentrations, can reduce the consumption of electricity associated with aeration, resulting in lowered operating costs (Daigger & Littleton, 2000; Jimenez et al., 2010; L.-K. Ju, Huang, & Trivedi, 2007; Keene et al., 2017). Even though optimization of an oxidation ditch for SBNR has its benefits, long-term reliability of these optimizations has yet to be established, exemplified by the lack of literature on microbial community response to modifications for SBNR. Microbial community stability and robustness coupled with successful SBNR performance could indicate that optimizations can result in reliable processes. Further insight into the environmental and meteorological factors that affect community composition is needed to determine whether a community will stabilize over time. Therefore, studying the activated sludge microbial community from an ecological perspective and determining factors that affect the community could help validate optimization as well as contribute to successful operational practices (Rittmann et al., 2006).

Furthermore, there are still gaps in the understanding of how some microorganisms contribute to SBNR in unconventional systems, especially the PAO community (Coats, Brinkman, & Lee, 2017; Oehmen et al., 2007; Seviour, Mino, & Onuki, 2003; Stokholm-Bjerregaard et al., 2017b). PAOs are traditionally found in facilities designed for EBPR and are commonly studied on a laboratory-scale, however very few studies have investigated the structure and function of PAOs in full-scale, non-EBPR facilities (Albertsen, Hansen, Saunders, Nielsen, & Nielsen, 2012; Mehlig et al., 2013). Examining this community in an unconventional environment is important because PAOs and DPAOs are critical for successful optimization of SNDP. In our preliminary research, PAOs were found in oxidation ditches of two non-EBPR facilities, in which CAP was detected in relatively high abundance. Since these facilities operate oxidation ditches for N removal, there is a possibility that the PAOs detected could be classified as DPAOs. There has yet to be a conclusive study as to the entire functionality of both observed and potential PAOs, which is especially true for CAP. Due to diversity on the clade level, CAP can vary in morphology, utilize a variety of carbon sources, and participate in N and P removal (Flowers, He, Yilmaz, Noguera, & McMahon, 2009; Skennerton, Barr, Slater, Bond, & Tyson, 2014), however it is still unclear as to which clade performs which function (Camejo et al., 2016; Saad et al., 2016; A. N. Zhang, Mao, & Zhang, 2016). Literature has stated that it is difficult to identify the PAO community based solely on genomic data because the genes that allow PAOs to perform biological P removal span more than just the PAO community. For that reason, there is a need for studies that assess the active microbial community through investigation of gene expression (i.e. RNA) during various environmental conditions, including PAOs in unconventional environments (Oyserman,

Noguera, Glavina Del Rio, Tringe, & McMahon, 2016; Stokholm-Bjerregaard et al., 2017b). Therefore, this study also aims to characterize the active PAO and DPAO community in unconventional operational strategies and determine environmental factors that encourage PAOs and DPAOs to thrive. The results of our study will hopefully contribute to the current body of SNDP knowledge, thereby allowing for biological P removal to be expanded to facilities currently thought unable to accommodate the process.

1.4 Research Objectives, Hypotheses, and Rationale

To address the aforementioned gaps and to advance the knowledge of process and microbial stability during optimization for SNDP in oxidation ditches, the overarching goals for this research are (1) assess whether optimizations of oxidation ditches that allow for SNDP cause changes in the corresponding WRRF's microbial community and whether the community will eventually stabilize; (2) characterize the active microbial community during SNDP; and (3) determine environmental factors that influence microbial composition and putative function. Using the collective data from these goals we intend to answer how microbial communities adapt to unconventional operations of oxidation ditches for SNDP and whether the community and process stabilize over time after an optimization.

The overall goal of this research was addressed by integrating full-scale and laboratory-scale studies, through the following objectives:

Objective I. To understand if and how microbial community structure and putative function shifts in response to process optimization for SNDP in oxidation ditches. If a significant

shift is detected in response to optimization, evaluate whether various wastewater characteristics influenced the shift and their implications for long-term process stability.

Hypothesis to be tested- Microbial community composition and function will change to accommodate unconventional operational configurations in an oxidation ditch. As the community structure shifts, so will the wastewater characteristics with respect to BNR. Therefore, a correlation between structure stability and process stability will be observed.

Rationale- It is well established that microbial communities adapt to environmental changes in order to survive (Allison & Martiny, 2008; Vuono et al., 2014; Werner et al., 2011). Since microbial communities are heavily influenced by DO concentrations (Park & Noguera, 2004; Yadav, Khardenavis, & Kapley, 2014; Zhou et al., 2012) and the primary focus of the optimization in this study is aeration modifications, the community composition and functionality in the optimized WRRF will most likely shift in response to the process optimizations for BNR. However, it should be noted that microorganisms in any activated sludge process undergo a natural temporal shift (Griffin & Wells, 2017; F. Ju, Guo, Ye, Xia, & Zhang, 2014; Kim, Jeong, Wells, & Park, 2013). Therefore, to rule out the effects of temperature on community shift, we evaluated two other WRRFs that did not undergo optimizations. For those two facilities, we expect any shift in microbial composition to be due to seasonal variation. Differences in community structure between all three WRRFs may be correlated to the operational configurations and wastewater characteristics of each facility.

Objective II. To evaluate which members of the microbial community are active in the optimized oxidation ditch and identify those microorganisms with potential to contribute to SNDP.

Hypothesis to be tested- Some microorganisms will be revealed to be more active during unconventional BNR than previously thought.

Rationale- In our preliminary research, we detected unique, nutrient-removing microorganisms in a WRRF operating an unconventional configuration for nutrient removal. Considering this, we expect that some of these microorganisms are more functionally involved with BNR than previously thought. For example, PAOs are not traditionally associated with extended aeration oxidation ditches, but during our preliminary investigation, we detected them in relatively high abundance in the optimized facility of this study. Investigating which microorganisms are active in this facility during BNR will result in a deeper understanding of SNDP, especially for the PAO community.

Objective III. To better understand the presence of PAO and DPAOs in the optimized oxidation ditch, using laboratory-scale bioreactors, determine whether a PAO-enriched microbial community could accommodate both nitrate and P removal in the presence of low DO concentrations and compare the microbial community during the EBPR period to the resulting denitrifying EBPR (dEBPR) community.

Hypothesis to be tested- Investigating activity during EBPR and dEBPR will reveal microorganisms who are potentially contributing to each process and how the community changed to accommodate dEBPR.

Rationale- Over the years, certain bacteria have been proposed as putative PAOs and DPAOs; some have been extensively investigated, while others have not. Various potential PAOs have been found in high abundance in our laboratory-scale EBPR bioreactors, including those that have not been widely investigated. The community in the laboratory-scale bioreactors has been successfully removing P for three years and can be assumed to have functional stability. Therefore, these bioreactors were used to investigate the microbial community involved in EBPR and dEBPR. RNA analysis will provide insight as to which bacteria are active during EBPR and which are active during dEBPR, which may also suggest some PAOs as DPAOs.

1.5 Dissertation Organization

This dissertation is organized into six chapters. Chapter 1 provides a general background on optimizations for BNR, including the putative microorganism involved. This chapter also presents knowledge gaps in this field as well as our research goals and objectives to address the gaps. Chapters 2 through 5 are the four main chapters that address the research objectives. Each chapter is written in a manuscript format, and includes sections for the introduction, methodology, results and discussion, and conclusions. Chapter 6 presents a final discussion on the overall observations and conclusions made during this study and includes recommendations for future studies. References for the dissertation have been placed after the appendix.

Chapter 2. Microbial Community Structure of an Oxidation Ditch Undergoing Process Optimization for Nutrient Removal *(Manuscript in preparation for submission)*

Chapter 3. An Assessment of the Microbial Communities of Three Water Resource Recovery Facilities Using Different Strategies for Nutrient Removal

Chapter 4. An Investigation into the Potentially Active Microbial Community in an Oxidation Ditch Optimized for Biological Nutrient Removal

Chapter 5. Monitoring a Polyphosphate Accumulating Organism Community as it Transitions to Accommodate Denitrifying Enhanced Biological Phosphorus Removal in a Laboratory-scale Reactor

Chapter 6. A summary of the main conclusions observed throughout this research followed by recommendations for future work.

CHAPTER 2: MICROBIAL COMMUNITY STRUCTURE OF AN OXIDATION DITCH UNDERGOING PROCESS OPTMIZATION FOR NUTRIENT REMOVAL

Abstract

Optimizing the secondary treatment process of a water resource recovery facility (WRRF) can be a cost-effect strategy for meeting nutrient discharge limits. Biological nutrient removal (BNR) is commonly the focus of such optimizations and can be implemented in a single reactor, such as an oxidation ditch. Since it is an emerging practice, few studies have investigated the process stability and associated microbiology of an optimized oxidation ditch. Therefore, factors leading to long-term stability and the success of process optimization are yet to be established. In this study, we investigated an oxidation ditch of a WRRF over three years as it underwent aeration modifications to generate a dissolved oxygen gradient to enable simultaneous nitrification, denitrification, and enhanced biological phosphorus removal (SNDP). The facility's wastewater characteristics, microbial community, and potential for enhanced biological phosphorus removal (EBPR) was evaluated. Under the optimized operational strategy, the WRRF was able to achieve simultaneous nitrification and denitrification, while fluctuating orthophosphorus (P) concentrations were observed in the oxidation ditch. Supporting evidence provided by measured P-release and P-uptake rates obtained during batch testing suggests that this facility can accommodate EBPR, even though evidence of EBPR in the form of biomass P concentrations greater than synthesis requirements was not observed (see Chapter 4 section 4.3.1.2). Furthermore, the microbial community structure changed

over time and exhibited trends indicative of community stabilization, suggesting the optimized process may have reached a steady-state. The outcomes of this study suggest that optimization of oxidation ditches for SNDP has the potential to be a successful and reliable practice.

2.1 Introduction

Nitrogen (N) and phosphorus (P) from water resource recovery facilities (WRRFs) have long been established as contributors to eutrophication. Consequently, regulatory agencies are enforcing stringent effluent limits to reduce N and P in wastewater (Ambulkar, 2017; Carey & Migliaccio, 2009; USEPA, 2011). Stricter limits are triggering increased investments in new treatment infrastructure, and higher chemical and energy demands. As upgrading or adding infrastructure for nutrient removal can sometimes be an economic burden to many utilities, optimizing the secondary treatment process is gaining popularity as being more cost-effective (Collivignarelli & Bertanza, 1999; USEPA, 2015a). Biological nutrient removal (BNR) is usually the primary focus of such process optimizations (Daigger & Littleton, 2014; Grote, 2010).

A common approach for BNR process optimization, especially in oxidation ditches, involves altering the aeration patterns to generate a dissolved oxygen (DO) gradient (Daigger & Littleton, 2000; Jin et al., 2015; Zhou et al., 2015). This operational modification permits simultaneous biological nutrient removal (SBNR), such as simultaneous nitrification and denitrification (SND) or simultaneous nitrification, denitrification, and enhanced biological phosphorus removal (SNDP), to occur due to the creation of anaerobic, anoxic, and aerobic conditions in the biofloc microenvironment and the bioreactor macroenvironment (Daigger & Littleton, 2000; USEPA, 2015a; Yang et al.,

2011). The dynamic micro- and macroenvironments accommodate the syntrophic relationships necessary for the proliferation of known and novel nitrifiers, denitrifiers, and polyphosphate accumulating organisms (PAOs) (Camejo et al., 2016; Daims et al., 2015; Datta & Goel, 2010; C. Guo et al., 2013; Jin et al., 2015; Zhou et al., 2015). By enriching a functionally diverse community, these complex environments encourage a more stable BNR process (Flowers et al., 2013; Mccann, 2000). As oxidation ditches are widely used throughout the United States (USEPA, 2015a; Water Environment Federation, 2010), establishing reliable process optimization strategies by understanding the relationships between the environmental niches and the BNR microbial communities that emerge after establishing a DO gradient, is a worthwhile endeavor. Moreover, many oxidation ditches are operated in facilities serving populations less than 50,000 that may not have the resources for expensive upgrades (Bartlett et al., 2017; Hughes et al., 2005; USEPA, 2016; Water Environment Federation, 2010), leaving optimization a more favorable option. Despite the prevalence of the oxidation ditch and the existence of a few studies that investigated SBNR in ditches, a comprehensive understanding of how microorganisms adapt to optimizations and support long-term BNR process reliability remains elusive.

Daigger and Littleton (2000) investigated how the micro- and macroenvironments along with relevant microorganisms allow SBNR by studying seven WRRFs operating the Orbal® oxidation ditch process. They observed up to 90% total N and P removal in facilities lacking defined anaerobic, anoxic, or aerobic reactors, thus providing the basis for successful SBNR in unconventional designs. Littleton et al. (2003) extended this study by investigating the potential contributions of novel microorganisms to SBNR in Orbal® oxidation ditches. PAOs were detected in one of the facilities during batch testing for

enhanced biological phosphorus removal (EBPR). Since this study predated high-throughput sequencing, it was limited in identifying the relevant microorganisms involved in SBNR. Another study that detected PAOs in an Orbal® oxidation ditch exhibiting EBPR found that the outer channel provided an anoxic zone for P-release, while the aerated inner channels allowed for P-uptake (Zilles et al., 2002). Using fluorescence in situ hybridization (FISH), they determined that PAOs made up around 22% of the total microbial community in the activated sludge. Zhou et al. (2012) achieved SND in the outer channel of an Orbal® oxidation ditch by maintaining a low DO concentration of 0.20 mg/L. While testing aeration patterns, they observed changes in microbial diversity through PCR-DGGE. High-throughput amplicon sequencing was utilized by Xu et al. (2017) to compare the microbial communities performing SND in six different oxidation ditch facilities. They found *Nitrospira, Denitratisoma,* and uncultured members of *Nitrosomonadaceae* to be strongly correlated with geographical location, COD, and ammonia. They also found *Dechloromonas, Thauera, Rhodocyclaceae,* and *Comamonadaceae* to be strongly correlated with flow and temperature. Although these studies focused on SBNR in oxidation ditches, and characterized some of the BNR microbial community, none correlated community structure and function with BNR process stability. Additionally, microbial communities can vary among WRRFs due to different operational configurations, influent characteristics, and meteorological elements (Griffin & Wells, 2017; Lopez-Vazquez et al., 2008; Xia et al., 2016; Xu et al., 2017), further supporting the need for more full-scale studies.

Over the course of three years, we examined the microbial community structure of an extended aeration oxidation ditch as it underwent optimization for SNDP, with the

objective of evaluating the response of the microbial community to process optimization, and assessing whether the community stabilized over time. We hypothesized that the microbial community will change in structure and function to accommodate unconventional BNR, and that over time, the community, and thereby the optimized BNR process, will stabilize or reach steady-state. In this study, we show that establishing a DO gradient in an oxidation ditch generates the environmental niches necessary to exploit preexisting microorganisms of the activated sludge community to accommodate SBNR. This study is also the first of its kind to explore the long-term response of microbial communities to this type of optimization and could provide further insight to better process optimization for SBNR.

2.2 Methodology

2.2.1 WRRF Description and Sampling

The study was conducted at the city of Cookeville's WRRF, located in middle Tennessee. On average, the facility receives 7 million gallons per day (MGD) wastewater flow, and is designed to treat up to 14 MGD. Four oxidation ditches with surface aerators are operated in parallel in an extended aeration mode for biochemical oxygen demand (BOD) removal and nitrification. In anticipation of future nutrient effluent limits, the facility initiated optimizing the ditches to incorporate SNDP in April 2015, by modifying the surface aerator operations (Fig. 2-1). Instead of operating all six aerators concurrently, the facility alternates between pairs of aerators every four hours to create a DO gradient both laterally and vertically throughout each ditch, resulting in impermanent anoxic and aerobic zones.

Influent characteristics and pollutant removal efficiencies of the WRRF over seven years illustrate how the aforementioned operational change affected the overall process performance (Fig. 2-2 and Table 2-1). To further evaluate the SNDP process facilitated through optimization, influent and effluent samples and mixed liquor of a single oxidation ditch were collected during seven sampling events over three years. Sampling events were conducted once every summer and winter between 2015 and 2018, to account for the influence of temperature, except for summer 2018. During summer 2018, the facility was sampled twice to determine whether transient microorganisms and daily fluctuations in wastewater characteristics caused the microbial community to change within a season. Dissolved oxygen, pH, and temperature were measured at each location in the oxidation ditch using a HACH hand-held meter with rugged LDO and pH probes (HACH Company, Loveland, CO, USA). The sampling locations in the oxidation ditch (Fig. 2-1) were selected to represent varying DO concentrations. Therefore, samples were collected from the surface and the bottom of the ditch. Mixed liquor collected from each location was filtered onsite using 0.45 um syringe filters. Both unfiltered and filtered samples were immediately stored on ice and transported to laboratory for further analysis.

2.2.2 Analytical Techniques

Composite influent and effluent, as well as grab mixed liquor samples from oxidation ditch, were analyzed for ammonia, nitrate, total N, ortho-P, and total P, COD, volatile fatty acids (VFA), total suspended solids (TSS) and volatile suspended solids (VSS). Nutrients and COD were analyzed using Test N Tube kits (HACH Company, Loveland, CO, USA), except for nitrate, which was measured using ion chromatography (Dionex AS-DV, Thermo Scientific, Sunnyvale, CA, USA). VFAs were analyzed by gas

chromatography (Model 6890N; Agilent, Santa Clara, CA, USA) equipped with a flame ionization detector and a 0.53 mm internal diameter x 30 m Agilent DB-FFAP capillary column. TSS and VSS were analyzed following Standard Methods (APHA, American Water Works Association, Water Pollution Control Federation, & Water Environment Federation, 2012).

2.2.3 Batch Activity Tests

To confirm potential EBPR, batch kinetic tests were performed in the laboratory to determine P-release and P-uptake rates of the microbial community. The first batch test was conducted on mixed liquor collected prior to optimization (April 2015), while others were conducted post optimization from winter 2017 to summer 2018. Mixed liquor collected from the oxidation ditch was sparged with air for 15 minutes to assure complete soluble COD and P depletion. The sample was then sparged with nitrogen gas for two hours to simulate an anaerobic environment, following which air was reintroduced for a three-hour aerobic period. A solution of sodium acetate was added at the beginning of the anaerobic phase to attain an initial COD concentration of 130 mg/L. Samples were collected immediately after the addition of sodium acetate and every 15 minutes thereafter. The pH was maintained between 7.4 and 7.8. All samples were analyzed for VFA, COD, and ortho-P concentrations.

2.2.4 DNA Extraction, Library Preparation, 16s rRNA Amplicon Sequencing

Whole genomic DNA was extracted from mixed liquor samples collected from the oxidation ditch using DNeasy PowerSoil kit (Qiagen, Valencia, CA, USA). Additional sample details can be found in Table 2-2. DNA library preparation and amplicon sequencing of the v4 region of 16s rRNA was carried out on the Illumina MiSeq platform

according to the manufacturer's protocol (16S Metagenomic Sequencing Library Preparation protocol, Illumina, San Diego, CA, USA). Briefly, barcoded primer sets 515f and 806r (Integrated DNA Technologies, Coralville, IA, USA) were used to amplify the v4 region. A second round of PCR was performed to add sample-specific indices to each sample. A bead clean-up was performed after each PCR reaction to remove adapter/index dimers. Following library quantification using either a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA) or Pico Green assay (Invitrogen, Carlsbad, CA, USA), we normalized and pooled libraries for sequencing on a MiSeq Reagent V2 flow cell (2 x 250bp reads).

2.2.5 Amplicon Sequence Processing

Amplicon processing was performed using mothur v1.39.5 (Schloss et al., 2009), along with the corresponding MiSeq SOP (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013) (accessed on 12/30/2018). Briefly, after raw paired-end reads were assembled and primer sequences removed, sample sequences were filtered based on read length and ambiguity. Sequences from 245 to 275 nucleotides long, containing no ambiguous base calls, and no homopolymers longer than 8 nucleotides, were kept. Representative operational taxonomic units (OTUs) were aligned to the SILVA v132 reference database (Quast et al., 2013). Sequences were pre-clustered based on two nucleotide differences and chimeras were removed using VSEARCH v2.4.4 (Rognes, Flouri, Nichols, Quince, & Mahé, 2016). After assigning taxonomy using the Microbial Database for Activated Sludge (MiDAS) (McIlroy et al., 2015), sequences that were identified as chloroplast, mitochondrial, eukaryotic or of unknown origin were removed. Remaining sequences, representing *total* community structure, were then clustered based on 97% similarity. OTUs with ≤ 3 reads were removed (Keene et al., 2017), resulting in

6,208,281 remaining sequences. Samples were then subsampled at 11,842 sequences to remove heterogeneity in sequencing depth (Weiss et al., 2017) resulting in > 91.8% coverage for independent libraries (total of 9,567 OTUs).

2.2.6 BNR-Relevant Microbial Community Selection

In this study, *BNR-relevant* community structure includes microorganisms with the potential to contribute to BNR. The putative BNR-relevant OTUs were selected based on a genus-level investigation of the total microbial community. The MiDAS taxonomic database (McIlroy et al., 2015) among other resources (Fahrbach, Kuever, Meinke, Kämpfer, & Hollender, 2006; Lucker & Daims, 2014; Prosser, Head, & Stein, 2014; Stokholm-Bjerregaard et al., 2017a) was utilized for developing a list of microorganisms that have been observed performing a role in N or P removal. To generate a second dataframe representing the BNR-relevant community, we selected 557,026 sequences from the total community, identified according to the criteria above. Each BNR-relevant community was then subsampled at 862 sequences to generate a second dataset with > 95.7% coverage (total of 276 OTUs).

2.2.7 Statistical Analysis

Microbial community structure was evaluated separately for the total and the BNR-relevant community to determine whether diversity and composition changed as a result of process optimization. The alpha diversity for each community was characterized using R Studio version 1.1.463 (RStudio Team, 2016) to calculate inverse Simpson diversity for each sample collected post optimization using packages *lme4* (Bates, Mächler, Bolker, & Walker, 2015) and *brms* (Bürkner, 2017, 2018). Linear mixed effects models (function *lmer*) were used, assuming a Gaussian distribution, to test for differences in alpha diversity

of the total communities with surface and bottom of the oxidation ditch nested within sampling location (A-F; Fig. 2-1), treated as a random effect, to control for sample non-independence. Bayesian mixed effects models (function *brm*) were used to test for differences in alpha diversity of the BNR communities assuming a Gaussian distribution and sampling location (A-F; Fig. 2-1) treated as a random effect to control for sample non-independence.

Beta diversity analyses were conducted on the OTU level utilizing RStudio with package *vegan* (Oksanen et al., 2019) and Primer-E version 7.0.9 (Quest Research Limited, Auckland, New Zealand) to separately analyze differences in composition within the total community and the BNR-relevant community. A Bray-Curtis dissimilarity matrix was generated for each community from subsampled relative abundance data. To determine the effect of OTU presence/absence apart from relative abundance, each relative abundance table was transformed into a presence/absence table and another Bray-Curtis matrix was generated. Using Primer7, all matrices were visualized with a non-metric multi-dimensional scaling (NMDS) plot and SIMPROF was performed on the clusters to determine which samples had a statistically similar ($p \leq 0.05$) community composition. The function *adonis* in *vegan* was used to conduct permutational multivariate analyses of variance (PERMANOVAs) based on Bray-Curtis dissimilarity values. This tested for global differences by season, year, and an interaction between season and year in total and BNR communities and accounted for possible non-independence between samples by designating sampling location as a random effect using the *strata* parameter. Pairwise comparisons among sampling events and seasons were conducted using an analysis of similarities (ANOSIM). A SIMPER analysis was also performed to better understand

which OTUs, and therefore genera, were contributing to the clustering observed in NMDS plots. Differences in the putative denitrifier communities were assessed by calculating beta diversity using the *vegdist* function in the vegan package to generate a Bray-Curtis dissimilarity matrix. The *betadisper* function was used to test for homogeneity of variances as a proxy for 'community stability' between sampling events by season and year.

Correlations between wastewater characteristics and microbial community structures were measured with a two-tailed, Spearman's rank test using RStudio and visualized with the ggplot2 package (Wickham, 2016). When graphing the wastewater characteristics of the oxidation ditch, measurements were fourth-root transformed to reduce skewness.

2.3 Results and Discussion

2.3.1 Process Optimization of Oxidation Ditch for SNDP

2.3.1.1 Improvements in Biological Nutrient Removal

Influent flow and temperature, as well as BNR efficiencies of City of Cookeville's WRRF pre- and post-process optimization are depicted in Figure 2-2. Raw data for the winter and summer seasons, and for specific sampling events can be found in Tables 2-1, 2-3, 2-4. Values for the winter season were averaged across January, February, and March, while those for the summer season were averaged across July, August, and September, to represent typical flow and temperature characteristics during sampling events. Flow to the facility was typically higher during the winter seasons, with the highest flow occurring during winter 2018. Influent temperatures averaged 13 and 22^{o}C for winter and summer, respectively.

An assessment of the WRRF's overall performance in removing nutrients combined with a closer examination of the processes occurring in the oxidation ditch suggested SNDP. Soon after aeration modifications were implemented, DO and nutrient concentrations varied throughout the ditch (Fig. 2-3a.). Additionally, a drop in pH was observed at the bottom of the ditch, where there was typically lower DO and elevated ammonia and ortho-P concentrations (Fig. 2-3b). Prior to optimization, the WRRF's nitrification efficiency was above 99%, and even with a DO gradient in the ditch, it remained above 95% post optimization. Naturally, nitrification also resulted in an increase in nitrate concentrations (Tables 2-3 and 2-4), and for most sampling events the facility discharged more nitrate than it received. However, with the exception of summer 2017, the nitrate concentration in the facility's effluent was less than the maximum concentration in the ditch, indicating potential denitrification. After optimization, total N removal increased from 37% to 76% during winter and from 35% to 89% during summer. Total P removal during the winter changed minimally after optimization, however during the summer seasons it increased from 46% to 66%. Furthermore, samples collected from the ditch's aerated zones had lower ortho-P concentrations than those collected from locations with low DO, indicating EBPR potential. The ortho-P concentrations of the less aerobic samples frequently surpassed the amount entering the facility, suggesting PAO activity.

2.3.1.2 Potential of Activated Sludge to Perform EBPR

The potential for EBPR by the activated sludge was investigated through batch tests to determine trends and kinetic rates (Table 2-5, Fig. 2-4). Anaerobic P-release was observed immediately upon the addition of acetate (HAc). P-uptake, however, was delayed after air was introduced, most likely due to the presence of HAc at the beginning of the

aerobic phase. Once the HAc was depleted, P-uptake began. Trends observed during batch tests suggested EBPR potential and the existence of a PAO community.

With the exception of winter 2018, the ratios of P-release to HAc-uptake in post-optimization batch tests were comparable to findings from full-scale studies conducted at WRRFs designed for EBPR (Gu, Saunders, Neethling, Stensel, & Blackall, 2008; He, Gu, & McMahon, 2008; Lopez-Vazquez et al., 2008). Winter 2018 had a P-release to HAc-uptake ratio of 0.21, with P-release and uptake rates of 2.41 and 1.76 mg P/g VSS·hr, respectively, and an HAc-uptake rate of 12.34 mg HAc/g VSS·hr. Interestingly, this sample was collected during the sampling event with the highest DO levels measured in the oxidation ditch (> 2 mg/L, Fig. 2-5), due to increased flow into the facility. The highly aerobic environment may have affected the growth rate of PAOs, thereby impacting EBPR performance during the batch test. Considering the elevated rate of HAc consumption, the PAO microbial community may also have been starved due to being outcompeted in the highly aerobic ditch. Therefore, in order to survive, they may have altered their metabolism akin to that of the glycogen accumulating organisms (GAO) by utilizing glycogen instead of poly-P to consume HAc (Acevedo et al., 2012; Welles et al., 2015), which could explain a deteriorated EBPR performance. An increase in HAc-uptake along with both a decline in P-release and uptake could also indicate a more dominant role of denitrifiers in the activated sludge community during winter 2018. Nevertheless, an immediate anaerobic P-release during the batch tests reveals that Cookeville's WRRF has the potential for EBPR, even though the facility is not designed for EBPR.

2.3.2 Microbial Diversity and Composition During Process Optimization

2.3.2.1 Total Microbial Community Structure

The inverse Simpson index was used to quantify diversity of the total microbial community, which exhibited an overall significant difference among sampling events (χ^2= 152, p<0.001, Fig. 2-6). The diversity was found to be relatively high, which is typical of an activated sludge process (F. Ju & Zhang, 2015; Saunders, Albertsen, Vollesen, & Nielsen, 2016). Higher diversity during the summer seasons was also not surprising, considering temperature can have an impact on the community structure (Wang, Hu, Xia, Wen, & Ding, 2012; Xu et al., 2017). A decrease in microbial diversity was observed from winter 2016 to winter 2018, with the least diverse community existing during winter 2018, which could have been due to the high DO concentrations in the oxidation ditch (Fig. 2-5).

Beta diversity measures of the total microbial community displayed similar clustering patterns for the relative abundance and the presence/absence datasets (Fig. 2-7), indicating that clusters were not overly affected by sampling, PCR bias, or DNA sequence read abundance. The similar patterns rather suggest that rare taxa may be contributing to the differences in community composition at the species level. If changes in abundances were the driving factor, the presence/absence NMDS would not show distinct clusters. PERMANOVA results specified that community composition was significantly different based on year (F=35.6, p<0.001, R^2 = 22.7%), season (F=28.1, p<0.001, R^2 = 17.9%) and that an interaction between year and season (F=11.1, p<0.001, R^2 = 7.1%) occurred, indicating that the differences in the microbial communities between seasons varied across years. Further analysis (ANOSIM; R= 0.998 – relative abundance, 0.956 – presence/absence, p<0.001) revealed that each sampling event, except the two from summer 2018, displayed a distinct microbial composition. The summer 2018 sampling events, which occurred two weeks apart, were not well-separated by the presence/absence

dataset (R=0.591, p<0.001) (Ramette, 2007), suggesting temporal clustering for the other events in Figure 2-7 was not due to the daily dynamic conditions of the activated sludge environment and that there may not have been a high degree of changes within a season. Furthermore, increased separation between summer 2018 and 2018b clusters based on relative abundance compared to presence/absence may indicate stable community membership with day-to-day relative abundance fluctuations.

The NMDS plots suggest that the community composition may have changed in response to optimization, but then, after enough time, stabilized based on season. However, it is unclear whether the transient and rare microorganisms will continue to contribute to community separation among sampling events, thereby never fully allowing total community structure to stabilize, i.e. clusters will be close together but never significantly overlap. Another possibility could be that the transient and rare microorganisms only temporarily contributed to community separation while the essential microbes adapted to the new niches created by the optimization, i.e. clusters, will eventually overlap for each season. Either way, the observed sample clustering patterns in conjunction with relatively high alpha diversity suggested that the total community structure may have been heavily defined by the rare species during the post-optimization stabilization process and community composition should be investigated further.

The Proteobacteria constituted the numerically dominant phylum in all samples, averaging 40% of the total community throughout the study, followed by the Bacteroidetes (23%) and Planctomycetes (7%). This was consistent with other studies that investigated microbial community composition of activated sludge (Wu et al., 2019; T. Zhang, Shao, & Ye, 2012). The top 25 most abundant genera included *Haliangium* and *Nitrospira*, which

are well-established contributors to total N removal (Daims et al., 2015; McIlroy et al., 2016), and *Candidatus* Accumulibacter (CAP) and *Dechloromonas,* which may potentially contribute to both total N and P removal (Camejo et al., 2016; Islam et al., 2017) (Fig. 2-8). Each of the top 25 genera only contributed anywhere from 0.7 to 5% of the total community composition and was mostly consistent across sampling events. Furthermore, a SIMPER test determined that a multitude of different species contributed to the clustering in Figure 2-7, with the top contributors differing for each event. These outcomes substantiate the previous assessment that rare taxa, specifically at the genus and species level, may be contributing to differences in total community composition and may help explain the lengthy timespan it took for the community composition to show signs of stabilization (Kim et al., 2013).

2.3.2.2 BNR-Relevant Microbial Community Structure

Alpha diversity for the BNR-relevant community was lower than that of the total community. Diversity dropped significantly ($p<0.05$; Fig. 2-9) in winter 2018 even though the oxidation ditch was operating under the optimized strategy. During this time, high flow into the facility generated turbulence that resulted in a highly aerated ditch (Table 2-4), thereby losing the bioreactor macroenvironments needed for the enrichment of functionally diverse microbial communities (Daigger & Littleton, 2000; Flowers et al., 2013; Mccann, 2000). Lastly, the two summer 2018 events were not significantly different, suggesting that the diversity of the BNR-relevant community may not change much from day to day.

Distinct clustering was observed for the microbial community by both season and year (Fig. 2-10), possibly suggesting that rare species may have contributed to the differences in community composition following process optimization. PERMANOVA

results indicated that BNR-relevant community composition was significantly different based on year ($F= 29.1$, $p<0.001$, $R^2 = 17.6\%$), season ($F= 37.5$, $p<0.001$, $R^2 = 22.6\%$), and that an interaction between year and season ($F= 16.7$, $p<0.001$, $R^2 = 10.1\%$) occurred. ANOSIM revealed significant clustering related to sampling event for relative abundance and presence/absence datasets (R=0.993, 0.835 respectively, p<0.001). However, the two summer 2018 events for the presence/absence data were considered barely separable (R=0.169, p=0.003) (Ramette, 2007) and supports the notion that the microbial communities may not change significantly on a daily basis. The microbial community structure showed strong changes over the entire three-year study period following optimization, regardless of season (Fig. 2-10). However, there was a slight separation based on season for both datasets (R=0.630, 0.491 p<0.001, Fig. 2-10), indicating some overlap between the community structures during the summer and winter. Furthermore, distinct composition was observed between sampling events within the first year of optimization, but, for more recent events, similar composition among seasons was observed in terms of species present and their relative abundance (Fig. 2-10). Thus, the overlapping clusters for winters 2017 and 2018 and for summers 2016 through 2018b (Fig. 2-10), suggest that the BNR-relevant community could be stabilizing. The composition of this community also seems to have stabilized in earlier sampling events unlike the total community, possibly due to the fact that the BNR-relevant community lacked the vast number of transient microorganisms that the total community included.

The BNR-relevant community consisted of seven phyla with Proteobacteria as the most dominant, followed by Nitrospirae, Actinobacteria, Cyanobacteria, Firmicutes, Chloroflexi, and Gemmatimonadetes. Proteobacteria constituted an average of 81% of the

community across sampling events, whereas Nitrospirae, the second most dominant phyla, averaged 14% of the community. Interestingly, the nitrifying genus *Nitrosomonas* was reduced from 5% to less than 0.3% of the BNR-relevant community after optimization (Fig. 2-11) even though the facility continued to perform satisfactory nitrification (Fig. 2-2). This suggests that some members of *Nitrosomonas* may not respond favorably to a DO gradient, an observation supported by other studies with low DO conditions (Fitzgerald, Camejo, Oshlag, & Noguera, 2015; Keene et al., 2017). Furthermore, an OTU-level analysis of the putative denitrifying community (Fig. 2-12) revealed a significant difference between sampling events in terms of sample homogeneity (*betadisper*, F= 2.62, p<0.05), with winter 2016 and winter 2018 showing an increase in heterogeneity among samples. This is noteworthy because variation in community composition can allow improved functional redundancy (Allison & Martiny, 2008), exemplifying the benefits of implementing a DO gradient and associated bioreactor macroenvironments. Additionally, putative PAOs were detected before optimization (Fig. 2-8 and 2-11), further signifying the possibility that a non-EBPR activated sludge process can be modified to exploit PAOs for P-removal. PAO presence in high abundance (Fig. 2-8) prior to optimization also suggests they may have a larger role in the activated sludge community than removing P.

2.3.3 Wastewater Characteristics and Microbial Community

Correlations between wastewater characteristics of the oxidation ditch and various attributes of the total and BNR-relevant microbial communities were evaluated using Spearman's Rank correlation (Figs. 2-13, 2-14, 2-15). Among the wastewater characteristics, ammonia and ortho-P were strongly correlated with each other (r_s= 0.66) and negatively correlated with DO (r_s= -0.46, -0.51 respectively). Since there were areas at

the bottom of the oxidation ditch that lacked DO and exhibited a drop in pH, the relationship between ammonia and ortho-P concentrations with DO indicates possible in-basin fermentation.

Inverse Simpson diversity and species richness of the total community positively correlated with temperature (r_s= 0.48, 0.54 respectively), however these metrics were more negatively correlated with DO (r_s= -0.54, -0.71). As for the BNR-relevant community, species richness exhibited a similar correlation with temperature (r_s= 0.79) and DO (r_s= -0.64). Alpha diversity metrics for both communities were positively correlated with temperature and negatively correlated with DO concentrations, which explains the drop in species diversity observed for winter 2018 (Figs. 2-6 and 2-9).

Analysis of specific BNR-relevant genera revealed relationships with wastewater characteristics. Many of the putative denitrifying genera had a moderate to strong correlation with temperature, such as *Iamia*, *Hyphomicrobium*, *Thiothrix*, *Candidatus* Obscuribacter, and *Zoogloea* (r_s= 0.55, 0.58, 0.74, -0.56, and -0.76). The existence of positive and negative correlations between different denitrifying genera and temperature affirms that functional redundancy can be beneficial. Some denitrifiers thrive in cold environments while others thrive in warm environments, allowing this community to perform denitrification year-round. Furthermore, multiple putative denitrifying genera were correlated with each other, illustrating potential shared and competitive niches, such as *Iamia* and *Bradyrhizobium* (r_s= 0.83) and *Hyphomicrobium* with *Bradyrhizobium* and *Iamia* (r_s= 0.74). It is noteworthy that the two most abundant genera with the potential to denitrify, *Haliangium* and CAP, were negatively correlated (r_s= -0.65). Considering CAP is a well-known PAO and has been studied as a denitrifying PAO (DPAO) (Camejo et al.,

2016), they may compete with *Haliangium* for a similar niche, competition which may be influenced by season (Fig. 2-11). *Candidatus* Obscuribacter, another potential DPAO, was strongly correlated with flow (r_s= 0.86) and therefore may be a transient microorganism.

2.4 Conclusion

Oxidation ditches designed for BOD_5 and ammonia removal have been optimized to incorporate a DO gradient, creating the bioreactor macroenvironments needed to accommodate SNDP. However, the changes in the microbial community as a response to this type of process optimization are not well understood or documented. To contribute towards this knowledge gap, the primary goal of our three-year study was to assess the response of the microbial community of an oxidation ditch to process optimization for SNDP and determine whether the community stabilizes over time. The DO gradient in the optimized oxidation ditch allowed for successful SND without deterioration in nitrification performance, even with changes in the microbial community. The composition of the putative denitrifying community changed in response to the optimized bioreactor macroenvironment, potentially becoming more functionally redundant and stable. Further, even though the facility receives influent with relatively low P, the varying concentrations of P throughout the ditch, the kinetic rates measured from batch tests, and the detection of putative PAOs in relatively high abundance, suggest that the oxidation ditch is maintaining an environment that could be utilized for EBPR. Shifts in microbial community composition over time in both total and BNR-relevant communities, showed a trend that may indicate stabilization of community structure. These findings suggest that optimization of oxidation ditches for SNDP has the potential to be a successful practice. To better understand whether these microbial community changes were due to optimization

or a natural phenomenon of activated sludge, other WRRFs operating similar secondary treatment processes as Cookeville, but did not undergo optimization, should also be investigated. Information from this study is encouraging for municipalities that may need cost-effective solutions to meet new effluent limits, since optimizing an oxidation ditch as previously described calls for little to no financial investment.

CHAPTER 3: AN ASSESSMENT OF THE MICROBIAL COMMUNITIES OF THREE WATER RESOURCE RECOVERY FACILITIES USING DIFFERENT STRATEGIES FOR NUTRIENT REMOVAL

Abstract

Biological nutrient removal (BNR) is a microbial-driven process utilized by many water resource recovery facilities and is increasingly becoming the focus of optimizations for facilities trying to improve or accommodate total nitrogen or total phosphorus removal. Conventionally, BNR is achieved through the use of separate reactors that allow for anaerobic, anoxic, and aerobic environments. Simultaneous nitrification, denitrification, and enhanced biological phosphorous (SNDP) removal is a type of BNR process in which these different environments are achieved in a single reactor, such as an oxidation ditch, through operational changes including, but not limited to, aeration modifications. Characterizing the microbial communities of the BNR process can contribute to better strategies for efficient optimizations. However, even though many studies have investigated these microbial communities, they are still not completely understood. Furthermore, studies are lacking that pertain to the response of the microbial communities during optimizations for SNDP. In this study, we continued our investigation on how a microbial community of the activated sludge process responded to optimizations of an oxidation ditch for SNDP through aeration modifications. Two additional facilities were investigated in order to establish how a microbial community might change over time without the influence of major operational modifications since dynamic influent characteristics and seasonal temperature changes can impact community structure. One of

these facilities conventionally operated an oxidation ditch and the other facility was designed and operated for BNR. Wastewater characteristics and the diversity, structure, and core members of the microbial communities of each facility were investigated. Even though the three facilities had similar influent characteristics, due to differences in operational parameters, each facility exhibited different nutrient removal efficiencies. Additionally, we found that shifts in the microbial community were different for the optimized facility when compared to the other two facilities. Furthermore, diversity and the core community structure of the optimized facility were more similar to the BNR-designed facility than the facility conventionally operating an oxidation ditch. The outcomes of this study not only support the idea that an oxidation ditch can be modified to accommodate BNR, but that the microbial community of the activated sludge process can shift in response to optimizations, further supporting the potential success of this type of optimization strategy.

3.1 Introduction

Biological nutrient removal (BNR) is a widely used engineered process in water resource recovery facilities (WRRFs) that employs microorganisms for nitrogen (N) and phosphorus (P) removal (Metcalf & Eddy Inc, 2014). Traditionally, this process involves multiple, separate reactors to generate anerobic, anoxic, and aerobic environments to allow nitrification, denitrification, and enhanced biological phosphorus removal (EBPR) to occur efficiently. Since its first discovery, many researchers have investigated the microbial communities of BNR and as understanding of these microorganisms has improved, many alternative BNR processes have been developed and implemented (Ferrera & Sánchez, 2016; Flowers et al., 2013; Grote, 2010; Keene et al., 2017; Metcalf & Eddy Inc, 2014).

One such alternative to traditional BNR is enabling simultaneous nitrification, denitrification, and enhanced biological phosphorus removal (SNDP), which is a type of BNR that is achieved in a single reactor. This process has been studied extensively; however, factors that shape and impact the microbial community of SNDP and their associated mechanisms are still not completely understood (Camejo et al., 2016; Daigger & Littleton, 2014; L.-K. Ju et al., 2007; R. J. Zeng et al., 2003). Evaluating such factors can help in future designs of WRRFs and in addressing challenges operators encounter, particularly when a WRRF plans to optimize their secondary treatment operations to accommodate or improve BNR processes like SNDP.

Simultaneous nitrification, denitrification, and enhanced biological phosphorus removal (SNDP) is a process in which aerobic, anaerobic, and/or anoxic zones are achieved in a single reactor through implementation of a bioreactor macroenvironment and the existence of floc microenvironments (Daigger & Littleton, 2000, 2014). Optimizations for SNDP are useful for WRRFs operating single, secondary treatment reactors, such as oxidation ditches, where such facilities may need to incorporate denitrification and EBPR. Optimizing the secondary treatment process, in general, is becoming an increasingly utilized practice because it can allow WRRFs to meet nutrient criteria while remaining cost-effective (Collivignarelli & Bertanza, 1999; USEPA, 2015a). To be economical and avoid the addition of chemicals or building new infrastructure, a key focus of SNDP optimizations is to modify operational parameters, such as aeration patterns or solids retention time. Modifying aeration patterns, specifically, can lower operating costs due to less energy consumption (Daigger & Littleton, 2000; Jimenez et al., 2010; Keene et al., 2017) and can allow for BNR or SNDP to occur in a single reactor, such as an oxidation

ditch. Many studies focusing on oxidation ditches have demonstrated the potential success of these practices for improved total N or total P removal (Datta & Goel, 2010; C. Guo et al., 2013; Zhou et al., 2012, 2015). An important approach to better understanding the treatment efficiency of SNDP in an oxidation ditch is to investigate the microbial communities involved. One study testing various aeration modes in an Orbal® oxidation ditch to improve total N removal detected aerobic and anaerobic bacteria in the outer channel of the ditch (Zhou et al., 2012). This finding demonstrated that a single reactor can be modified to simultaneously support anaerobic and aerobic microorganisms, which is important since both types of microorganisms are needed for SNDP. Another study investigating an Orbal® oxidation ditch observed the occurrence of EBPR in the ditch due to the outer channel providing an anoxic zone for P-release and the aerated inner channels allowing for P-uptake (Zilles et al., 2002). This study also detected polyphosphate accumulating organisms (PAO), which is important since PAOs are crucial for SNDP. Although these studies investigated microorganisms in BNR, they failed to reveal how the microbial community responded to aeration optimizations, and whether the microbial community eventually stabilized under an unconventional operational strategy. Determining whether a microbial community has stabilized, or reached a steady state, in response to optimizations can help an operator to know whether their process modifications were successful and provides encouragement for other facilities to try optimization.

A common approach to characterizing the microbial communities of the activated sludge process is to investigate diversity and community structure, which can be useful when tracking changes in the community over time and for determining whether the community has reached a steady state, particularly after optimization. Not only it is

important to determine whether a community has stabilized, it is also important to determine what community members are crucial to a particular BNR process, such as SNDP. Identifying which microorganisms are consistently present during successful nutrient removal processes can provide insight as to who may be important to the process. Therefore, investigating the core microbial community of activated sludge can help determine which microorganisms are potentially important to an atypical BNR process by either revealing microorganisms already known for contributing to BNR or by suggesting potentially novel BNR microorganisms whose function may be unknown. Unfortunately, there is a lack of research focusing on the core communities of BNR, and even less investigating how a facility's core community responds to optimizations for BNR. A few studies, however, have provided the groundwork for this type of investigation. One of the first studies to investigate the core community looked at 14 WRRFs spanning Asia and North America (T. Zhang et al., 2012). Even though the secondary treatment process differed, they discovered that 70 out of 744 genera were shared among all facilities, including the putative denitrifier, *Zoogloea* (Larsen, Nielsen, Otzen, & Nielsen, 2008), and putative denitrifying PAO (DPAO), *Dechloromonas*, which were among the most abundant genera for most samples. Another study investigated 13 Danish WRRFs and detected 63 OTUs that made up the shared core community among these facilities, with the putative DPAO, *Tetrasphaera* (Kristiansen et al., 2013), as the most abundant member (Saunders et al., 2016). A more recent study focusing on 13 Chinese WRRFs, nine of which operated oxidation ditches, found that 1757 OTUs comprised the shared core community (Hou et al., 2019). Since the WRRFs studied are located in two different regions of China, the higher number of core OTUs suggests that there may be a larger core community among

similar process types, particularly among oxidation ditches. Finally, the largest core community study thus far, investigated 269 WRRFs spanning every continent except Antarctica and established 28 OTUs as the shared core community (Wu et al., 2019). They also estimated that 99.999% of the activated sludge community has not yet been cultured, which suggests that designing or optimizing reactors for the microbial-driven BNR process is still somewhat of a black box for the engineer and reinforces the need for more microbial studies.

Establishing a core community among WRRFs designed to accommodate the BNR process could distinguish which microorganisms are present during efficient N and P removal and therefore provide insight as to what factors may support a successful BNR community in a facility optimizing for BNR. Furthermore, environmental disturbances in a reactor that are temporary, such as fluctuations in pH or increased DO due to large amounts of rain, may not permanently impact a resilient core community (Allison & Martiny, 2008). However, process optimizations like aeration modifications could cause a core microbial community to permanently change since the "environmental disturbance" is long-term or permanent. Some studies, though very few, have started to investigate correlations between process efficiency and core communities (Mielczarek, Nguyen, Nielsen, & Nielsen, 2013; Wu et al., 2019). For example, Wu et. al 2019 observed on a global scale that the putative DPAO *Dechloromonas* (Kong, Xia, Nielsen, & Nielsen, 2007), was part of the global activated sludge core community and was positively correlated with TN removal. Therefore, investigating diversity, community structure, and the core community for both the activated sludge community and the BNR community is important for optimization purposes, because correlating these communities with

environmental conditions and wastewater characteristics can help tease out factors important to a stable community and thereby, a reliable BNR process. Identifying the similarities and differences between the microbial communities of optimized processes and those of non-optimized processes can provide information and guidance for when a facility needs to exploit the activated sludge to improve or accommodate BNR.

In this study, we examined how the diversity, community structure, and core community of an optimized facility changed over time and compared it with the communities of two geographically similar facilities, which served as references for traditionally operated WRRFs. The reference facilities provided a baseline to show how the activated sludge community shifts over time without the influence of a significant operational modification. The dynamic fluctuations in influent characteristics and seasonal temperature changes can shape the activated sludge microbial community (Griffin & Wells, 2017; Wu et al., 2019) and therefore, these two reference facilities served as a way to account for those types of shifts. Specifically, we investigated the microbial community of an extended aeration oxidation ditch as it underwent optimization for SNDP. This optimization involved modifying aeration patterns to achieve a dissolved oxygen gradient throughout the ditch. Therefore, we were able to monitor the microbial community of the activated sludge as it shifted from a fully aerobic environment to a more complex environment with anaerobic, anoxic, and aerobic zones. Additionally, we investigated the microbial communities of the activated sludge of two other WRRFs that did not undergo optimization. One facility operates an oxidation ditch in a traditional manner, for nitrification and BOD_5 removal, and represents how the optimized facility originally operated before process modifications. The other facility operates an A_2O process, with an

oxidation ditch for the aerobic zone, in order to achieve TN and TP removal, and represents what the optimized facility was trying to achieve with the aeration modifications. Our goals were (1) to determine whether the community structure patterns observed in the optimized facility were due to a microbial response to optimization or due to a typical shift of the activated sludge community and (2) to determine whether the optimized facility's core community changed and characterize the core community shared between the optimized facility and the reference facilities. We hypothesized that the microbial community of the facility undergoing aeration modifications did change in response to optimization and that the resulting core community will be similar to that of the A_2O facility.

3.2 Methodology

3.2.1 WRRF Description and Sampling

This study was conducted at three different WRRFs located in middle and east Tennessee. The facility undergoing optimization, located in Cookeville, TN, treats an average of 7 million gallons per day (MGD) of wastewater flow, and is designed to treat 14 MGD. Originally designed for BOD_5 removal and nitrification, this facility operates four parallel oxidation ditches with brush aerators and an extended aeration variant of the activated sludge process as their secondary treatment. They began modifying the aeration patterns of their oxidation ditches to accommodate simultaneous nitrification, denitrification, and enhanced biological phosphorus removal (SNDP) in April 2015, which resulted in a dissolved oxygen (DO) gradient laterally and vertically throughout the ditches. The facility located in Etowah, TN operates under a 1.2 MGD design flow with an average daily flow of 1.15 MGD and has two oxidation ditches with brush aerators and extended aeration variant of the activated sludge process as their secondary treatment. They are not

designed nor optimized for SNDP and only remove ammonia and BOD_5. The facility designed for BNR, located in Maryville, TN, operates under a 17 MGD design flow with an average daily flow of 13.2 MGD. This facility has an A_2O process as their secondary treatment, which includes separate anaerobic and anoxic chambers ahead of two oxidation ditches with spindle surface aerators and extended aeration variant of the activated sludge process.

Samples were collected once every summer and winter season over a course of three years for the Cookeville WRRF and over two years for the Etowah and Maryville WRRFs. One exception to the sampling routine was that the Cookeville facility was sampled twice during the summer of 2018. Composite samples were collected representative of the influent and effluent for each facility. Using a HACH hand-held meter with rugged LDO and pH probes (HACH Company, Loveland, CO, USA), DO, pH, and temperature were measured throughout the secondary treatment process to determine sampling locations for mixed liquor collection (Figs. 2-1, 3-1). These sampling locations were selected to represent varying DO concentrations. Due to stratification, samples were collected from the surface and the bottom of the oxidation ditch at Cookeville's WRRF. Etowah's ditch did not have significant stratification; however, samples were collected from the surface and bottom for comparison with the optimized facility. Mixed liquor was collected from a single oxidation ditch for Cookeville and Etowah and from the three reactors in a single A_2O treatment train for Maryville (Figs. 2-1, 3-1). Mixed liquor collected from each location was filtered onsite using 0.45 um syringe filters. Both unfiltered and filtered samples were immediately stored on ice and transported to laboratory for further analysis. All samples were analyzed for various water quality

parameters and DNA was extracted from the unfiltered mixed liquor samples collected from the locations of the secondary treatment process.

3.2.2 Analytical Techniques

Composite facility influent and effluent samples, as well as grab mixed liquor samples from the activated sludge, were analyzed for ammonia, nitrate, total N, ortho-P, and total P, COD, volatile fatty acids (VFA), total suspended solids (TSS) and volatile suspended solids (VSS). Nutrients and COD were analyzed using Test N Tube kits (HACH Company, Loveland, CO, USA), except for nitrate, which was measured using ion chromatography (Dionex AS-DV, Thermo Scientific, Sunnyvale, CA, USA). VFAs were analyzed by gas chromatography (Model 6890N; Agilent, Santa Clara, CA, USA) equipped with a flame ionization detector and a 0.53 mm internal diameter x 30 m Agilent DB-FFAP capillary column. TSS and VSS were analyzed following Standard Methods (APHA et al., 2012).

3.2.3 Batch Activity Tests

To investigate and compare EBPR potential of the activated sludge of each WRRF, batch kinetic tests were performed in the laboratory to determine P-release and P-uptake rates by the respective microbial communities. Mixed liquor collected from the oxidation ditch of each facility was treated, monitored, and analyzed as described in Chapter 2 section 2.2.3.

3.2.4 DNA Extraction, Library Preparation, 16s rRNA Amplicon Sequencing

Whole genomic DNA was extracted from the activated sludge samples using DNeasy PowerSoil kit (Qiagen, Valencia, CA, USA); sample details can be found in Tables 2-2 and 3-1. DNA library preparation and amplicon sequencing of the v4 region of 16s

rRNA was carried out on the Illumina MiSeq platform according to the manufacturer's protocol (16S Metagenomic Sequencing Library Preparation protocol, Illumina, San Diego, CA, USA) and briefly detailed in Chapter 2 section 2.2.4.

3.2.5 Amplicon Sequence Processing

Amplicon processing was performed using mothur v1.39.5 (Schloss et al., 2009), along with the corresponding MiSeq SOP (Kozich et al., 2013) (accessed on 12/30/2018). Details are described in Chapter 2 section 2.2.5. OTUs with ≤ 3 reads were removed (Keene et al., 2017), resulting in 10,333,866 remaining sequences, which represent *total* community structure. Samples were then subsampled at 11,769 sequences to remove heterogeneity in sequencing depth (Weiss et al., 2017) resulting in > 91.5% coverage for independent libraries (total of 12,152 OTUs).

3.2.6 BNR-relevant Microbial Community Selection

BNR-relevant community structure included microorganisms with the potential to contribute to BNR. Details as to how this community was selected can be found in Chapter 2 section 2.2.6. To generate a second dataframe representing the BNR-relevant community, we selected 1,106,954 sequences from the total community, identified according to the criteria previously described in section 2.2.6. Each BNR-relevant community was then subsampled at 877 sequences to generate a second dataset with > 95.7% coverage (total of 353 OTUs).

3.2.7 Statistical Analysis

Wastewater characteristics of the influent for each facility were evaluated using either a one-way ANOVA with Tukey post hoc or Kruskal-Wallis test depending on distribution of data. The Shapiro-Wilk test was used to determine normality. Additionally,

batch test ratios between facilities were evaluated using a one-way ANOVA with Tukey post hoc due to normal distribution. When graphing the wastewater characteristics of the secondary treatment processes, measurements were fourth-root transformed to reduce skewness.

Microbial community structure was evaluated separately for the total and the BNR-relevant communities to determine whether diversity and composition differed between facilities. The alpha diversity for each community was characterized using R Studio version 1.1.463 (RStudio Team, 2016) with *vegan* package (Oksanen et al., 2019) to calculate Shannon and inverse Simpson diversity for samples collected from each facility. Shannon diversity was transformed for both community datasets using the exponential (*exp)* function in R to attain a normal distribution; inverse Simpson diversity was normalized using the Box-Cox power transformation for only the BNR-relevant community dataset. Two diversity metrics were estimated in order to account for the bias in each; Shannon diversity is weighted towards rare species and the Simpson index is weighted towards dominant species. Linear mixed effects models (function *lmer*) were used from the *lme4* package in R (Bates et al., 2015), assuming a Gaussian distribution, to test for differences in alpha diversity of the total and BNR-relevant communities with lateral sampling location (Figs. 2-1, 3-1) treated as a random effect, to control for sample non-independence.

Beta diversity among all three facilities was analyzed on the OTU-level utilizing RStudio with package *vegan* and Primer-E version 7.0.9 (Quest Research Limited, Auckland, New Zealand) to investigate differences in composition within the total community and the BNR-relevant community. Separate Bray-Curtis dissimilarity matrices were generated for the total and BNR-relevant communities using subsampled relative

abundance data. To determine the effect of OTU presence/absence, each relative abundance table was transformed into a presence/absence table and additional Bray-Curtis matrices were generated. All four matrices were visualized with a non-metric multi-dimensional scaling (NMDS) plot using Primer7. SIMPROF was performed on the clusters to determine which samples had a statistically similar ($p \leq 0.05$) community composition. The function *adonis* in *vegan* was used to conduct permutational multivariate analyses of variance (PERMANOVAs) based on Bray-Curtis dissimilarity values and the *betadisper* function was used to test for homogeneity of variances in sample clusters. PERMANOVA tested for global differences by sampling event, season, facility, and an interaction between season and facility in total and BNR communities and accounted for possible non-independence between samples within a facility by designating sampling location as a random effect using the *strata* parameter. Pairwise comparisons among sampling events, seasons, and facilities were conducted using an analysis of similarities (ANOSIM).

A core microbial community was investigated based on the total microbial dataset following the methodology used in Wu et. al 2019. To be defined as part of the core community, an OTU had to meet three sets of criteria. First, the overall average of relative abundance across all samples was calculated for each OTU and those OTUs that made up at least 0.1% of the overall average abundance were selected. Second, each of the previously selected OTUs had to be present in at least 80% of samples (Saunders et al., 2016). Finally, the third set of criteria involved a two-step analysis. For each sample individually, the top 80% abundant OTUs were selected. Then, out of those OTUs, each one had to be in at least half of all samples while still remaining among the top 80% abundant OTUs for those samples.

Correlations between wastewater conditions, alpha diversity metrics, and core BNR-relevant genera were measured with a two-tailed, Spearman's rank test using RStudio and visualized with the ggplot2 package (Wickham, 2016). Wastewater characteristics and beta diversity measurements were correlated with a distance-based redundancy analysis (dbRDA) using the *capscale* function in *vegan* and visualized with *ggord* (Beck, 2017). Relationships between core communities and wastewater characteristics were also investigated using *capscale*.

3.3 Results and Discussion

3.3.1 Wastewater Characteristics

3.3.1.1 Trends Observed During Sampling Events

Influent and effluent characteristics varied among the facilities and sampling events (Tables 2-3 and 3-2). Interestingly, ammonia was the only influent water quality parameter that was significantly different between facilities (Kruskal-Wallis; χ^2=6.513, p=0.039), with Maryville's influent exhibiting a higher concentration than the other two facilities. This suggests, perhaps, that differences observed between these facilities are defined more by the operational conditions of each facility, instead of the influent water quality parameters measured in this study. Since the secondary treatment process configuration and operation were slightly different for each facility, varying environmental conditions were observed as reflected in Figures 2-3 and 3-2. Actual water quality data for the activated sludge samples can be found in Tables 2-4 and 3-3. These tables show that pH measured at or above 7.00 for most sampling locations at Etowah and Maryville, with Maryville's anaerobic zone only dropping to 6.93. Cookeville, on the other hand, measured as low as 6.22 at the bottom of the oxidation ditch and frequently exhibited pH values less

than 7.00 (Table 2-4). Dissolved oxygen concentrations for each facility's oxidation ditch are depicted in Figure 3-3. Etowah's oxidation ditch exhibited the highest DO compared to the other facilities for each sampling event, with the exception of winter 2017. During this sampling event, due to a mechanical malfunction, each oxidation ditch had only a single active brush aerator. The oxidation ditches of Cookeville and Etowah experienced an overall increase in DO for the winter 2018 sampling event, due to heavy rains immediately prior to sampling, whereas Maryville's oxidation ditch did not show an increase in DO. Less than 0.25 mg/L of DO were measured at the Maryville facility in both anaerobic and anoxic zones across all sampling events and even displayed a slight lateral DO gradient throughout its oxidation ditch. This varied DO for Maryville was observed for all sampling events and is most likely due to the placement of their mixers, which are located at one end of the ditch and therefore the DO decreased for samples taken further downstream. Interestingly, neither DO nor MLSS varied vertically throughout the ditch, indicating little to no stratification. Cookeville also experienced a DO gradient for all sampling events, but the gradient was both lateral and vertical throughout the ditch with stratification in the activated sludge increasing at sampling locations further downstream from the active brush aerators.

Since the oxidation ditches in Etowah are traditionally operated for nitrification and BOD_5 removal, it was not surprising that we observed close to 100% ammonia removal for all sampling events (Table 3-2), except for winter 2017. During this event, the facility was able to achieve 87% ammonia removal, even with the reduced DO concentration in the ditch. As a result of high nitrification efficiency, elevated nitrate concentrations were observed in Etowah's oxidation ditch and effluent, when compared to the facility influent.

Total N removal was less than 35% for all events, except winter 2017. The increase in nitrate and low total N removal suggests that little to no denitrification was occurring in the oxidation ditch. Furthermore, with the exception of winter 2017, samples collected from Etowah did not demonstrate ortho-P fluctuations throughout the ditch, which suggests there was no EBPR occurring. Except for winter 2017, at least 98% ammonia removal, 92% total N removal, 74% ortho-P removal and 78% total P removal were observed for Maryville during each sampling event, as expected from a process designed for BNR. Total N and P removal by the facility was much less for the winter 2017 sampling event compared to other events, which may be due to a rain event that occurred during sampling; therefore, the composite influent sample may have been diluted as a result of possible inflow and infiltration. Regardless, nutrient concentrations measured in Maryville's oxidation ditch reflected the occurrence of nitrification, denitrification, and EBPR for all sampling events (Table 3-2). Cookeville achieved at least 96% ammonia removal for all events except winter 2017. Additionally, it achieved at least 89% total N removal during the summer sampling events and 52% for the winter sampling events (Table 2-3), demonstrating more efficient total N removal than Etowah. Furthermore, Cookeville achieved 77% total N removal during the winter 2018 event, an event in which it experienced very high (>2mg/L) DO concentrations throughout the oxidation ditch, indicating perhaps that the DO gradient in the oxidation ditch still allowed for denitrification to occur. Cookeville WRRF also exhibited some biological P removal (Table 2-3). Fluctuations in P concentrations relative to DO concentrations throughout the ditch reflect P-release and uptake and support the potential for this facility to perform EBPR.

3.3.1.2 Potential of Activated Sludge to Perform EBPR

The activated sludge from each facility was investigated for EBPR potential through batch tests to determine trends and kinetic rates (Tables 2-5, 3-4, Figs. 2-4, 3-4). The batch tests from Etowah and Maryville were compared with Cookeville to assess whether Cookeville's rates were comparable to a traditionally-operated oxidation ditch or a BNR-designed facility. Anaerobic P-release was observed immediately upon the addition of acetate (HAc) for all three facilities, even though not as pronounced for Etowah (Figs. 2-4, 3-4). P-uptake was also observed for all three facilities; however, it was not immediate upon the introduction of air, most likely due to the presence of HAc at the beginning of the aerobic phase. After the HAc was depleted, P-uptake began. Unfortunately, HAc was not completely consumed for either of the summer batch tests for Etowah, therefore P-uptake could not be measured for these two tests. Since Etowah's oxidation ditch is a highly aerated environment, the microbial community is not subjected to performing feed uptake in anerobic conditions, and therefore may be dominated by obligate aerobes. The P-release rates for Etowah were less than 0.5 mg P/ g VSS*hr and P-release/HAc-uptake ratios were less than 0.08 for all batch tests except winter 2017. During the winter 2017 batch test, Etowah exhibited a P-release/HAc-uptake ratio of 0.18 mg P/mg HAc. These low measurements, when compared to EBPR-designed facilities (see below), were not surprising since Etowah operates a highly aerobic oxidation ditch. P-release rates for Maryville batch tests ranged from 2.77 to 3.82 mg P/ g VSS*hr and P-release/HAc-uptake ratios ranged from 0.34 to 0.48 mg P/mg HAc, which is comparable to other A_2O studies (Gu et al., 2008). Batch test results for Cookeville are presented in Chapter 2 section 2.3.1.2. All batch tests exhibited trends similar to those observed for Maryville and other

facilities designed for EBPR (Gu et al., 2008; He et al., 2008; Lopez-Vazquez et al., 2008). In brief, P-release rates ranged from 2.14 to 3.54 mg P/ g VSS*hr and P-release/HAc-uptake ratios ranged from 0.20 to 0.59 mg P/mg HAc. P-release/HAc-uptake ratios calculated from the batch tests for Etowah were significantly lower than Maryville and Cookeville (ANOVA; $F=11.332$, $p<0.01$), whereas no significance was detected between Maryville and Cookeville. The differences in activity between Etowah, Maryville, and Cookeville suggested that the microbial community for each facility might differ as well.

3.3.2 Microbial Community

3.3.2.1 Total Microbial Community Structure

The Shannon and inverse Simpson indices were used to quantify the diversity of the total microbial community in and among the WRRFs. Both indices exhibited an overall significant difference among facilities (χ^2 = 64.9, 56.6 respectively, $p<0.001$) and among seasons (χ^2= 211.6, 146.1 respectively, $p<0.001$) (Fig. 3-5). There was a significant overall interaction between facilities and seasons for Shannon diversity (χ^2=6.26, $p<0.05$), meaning that differences were detected in Shannon diversity between facilities during the winter and during the summer. This was not initially the case for inverse Simpson diversity. However, upon visualizing the diversity metrics (Fig. 3-5), inverse Simpson trends suggested potential for pairwise differences. Therefore, a Tukey's post-hoc test was conducted and detected significant pairwise interactions between facility and season for both Shannon and inverse Simpson diversity. Investigating the pairwise test without rejecting the null of the overall test has been supported by literature (Hsu, 1996). Diversity during the summer was generally higher than during the winter for each facility.

Furthermore, for each sampling event, diversity for Cookeville was typically higher than Etowah, but similar to Maryville. This is interesting because the dynamic conditions of Cookeville's oxidation ditch due to the DO gradient, may have selected for a more diverse microbial community by providing a more complex environment like Maryville's A_2O process. Lastly, each facility exhibited the lowest diversity for the total community during winter of 2018, when all three facilities experienced large rain events prior to sampling, possibly causing a washout of the rare or transient microorganisms (Saunders et al., 2016).

Visualization of the Bray Curtis matrices displayed similar clustering patterns for both the relative abundance and the presence/absence datasets (Fig. 3-6), indicating that clusters were not overly affected by sampling, PCR bias, or DNA sequence read abundance. These similar patterns also suggest that rare species may be contributing to the differences in community composition. If shifts in abundances were the main factor shaping the cluster patterns, then those patterns would not be observed in the presence/absence NMDS. Therefore, during optimization, the microbial community of Cookeville may have experienced major shifts in less abundant or possibly rare species while the community was adjusting to the new operational parameters. A global PERMANOVA test detected significant differences in community composition based on sampling event (F=47.5, R^2 = 86.3% - relative abundance, F=12.9, R^2 = 63.2% - presence/absence; $p<0.001$). Additional analysis for pairwise comparisons supported that all samples clustered together based on event (ANOSIM; R= 0.999 – relative abundance, 0.987 – presence/absence, $p<0.001$), with one exception. The two summer 2018 sampling events for Cookeville were not well-separated by the presence/absence dataset (R= 0.588, $p<0.001$) (Ramette, 2007). This was expected and explained in detail in Chapter 2 section

2.3.2.1. Additionally, significant differences in community composition based on facility were detected (PERMANOVA; F=29.3, R^2 = 29.1% - relative abundance, F=24.1, R^2 = 25.2% - presence/absence; p<0.001). Pairwise analysis between facilities revealed some similarity in community composition between Cookeville and Maryville by detecting slight overlapping among sample clusters (ANOSIM; R= 0.761 – relative abundance, 0.613 – presence/absence, p<0.001). The community structure of Etowah, however, was quite distinct from Cookeville and Maryville (ANOSIM; R> 0.950 for all pairwise, p<0.001). Composition across all facilities based solely on season was not distinct (ANOSIM; R=0.304 – relative abundance, 0.194 – presence/absence), but interactions detected between season and facility (PERMANOVA; F=12.8, R^2 = 6.0% - relative abundance, F=5.4, R^2 = 4.0% - presence/absence, p<0.001) further supported similarities in community structure between Cookeville and Maryville. For example, the summer microbial communities for Cookeville and Maryville exhibited the most similar structure out of all sample comparisons (ANOSIM; R= 0.689 – relative abundance, 0.516 – presence/absence, p<0.001). It was not surprising that the total community differed between Etowah and Maryville since they operate their secondary treatment process very differently. However, since the majority of samples from Cookeville were collected after operational changes, the response of Cookeville's community may have resulted in a more similar structure to that of Maryville's community. This timeframe for sample collection could also explain the lack of similarities detected between Cookeville and Etowah's total community.

The differences in community structure between facilities for each sampling event suggests that the operational parameters and influent characteristics of each WRRF might shape distinct communities. Furthermore, sampling events for each reference facility

displayed a distinct microbial community structure. The lack of overlap within each reference facility could be due to sample size, in that more sampling events are needed since wastewater activity is very dynamic due to varying parameters, such as influent water quality and flow (Griffin & Wells, 2017). Another explanation could be that this is a typical trend, in that the total community structure of the activated sludge inevitably changes over time, possibly due to the existence of rare and transient microorganisms (F. Ju et al., 2014). Hence, addressing the question posited in Chapter 2, the total microbial community is unable to fully stabilize due to the constant influence of rare and transient microorganisms. Additional sampling events could help to answer these questions. However, if rare or transient species are shaping the distinct community structures, then community membership should be further investigated to identify potentially important microorganisms whose presence may reflect community and process stability.

Proteobacteria was the most numerically dominant phylum, comprising of at least 35% relative abundance for each WRRF, followed by Bacteroidetes with around 20% relative abundance for each facility (Fig. 3-7). These two phyla are commonly found in the activated sludge microbial community and have been reported as dominant by other full-scale studies (Wu et al., 2019; T. Zhang et al., 2012). The top 30 abundant genera differed slightly for each WRRF (Fig. 3-8). For example, *Nitrospira* and *Dechloromonas* ranked 6th and 7th, respectively, for Maryville and Etowah, but ranked 15th and 26th, respectively, for Cookeville. This was interesting because *Nitrospira* is a well-known nitrifier and was expected to be more dominant in Cookeville since this facility continued successful ammonia removal after aeration modifications and no other known nitrifier was identified even among the top 50 genera of Cookeville. Furthermore, *Dechloromonas* is a putative

DPAO and therefore it is surprising that this genus was more abundant in Etowah than Cookeville. *Haliangium*, on the other hand, is a well-known contributor to denitrification with some strains exhibiting potential for sulfate reduction (McIlroy et al., 2015, 2016), and was one of the top ten most abundant genera in all three facilities, even in Etowah which does not operate for denitrification. Additionally, *Candidatus* Accumulibacter was among the top 30 genera for Cookeville and Maryville, but ranked 69 for Etowah, indicating that this putative DPAO may thrive in an environment with varied DO concentrations. These findings warranted a closer look into the BNR-relevant community. Furthermore, since the rankings of each genus shifted between WRRFs, establishing a core community among all three facilities could identify microorganisms crucial to the activated sludge process.

3.3.2.2 BNR-relevant Community Structure

The Shannon and inverse Simpson diversity metrics were lower for the BNR-relevant community than for the total community (Fig. 3-9). Both indices exhibited an overall significant difference among facilities (χ^2 = 139.2, 111.1 respectively, $p<0.001$), with diversity values for the Cookeville WRRF measuring at or higher than Etowah and Maryville. Overall significance between seasons, however, was only detected for Shannon diversity (χ^2 = 39.8, $p<0.001$). Therefore, the inverse Simpson index was not overallsignificantly different between the summer and winter. Interactions were also detected between seasons and facilities for both Shannon and inverse Simpson diversity measures (χ^2 = 6.1, 11.9 respectively, $p<0.001$), which means that differences were detected in diversity of the BNR-relevant community between facilities during the winter and during the summer. These interactions supported the observation that Cookeville's

diversity was typically higher than the other facilities during summer and during winter. This is interesting because for the total community, Cookeville exhibited diversity values similar to Maryville, but when focusing on just the BNR-relevant community, Cookeville exhibited generally higher diversity than Maryville. Therefore, perhaps the DO gradient in Cookeville's oxidation ditch allows for a more diverse BNR-relevant community than the designed A_2O process. Similar to the total community, diversity dropped during winter 2018 for the BNR-relevant community in all three facilities. However, among all sampling events, the lowest BNR-relevant diversity was exhibited during the summer of 2018 for Etowah's WRRF, when the other two facilities showed an increase in diversity. Interestingly, Etowah began adding a solids-reducing, microbial-based agent, Revive® (Bio-Chem Industries, Dalton, GA, USA), to their activated sludge at the beginning of 2018. Since this product is designed to reduce grease and sludge, it is very possible that it affected the activated sludge community during the summer 2018 sampling event. Furthermore, the total community of Etowah did not exhibit an extreme decrease in alpha diversity for summer 2018, implicating that Revive® may have targeted some of the members of the BNR-relevant community.

Visualizations of the Bray Curtis matrices for the BNR-relevant community displayed similar clustering patterns for both the relative abundance and the presence/absence datasets (Fig. 3-10), indicating that clusters were not overly affected by sampling, PCR bias, or DNA sequence read abundance. A PERMANOVA test detected significant differences in community composition based on sampling event (F=44.2, R^2 = 85.5% - relative abundance, F=14.3, R^2 = 65.6% - presence/absence; $p<0.001$). Further analysis conducted for pairwise comparisons indicated that most sample clusters were

separated based on event (ANOSIM; R= 0.998 – relative abundance, 0.941 – presence/absence, p<0.001); the few exceptions were among the Cookeville sampling events which are explained in Chapter 2 section 2.3.2.2. Like the total community, the BNR-relevant community structure was distinct for most sampling events, which is interesting since all three facilities have similar influent nutrient profiles. This observation suggests that operational configurations may drive BNR-relevant community structure and that perhaps some BNR-relevant genera might be rare or transient (Saunders, Albertsen, Vollertsen, & Nielsen, 2015). Significant differences in community structure were also detected between facilities (PERMANOVA; F=45.1, R^2 = 22.9% - relative abundance, F=37.2, R^2 = 24.7% - presence/absence; p<0.001). However, even though individual sampling events exhibited distinct community structure, a pairwise analysis revealed separate, but overlapping community structure (Ramette, 2007) between Cookeville and Maryville when all events were grouped together (ANOSIM; R= 0.574 – relative abundance, 0.580 – presence/absence, p<0.001). Community composition for each sample was not well-separated based solely on season (ANOSIM; R=0.394 – relative abundance, 0.334 – presence/absence), but interactions were detected between season and facility (PERMANOVA; F=15.6, R^2 = 7.9% - relative abundance, F=6.2, R^2 = 4.1% - presence/absence, p<0.001). Pairwise analyses for this interaction indicated that samples from Cookeville and Maryville exhibited slightly similar BNR-relevant community structure during the winter (ANOSIM; R=0.680 – relative abundance, 0.754 – presence/absence; p<0.001) and the summer (ANOSIM; R=0.753 – relative abundance, 0.538 – presence/absence; p<0.001). Similarities observed between these two facilities may be a result of the how Cookeville's BNR-relevant community responded to the

aeration modifications. Since Cookeville optimized to accommodate BNR, it is encouraging to observe this facility exhibiting similarities in BNR-relevant community structure with a facility that is designed for BNR. Winter samples from Etowah and Cookeville also exhibited some overlapping community structure based on relative abundance (ANOSIM; $R=0.676$, $p<0.001$), which might indicate that these two facilities have similar dominant BNR-relevant species during the winter. Lastly, Maryville exhibited some similarities between the BNR-relevant community structures from the summer and winter (ANOSIM; $R=0.681$ – relative abundance, 0.563 – presence/absence; $p<0.001$), unlike Etowah's community which had no significant overlap between summer and winter (ANOSIM; $R>0.920$). These results might implicate that Maryville's BNR-relevant community structure is somewhat stable between seasons, which might be indicative of a stable BNR process.

The BNR-relevant community composition was slightly different between each facility (Fig. 3-11). *Dechloromonas, Nitrospira, Haliangium,* and *Candidatus* Accumulibacter were among the top five BNR-relevant genera for each facility; however, these genera differed in ranking between each facility. This was not surprising for Maryville or Cookeville since both facilities operate for BNR. However, it was interesting for Etowah since they operate a highly aerobic oxidation ditch and therefore lack an anerobic zone, which is usually associated with some of these genera. *Tetrasphaera,* a well-known putative DPAO (Kong, Nielsen, & Nielsen, 2005; Kristiansen et al., 2013; Nielsen, McIlroy, Albertsen, & Nierychlo, 2019), was observed in higher abundance in Maryville and Cookeville than in Etowah, where it was barely detected. Cookeville experienced an increase in the relative abundance of *Haliangium* and a more uneven BNR-relevant

community during winter 2018, which was also the sampling event that exhibited a decrease in diversity. Furthermore, *Haliangium* is a putative denitrifier and the relatively high relative abundance of this genus during winter 2018 may explain why total N removal was still achieved even though DO concentrations were elevated in the oxidation ditch. For Etowah, however, summer 2018 was the sampling event with the most uneven community, exhibiting an increase in *Dechloromonas*. Maryville WRRF exhibited a shift in composition for summer 2017 with an increase in the GAO, *Defluviicoccus* (Lanham et al., 2013), and the PAOs, *Candidatus* Accumulimonas and *Candidatus* Obscuribacter (Stokholm-Bjerregaard et al., 2017a), however facility operations in terms of nutrient removal did not differ from other sampling events, possibly indicating functional redundancy among the PAOs for this facility. As mentioned previously, the rankings of the various genera were different for each facility and even shifted among events, therefore establishing a core community could reveal the potentially important microorganisms of the activated sludge process. Furthermore, a defined core community could also be useful to better understand which BNR-relevant species or genera are shared among all three facilities, especially since the secondary treatment process is operated differently at each WRRF.

3.3.2.3 Core Community Structure

A core community was established among the three WRRFs across all sampling events (Fig. 3-12). This community consisted of 139 OTUs, with BNR-relevant members such as *Dechloromonas, Nitrospira, Haliangium,* and *Candidatus* Accumulibacter ranking among the top 20 genera. Observing these BNR-relevant genera as core members for Etowah is interesting because as stated previously, some of these genera are conventionally

associated with anerobic zones. Regardless, it is promising to detect these BNR-relevant genera as core members of Etowah because it demonstrates that the activated sludge of this facility has the potential to be exploited to accommodate BNR like Cookeville. The core community structure differed between facilities when based on relative abundance (ANOSIM; R= 0.818, p<0.001), but less so when based on presence/absence (ANOSIM; R= 0.394, p<0.001), which is to be expected considering the criteria used to determine the core community. Interestingly, Etowah's core community structure was still distinct from the core structure of Cookeville (ANOSIM; R= 0.926 – relative abundance, 0.791 – presence/absence, p<0.001) and Maryville (ANOSIM; R= 0.832 – relative abundance, 0.682 – presence/absence, p<0.001), whereas the core community structure between Cookeville and Maryville was less distinct and practically inseparable in terms of presence/absence (ANOSIM; R= 0.768 – relative abundance, 0.108 – presence/absence, p<0.01). Therefore, even though a core community was established among all three WRRFs, the two facilities that select for BNR exhibited slightly more similar core community structure with each other than with the facility that traditionally operates an oxidation ditch. Again, Maryville's core community structure exhibited overlap between seasons (ANOSIM; R= 0.398 – presence/absence, p<0.01), whereas Etowah's core community structure did not (ANOSIM; R=0.910 - presence/absence, p<0.01). An NMDS visualizing the overall core community structure for each facility (Fig. 3-13) shows that the samples collected from Cookeville prior to optimization did not cluster with Maryville like the other Cookeville samples, suggesting that the core community structure may have been different prior to optimization. However, due to a lack of microbial data predating optimization, we cannot say if Cookeville's pre-optimization community was more like

Etowah's. Future studies which can collect samples for at least a year prior to operational changes could potentially contribute to a better understanding of this shift.

Additionally, the microorganisms of the winter and summer sampling events were evaluated separately to determine whether there was a distinct core community based on season since temperature can impact the microbial community of the activated sludge (Fig. 3-14). The core community for the winter sampling events encompassed 133 OTUs and for the summer sampling events, consisted of 134 OTUs. Interestingly, the winter and summer core communities only shared 91 OTUs, meaning each seasonal community consisted of roughly 45 core OTUs unique to that season. Since all three facilities continuously achieve their respective nutrient removal efficiencies year-round, the differences in core communities between seasons could indicate seasonal functional redundancy on the species-level. Furthermore, *Candidatus* Accumulimonas and *Haliangium* were detected as core genera during both seasons, however both genera had different species as core members in each season, which supports the idea of seasonal functional redundancy. Moreover, the genera *Pseudomonas* and *Candidatus* Obscuribacter, both putative PAOs (Stokholm-Bjerregaard et al., 2017a), were only present in the winter core community and therefore may be more important to the EBPR or SNPD process during the winter months.

3.3.3 Wastewater Characteristics and Microbial Community

Correlations between wastewater characteristics of each facility's secondary treatment process were evaluated using Spearman's rank correlation test (Figs. 3-15, 3-16, 3-17). Among the wastewater characteristics, ammonia and DO were negatively correlated for each facility, with Etowah exhibiting the highest correlation (r_s= -0.80) and Cookeville

exhibiting the lowest (r_s= -0.45). This was expected since ammonia removal requires an aerobic environment and the oxidation ditch of Etowah had higher DO concentrations than the other two facilities. Ammonia and ortho-P were strongly correlated with each other for Cookeville and Maryville (r_s= 0.65, 0.75), but barely correlated for Etowah (r_s= -0.28). This is most likely due to the lack of a DO gradient in Etowah, allowing for little variation in ortho-P concentrations throughout the oxidation ditch to correlate with the high ammonia removal (Table 3-3, Fig. 3-2a). Ammonia and ortho-P were negatively correlated with pH for Cookeville (r_s= -0.54, -0.65 respectively) and for Maryville (r_s= -0.62, -0.54 respectively). Only ortho-P was correlated with pH for Etowah (r_s= -0.52). Again, since there is little variation in pH, DO, and ortho-P within Etowah's oxidation ditch, this correlation may be due to seasonal variation, as opposed to potential in-basin fermentation like Cookeville. Overall, Cookeville exhibited correlations between wastewater characteristics that were more similar to Maryville than Etowah, indicating that the environmental conditions of Cookeville's optimized oxidation ditch might mimic the dynamic conditions of Maryville's A_2O process more than a traditionally-operated oxidation ditch.

Alpha diversity metrics for the total and BNR-relevant communities were also investigated for correlations with wastewater characteristics (Figs. 3-15, 3-16). Interestingly, diversity measurements for the total community in Cookeville were more strongly correlated with DO than temperature, unlike Etowah and Maryville. Cookeville exhibited a strong negative relationship between alpha diversity and DO, especially for Shannon and inverse Simpson metrics (r_s = -0.72, -0.64 respectively), and only a moderately positive relationship with temperature, with r_s=0.51 for Shannon and r_s= 0.49

for inverse Simpson. The two reference facilities, however, were very strongly correlated with temperature; Shannon diversity exhibited rho values of 0.94 and 0.91 for Etowah and Maryville, respectively, and inverse Simpson exhibited rho values of 0.92 and 0.91, respectively. As to the relationship with DO, rho values for all alpha metrics were less than -0.20 for Etowah and less than -0.40 for Maryville. Therefore, the alpha diversity of Cookeville's microbial community, which experienced the implementation of a DO gradient, exhibited a stronger relationship with DO than the communities of the two facilities that did not modify their DO patterns. Since there was a moderate relationship detected with temperature, it is possible that given enough time, the alpha diversity of Cookeville WRRF might shift and exhibit a stronger relationship with temperature like the reference facilities, possibly indicating that the community has stabilized in response to the optimization.

The BNR-relevant community exhibited slightly different correlations than the total community (Fig. 3-16). Richness and Shannon diversity for the BNR-relevant community were correlated with temperature (r_s= 0.70, 0.48 respectively) for Cookeville's WRRF, but inverse Simpson was not (r_s= 0.16). Since Shannon diversity is weighted towards rare species, these findings could reflect that a higher number of BNR-relevant species exist during the summer than the winter, and that rare species contribute more to BNR diversity during the summer. Furthermore, inverse Simpson was only slightly correlated with DO (r_s= -0.36), and Shannon diversity and richness were moderately correlated with DO (r_s= -0.48, -0.60 respectively), indicating that BNR diversity may have been affected by DO concentrations, but less so than the total community diversity. Similar to Cookeville, the BNR diversity of Etowah exhibited correlations for richness and

Shannon diversity with temperature (r_s= 0.88, 0.38 respectively). Unlike the total community of Etowah, evenness, Shannon diversity, and inverse Simpson diversity of the BNR-relevant community had moderate to strong correlations with DO (r_s= -0.58, -0.48, -0.69 respectively), suggesting perhaps that DO concentrations may have affected Etowah's BNR diversity. Considering Etowah added the Revive® agent to its activated sludge at the beginning of 2018, the observed differences in Etowah's total and BNR diversity correlations may have been due to this chemical specifically altering the BNR community structure. Alpha diversity of the BNR-relevant community for Maryville continued to exhibit strong relationships with temperature and weak relationships with DO. Specifically, temperature was positively correlated with richness, Shannon and inverse Simpson diversity (r_s= 0.85, 0.77, and 0.64, respectively). These findings further indicate that Maryville may have a stable community and that Cookeville may eventually exhibit these correlations detected in Maryville, which could support the stabilization of Cookeville's community.

The BNR-relevant OTUs detected in the core community were selected, grouped by genera, and then correlated with various wastewater characteristics for each WRRF in order to determine environmental conditions that may be linked to potential BNR-related microbial function (Fig. 3-17). In the Cookeville WRRF, *Candidatus* Accumulimonas was positively correlated with temperature (r_s= 0.60), whereas *Candidatus* Obscuribacter was negatively correlated with temperature (r_s= -0.56). Furthermore, these putative PAOs (Nielsen et al., 2019; Stokholm-Bjerregaard et al., 2017a) were also negatively correlated with each other (r_s= -0.71), suggesting they could potentially share the same niche but thrive in different seasons. Their presence as core members in Cookeville's oxidation ditch

combined with these correlations supports a potentially dynamic and resilient PAO community. In Etowah's facility, *Dechloromonas* and *Candidatus* Accumulimonas were positively correlated with temperature (r_s= 0.73, 0.83 respectively) and nitrate (r_s= 0.57, 0.87 respectively), which suggests that *Candidatus* Accumulimonas may thrive better during the summer. Furthermore, *Candidatus* Accumulibacter, a well-established PAO (Nielsen et al., 2019; Stokholm-Bjerregaard et al., 2017a), was negatively correlated with *Dechloromonas, Candidatus* Accumulimonas, and nitrate (r_s= -0.75, -0.74, and -0.77 respectively), begging the question of what is the functionality of *Candidatus* Accumulibacter in a highly aerobic oxidation ditch. In Maryville's facility, *Candidatus* Obscuribacter, was correlated with two other DPAOs, *Dechloromonas* (r_s= 0.70) and *Candidatus* Accumulibacter (r_s= 0.55), suggesting these three genera may rely on each other to perform various roles in Maryville's A_2O process. Additionally, *Nitrospira* and unclassified members of *Nitrosomonadaceae,* both of which are part of the nitrifying community, exhibited strong relationships with temperature (r_s= -0.86, 0.80 respectively). These correlations reflect the greater abundance of core *Nitrospira* OTUs detected in Maryville during the winter and the greater abundance of core *Nitrosomonadaceae* OTUs during the summer.

Beta diversity based on relative abundance was also correlated with environmental parameters using the *capscale* function in R for the total, BNR-relevant, and core communities (Figs. 3-18, 3-19, 3-20). The step-wise model with the lowest AIC value and overall significance (ANOVA; F=10.15, p=0.001) for the total community included all water quality parameters except for ammonia, which was not surprising since all three facilities operate for ammonia removal. Additionally, each of these water quality

parameters were also individually significantly correlated with the total community structure (ANOVA; $p<0.01$). This model detected strong correlations for beta diversity with temperature and nitrate (ANOVA; F=19.15, 6.53- respectively, p=0.001). The strong temperature correlation was expected since we sampled each facility during the winter and the summer and microbial community structure is influenced by temperature. Interestingly, nitrate and DO were both positively correlated with the total community structure of Etowah, the facility operating a highly aerobic secondary treatment process and not selecting for denitrification. Since this facility did not operate for BNR, it was not surprising that higher levels of nitrate and DO concentrations were more associated with variations in Etowah's total community structure compared to the other facilities and may have potentially shaped the community, as is further supported by the additional capscale analyses (Fig. 3-19, 3-20). Additionally, the communities from two sampling events for Cookeville that occurred within the first year of optimization exhibited slightly more positive correlation with nitrate than the communities from other Cookeville sampling events. This may have been due to the community still adapting to performing denitrification. Interestingly, the total community capscale analysis was the only dataset to demonstrate this correlation. The BNR-relevant capscale analysis displayed a few similar correlations to that of the total community, except this step-wise model with the lowest AIC and overall significance (ANOVA; F=11.99, p=0.001) included all parameters except DO and ortho-P. Each of these water quality parameters were also individually significantly correlated with the BNR-relevant community structure (ANOVA; $p<0.01$). Like the total community, temperature and nitrate were strongly correlated to the beta diversity of the BNR-relevant community (ANOVA; F=24.69, 7.29 – respectively,

p=0.001). The BNR-relevant community structure for the first two sampling events for Cookeville was not explained by nitrate (Fig. 3-19) as was the case for the total community (Fig. 3-18). The lack of correlation between nitrate and Cookeville's BNR-relevant community was surprising. This could indicate that more microorganisms contribute to BNR than are currently known, and that our BNR-relevant list may be lacking. Lastly, like the total community, the step-wise model with lowest AIC and overall significance (ANOVA; F=12.10, p=0.001) comparing water quality parameters with the core community included all parameters except for ammonia. However, like the BNR-relevant capscale, the core microbial structure of Cookeville's first two sampling events did not correlate with nitrate. For all three capscale analyses, temperature explained over 50% of the clustering patterns and nitrate explained over 20%.

3.4 Conclusion

The outcomes from the aeration modifications implemented in Cookeville's WRRF demonstrate that an oxidation ditch can be optimized to accommodate BNR. Comparing the microbial community of Cookeville with other facilities also demonstrated that the corresponding microbial community can respond favorably to this type of optimization. Since influent water quality characteristics, particularly in regard to nutrient concentrations, were mostly similar between facilities, this suggests that perhaps the operational conditions of each facility were a stronger determinant of the resulting microbial communities. Cookeville exhibited a DO gradient that accommodated total N removal more like the BNR-designed facility in Maryville rather than the traditional oxidation ditch in Etowah. Additionally, EBPR batch tests demonstrated that Cookeville's activated sludge exhibited P-release/HAc uptake ratios more similar to Maryville than

Etowah, which was also supported by findings from other studies on EBPR-designed facilities. Shifts in the microbial community for Cookeville were different when compared to the other two facilities, suggesting that the community responded to optimization. Furthermore, diversity and core community structure of the optimized facility exhibited more similarities with the BNR-designed facility than the facility conventionally operating an oxidation ditch. Lastly, dissolved oxygen was strongly correlated with the diversity of the optimized facility, but not with the diversity of the two reference facilities, which were strongly correlated with temperature. Since aeration modifications were the key focus of Cookeville's process optimization and there was a moderate relationship detected between diversity and temperature for this facility, it is possible that given enough time, the alpha diversity of Cookeville WRRF might shift and exhibit a stronger relationship with temperature like the reference facilities. This could possibly indicate that the Cookeville microbial community has stabilized in response to the optimization. This study provides evidence that the microbial community of the activated sludge process has the potential to shift to accommodate BNR and therefore promotes this type of operational strategy as a possibility for other WRRFs to incorporate.

CHAPTER 4: AN INVESTIGATION INTO THE POTENTIALLY ACTIVE MICROBIAL COMMUNITY IN AN OXIDATION DITCH OPTIMIZED FOR BIOLOGICAL NUTRIENT REMOVAL

Abstract

Microbial communities of the biological nutrient removal (BNR) process in water resource recovery facilities have been extensively studied using DNA-based methods. Through this type of workflow, researchers have been able to identify the microorganisms present during the BNR process and identify their putative contribution based on genetic potential. While DNA-based studies are a useful starting point for evaluating the community of BNR, gene expression, or RNA-based, investigations allow for detection of microorganisms who are potentially active during BNR processes. This study combines both DNA and RNA-based approaches to characterize the active microbial community of an activated sludge process and the potential contributions to BNR. Specifically, over a course of two weeks, we evaluated the microbial community of an oxidation ditch that had been optimized with aeration modifications to accommodate simultaneous nitrification, denitrification, and enhanced biological phosphorus removal (SNDP). In this study, we focused on how the resulting DNA and RNA-based communities differed and correlated the active community with various wastewater characteristics in order to identify microorganisms with potential importance to the SNDP process and that warrant further investigation. One such group that warrants further investigation are the polyphosphate accumulating organisms (PAOs). We detected active PAOs in high abundance during both sampling events, however the wastewater characteristics of the facility did not reflect the

traditional conditions for these microorganisms to contribute to SNDP. Furthermore, the DNA and RNA-based communities were correlated differently with the various wastewater characteristics considered in this study, suggesting that further investigations are needed to truly establish the active community of the BNR process and the corresponding relationships with environmental conditions.

4.1 Introduction

Many studies have posited that linking the microbial structure and function of the activated sludge community could improve design and optimization of a water resource recovery facility's (WRRF) operational processes, especially for biological nutrient removal (BNR) (Carvalho, Lemos, Oehmen, & Reis, 2007; Kong et al., 2007; Mao et al., 2016; Xia, Wen, Zhang, & Yang, 2018; Yu & Zhang, 2012). So far, most studies have primarily investigated structure and potential function of the activated sludge community through DNA-based analyses, which has become easier and accessible with modern high-throughput sequencing workflows (Ferrera & Sánchez, 2016; Goodwin, McPherson, & McCombie, 2016; Wu et al., 2019). High-throughput sequencing has contributed to estimating community diversity, identifying unknown microorganisms with potentially important roles in the activated sludge process, and helping to reveal correlations between diversity or abundance with wastewater characteristics (Xia et al., 2018). One study, for example, used this method to assess the abundance and distribution of polyphosphate accumulating organisms (PAOs) across Danish enhanced biological phosphorus removal facilities and found the most abundant PAO to be *Tetrasphaera* (Stokholm-Bjerregaard et al., 2017a). Additionally, Keene et al. (2017), used high-throughput sequencing during their study on the impacts of low dissolved oxygen during BNR and were able to detect

shifts in the nitrifying microbial community on the species level. Nonetheless, while DNA-based investigations are quite useful for identifying microorganisms present in the secondary treatment process and proposing their genetic potential to contribute to various processes, such as BNR, studying RNA can reveal the microorganisms that are active or soon-to-be active during BNR (Blazewicz, Barnard, Daly, & Firestone, 2013; Metch et al., 2019; Vuono et al., 2016). Fewer studies, however, have investigated the activity of the activated sludge community, in other words, RNA-based analyses. This process can be more challenging than DNA-based workflows due to the time-sensitive nature of RNA degradation and handling fragility, however RNA-based studies are becoming more attainable through recent technologies, such as preservation solutions and kits with streamlined workflows (Karst et al., 2018b; Liu et al., 2019; Sato et al., 2019; Vieira et al., 2018). One method for studying microbial activity involves synthesizing a complementary strand of DNA (cDNA) from RNA sequences, which can then be treated like a DNA sequence. One downfall with RNA investigations is that output can be misleading due the variation in gene counts for different bacteria and due to differing RNA synthesis and degradation rates (Blazewicz et al., 2013; Klappenbach, Dunbar, & Schmidt, 2000). However, proposing microorganisms that are active during a certain set of environmental conditions or operational parameters could be useful for determining characteristics of a productive activated sludge process. Furthermore, by revealing which functionally-relevant microorganisms are active, this type of investigation can also help narrow down which functional genes may warrant further examination. One example of an activated sludge process that could benefit from cDNA-based analyses is BNR, particularly when

trying to establish the microorganisms that are active during unconventional BNR processes that result from optimization.

Biological nutrient removal is a widely studied, microbial-driven activated sludge process. This process has been explored considerably by DNA-based studies (C. Guo et al., 2013; Keene et al., 2017; Saunders, Larsen, & Nielsen, 2013; Stokholm-Bjerregaard et al., 2017a; T. Zhang et al., 2012), however, there is still a need for identifying active microorganisms during optimal performance and after operational modifications (Sato et al., 2019; Yasong et al., 2019). Furthermore, the active microbial community associated with a successful BNR process might slightly shift from day-to-day for a single WRRF due to the dynamic conditions of wastewater. Additionally, the active BNR community may also differ depending upon a facility's operational parameters, further indicating the need for more studies that link the active community with environmental conditions of the activated sludge. There have been a few studies that looked at microbial activity during BNR and illustrated the usefulness and application of a cDNA-based approach. One study assessed the response of the activated sludge microbial community to ammonia-starvation periods while exposed to various temperatures (Metch et al., 2019). They found that the relative abundances of detected phyla differed between the cDNA-based and DNA-based community structures. The DNA-based dataset portrayed Bacteroidetes as a prominent phylum during the starvation period, but the cDNA dataset showed this phylum as not very active throughout the study. Furthermore, relative abundance for the cDNA-based community showed Planctomycetes and Cyanobacteria as more active during the starvation periods than the DNA-based community indicated. Another study investigated microbial activity during operational modifications of a pilot-scale A_2O process, including

carbon source supplementation and multipoint nitrification liquor recycling (Yasong et al., 2019). They focused on functional gene expression related to nitrification and denitrification. They found that modifying the carbon source improved nitrogen removal in the short-term and increased nitrifier and denitrifier abundance. However, over time they observed inhibited gene expression from the nitrite oxidizers and the denitrifiers, eventually making this modification unfavorable. These studies provide encouragement that investigating the active microorganisms during BNR can provide new insight on the factors that may enable a stable and resilient microbial community and thereby an improved secondary treatment process.

In this study, we looked at a full-scale WRRF operating an oxidation ditch under an optimized strategy. This facility was optimizing their oxidation ditch to accommodate simultaneous nitrification, denitrification, and enhanced biological phosphorus removal (SNDP) in order to improve nutrient removal capability without adding or upgrading infrastructure. This optimized strategy involved modifying aeration patterns that allowed for a dissolved oxygen (DO) gradient, both laterally and vertically, in the oxidation ditch. This gradient generated aerobic, anoxic, and anaerobic zones throughout the ditch. Since it is well established that microbial communities are affected by variations in dissolved oxygen (Daigger & Littleton, 2014; Keene et al., 2017; Zhou et al., 2015), studying the active community of this oxidation ditch will help to better understand how the community responds to such dynamic conditions. We previously conducted a DNA-based study and now turn to a cDNA-based investigation to determine which microorganisms were active during two summer sampling events. These two events took place three years after the WRRF began aeration modifications and were selected for cDNA-based analysis with the

hope that the community had stabilized enough to gather information that would reflect active microorganisms during unconventional BNR in an oxidation ditch. We hypothesized that the active community will exhibit differences from the DNA-based community, but that the similarities that are detected will also support observations from the previous study on this facility (see Chapter 2). Our goals were (1) to compare the composition and structure of the community established from cDNA-based analysis to the community established from DNA-based analysis and (2) to correlate these communities with various wastewater characteristics. This study reveals some microorganisms and environmental conditions that warrant a closer look for future investigations regarding functional activity during BNR.

4.2 Methodology

4.2.1 Sampling, Analytical Techniques, and DNA Extraction

This study focused on two sampling events that occurred over the course of two weeks during the summer of 2018 at the WRRF located in Cookeville, TN. These events took place three years after the facility implemented aeration modifications in the oxidation ditch. This facility and the two corresponding events were selected for cDNA-based analysis in order to better understand the microbial community in an optimized oxidation ditch. Investigating activity may support the interesting observations from the DNA-based analysis, such as whether the PAOs that were detected in such high abundance during previous events are active. This type of investigation can also provide suggestions for further investigation. Summer 2018, specifically, was chosen as it was the last season to sample for this study and it was our hope that the community had stabilized enough that the information gathered would reflect active microorganisms during unconventional BNR

in an oxidation ditch. A detailed description of the facility, sampling strategy, analytical techniques, batch kinetic tests, and DNA extraction and sequencing protocol can be found in Chapter 2 sections 2.2.1 through 2.2.4.

Due to the high abundance of PAOs detected in previous DNA-based investigations of Cookeville WRRF, the facility was additionally sampled to assess the possibility of enhanced biological phosphorus removal (EBPR) during sludge wasting and whether facility influent provided enough readily biodegradable carbonaceous oxygen demand (rbCOD) to support traditional PAO activity. Samples for this analysis were collected in May and November of 2019. Composite samples representing the influent and effluent of the facility were collected over the 24-hour sampling period. Using a sludge judge, samples were also collected from two clarifiers, number 3 and 4; since only clarifier 4 was used for wasting, clarifier 3 was used as a reference for comparison. Before wasting could begin, the return activated sludge (RAS) pump to clarifier 4 was turned off for a 24-hour period to allow solids to build up for a more efficient wasting process. Samples from both clarifiers were collected prior to the RAS pump shutdown and then collected again the next day prior to the waste activated sludge (WAS) pump being turned on. Samples from the clarifiers were analyzed for total and volatile suspended solids following Standard Methods (APHA et al., 2012), and for total P, ortho-P, and soluble COD using Test N Tube kits (HACH Company, Loveland, CO, USA). Composite samples were also analyzed for total P and ortho-P and were used to measure rbCOD of the facility following the flocculation-filtration method (Mamais, Jenkins, & Prrr, 1993; Metcalf & Eddy Inc, 2014).

4.2.2 RNA Extraction, cDNA Synthesis and Sequencing

The activated sludge samples that were collected from each sampling location in the oxidation ditch for DNA extractions were also used for RNA extractions (Fig. 2-1, Table 2-2). Immediately after collecting each sample, 1ml of sludge was transferred to a clean tube and centrifuged for 1 minute at 3300 g. After supernatant was decanted, 1mL of RNAlater® Stabilization Solution (Thermo Fisher Scientific, Sunnyvale, CA) was added and the tube was inverted to resuspend cells in solution. The tube was then placed on ice for transportation to the laboratory where samples were stored in 4 deg C for up to a week, as suggested by RNAlater® manufacturer. Tubes were then placed in -80 deg C for long-term storage.

Once both sampling events were complete, RNA was extracted from all samples following the manufacturer's protocol for the RNeasy PowerMicrobiome kit and the RNeasy MinElute Cleanup kit (Qiagen, Valencia, CA), which helped to remove DNA contamination. Concentrations of RNA were quantified for each sample using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Sunnyvale, CA). Reverse transcription for cDNA synthesis was then carried out following the manufacture's protocol for GoScript® Reverse Transcription System (Promega, Madison, WI). During cDNA synthesis, a blank was taken through the process and included all components except reverse transcriptase. This blank served as a negative control to verify the absence of DNA contamination. After cDNA synthesis was complete, the v4 region of the 16s rRNA gene was targeted using 515F/806R primers and amplified using endpoint PCR. Each amplified sample was visualized by gel electrophoresis to detect the presence of 250bp cDNA-based amplicons. The absence of amplicons in the negative control confirmed the lack of DNA contamination, or if there was contamination, it was at a non-detectable level. After

verifying the presence of cDNA, library preparation and sequencing were conducted following the same protocol for DNA, as described in Chapter 2 section 2.2.4.

4.2.3 Amplicon Sequence Processing

DNA and cDNA amplicons were processed together using mothur v1.39.5 (Schloss et al., 2009), along with the corresponding MiSeq SOP (Kozich et al., 2013) (accessed on 12/30/2018). Details are described in Chapter 2 section 2.2.5. OTUs with ≤ 3 reads were removed (Keene et al., 2017), resulting in 3,315,354 remaining sequences, which represent *total* community structure. Samples were then subsampled at 26,064 sequences to remove heterogeneity in sequencing depth (Weiss et al., 2017) resulting in > 96.5% coverage for independent libraries (total of 7026 OTUs).

4.2.4 BNR-relevant Microbial Community Selection

BNR-relevant community structure includes microorganisms with the potential to contribute to BNR and details as to how this community was selected can be found in Chapter 2 section 2.2.6. To generate a second dataframe representing the BNR-relevant community, we selected 392,092 sequences from the total community, identified according to the criteria previously described in Chapter 2 section 2.2.6. Each BNR-relevant community was then subsampled at 1850 sequences to generate a second dataset with > 98.2% coverage (total of 245 OTUs).

4.2.5 Statistical Analysis

Differences between cDNA-based and DNA-based microbial community structures were evaluated for the total and the BNR-relevant communities. Beta diversity was analyzed on the OTU-level utilizing RStudio version 1.1.463 (RStudio Team, 2016) with package *vegan* (Oksanen et al., 2019) and Primer-E version 7.0.9 (Quest Research

Limited, Auckland, New Zealand) to investigate differences in cDNA and DNA composition within the total community and within the BNR-relevant community. Separate Bray-Curtis dissimilarity matrices were generated for the total and BNR-relevant communities using subsampled relative abundance data. To determine the effect of OTU presence/absence, each relative abundance table was transformed into a presence/absence table and additional Bray-Curtis matrices were generated. Matrices were visualized with a non-metric multi-dimensional scaling (NMDS) plot using Primer7. SIMPROF was performed on the clusters to determine which samples had a statistically similar ($p \leq 0.05$) community composition. The function *adonis* in *vegan* was used to conduct permutational multivariate analyses of variance (PERMANOVA) based on Bray-Curtis dissimilarity values and the *betadisper* function was used to test for homogeneity of variances in sample clusters. PERMANOVA tested for global differences by sequence type (cDNA v DNA) and sampling event in total and BNR communities while accounting for possible non-independence between samples by designating sampling location as a random effect using the *strata* parameter. Pairwise comparisons were conducted using an analysis of similarities (ANOSIM). Additionally, a SIMPER analysis was performed to better understand which OTUs, and therefore species and genera, were contributing to the clustering observed in NMDS plots.

Correlations between wastewater conditions and the BNR-relevant community were measured with a two-tailed, Spearman's rank test using RStudio and visualized with the ggplot2 package (Wickham, 2016). Wastewater characteristics and beta diversity measurements were correlated with a distance-based redundancy analysis (dbRDA) using the *capscale* function in *vegan* and visualized with *ggord* (Beck, 2017).

4.3 Results and Discussion

4.3.1 Wastewater Characteristics

4.3.1.1 Facility Influent and Oxidation Ditch

The influent characteristics of the WRRF significantly differed between the two sampling events of this study (Wilcoxon; $p = 0.018$) (Table 2-3). The composite influent sample from Summer 2018a measured unusually high nutrient concentrations due to maintenance on the waste reactor in which waste activated sludge residue was recycled back through the facility. This maintenance resulted in an increase in nitrogen and phosphorus loading into the facility. Most measurements taken from the oxidation ditch, however, reflected typical variations in chemical parameters when compared to other sampling events (Table 2-4). Nitrate was the exception where concentrations varied throughout the ditch, but the actual measurements for Summer 2018a were at least twice as much as other summer sampling events. This was most likely due to the influx of nitrate from the waste reactor considering nitrate in the facility influent was more than five times that of Summer 2018b. Temperature and soluble COD concentrations were relatively consistent throughout the oxidation ditch for each event. Dissolved oxygen, pH, ammonia, and ortho-P concentrations showed similar trends throughout the ditch, fluctuating between sampling locations laterally and vertically. Like other events, ortho-P fluctuations in the ditch suggested the possibility of EBPR where increases in ortho-P concentrations corresponded with decreases in DO concentrations.

4.3.1.2 Potential for Facility to Support EBPR

Phosphorus required for cellular maintenance measures approximately 1.5 to 2.5% of dried biomass (Madigan, Martinko, Bender, Buckley, & Stahl, 2015; Metcalf & Eddy

Inc, 2014). If phosphorus is detected in excess of what is needed for cellular growth, then it is considered "luxury uptake" (Levin & Shapiro, 1965). In conventional EBPR, dried biomass can consist of 4 to 8% P in full-scale systems and over 10% in laboratory-scale systems (Gebremariam, Beutel, Christian, & Hess, 2011). The waste activated sludge from Cookeville's WRRF measured only 1.92% P of the dried biomass for the first sampling event and 2.15% P for the second event (Table 4-1), indicating that luxury uptake, and therefore EBPR, was not exhibited based on this analysis during the wasting process at this facility. Furthermore, traditionally, an rbCOD/TP ratio of at least 15 is needed to support a productive EBPR process (Barnard, Dunlap, & Steichen, 2017; Metcalf & Eddy Inc, 2014) and composite samples from these events revealed that only 7.6 and 7.9 rbCOD/TP was entering the facility (Table 4-1). This additional investigation, while limited in data points, further supports the need to better understand the activity of PAOs in non-EBPR secondary treatment processes, such as oxidation ditches.

4.3.1.3 Potential of Activated Sludge to Perform EBPR

Kinetic batch tests to determine P-release and uptake rates were conducted on the activated sludge collected from both sampling events in this study (Table 2-5). Summer 2018b exhibited the slowest acetate (HAc) uptake rate of 6.01mg HAc/g VSS·hr and the highest P-release/HAc uptake ratio 0.59 when compared to all other post-optimization batch tests. The batch test for Summer 2018a, on the other hand, resulted in a P-release/HAc uptake ratio of 0.31, the second lowest among all sampling events. The lower rates and ratios for Summer 2018a could be due to the high concentrations of nitrate in the oxidation ditch. Some studies have reported that nitrate can be inhibitory for EBPR (Guerrero et al., 2011; Zheng et al., 2014), at least until the community adapts, which it

may not have had ample time to do since this was a relatively recent shock to the system for this sampling event. Nevertheless, these ratios were still comparable to batch tests from studies focused on EBPR-designed facilities (Gu et al., 2008; Lopez-Vazquez et al., 2008). Interestingly, even though batch testing and ortho-P fluctuations in the oxidation ditch show EBPR potential of the activated sludge, our additional investigation of Cookeville's WRRF regarding the possibility of EBPR during sludge wasting and rbCOD in the influent depicted a slightly different story in that these parameters did not reflect the wastewater characteristics supportive of EBPR (Table 4-1). However, as presented in Chapter 2, PAOs were detected in high abundance for all sampling events based on DNA analysis, therefore RNA was evaluated to determine whether these PAOs were active in this unconventional environment.

4.3.2 Microbial Community

4.3.2.1 Community Composition Based on DNA and Potential Activity

The community composition differed between the DNA and cDNA datasets for both the total and BNR-relevant communities. However, it should be noted that, like DNA-based analyses, cDNA-based analyses can also result in misleading output. This can be attributed to the variation in gene counts for different bacteria and the different rates among bacteria for RNA synthesis and degradation (Blazewicz et al., 2013; Klappenbach et al., 2000). Nevertheless, detecting microorganisms that are active or potentially active can support or suggest differences in observations made from DNA-based analysis as well as provide information regarding microorganisms that may warrant further examination. Proteobacteria was the most dominant phyla for all samples and communities (Fig. 4-1), which was expected since other studies on the activated sludge community in an oxidation

ditch have also reported this trend (Jin et al., 2015; Xu et al., 2017). However, for the total community, Bacteroidetes was the second largest phyla detected in the DNA samples, whereas Planctomycetes was the second largest phyla detected in the cDNA samples. This is interesting because even though all roles of Planctomycetes in the activated sludge process are not yet completely understood, some members contribute to the global nitrogen and carbon cycles and this phylum harbors anaerobic ammonium oxidation bacteria (Che et al., 2017; Chiellini et al., 2013; J. Guo et al., 2017). Furthermore, Metch et. al 2019 also observed more Planctomycetes in the active community than were represented in the DNA-based analysis, which could be due to biases mentioned above or the possibility that they are more active and important to the activated sludge process than currently thought. Additionally, the nitrifying phylum, Nitrospirae, made up less than 2% for the DNA and cDNA-based datasets, which was slightly lower than the DNA-based observations from Xu et. al, 2017, which detected closer to 4% Nitrospirae among the studied Carrousel oxidation ditches with anaerobic, anoxic, and aerobic zones. The top 25 genera for the total community show *Haliangium*, a well-known denitrifier (McIlroy et al., 2016), as the most active genera during the studied sampling events, as opposed to the unclassified members of *Comamonadaceae,* which have been listed as the most dominant genera in previous DNA-based total community datasets (Figs. 2-8, 3-8, 4-2). Other BNR-relevant genera that differed between the cDNA and DNA samples were the putative denitrifiers *Zoogloea*, *Candidatus* Promineofilum, and *Iamia*, which were revealed as more active than the DNA-based dataset indicated (Fig. 4-3). The putative nitrifier *A0837*, glycogen accumulating organism (GAO) *CCM19a*, denitrifier *Sulfuritalea*, and PAO *Candidatus* Accumulimonas, on the other hand, were found to be less active than would be predicted by the DNA-based

community analysis. It is interesting to observe a mix of BNR-relevant genera as active or soon-to-be active in an unconventionally-operated oxidation ditch because it may indicate that the modifications of aeration patterns allowed for a community with potential to contribute to BNR or SNDP in an oxidation ditch. Furthermore, for these sampling events, *Candidatus* Accumulibacter was ranked fifth in relative abundance for the total active community but 37th for the DNA-based community. Interestingly, other putative PAOs such as *Candidatus* Accumulimonas, *Candidatus* Obscuribacter, and *Tetrasphaera* were also detected as active. Considering this facility is not designed for EBPR, functional gene expression by the PAO community should be investigated in future studies to better understand their roles in the activated sludge process.

4.3.2.2 Beta Diversity Based on DNA and Potential Activity

Visualizations of the Bray Curtis matrices for the total and BNR-relevant communities displayed separation between the DNA-based and the cDNA-based dataset (Fig. 4-4, 4-5). A global PERMANOVA test detected a significant difference based on sequence type (cDNA or DNA) for the total community (F=121.4, R^2 = 61.7%, p=0.001- relative abundance, F=10.1, R^2 = 18.3%, p=0.001- presence/absence) and BNR-relevant community (F=83.0, R^2 = 52.9%- relative abundance, F=13.3, R^2 = 25.5%- presence/absence, p=0.001). When including both DNA and cDNA datasets, there was a significant difference between sampling events for the total community (PERMANOVA; F=27.3, R^2 = 13.9%- relative abundance, F=3.9, R^2 = 6.9%- presence/absence, p=0.001) and BNR-relevant community (PERMANOVA; F=31.5, R^2 = 20.0%- relative abundance, F=2.6, R^2 = 4.6%- presence/absence, p<0.05). Pairwise analysis using ANOSIM further revealed that the active total community structure strongly differed between sampling

events when based on relative abundance (R= 1.0, p=0.001), but were overlapping for community membership when assessing the presence/absence dataset (R= 0.597, p=0.001) (Ramette, 2007). Like the active community, the DNA-based structure exhibited the same pattern between events (ANOSIM; R= 1.0- relative abundance, 0.556- presence/absence, p=0.001). As mentioned in Chapter 2, these two events were not well-separated by the presence/absence dataset, especially when compared to other sampling events for Cookeville, for which ANOSIM values reflected complete separation. Transient microorganisms entering the activated sludge from the facility's influent may have contributed to differences in the total community structure. Similar to the DNA-based community, the active community differed more between these two events based on relative abundance compared to presence/absence, which supports the idea of stable community membership for a season, with day-to-day relative abundance fluctuations. Furthermore, the active BNR-relevant community structure was slightly similar between events based on relative abundance, but almost completely inseparable based on community membership (ANOSIM; R= 0.835- relative abundance, 0.150- presence/absence, p<0.05). The DNA-based BNR-relevant structure was completely separated based on relative abundance, but inseparable based on community membership (ANOSIM; R= 1.0- relative abundance, 0.122- presence/absence, p<0.05). These results reflect that the potentially active BNR-relevant community structure may also shift somewhat like the active total community, in that abundances may fluctuate, but community membership remains relatively the same within a season. This idea warrants further investigation because presence/absence of community members may be more definitive of a stable community than relative abundance. Studying an activated sludge

process with ample time before and after optimization for BNR, may help to establish whether this metric is useful for determining stability of the microbial community and therefore, the BNR process.

Since the active and DNA-based community structures differed, yet exhibited similar clustering patterns, a SIMPER analysis was conducted to better understand which OTU's had the most impact. The top OTU contributing to the dissimilarity between cDNA and DNA-based total community structures belonged to the genus *Candidatus* Accumulibacter, which contributed 1.49%, followed by OTUs belonging to *B63* (1.47%) and *Terrimonas* (1.35%). The OTU belonging to *Candidatus* Accumulibacter was more abundant in the cDNA-based dataset, whereas the OTUs belonging to *B63* and *Terrimonas* were more abundant in the DNA-based dataset. Additional contributors to the top 25% dissimilarity between the cDNA and DNA-based total communities included OTUs belonging to BNR-relevant genera *Nitrospira*, *Haliangium*, *Zooglea*, *Dechloromonas*, and *Iamia*. These BNR-relevant OTUs were all detected in higher abundance in the active total community than in the DNA-based total community. When assessing just the BNR-relevant community dataset, only three OTUs contributed to the top 25% dissimilarity. Those OTUs belonged to genera *Candidatus* Accumulibacter (11.5%), *A0837* (7.7%), and *Sulfuritalea* (5.4%), all of which have potential to contribute to total N removal (McIlroy et al., 2015). It is interesting that *Candidatus* Accumulibacter, a commonly classified PAO or DPAO (Camejo et al., 2016; Flowers et al., 2009), had the most impact on the dissimilarity between the active community and the DNA-based community, especially since this facility did not exhibit strong properties indicative of the EBPR process.

Even though characteristics of Cookeville's WRRF did not reflect the occurrence of EBPR, the kinetic batch tests revealed that the activated sludge of this facility has the potential to perform EBPR. However, since the rates resulting from these tests differed between the two sampling events, another Bray Curtis dissimilarity matrix based on relative abundance was generated for the cDNA-based and the DNA-based putative PAO communities (Fig. 4-6). The PAO community structure was found to be significantly different based on sequencing type and sampling event when evaluated on the OTU-level (PERMANOVA; F=267.8, R^2 = 71.9%- type, F=37.5, R^2 = 10.1%- event, p=0.001). Pairwise analysis revealed that the DNA-based PAO community was completely separated between events but that the active putative PAO community was slightly overlapping (ANOSIM; R= 0.995- DNA, 0.527- cDNA, p=0.001). This demonstrates that the active community with potential to perform EBPR differed between the two sampling events and might be related to the differing EBPR activity seen in the batch tests. A SIMPER analysis comparing the 43 OTUs in the active putative PAO community found that only two OTUs contributed to 56.1% of the dissimilarity between sampling events and they belonged to the genera *Candidatus* Accumulibacter (34.4%) and *Dechloromonas* (21.7%). This observation again supports the need for more studies to characterize the role of *Candidatus* Accumulibacter and other PAO-genera in activated sludge processes outside of the conventional EBPR-designed facility.

4.3.3 Correlations Between Wastewater Characteristics and Microbial Communities

Correlations between the BNR-relevant community and wastewater characteristics of the oxidation ditch were evaluated using Spearman's rank correlation for the cDNA and DNA-based communities (Figs. 4-7, 4-8). The outcomes of these analyses differ in many

respects, in that some correlations were detected or weighted differently for the DNA dataset than the cDNA dataset. Nitrate was very strongly negatively correlated with the putative denitrifying and PAO communities (r_s = -0.82, -0.85, respectively) based on the DNA dataset, whereas the cDNA dataset showed these relationships less correlated (r_s = -0.61, -0.44, respectively). The putative denitrifying community was strongly correlated with pH for the cDNA dataset (r_s = 0.79) suggesting that as pH increased, so did the active denitrifiers. This is interesting because literature has reported that as pH increases, so can denitrification rates, such as nitrate reduction, until the optimal point is reached (Čuhel et al., 2010; Glass & Silverstein, 1998). However, this correlation between putative denitrifiers and pH was weaker for the DNA-based dataset (r_s = 0.43). Considering pH is an important and manageable environmental factor at a WRRF, this relationship for the active community suggests it deserves further consideration, however it might have been overlooked when based solely on the DNA dataset. On the other hand, the denitrifiers were correlated with ortho-P in the DNA-based dataset (r_s = 0.56), but not at all for the cDNA-based dataset (Fig. 4-7). Additionally, the putative denitrifier and PAO communities were very strongly correlated with each other for the DNA-based dataset (r_s = 0.93), but less so for the cDNA-based dataset (r_s = 0.60) (Fig. 4-7). However, since this correlation was detected for both datasets, there may be an important relationship between the denitrifiers and PAOs during BNR in an oxidation ditch. Therefore, future studies investigating functional genes of these BNR-relevant communities may reveal meaningful correlations with the varying wastewater characteristics in an oxidation ditch; correlations that could help diagnose and solve SNDP process failure.

Specific BNR-relevant genera from the cDNA and DNA-based communities were also analyzed for correlations with wastewater characteristics. The DNA-based dataset showed *Candidatus* Accumulibacter and *Sulfuritalea* strongly negatively correlated with nitrate (r_s = -0.88, -0.79, respectively), however the cDNA-based dataset only showed moderate to weak correlations (r_s = -0.49, -0.12, respectively). This correlation is interesting since both *Candidatus* Accumulibacter and *Sulfuritalea* have potential to contribute to denitrification (McIlroy et al., 2015). Furthermore, since higher abundance was strongly correlated with lower nitrate concentrations for the DNA-based community, perhaps these two microorganisms contributed to nitrate reduction in the oxidation ditch, especially *Candidatus* Accumulibacter since there was a moderate correlation with nitrate detected in the cDNA-based dataset. Additionally, the DNA-based dataset detected very strong correlations between *Candidatus* Accumulibacter and *Sulfuritalea*, *Zoogloea, Haliangium,* and the putative GAO, *Candidatus* Contendobacter (r_s = 0.81, 0.80, 0.72, 0.83, respectively), all of which have potential to contribute to denitrification (McIlroy et al., 2015). However, none of these correlations were detected as more than moderate in the cDNA dataset (r_s = 0.47, 0.29, 0.45, 0.52, respectively). *Thauera*, *Haliangium*, and *Skermania,* all putative denitrifiers (McIlroy et al., 2015), were correlated with pH in the cDNA dataset (r_s = 0.59, 0.70, -0.47, respectively) and less correlated with pH in the DNA dataset (r_s = 0.32, 0.49, 0.03, respectively), which was not surprising since the overall putative denitrifying community also reflected this difference. *A0837,* a genus linked to ammonia oxidation (Prosser et al., 2014), exhibited moderate correlations with ortho-P and nitrate in the cDNA dataset (r_s = 0.47, -0.66, respectively), but these relationships were dwarfed in the DNA dataset (r_s = 0.01, -0.04, respectively). Furthermore, this genus was

positively correlated with *Nitrospira*, a well-known nitrifier (Pester et al., 2014), in the active community ($r_s = 0.47$), but negatively correlated in the DNA-based community ($r_s = -0.37$). Conversely, the putative denitrifiers, *Denitratisoma* and *Lautropia* (Fahrbach et al., 2006; McIlroy et al., 2015), were found to have more significant relationships in the DNA-based dataset than in the cDNA-based dataset. Most noteworthy, *Denitratisoma* had moderate to strong correlations with *Candidatus* Accumulibacter, *Candidatus* Accumulimonas, and nitrate ($r_s = 0.59, 0.65, -0.59$, respectively), however none of these relationships were detected in the active community ($r_s = 0.17, 0.01, -0.1$, respectively). *Lautropia* also exhibited a moderate relationship with nitrate in the DNA-based community ($r_s = -0.53$), but not the cDNA-based community ($r_s = -0.1$). These results illustrate how different and potentially misleading DNA-based and cDNA-based correlations can be for genus-level investigations, further supporting the need for more in-depth investigations into these microbial communities.

Beta diversity based on relative abundance from the cDNA and DNA datasets was also correlated with environmental parameters using the *capscale* function in R for the total and BNR-relevant communities (Figs. 4-9, 4-10). The step-wise models with the lowest AIC value for the total community included wastewater characteristics nitrate and ortho-P for the cDNA dataset and ammonia, nitrate, and ortho-P for the DNA-based dataset, with overall significance detected (ANOVA; F= 10.1-cDNA, 8.7-DNA, $p=0.001$). Nitrate and ortho-P were significantly correlated with the total cDNA-based community structure (ANOVA; $p<0.01$), whereas nitrate and ammonia were significantly correlated with the total DNA-based community (ANOVA; $p<0.05$). Both datasets showed nitrate as the strongest explanatory variable for total community structure, explaining at least 80% of the

clustering patterns for the studied sampling events. The step-wise models with the lowest AIC value for the BNR-relevant community included wastewater characteristics nitrate and COD for the cDNA dataset and ammonia, nitrate, and pH for the DNA-based dataset, with overall significance detected (ANOVA; F= 6.2-cDNA, 10.2-DNA, p=0.001). Nitrate and COD were significantly correlated with the BNR-relevant cDNA-based community structure (ANOVA; $p<0.05$), whereas nitrate and ammonia were significantly correlated with the BNR-relevant DNA-based community (ANOVA; $p<0.05$). Again, nitrate explained more that 80% of the BNR-relevant structure for both cDNA and DNA datasets. Within the context of the variables chosen for evaluation, it was not surprising that nitrate explained a large percentage of the total and BNR-relevant community structures for each dataset since nitrate concentrations were unusually high for the summer 2018a sampling event. This is further illustrated in the capscale plots, where the samples from summer 2018a cluster around the positive nitrate vector more so than the samples from summer 2018b (Figs. 4-9, 4-10). Overall, these observations might suggest that since the evaluated wastewater characteristics vary throughout the oxidation ditch, activity of the total and BNR-relevant communities may also shift throughout the ditch, however more sampling events are needed to confirm. Analyzing BNR-relevant functional gene expression of the community and the corresponding environmental parameters could reveal how this community changes throughout the ditch to accommodate SNDP in the various zones of the DO gradient.

4.4 Conclusion

Utilizing cDNA-based methods to characterize the active microbial community during BNR can reveal potentially important microorganisms that may contribute to the

process by demonstrating those that are active or soon-to-be active, whereas DNA-based methods capture active, potentially active, and deceased microorganisms. This is crucial when attempting to decipher the roles of BNR-relevant microorganisms that are present, especially in high abundance, in an unconventional BNR process, like SNDP in an optimized oxidation ditch. In this study, we detected a high abundance of active putative PAOs, some with the potential to denitrify, in the oxidation ditch of Cookeville WRRF, a facility that did not exhibit the traditional conditions for supporting such microorganisms. Batch testing of the activated sludge for P-release and uptake from both summer events resulted in ratios that were comparable to a typical, designed EBPR process. However, the rbCOD of the facility influent along with intracellular P estimated from the wasting process did not indicate the occurrence of EBPR at the Cookeville WRRF. These outcomes suggest that the detected PAOs can survive in an unconventional BNR process, but their roles during these conditions warrant further investigation. Additionally, the active putative denitrifiers were strongly correlated with pH in that they were more abundant when pH increased. This was a relationship not detected with the DNA-based dataset and is one example of potentially important information for an operator when diagnosing a failed BNR process. The results of this study further illustrate the need for more investigations into the active microbial community of the activated sludge process in order to provide useful information for a WRRF operator as well as provide suggestions for further research related to investigating unusual roles of microorganisms, especially during SNDP.

CHAPTER 5: MONITORING A POLYPHOSPHATE ACCUMULATING COMMUNITY AS IT TRANSITIONS TO ACCOMMODATE DENITRIFYING ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL IN A LABORATORY-SCALE REACTOR

Abstract

Identifying the microorganisms that contribute to biological nutrient removal (BNR) in water resource recovery facilities (WRRFs) can provide useful information for operators during daily management of the facility as well as for engineers when designing or optimizing a secondary treatment process for BNR. To contribute to this body of knowledge, we previously investigated the microbial community of a full-scale oxidation ditch that underwent aeration modifications to accommodate simultaneous nitrification denitrification, and enhanced biological phosphorus removal. During that study, we detected active, putative polyphosphate accumulating organisms (PAOs) in high abundance, but did not observe the traditional conditions associated with these microorganisms. These outcomes warranted a closer investigation into the PAO community. Therefore, for this study, we enriched activated sludge of a laboratory-scale bioreactor for PAOs by subjecting the reactor to a traditional enhanced biological phosphorus removal (EBPR) environment. The reactor was then transitioned to incorporate anerobic and anoxic/microaerobic stages to represent dissolved oxygen conditions of the full-scale optimized oxidation ditch. The nutrient removal performance and the microbial community was monitored throughout the study to evaluate whether the community could perform satisfactory nutrient removal under these conditions and to assess the shifts in

community to better understand which microorganisms thrive in the new environment. The goal of this study was to better understand the PAO community during denitrifying EBPR (dEBPR). We found that *Dechloromonas* and the putative glycogen accumulating organism, *CPB_C22&F32*, exhibited more potential importance during the dEBPR process than the well-known PAO, *Candidatus* Accumulibacter.

5.1 Introduction

Excessive discharge of nitrogen (N) and phosphorus (P) from both point and nonpoint sources contribute to eutrophication of receiving water bodies and has been an environmental concern for some time (Dodds & Smith, 2016; Sharpley et al., 2014; USEPA, 2015b). To address the impact from point sources, specifically water resource recovery facilities (WRRF), regulatory agencies have been implementing stricter limits on nutrient discharge (USEPA, 2011, 2015a). In response to this, optimizing existing processes for biological nutrient removal (BNR) has become increasingly popular since it has been shown to reduce nutrients in effluent without expensive upgrades (Collivignarelli & Bertanza, 1999; USEPA, 2015a). Biological nutrient removal is a microbial-driven process that is traditionally achieved through the use of multiple reactors where activated sludge is cycled through anerobic, anoxic, and aerobic zones in order to remove N and P. However, unconventional implementations of BNR have emerged in facilities operating single reactors for their secondary treatment process, like an oxidation ditch. One strategy for optimizing a single reactor for BNR is to modify aeration patterns in a way that generates a dissolved oxygen gradient, providing the anaerobic, anoxic, and aerobic zones that BNR-designed facilities achieve (Daigger & Littleton, 2000). Creating this complex environment can allow for the activated sludge to carry out simultaneous nitrification,

denitrification, and enhanced biological phosphorus removal (SNDP) in an oxidation ditch. This unconventional application of the BNR process has been successful for some facilities (Daigger & Littleton, 2014), however process failures still occur and the microorganisms involved, along with their corresponding roles, still remain elusive. Since BNR is a biological process, understanding the microbial community structure and potential function, and how it relates to wastewater characteristics and operational conditions is relevant to improving process efficiency.

Investigating the microbial community responsible for BNR in the activated sludge process can provide insight to improve operational parameters of a facility's secondary treatment (Carvalho et al., 2007; Xia et al., 2018). Furthermore, as WRRF move towards optimization for BNR (USEPA, 2015a), understanding how these communities might respond to various new conditions can be useful knowledge to have before implementation in the field. Our current knowledge of the BNR microbial community is based on many studies that have proposed various microorganisms as nitrifiers, denitrifiers, and PAOs (Guerrero, Guisasola, & Baeza, 2015; McIlroy et al., 2016; Mielczarek et al., 2013; Oehmen et al., 2007; Siripong & Rittmann, 2007; Yao & Peng, 2017). However, most of these studies focus on WRRFs designed for BNR with separate anaerobic, anoxic and aerobic zones. Studies are lacking that have specifically looked at this community during an unconventional application of the BNR process, like SNDP in an oxidation ditch. As more WRRFs, especially the small to medium-sized facilities operating oxidation ditches, are evaluating optimization to incorporate SNDP instead of designing for BNR, the microbial communities' response with respect to function and process efficiency is not still well understood and needs to be addressed. Understanding the microbial communities of

BNR processes like SNDP is important since the goal of many optimizations is to add or improve total nitrogen (N) and total phosphorus (P) removal (Daigger & Littleton, 2014; USEPA, 2015a). To address this, the previous three chapters have focused on full-scale WRRFs. An observation from these chapters were the high relative abundance of known PAOs, which were also demonstrated to be active in the optimized oxidation ditch. Very few studies, however, have investigated their role in a full-scale, non-EBPR facility, even when found in relatively high abundance (Mehlig et al., 2013). Therefore, these observations suggested that the roles of PAOs in this atypical environment may be more encompassing then currently known. We do know, however, that *Candidatus* Accumulibacter phosphatis (CAP), which was detected in high abundance in the optimized oxidation ditch, is a PAO that has been the focus of many full-scale and laboratory-scale studies performing EBPR and has also been suggested as a contributor to denitrification during EBPR (Camejo et al., 2016; Coats et al., 2017; Flowers et al., 2009). With this in mind, we decided to investigate the PAO and DPAO community through DNA and RNA analysis to shed light on which members might be important to EBPR or denitrifying EBPR (dEBPR).

Evaluating the microorganisms of activated sludge through DNA-based analysis can provide insight as to the potential of a microbial community, however RNA-based studies are also useful since they can reveal which microorganisms are active. In order to study RNA transcripts, one approach is to synthesize RNA into complementary DNA (cDNA), allowing those sequences to then be processed and analyzed like DNA. This workflow can be slightly more challenging than traditional DNA methods due to the sensitivity and instability of RNA, however advances in this field have made this process

more accessible through the advent of stabilization solutions and kits with simplified workflows (Karst et al., 2018a; Liu et al., 2019). The output from RNA investigations can be misleading due the variation in gene counts among bacteria and due to differing RNA synthesis and degradation rates (Blazewicz et al., 2013; Klappenbach et al., 2000). However, identifying microorganisms that are active in the BNR process can provide direction for future studies. Even though studies on cDNA-based, or active, microbial communities in the activated sludge are currently limited, a few have shown the usefulness of this approach when investigating the BNR process. A laboratory-scale study analyzed cDNA using qPCR and high-throughput sequencing to focus on the active microorganisms of dEBPR and observed that CAP and the denitrifier *Thauera* were the main nitrite-reducers in their system (Vieira et al., 2018). Furthermore, *Competibacter*-related species were observed to be highly active and largely contributing to nitrous oxide reduction. This is interesting since some *Competibacter* species are known as glycogen accumulating organisms (GAO) and traditionally thought to be a PAO competitor (Oehmen et al., 2007). This study suggested that *Competibacter* species and CAP might actually work together to achieve efficient dEBPR under the proper conditions. Another study that also investigated denitrification and EBPR originally used a DNA-based approach to determine how different clades of CAP responded to the electron acceptors oxygen, nitrate, and nitrite (Camejo et al., 2016). Through DNA-based analysis, they were able to characterize these close taxonomically-related groups of CAP based on shifts in abundances throughout the study, but it wasn't until their RNA-based investigation that they were able to evaluate actual activity (Camejo, Oyserman, McMahon, & Noguera, 2019). By analyzing cDNA, they were able to establish that a specific strain of CAP, UW-LDO-IC, is capable of

simultaneously reducing oxygen and various nitrogen species. These studies support the usefulness of RNA-based microbial investigations, as well as the need for further study into the BNR community, particularly the PAO members, to decipher links between structure and function. Correlating wastewater characteristics with structure and function of the active community can help to establish parameters that allow for a productive BNR microbial community.

This study is a continuation of research investigating the microbial community of an oxidation ditch that underwent aeration modifications to generate a DO gradient for SNDP. The previous RNA-based study of this WRRF suggested that CAP was an abundant active microorganism in the activated sludge community. This was an interesting observation since nitrification and denitrification were confirmed for this facility (Tables 2-3, 2-4), but the amount of total P in the waste activated sludge along with influent rbCOD did not indicate the occurrence of EBPR (Table 4-1). However, ortho-P fluctuations throughout the oxidation ditch and kinetic batch tests showed that this facility's activated sludge is capable of immediate P-release and uptake, especially when subjected to a traditional laboratory-scale EBPR environment. These outcomes warranted further investigation as to the function of CAP and other PAOs during an activated sludge process outside of the traditional EBPR-designed facility, particularly in oxidation ditches where they have been understudied. Furthermore, assessing shifts in the CAP and PAO community when the sludge is subjected to an environment that allows for denitrification and EBPR, can provide insight as to the functionality of these microorganisms and a better understanding on how to exploit them for BNR. Since it was not possible to conduct a PAO-focused analysis in a full-scale WRRF, we relied on a laboratory-scale study to

address this objective. Here we seeded a laboratory-scale bioreactor with activated sludge from the Cookeville WRRF and enriched the sludge with PAOs by subjecting it to a traditional EBPR environment. We then transitioned the bioreactor environment to an anerobic and anoxic/microaerobic environment to better mimic the variations in DO concentrations that were observed in the optimized oxidation ditch at the Cookeville facility. The bioreactor was monitored throughout this transition with the goals of (1) assessing whether the microbial community could accommodate both nitrate and phosphorus removal in the presence of low DO concentrations and (2) characterizing the microbial community during the EBPR period and comparing it to the resulting dEBPR community. By utilizing both DNA and cDNA data, the total and BNR-relevant microbial communities were investigated and allowed for a closer look into how putative PAOs and denitrifying PAOs (DPAOs) shifted throughout the study.

5.2 Methodology

5.2.1 Reactor Setup and Operation

Two laboratory-scale 2L sequencing batch reactors, one as the test reactor and the other as the control, were seeded with activated sludge from an oxidation ditch located at the Cookeville WRRF. The period covered by this study spanned 242 days, including periods of stable EBPR and dEBPR. Both reactors were set up to continually run four cycles per day at room temperature and were continuously mixed except during settling and decanting. Each cycle was six hours long and included a two-minute feeding time, two-hour anaerobic stage, a three-hour aerobic or anoxic/microaerobic stage, and a one-hour settling stage in which 800 mL of each reactor's supernatant was decanted during the last five minutes. The reactors were fed with synthetic wastewater, including a carbon source

and a nutrient source. The carbon source included sodium acetate trihydrate and yeast extract and the nutrient source included ammonia chloride, potassium phosphate, magnesium chloride, calcium chloride, trace minerals (Hesselmann, Werlen, Hahn, van der Meer, & Zehnder, 1999), and allylthiourea to suppress nitrification. The synthetic feed in the influent resulted in 150 mg/L COD, 4 mg/L PO_4-P, and 15 mg/L NH_3-N in each reactor at the beginning of each cycle. During the anaerobic stage, each reactor was sparged with nitrogen gas and the subsequent aerobic stage was achieved by sparging air back into the reactors. The pH was maintained between 7.4 and 7.8 using sulfuric acid (1 N H_2SO_4) and sodium hydroxide (1 N NaOH). The aforementioned programs were automated using the computer software LABVIEW 2010 (National Instruments, Austin, TX, USA).

For the first 86 days of the study, defined as "Pre-Nitrate", both reactors were operated for classic EBPR with an anaerobic stage for P-release and an aerobic stage for P-uptake. During this period, 125mL of mixed liquor was wasted each day which resulted in a solids retention time (SRT) of 16 days (Lv et al., 2014; R. J. Zeng et al., 2003). On day 87, the test reactor was modified to incorporate an anoxic/microaerobic stage in place of the aerobic stage by removing the air pump and adding sodium nitrate after the anaerobic stage, resulting in 20 mg/L NO_3-N in the reactor (Camejo et al., 2016; Keene et al., 2017). Since the continuous mixing alone did not generate the desired low DO concentrations (< 0.25 mg/L) in the test reactor, on day 96, the air pump was re-introduced and nitrate input was lowered to 10 mg/L NO_3-N in the reactor. Dissolved oxygen was measured daily at the end of the anoxic/microaerobic phase in order to maintain concentrations less than 0.25 mg/L. The period involving adjustments to DO and nitrate concentrations is referred to as the "Transitional" phase and spanned from day 87 to 145. After a few weeks of consistently

low DO concentrations, on day 146, nitrate was increased back to 20 mg/L NO_3-N in the reactor. The period spanning day 146 to 242 is referred to as the "Post-Transitional" phase. During the post phase, only 150 mL of mixed liquor was wasted from the test reactor each week due to the low growth rate of the microbial community, resulting in an SRT of 93.5 days, which was comparable to a similar laboratory study that maintained an SRT of 80 days (Camejo et al., 2016). Even though both studies utilized 2L reactors, our reactors may have required a longer SRT since they received 300mg/L COD in the influent, whereas Camejo et al. had a 500 mg/L COD influent concentration. Since the optimized facility from our full-scale study receives low soluble COD influent, averaging 62.3 mg/L across sampling events, leaving the COD as low as possible while maintaining the PAO population in the laboratory reactors was more desirable than lowering the SRT.

Both reactors were monitored weekly during this study. Once a week, samples were collected at the beginning of a cycle after the feed was introduced, then at the end of the anaerobic stage, and then again at the end of the aerobic stage. Once nitrate was introduced, additional samples were collected from the test reactor at the beginning of the anoxic/microaerobic stage. A sample was collected immediately following nitrate input and then again fifteen minutes later. Samples were analyzed for total and volatile suspended solids (TSS/VSS), COD, ortho-P, ammonia, and nitrate. Samples were also collected on monitoring days for DNA and RNA extractions. Lastly, DO was measured daily at the end of the aerobic or anoxic/microaerobic stage and pH probes and meters were periodically checked and calibrated.

5.2.2 Kinetic Study Procedures

Four kinetic studies were conducted in order to check performance of the mixed liquor to remove nutrients. The first was conducted in 2016 to determine whether the microbial communities of the reactors were performing EBPR comparable to that described in the literature. This kinetic study is being reported here to illustrate that the reactors had shown productive EBPR over a long period of time. The next kinetic investigation was conducted in 2018 on day 86 of this study after three months of rigorous monitoring and continual observations of productive EBPR, during which the reactors were deemed at a steady-state. The third kinetic study only included the test reactor and occurred on day 88, the day after nitrate was introduced and air pump removed. Finally, the last kinetic study for both reactors was conducted on day 199, which occurred after three weeks of productive dEBPR for the test reactor and included four monitoring days. Samples were collected every fifteen minutes throughout an entire cycle. The kinetic study for the test reactor on day 88 and the control reactor on day 199 were the exceptions in which samples were prioritized and only collected for rate calculations. Samples were filtered using a 0.45um syringe filter and analyzed for acetic acid (HAc), COD, ortho-P, ammonia, and nitrate, where applicable. Mixed liquor was also collected for TSS/VSS measurements and for DNA and RNA extractions. Dissolved oxygen and temperature were measured every fifteen minutes using a HACH laboratory-scale LDO probe (HACH Company, Loveland, CO, USA); pH was also recorded every fifteen minutes. The oxidation/reduction potential (ORP), however, was measured at various time intervals due to lengthy stabilization period of probe.

5.2.3 Analytical Techniques

Nutrients and COD were analyzed using Test N Tube kits (HACH Company, Loveland, CO, USA), except for nitrate, which was measured using ion chromatography (Dionex AS-DV, Thermo Scientific, Sunnyvale, CA, USA). Acetic acid (HAc) was analyzed by gas chromatography (Model 6890N; Agilent, Santa Clara, CA, USA) equipped with a flame ionization detector and a 0.53 mm internal diameter x 30 m Agilent DB-FFAP capillary column. TSS and VSS were analyzed following Standard Methods (APHA et al., 2012).

5.2.4 DNA and RNA Extractions, cDNA Synthesis, Sequencing

A detailed description on DNA extraction and sequencing can be found in Chapter 2 sections 2.2.1 through 2.2.4 and for information pertaining to samples collected for DNA and RNA extractions, refer to Table 5-1. Briefly, samples for DNA extractions were collected monthly prior to the introduction of nitrate in the test reactor. Once the Transitional phase began, samples for DNA extraction were collected weekly during monitoring. Samples for RNA extraction were not collected until day 86 during the EBPR kinetic study and were then collected weekly during monitoring. RNA was extracted from samples collected at the end of the anaerobic stage and from the end of the anoxic/microaerobic stage in order to determine whether any differences in active community structure existed between the two stages. Immediately after collecting each sample, 1ml of sludge was transferred to a clean tube and centrifuged for 1 minute at 3300 g. After supernatant was decanted, 1mL of RNAlater® Stabilization Solution (Thermo Fisher Scientific, Sunnyvale, CA) was added and the tube was inverted to resuspend cells in solution. The tube was then stored at 4 deg C for up to a week, as suggested by the RNAlater® manufacturer. Tubes were then placed at -80 deg C for long-term storage. Once

the last monitoring day was complete, RNA was extracted and reverse transcribed into cDNA following the protocol described in Chapter 4 section 4.2.2. Library preparation and sequencing of the cDNA was conducted following the same protocol for DNA, as described in Chapter 2 sections 2.2.1 through 2.2.4.

5.2.5 Amplicon Sequence Processing

DNA and cDNA amplicons were processed using mothur v1.39.5 (Schloss et al., 2009), along with the corresponding MiSeq SOP (Kozich et al., 2013) (accessed on 12/30/2018). Details are described in Chapter 2 section 2.2.5. Two different datasets were generated, one from processing just DNA amplicons from both reactors and the other from processing cDNA and DNA amplicons from the test reactor, with four cDNA samples from the control reactor. For the cDNA dataset, both anaerobic and anoxic/microaerobic samples were included. OTUs with ≤ 3 reads were removed (Keene et al., 2017), resulting in 4,131,081 remaining sequences for the DNA-based dataset and 4,625,717 for the cDNA-based dataset, which represent *total* community structure. Samples were then subsampled at 46,949 sequences for the DNA dataset and 48,866 for the cDNA dataset to remove heterogeneity in sequencing depth (Weiss et al., 2017). This resulted in > 99.5% coverage for each independent library with a total of 953 OTUs for the DNA dataset and 1189 OTUs for the cDNA dataset.

5.2.6 BNR-relevant Microbial Community Selection

BNR-relevant community structure includes microorganisms with the potential to contribute to BNR and details as to how this community was selected can be found in Chapter 2 section 2.2.6. To generate additional dataframes representing the BNR-relevant community, we selected sequences from the total community. For the DNA-based dataset,

1,350,972 sequences were selected and for the cDNA-based dataset, 2,165,532 sequences were selected. These sequences were identified according to the criteria previously described in Chapter 2 section 2.2.6. Each BNR-relevant community was then subsampled at 5781 for the DNA dataset and 19,143 for the cDNA dataset with > 99.8% coverage and a total of 68 and 100 OTUs, respectively.

5.2.7 Statistical Analysis

Differences between the microbial communities of each reactor based on DNA and cDNA samples were evaluated for the total and the BNR-relevant communities. The alpha diversity for each community was characterized using R Studio version 1.1.463 (RStudio Team, 2016) with package *vegan* (Oksanen et al., 2019) to calculate inverse Simpson and Shannon diversity for each sample collected. A t-test in R was used to compare alpha diversity between phases of each reactor. Beta diversity was analyzed on the OTU-level utilizing Primer-E version 7.0.9 (Quest Research Limited, Auckland, New Zealand) to investigate differences between reactors for the total community and the BNR-relevant community. Separate Bray-Curtis dissimilarity matrices were generated for the total and BNR-relevant communities using subsampled relative abundance data. To determine the effect of OTU presence/absence, each relative abundance table was transformed into a presence/absence table and additional Bray-Curtis matrices were generated. Matrices were visualized with a non-metric multi-dimensional scaling (NMDS) plot using Primer7. Analysis of similarities (ANOSIM) was used to determine how similar or different community structures were between reactors and sequence type (DNA and cDNA). Additionally, a SIMPER analysis was performed to better understand which OTUs, and therefore genera, were contributing to the clustering observed in NMDS plots. Linear

regression using the *lm* function in R was used to compare the relative abundance of various genera between reactors during each phase.

Correlations between wastewater conditions and the BNR-relevant community were measured with a two-tailed, Spearman's rank test using RStudio and visualized with the ggplot2 package (Wickham, 2016). Characteristics included P-removal, nitrate removal, COD removal, DO concentrations measured at the end of the aerobic or anoxic/microaerobic stages, and nitrate concentrations due to feed input at the beginning of the anoxic/microaerobic stage. Wastewater characteristics and beta diversity measurements were correlated with a distance-based redundancy analysis (dbRDA) using the *capscale* function in *vegan* and visualized with *ggord* (Beck, 2017).

5.3 Results and Discussion

5.3.1 Sequencing Batch Reactor Performance

Weekly monitoring of each reactor prior to the introduction of nitrate reflected conventional EBPR characteristics, with high COD and ortho-phosphorus removal efficiencies (Fig. 5-1). COD consumption and ortho-P release was observed during the anaerobic stage, with ortho-P concentrations at the end of the anaerobic stage typically measuring more than three times that of the concentrations measured at the beginning of the anaerobic stage. During the Pre-Nitrate EBPR phase, ortho-P removal for both reactors consistently exceeded 97%. For the control reactor, ortho-P removal remained above 97% except for a couple days when dissolved oxygen was low during the aerobic stage. This periodically occurred due to clogged stones used for sparging air. Once the stone was cleaned or replaced, P-removal efficiency was restored. During the Transitional phase, the test reactor did not simultaneously achieve high efficiency ortho-P and nitrate removal.

Nitrate removal higher than 98% was achieved shortly after the introduction of nitrate and removal of air pump, but P-removal dropped to around 32%. After the re-introduction of DO, which averaged 1.76 mg/L for a week before it was maintained at <0.25 mg/L, and lowering nitrate concentration in the reactor to 10 mg/L NO_3-N, P-removal was immediately restored to 99% but nitrate removal dropped to 30%. Interestingly, once nitrate removal returned to 100% (day 115), P-removal dropped again and remained at less than 30% until nitrate was increased to 20 mg/L NO_3-N on day 146. Following this last adjustment, there were a few more weeks of acclimatization until day 177 when productive dEBPR was first observed with nitrate removal at 96% and P-removal at 100%.

5.3.2 Kinetic Studies

Throughout this study, tests were conducted lasting an entire six-hour cycle in order to determine kinetic rates, such as P release and uptake, of the microbial community in each reactor. Measuring kinetic rates allows for us to compare the performance of these reactors to other laboratory and full-scale studies, which can support the potential of the observed microorganisms and associated characteristics in these reactors to be applicable to the field. Specifically, we wanted to better understand which microorganisms are present and potentially active during productive EBPR compared to productive dEBPR and the calculated rates were utilized to confirm a productive process. Kinetic tests conducted during the Pre-Nitrate period further illustrated that both reactors exhibited typical EBPR characteristics (Table 5-2, Fig. 5-2). During the anaerobic stage, ORP measurements reached -104.2mV for the test reactor and -131.6mV for the control reactor, which supports an anerobic environment (Dabkowski, 2012; Gerardi, 2007). Ortho-P release was immediate upon the addition of acetate and plateaued once acetate was completely

consumed, which was within thirty minutes. Once DO was introduced into each reactor, the uptake of ortho-P began and was completely consumed within ninety minutes. Rates calculated for this kinetic test (day 86) were similar for both reactors, with P-release/HAc uptake of 0.30 and 0.29 and P-uptake rates of 10.20 and 8.37 mg P/g VSS*hr for the test and control, respectively. The ratios were comparable to what has been reported in other laboratory-scale studies (Brdjanovic et al., 1998; Hesselmann, Von Rummell, Resnick, Hany, & Zehnder, 2000; Oehmen, Vives, Lu, Yuan, & Keller, 2005). After productive EBPR was confirmed for both reactors, nitrate was introduced into the test reactor on day 87.

The kinetic test conducted on the test reactor, after nitrate was introduced and the air pump removed, served as a baseline for comparison of how nitrate and ortho-P removal subsequently failed or improved (Table 5-2, Fig. 5-3). The control reactor was not monitored on this particular day since this kinetic test was conducted two days after the Pre-Nitrate kinetic study and the control reactor had not been subjected to any operational changes. Acetic acid was still completely consumed in the anaerobic stage, but P-release/HAc uptake drastically decreased to 0.16 along with an almost non-existent P-uptake rate of 0.92 mg P/gVSS*hr. The test reactor then went through the Transitional phase, undergoing various DO and nitrate adjustments before settling on concentrations of 0.25 mg/L DO and 20 mg/L NO_3-N. After a few weeks of consistent productive dEBPR observations, another kinetic test (day 199) was conducted on both reactors (Fig. 5-4). ORP measurements supported the anaerobic stage with measurements reaching -197mV for the test reactor and -146.8mV for the control reactor. The anoxic/microaerobic stage for the test reactor was also supported by ORP measurements, which did not go above -68mV. In

contrast, the control reactor exhibited an ORP measurement of +43.5mV during this time, which was expected. During the anoxic/microaerobic stage of the test reactor, ortho-P and nitrate uptake rates measured 26.4 mg P/gVSS*hr and 6.4 mg NO_3-N/gVSS*hr, whereas rates from a similar laboratory-scale study (Camejo et al., 2016) measured almost half as much at 14 mgP/gVSS*hr and 2.1 mg NO_3-N/gVSS*hr. This difference may have been a result of a slightly different reactor set-up. Camejo et al., 2016 did not suppress nitrification for the daily operation of their reactors, therefore it is very likely their microbial community differed from our study. Interestingly, for this kinetic test (day 199), the test reactor exhibited a P-release/HAc uptake ratio of 0.59, which was the highest among all the kinetic tests conducted for this study.

5.3.3 Microbial Community Diversity and Structure

DNA sequencing conducted throughout this study prior to the transition to dEBPR, confirmed the high abundance of various putative PAOs, such as CAP and *Dechloromonas*, during the EBPR period. Specifically, these reactors averaged 46% putative PAO abundance out of the total community during the Pre-Nitrate phase, compared to the optimized full-scale WRRF which averaged 2.8% putative PAOs, demonstrating that the laboratory-scale reactors were enriched. Therefore, in conjunction with monitoring data and the kinetic studies, these results showed that an enriched PAO bioreactor performing productive EBPR is capable of accommodating dEBPR in the presence of nitrate and low DO. If and how the community changed to adapt to the new environmental conditions, including whether the putative PAOs shifted in abundance, was next to be investigated.

Inverse Simpson and Shannon diversity metrics were calculated for both the total and BNR-relevant microbial communities during productive EBPR in the Pre-Nitrate

phase and productive dEBPR in the Post-Transitional phase. These metrics were only measured for the DNA-based datasets due to the lack of cDNA samples collected during the EBPR phase. Both metrics revealed a significant increase (t-test; t= 3.9 – inv. Simpson, 3.5 – Shannon, $p<0.05$) in diversity between the two periods for the total community of the test reactor (Figure 5-5). These metrics were also calculated for samples collected from the control reactor for the same timespan as the test reactor, but no significant difference was detected for the total community between periods. This was interesting since we also observed a higher diversity for the Cookeville WRRF when compared to Etowah WRRF, suggesting that perhaps the mix of anoxic and micro-aerobic zones in the secondary treatment process allow for a more diverse microbial community. Contrary to this finding, however, both reactors exhibited a significant increase in diversity between the two periods for the BNR-relevant community (t-test; inverse Simpson: t=3.9-test, 2.5-control; Shannon: t=5.5-test, 3.4-control; $p<0.05$) (Fig. 5-6). This observation might not be as meaningful as the outcome for the total community since the BNR-relevant dataset may not include all OTUs that contributed to EBPR or dEBPR in this study, whereas the total community dataset included all OTUs that passed quality filtering.

The presence/absence NMDS showed similar patterns to the relative abundance NMDS for the DNA-based total and BNR-relevant communities, therefore only relative abundance NMDS plots are shown (Fig. 5-7). Specifically, both plots depicted the communities of the reactors clustering together during the Pre-Nitrate period and then separating over the duration of the study. For the total community, no significant difference was detected between the test and control reactors during the Pre-Nitrate phase in terms of relative abundance or presence/absence. However, the total community structure started to

exhibit differences between reactors during the Transitional phase with separate but overlapping clusters (Ramette, 2007) (ANOSIM; R= 0.641 – relative abundance, 0.561 – presence/absence, p=0.001). Finally, during the last phase, these communities were considered completely separate between the reactors (ANOSIM; R=0.929 – relative abundance, 0.999-presence absence, p=0.001). Interestingly, the total community structure significantly shifted from the Pre-Nitrate phase to the Post-Transitional phase for both the test and control reactors (ANOSIM; R> 0.950, p=0.005- relative abundance and presence/absence datasets for each reactor). In other words, the total community was similar between reactors before the introduction of nitrate, then the community changed over time for both reactors, but changed differently for the test reactor than the control reactor. The control reactor may have shifted due to the amount of mixed liquor that was removed during the Pre-Nitrate kinetic study. A SIMPER analysis revealed that the top two OTUs (OTU 2 and 5) contributing to dissimilarity between reactors during the Transitional phase both belonged to *Dechloromonas*, which contributed to 22.5% dissimilarity. The test reactor had more average abundance of OTU 5 (6430) than the control reactor (74), however both reactors had similar average abundances for OTU 2 (~6500). The average abundance for OTU 5 was much less during the Pre-Nitrate phase for the test reactor (7) and about the same for the control (43), suggesting this particular species of *Dechloromonas* may be important for dEBPR since it increased so drastically during the Transitional phase. Also noteworthy for the Transitional phase, OTU 3, a member of CAP, contributed to 5.24% dissimilarity between reactors during this period, with the control reactor exhibiting more average abundance of this OTU (2738) than the test reactor (275). This was a considerable decrease in abundance when compared to the Pre-Nitrate phase

for both reactors (18,298 – control, 22,769 -test). During the Post-Transitional phase, the top OTU contributing to dissimilarity again belonged to *Dechloromonas* (OTU 2) and contributed to 11.1% dissimilarity. This OTU exhibited similar abundances for the Pre-Nitrate and Transitional periods, but was more than twice as abundant for the test reactor (9451) than the control (3497) during the Post-Transitional phase. These observations for *Dechloromonas* on the species-level suggest that these two OTUs may have adapted differently during this study, but their increase in abundance during dEBPR suggests they have potential to be important to the process. Additionally, CAP member, OTU 3, continued to decrease for both reactors, but more so in the test reactor (142) than the control (1815). These observations were further supported when SIMPER was utilized to compare differences within each reactor over time. CAP and *Dechloromonas* contributed to 43.7% dissimilarity in the test reactor between the Pre-Nitrate EBPR and the Post-Transitional dEBPR periods, and contributed to 29.3% dissimilarity between these periods for the control reactor.

Upon seeing the impact that members from the BNR-relevant community had on the total community structure, the BNR-relevant dataset was analyzed to better understand how this specific community changed. The BNR-relevant community dataset exhibited similar trends as the total community, however there were a few differences. Similar to the total community, there was no significant difference in BNR-relevant community structures between the reactors during the Pre-Nitrate phase. For the Transitional phase, the communities of the two reactors exhibited an increase in separation, but still had some overlap (ANOSIM; R=0.547-relative abundance, 0.354-presence absence, p=0.001). However, the BNR-community differed from the total community in that during the Post-

Transitional phase, the two reactors remained somewhat alike based on relative abundance (R=0.671, p=0.001), but completely separate based on presence/absence (R=0.966, p=0.001). This suggests that during the Post-Transitional phase, some of the dominant species may have contributed to the similarities between the reactors and the rare species contributed to the differences. Furthermore, the BNR-relevant community of the test reactor was significantly different during the Pre-Nitrate phase compared to the Post-Transitional phase (ANOSIM; R=1-relative abundance, 0.931-presence/absence, $p<0.005$). However, the control reactor exhibited some overlap between the two phases based on relative abundance (R=0.595, p=0.001), but not presence/absence (R=0.994, p=0.005). These observations from analyzing the BNR-relevant dataset suggest that rare species may impact the ability of the community to perform different BNR processes and that further investigation into their activity, specifically their functional activity, could reveal microorganisms important to dEBPR.

For the cDNA-based microbial community, an NMDS was generated for the test reactor based on the dataset that included DNA and cDNA samples (Fig. 5-8). However, upon visualization, the cDNA samples from the anaerobic and anoxic/microaerobic stages were tightly clustered for each day they were collected. An ANOSIM test showed that the two stages, anaerobic and anoxic/microaerobic, did not significantly differ for the total or BNR-relevant communities. Therefore, the cDNA samples collected during the anoxic/microaerobic stage were selected for further analysis since the DNA samples were also collected at that time. Since there was only one cDNA sample for the Pre-Nitrate phase, we were unable to run a statistical test to compare that phase to other samples. The active total community exhibited some similarities in structure between the Transitional

and Post-Transitional phases (ANOSIM; R=0.441-relative abundance, 0.691-presence/absence, $p<0.05$). The active BNR-relevant community also exhibited similarities in structure between the transitional and Post-Transitional phases (ANOSIM; 0.363-relative abundance, 0.501-presence/absence, $p<0.05$). The cDNA and DNA datasets in the Post-Transitional phase for the test reactor were more similar to each other when based on relative abundance (ANOSIM; R=0.368, p=0.005) than presence/absence (ANOSIM; R=0.669, p=0.001), indicating that perhaps some of the active dominant species were adequately represented in the DNA-based dataset, whereas some of the active rare species were not.

The DNA-based analysis revealed that Proteobacteria was the most dominant phylum throughout the study for both reactors, averaging 64.1% of the total community for the test reactor and 55.4% for the control reactor. Bacteroidetes was the second-most dominant phylum, averaging 26.8% relative abundance for the test reactor and 28.9% for the control reactor. These observations were not surprising since they have been noted in other laboratory-scale studies (Coats et al., 2017; Lv et al., 2015; Vieira et al., 2018) Proteobacteria also made up the vast majority of the BNR-relevant community, averaging over 99% for both reactors. The top 35 most abundant genera for the total community was visualized to get a glimpse of whether dominant members shifted over time (Fig. 5-9). Both reactors showed similar community composition for samples collected during the Pre-Nitrate phase, which is supported by the beta diversity analysis. During this time, CAP comprised 48.8% of the total community in the test reactor and 39.0% in the control reactor, whereas *Dechloromonas* made up 0.6% and 1.3%, respectively. It was not surprising to observe CAP in such high abundance since these reactors were fed with acetate to enrich

for PAOs and they are considered prominent PAOs in literature, especially for laboratory-scale studies (Carvalheira, Oehmen, Carvalho, & Reis, 2014; He, Gu, & McMahon, 2006; Oehmen, Yuan, Blackall, & Keller, 2005; Tu & Schuler, 2013). The microbial community composition for both reactors changed after the Pre-Nitrate phase (Figs. 5-7, 5-9), even though the test reactor was the only one to receive the addition of nitrate and the control reactor had no changes introduced. The shift in the control reactor may have been due to the amount of sludge removed during the kinetic study. Either way, even though both reactors shifted, they each shifted differently, which is also supported by the beta diversity analysis. Notably, during the Post-Transitional phase, CAP decreased to 0.3% of the total community in the test reactor and 5% for the control reactor. *Dechloromonas*, on the other hand, increased to 29.0% in the test reactor and 7.6% in the control reactor.

Upon observing these shifts in potential denitrifying PAOs (DPAO), the BNR-relevant dataset was further analyzed to increase resolution for comparing phases and reactors (Fig. 5-10). Both *Dechloromonas* and CAP exhibited significant shifts between phases (Linear Regression; R^2=72.9%, 91.8%, F=21.1, 87.24- respectively; $p<0.01$). Pairwise comparisons confirmed that neither CAP nor *Dechloromonas* were significantly different between reactors during the Pre-Nitrate phase, however significance ($p<0.05$) was detected between reactors during the Transitional and Post-Transitional phases. Additionally, *Defluviicoccus*, a known GAO with potential to reduce nitrate (McIlroy et al., 2015), shifted from 3.3% to 0.08% of the BNR-relevant community in the test reactor but increased from 5.9% to 15.9% in the control reactor. This was interesting because the control reactor performance throughout most of the study was characteristic of typical EBPR, whereas the test reactor transitioned to accommodate nitrate reduction (Fig. 5-1).

This suggests perhaps that *Defluviicoccus* is more out-competed in a dEBPR environment than in an EBPR environments. Additionally, *Thauera*, which can contribute to denitrification (Thomsen, Kong, & Nielsen, 2007), and *CPB_C22&F32*, another GAO (Stokholm-Bjerregaard et al., 2017a), both increased in the test reactor from <0.1% to 14.8 and 2.2%, respectively. In the control reactor, these genera made up less than 0.5% of the BNR community and did not increase over time. The detection of these two genera during productive dEBPR in the test reactor is interesting because *CPB_C22&F32* belongs to the family *Competibacteraceae*, which includes *Candidatus* Competibacter, the genus that Vieira et al., 2018 detected in their study along with *Thauera*. In their study, they concluded that these two genera contributed to denitrification and that *Competibacter*-related species might have a symbiotic relationship with PAOs during dEBPR.

Even though both reactors showed signs of changes in the community composition, these observations did not establish whether there was a change in the active community. The cDNA-based dataset can provide insight as to which microorganisms are active or soon-to-be active, however due to disproportionate RNA production/degradation rates among bacteria, this approach can only *suggest* possible comparisons with the DNA-based community (Blazewicz et al., 2013; Klappenbach et al., 2000). The active community composition of the test reactor was investigated from day 86 until the end of the dEBPR phase on day 242 (Fig. 5-11). On the phylum level, the active community was comprised of 82.2% Proteobacteria and 10.9% Bacteroidetes, which was only slightly different from the DNA-based dataset. There was only one cDNA sample collected from the Pre-Nitrate phase, however, for this sample, *Dechloromonas* and CAP showed similar activity to what was represented in the DNA dataset, comprising 1.7% and 43.6% of the total community,

respectively. For the Post-Transitional phase, active *Dechloromonas* increased to 27% like in the DNA-based community, but active CAP only decreased to 4.5% instead of 0.3%. Further investigation into the active BNR-relevant community of the test reactor (Fig. 5-11) revealed *Defluviicoccus* as active in the sample collected during the Pre-Nitrate phase, making up 25.5% of the BNR community, but was barely detectable (0.1%) during Post-Transitional phase. Additionally, *Thauera* and *CPB_C22&F32* were not detected in the Pre-Nitrate sample, but were both observed as active during the Post-Transitional phase, comprising 16.4% and 9.8% of the BNR-relevant community, respectively.

The active BNR-relevant community was also investigated for *both* reactors specifically on days 86 (EBPR) and 199 (dEBPR) to see who was active during the kinetic studies conducted on those days (Fig. 5-12). The active BNR-relevant community during the first kinetic study exhibited similar community composition between reactors. However, the composition differed between the two reactors during the dEBPR kinetic study, where CAP made up 30.6% of the BNR community in the control reactor, but only 4.2% in the test reactor. Furthermore, *Defluviicoccus* was barely detectable (0.04%) in the test reactor during the dEBPR study, whereas *CPB_C22&F32* made up almost a quarter of the active BNR community (21.5%). Lastly, *Dechloromonas* made up less than 5% of the BNR-relevant community in each reactor during the first kinetic study but increased to more than 50% during the second kinetic study. These observations further suggest *Dechloromonas* and *CPB_C22&F32* may be important to the dEBPR process.

5.3.4 Correlation of Reactor Characteristics with Microbial Community

Spearman's rank correlation test was used to compare performance and environmental characteristics of the reactors with the microbial community. Only samples

collected after the initial addition of nitrate (day 87) were analyzed due to lack of samples ($n<5$) for the Pre-Nitrate phase. Each of the DNA-based analyses included alpha diversity measurements from the total community and selected BNR-relevant genera that exhibited moderate to strong correlations with the other factors. For the test reactor (Fig. 5-13a), nitrate input from feed and P-removal were positively correlated (r_s=0.52), supporting the observed improvement in P-removal when nitrate was added at 20 mg/L NO_3-N instead of 10 mg/L NO_3-N. Inverse Simpson and Shannon diversity metrics were negatively correlated with nitrate removal (r_s= -0.43, -0.40, respectively), meaning better nitrate removal was correlated with a less diverse total community. The putative DPAO, *Dechloromonas*, was positively correlated with nitrate removal (r_s=0.53), whereas the putative GAO, *Propionivibrio*, which has potential to reduce nitrate (Albertsen, McIlroy, Stokholm-Bjerregaard, Karst, & Nielsen, 2016), was negatively correlated with nitrate removal (r_s= -0.59). Interestingly, two putative PAOs, *Candidatus* Obscuribacter and *Gemmatimonas*, were negatively correlated with P-removal in the test reactor during the time that nitrate was present (r_s= -0.59, -0.74, respectively). Since the improvement in P-removal was correlated with increased nitrate, it may be that these two PAOs do not perform P-removal well in an anoxic environment, even though *Candidatus* Obscuribacter has the genetic potential for nitrate reduction (Soo et al., 2014). Furthermore, another putative GAO that has been observed reducing nitrate (McIlroy et al., 2015), *Defluviicoccus*, was negatively correlated with P-removal in the test reactor (r_s= -0.74). This is interesting because in the control reactor, which maintained EBPR, *Defluviicoccus* was among the top 4 BNR-relevant genera throughout the study period (Fig. 5-10b). However, in the test reactor, it was barely detectable during dEBPR, a time when an

increase in nitrate resulted in recovered P-removal performance, which theoretically seems as it would be an ideal environment for this GAO. On the other hand, the putative GAO, *CPB_C22&F32,* which as previously mentioned is in the *Competibacteraceae* family, was positively correlated with P-removal (r_s= 0.55), further supporting the possibility for it to contribute to, instead of inhibit, dEBPR. *CPB_C22&F32* was also positively correlated with *Dechloromonas* (r_s=0.63). Lastly, two putative denitrifiers, *Haliangium* and *Thauera,* were positively correlated with P-removal (r_s= 0.53, 0.54, respectively) and negatively correlated with *Defluviicoccus* (r_s= -0.71, -0.59, respectively), suggesting they may not compete with the DPAOs during denitrification, but perhaps contribute differently.

The control reactor did not exhibit as many strong correlation measurements as the test reactor and most were typical of an enriched EBPR environment (Fig. 5-13b). For example, P-removal and DO concentrations had a positive correlation (r_s= 0.49), which was not surprising since this reactor was operated at high DO conditions (>2 mg/L) and achieved successful EBPR throughout the study period. CAP and *Thauera* were positively correlated with COD removal (r_s= 0.64, 0.51, respectively), which is interesting to observe for *Thauera* because of the lack of nitrate in the reactor. Lastly, *Defluviicoccus* and *Dechloromonas* were positively correlated with each other in the control reactor (r_s= 0.55), but not for the test reactor (r_s= -0.09). This observation in conjunction with observations from SIMPER, suggest that a species-level investigation may help to characterize the relationship between these two genera. Lastly, the two putative GAOs, *Propionivibrio* and *CPB_C22&F32,* were strongly negatively correlated with each other in the control reactor (r_s= -0.68), but were not significantly correlated in the test reactor (r_s= -0.33).

The active BNR-relevant microbial community in the test reactor was also analyzed for the period following the introduction of nitrate (day 87) and only included samples collected from the aerobic stage (Fig. 5-13c). Like the DNA-based community of the test reactor, *Gemmatimonas* and *Candidatus* Obscuribacter were also negatively correlated with P-removal (r_s= -0.64, -0.64). Interestingly, the denitrifier, *Thauera*, was negatively correlated with nitrate removal (r_s= -0.41), unlike with the DNA-based dataset (r_s= 0.01). However, like the DNA-based dataset, *Thauera* along with *Haliangium*, were both positively correlated with P-removal (r_s= 0.72, 0.61) and *Defluviicoccus* was negatively correlated with P-removal (r_s= -0.78). *Dechloromonas*, was positively correlated with P-removal for the cDNA-based dataset (r_s= 0.61), but was not for the DNA-based dataset (r_s= 0.15). Additionally, *Dechloromonas* and *CPB_C22&F32* were strongly correlated with COD removal (r_s= 0.80, 0.75, respectively) and were very strongly correlated with each other (r_s=0.94). Also, just in the active community, *Dechloromonas* was negatively correlated with *Thauera* (r_s= -0.69) and *Defluviicoccus* (r_s= -0.77). Not surprisingly, *CPB_C22&F32* was also negatively correlated with *Defluviicoccus* (r_s= -0.87). Interestingly, the putative DPAO, *Tetrasphaera*, exhibited negative correlations with *Dechloromonas* (r_s= -0.51) and *CPB_C22&F32* (r_s= -0.64), but a very strong positive correlation with the putative denitrifier Iamia (r_s= 0.85). Since *Tetrasphaera* is a putative PAO, it may compete with *Dechloromonas* in the presence of nitrate.

Beta diversity calculated from the cDNA and DNA relative abundance datasets was correlated with environmental parameters using the *capscale* function in R for the total community of the test reactor (Fig. 5-14). The control reactor was also investigated, but none of the environmental variables exhibited a significant relationship with community

structure. Both DNA and cDNA-based datasets from the test reactor showed similar clustering patterns with the environmental parameters, unlike what was observed for the full-scale study described in Chapter 4 section 4.3.3. The similarities between the DNA and cDNA-based datasets for the laboratory-scale samples are most likely due to the controlled environment in the reactors, whereas samples collected from Cookeville's WRRF were exposed to a dynamic, changing environment. The step-wise model with the lowest AIC value included influent nitrate, ortho-P removal, COD removal, and DO concentration and was overall significantly correlated with the total community structure (ANOVA; $F= 3.05$, $p=0.001$). Ortho-P and COD removal had the strongest correlations with total community structure (ANOVA; $p<0.01$). The total community structure during the Post-Transitional phase, when productive dEBPR occurred, corresponded with higher P-removal and influent nitrate, whereas the transitional community was associated more with lower P-removal and influent nitrate. This outcome is not surprising since nitrate was added at 10mg/L NO_3-N during most of the Transitional phase and there was frequently poor P-removal. Therefore, the Post-Transitional community was most likely shaped by the higher nitrate concentrations and performed more efficient P-removal than the Transitional community. These observations demonstrate that a combination of nitrate and DO can support productive dEBPR.

5.4 Conclusion

Elucidating the microbial community responsible for denitrifying EBPR can contribute to better design and optimization strategies for a WRRF's secondary treatment process when implementing or improving BNR. Since the functionality of many microorganisms is still not understood, more studies are needed to help contribute to this

knowledge gap, especially in the wastewater field. Our primary goal of this laboratory-scale study was to investigate changes in the PAO microbial community as it transitioned from performing traditional EBPR to dEBPR in an anoxic/microaerobic environment. Using both DNA-based and cDNA-based analyses, we were able to track the community alongside changes to the bioreactor, which provided some insight into the shifts in community structure as well as which microorganisms were active throughout the study. We observed that the test reactor was able to accommodate dEBPR once the community acclimated to the presence of nitrate and low DO. Nitrate and P-removal was observed during the last weeks of this study. During this time, there was a significant decrease in CAP abundance and an increase in *Dechloromonas* and *CPB_C22&F32*, for both the DNA-based and cDNA-based communities. This study had the original intention to further characterize CAP, but found the putative DPAO, *Dechloromonas*, and putative GAO, *CPB_C22&F32*, to potentially be more important in the dEBPR environment. The shifts observed in these three genera suggest they warrant a much closer investigation into their actual function during all BNR processes. Furthermore, the decrease in CAP abundance in the presence of nitrate was unexpected since it was detected in such high abundance in the previously studied oxidation ditch at the Cookeville WRRF, where the DO gradient allowed for anaerobic, anoxic, and aerobic zones and varied nitrate concentrations throughout the ditch. Further study into the BNR-relevant community, including functional gene expression studies, could help establish CAP's presumably diverse role in the activated sludge process.

CHAPTER 6

6.1 Summary and Conclusions

The goals of this research were to (1) assess whether optimizations of oxidation ditches that allow for simultaneous nitrification, denitrification, and enhanced biological phosphorus removal (SNDP) cause changes in the corresponding WRRF's microbial community and whether the community will eventually stabilize; (2) characterize the active microbial community during SNDP; and (3) determine environmental factors that influence microbial composition and function. Using the collective data from these goals, we were able to contribute knowledge to how microbial communities adapt to unconventional operations of oxidation ditches for SNDP and propose that the microbial community and process can stabilize over time after an optimization.

The overall goals of this research were addressed by integrating full-scale and laboratory-scale studies and the individual objectives were accomplished through the following:

In Chapter 2, we investigated a water resource recovery facility (WRRF) located in Cookeville, TN that was originally designed to operate an oxidation ditch designed for BOD_5 and ammonia removal. This facility underwent optimization that involved modifying the aeration patterns of the oxidation ditches to incorporate a DO gradient, creating the bioreactor macroenvironments needed to accommodate SNDP. Changes in wastewater characteristics and the microbial community of the activated sludge were monitored for three years following this operational modification. We assessed the response of the microbial community in an oxidation ditch to this process optimization for SNDP and evaluated whether the community stabilized over time. The DO gradient in the

optimized oxidation ditch allowed for successful SND without deterioration in nitrification performance, even though there were changes in the microbial community. Furthermore, the composition of the putative denitrifying community changed in response to the optimized bioreactor macroenvironment, potentially becoming more functionally redundant and stable. This facility receives influent with relatively low rbCOD and P, however the varying concentrations of P throughout the ditch, the kinetic rates measured from batch tests, and the detection of putative polyphosphate accumulating organisms (PAO) in relatively high abundance, suggest that the oxidation ditch is maintaining an environment that could be utilized for EBPR. Additionally, changes were observed in the microbial community composition for both total and BNR-relevant communities, indicating a trend that may suggest stabilization of community structure. Also, the diversity of the microbial community correlated with DO concentrations, implicating that this environmental parameter most likely shaped the community during the stabilization process. This was a noteworthy observation since the optimization for this facility focused on changing the DO patterns throughout the oxidation ditch.

In Chapter 3, we continued to investigate how a microbial community of the activated sludge process responded to optimizations of an oxidation ditch for SNDP. Two other WRRFs were additionally investigated, which served as references to demonstrate how a microbial community might change over time without the influence of a major operational modification since dynamic influent characteristics and seasonal temperature changes can impact community structure. One facility, located in Etowah, TN, conventionally operated an oxidation ditch and the other facility, located in Maryville, TN, was designed and operated for BNR. All three facilities had similar influent characteristics,

but due to differences in operational parameters, each facility exhibited different nutrient removal efficiencies. The optimized oxidation ditch exhibited a DO gradient that accommodated total N removal more like the BNR-designed secondary treatment process rather than the traditionally-operated oxidation ditch. Comparisons between the microbial community of the Cookeville WRRF and the other facilities demonstrated that the corresponding microbial community responded favorably to this type of optimization in that the community exhibited characteristics similar to the BNR-designed facility. EBPR batch tests demonstrated that the activated sludge from Cookeville WRRF exhibited P-release/HAc uptake ratios more similar to the Maryville facility than to the Etowah facility, which was also supported by findings from other studies on EBPR-designed facilities. Furthermore, diversity and core community structure of the Cookeville WRRF exhibited more similarities with the BNR-designed facility than to the facility conventionally operating an oxidation ditch. Shifts in the microbial community for Cookeville WRRF were different when compared to the other two facilities, suggesting that the changes in the Cookeville's community were in response to optimization. Lastly, the diversity of the optimized facility was moderately correlated with temperature but strongly correlated with DO. This was not the case for the diversity of the two reference facilities, which were strongly correlated with temperature. This observation suggests that given enough time, the diversity of Cookeville WRRF might shift and exhibit a stronger relationship with temperature like the reference facilities, which could possibly indicate that the Cookeville microbial community has stabilized in response to the optimization.

In Chapter 4, we evaluated the active or potentially active microbial community in Cookeville's oxidation ditch three years after they implemented aeration modifications to

accommodate SNDP. DNA and cDNA-based methods were utilized to reveal microorganisms and environmental conditions that warrant a closer look for future investigations regarding functional activity during SNDP. We detected active putative PAOs and DPAOs in relatively high abundance in the oxidation ditch of Cookeville WRRF, a facility that did not exhibit the traditional environmental conditions or wastewater characteristics for supporting such microorganisms. For example, the rbCOD of the facility influent along with intracellular P-uptake estimated from the wasting process did not indicate the occurrence of EBPR at the Cookeville WRRF. However, batch testing of the activated sludge for P-release and uptake resulted in ratios that were comparable to a typical, designed EBPR process. These outcomes suggest that the detected PAOs and DPAOs can survive in an unconventional BNR process, but their roles or possible contributions to SNDP during these conditions warrant further investigation. Additionally, the active putative denitrifiers were strongly correlated with pH, a relationship that was not detected with the DNA-based dataset. This is one example of potentially important information an operator could use when diagnosing a failed BNR process.

The laboratory-scale study was addressed in Chapter 5 and was conducted in order to better understand the microbial community of SNDP, particularly putative PAOs and denitrifying PAOs (DPAO). In this chapter, we utilized sequencing batch reactors to investigate microorganisms that potentially contribute to denitrifying EBPR in the presence of low DO concentrations. Using both DNA and cDNA-based analyses, we monitored the community alongside changes to the bioreactor, which provided insight into the shifts in community structure as well as which microorganisms were active throughout the study. The activated sludge of the test reactor was able to accommodate dEBPR once the

community acclimated to the presence of nitrate and was reflected by the nitrate and P-removal observed during the last weeks of this study. During this time, *Candidatus* Accumulibacter abundance significantly decreased for both the DNA and cDNA-based dataset. This decrease in abundance during the presence of nitrate was unexpected since *Candidatus* Accumulibacter was detected in such high abundance in the full-scale oxidation ditch at the Cookeville WRRF, where the DO gradient allowed for anaerobic, anoxic, and aerobic zones and fluctuations in nitrate concentrations throughout the ditch. Also during productive dEBPR, abundances increased for the putative DPAO *Dechloromonas* and putative GAO *CPB_C22&F32*, for both the DNA-based and cDNA-based communities. This observation suggests that *Dechloromonas* and *CPB_C22&F32* are potentially more important in the dEBPR environment than previously thought. The shifts observed in these three genera warrant further investigation into their BNR-relevant functional gene expression to establish their actual function in unconventional BNR processes. Elucidating the microbial community responsible for denitrifying EBPR can contribute to better design and optimization strategies for the secondary treatment process of a WRRF.

In conclusion, these observations demonstrate that optimization of an oxidation ditch for SNDP has the potential to be a successful practice. Information from this study may encourage WRRFs that are searching for cost-effective solutions to meet new effluent limits to try optimization, especially for an oxidation ditch as previously described since this application calls for little to no financial investment. Furthermore, we demonstrated that the microbial community of the activated sludge can be exploited to accommodate different BNR processes. This study provides evidence that the microbial community of

the activated sludge process has the potential to shift to accommodate BNR and therefore promotes this type of operational strategy as a possibility for other WRRFs to incorporate. Additionally, it is interesting to observe a diverse group of BNR-relevant genera as active or soon-to-be active in an unconventionally-operated oxidation ditch because it demonstrates that the aeration modifications allow a community with potential to contribute to various BNR or SNDP processes to exist in an oxidation ditch. The results of this study demonstrate how investigating the microbial community of the activated sludge process can provide useful information for a WRRF operator or an engineer when optimizing or designing a BNR process.

6.2 Recommendations for Future Studies

- The microbial community of the activated sludge prior to optimization of the oxidation ditch in this study was not adequately characterized. Therefore, a similar study should be conducted that is able to monitor an oxidation ditch at least a year before optimization and include multiple sampling events per season. This would allow for better comparisons to be made of the community before and after process modifications. Sampling multiple times within a single season can help track the impact of transient microorganisms.
- There were not enough facilities sampled during this study to thoroughly assign a core community to a wastewater operational process. Future studies that can sample many WRRFs operating oxidation ditches for some form of BNR would be beneficial to establish whether our observations at Cookeville WRRF are duplicated among other optimized oxidation ditches.

- As mentioned, many times throughout the study, more RNA-based studies are needed that investigate BNR-relevant functional genes. Specifically, this could help characterize the activity and roles of PAOs and DPAOs, especially *Candidatus* Accumulibacter and *Dechloromonas*. Observing these two genera as active in an unconventionally-operated oxidation ditch is interesting, however it does not resolve what their function is in the activated sludge or BNR process. This is especially interesting since they were detected in a facility that did not exhibit characteristics of EBPR. Since this facility has low rbCOD concentrations in the influent, future studies could investigate this parameter specifically in relationship with these genera and determine if it impacts their metabolic function.

APPENDIX A: FIGURES

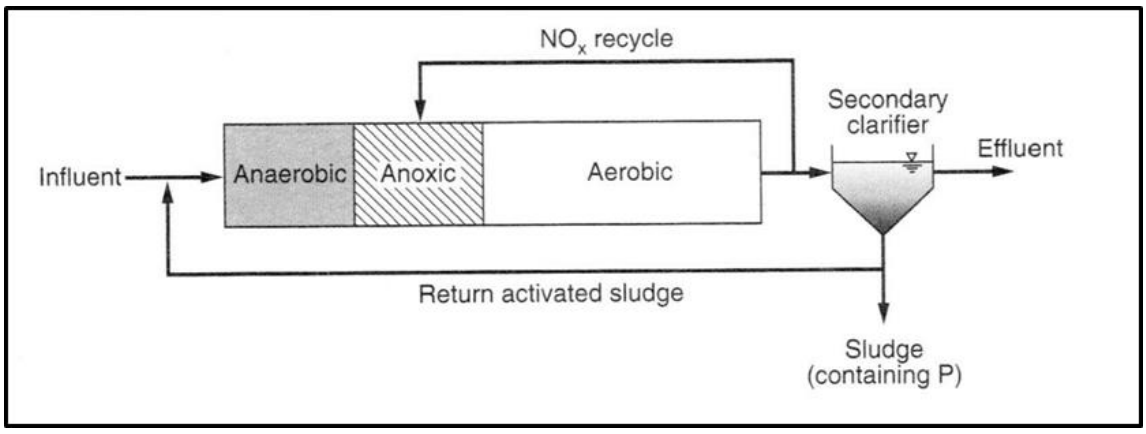


Figure 1.1: Anaerobic-anoxic-oxic (A_2O) design (from: Metcalf & Eddy).

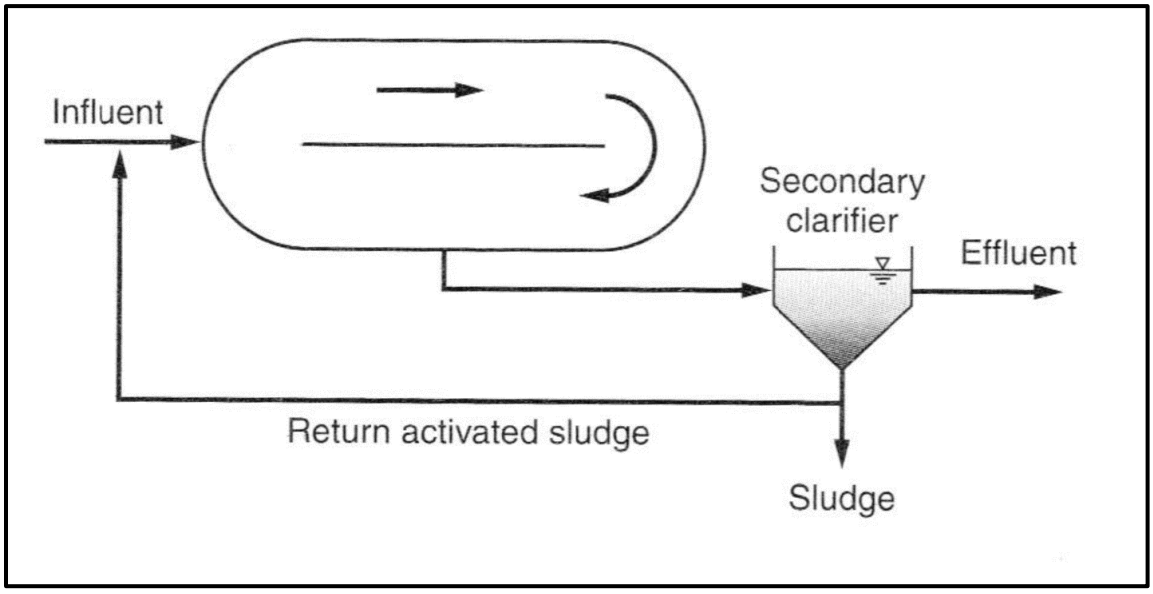


Figure 1.2: Traditional oxidation ditch (from: Metcalf & Eddy).

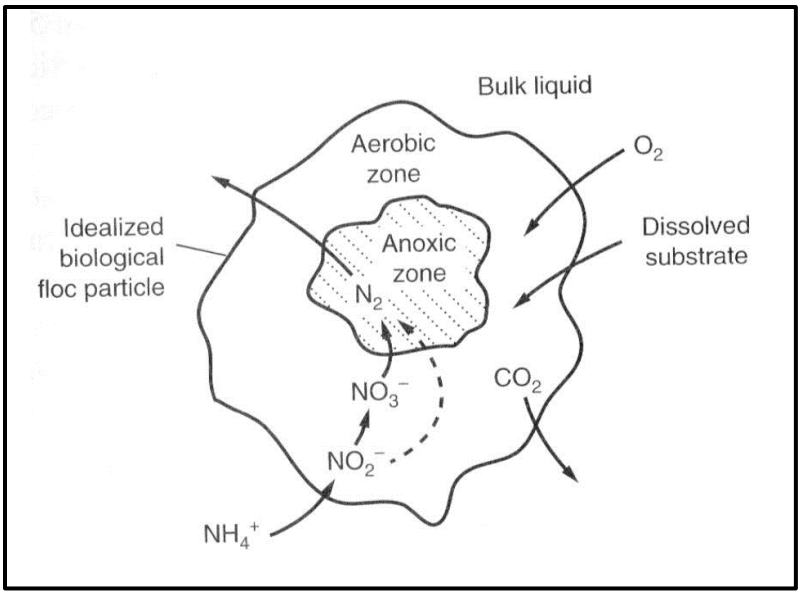


Figure 1.3: Microenvironment of a biological floc (from: Metcalf & Eddy).

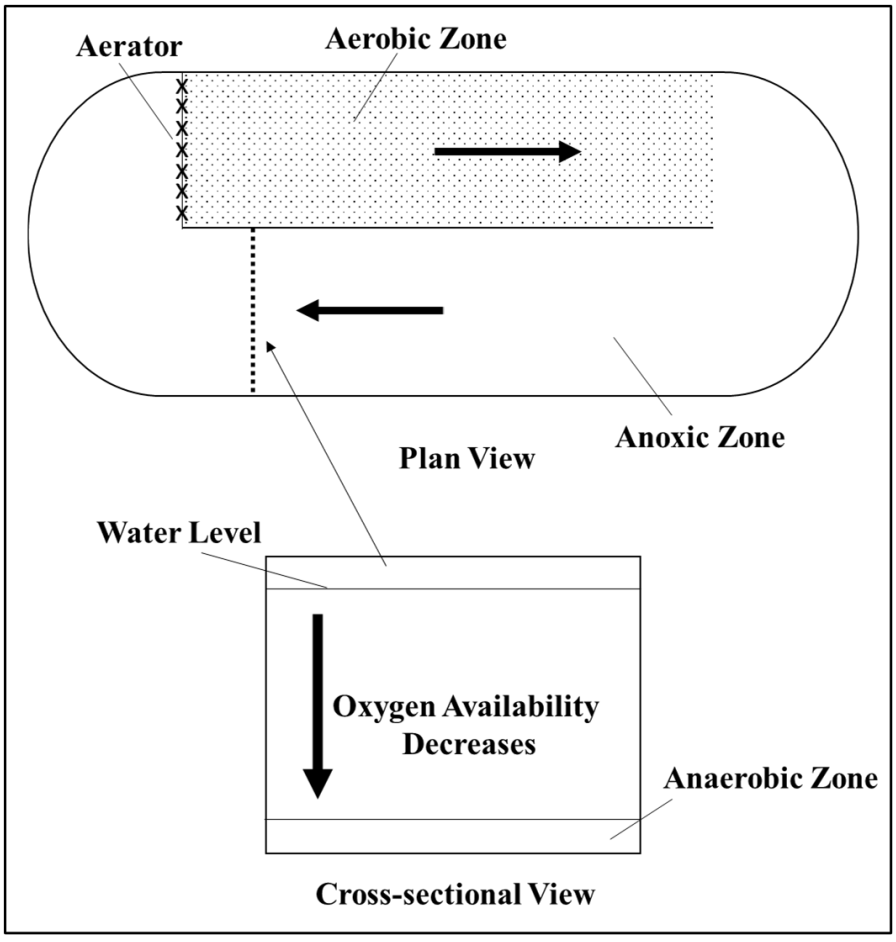


Figure 1.4: Macroenvironment of an oxidation ditch with brush-type aeration operating to generate a dissolved oxygen gradient.

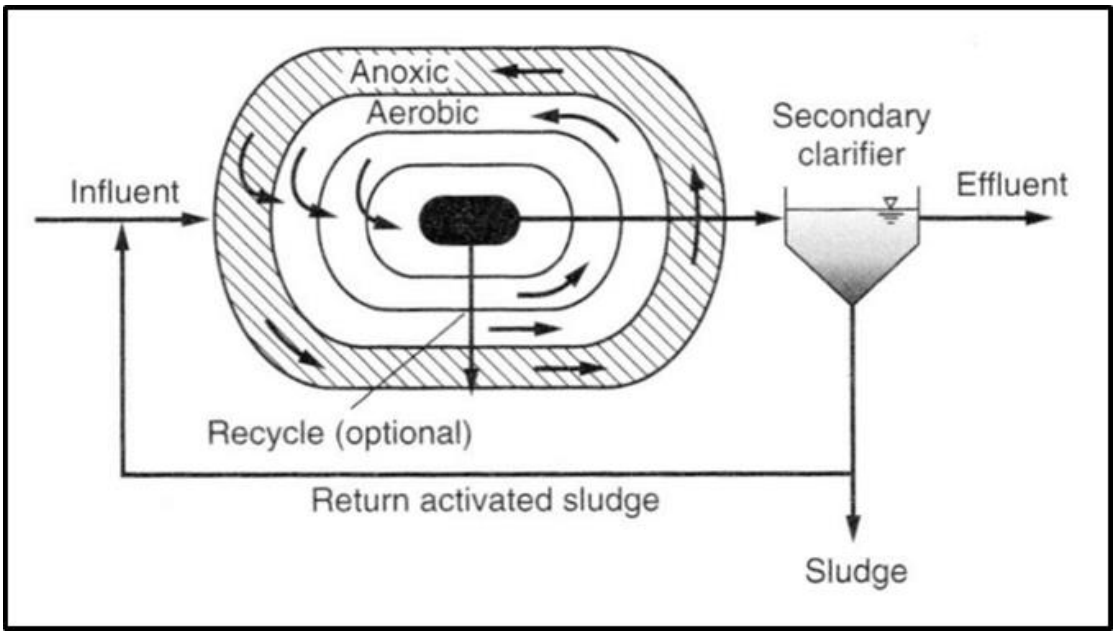


Figure 1.5: Orbal® oxidation ditch (from: Metcalf & Eddy).

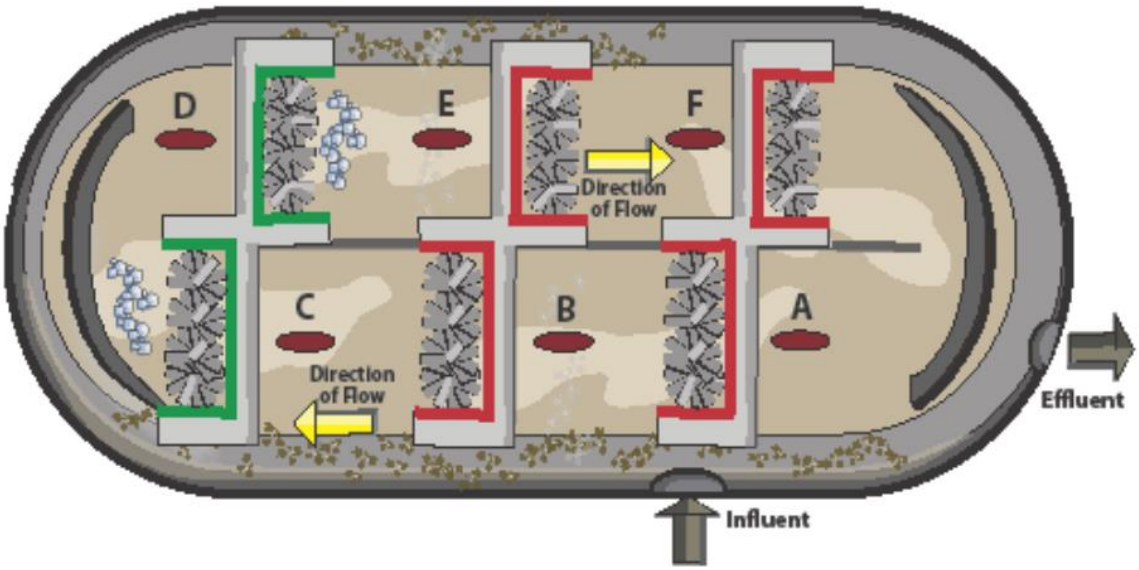


Figure 2-1: Aerial schematic of Cookeville's oxidation ditch during sampling events. Green indicates the pair of surface aerators in operation during sampling. Red dots (●) represent sampling locations. Samples were collected from the surface and the bottom of the ditch.

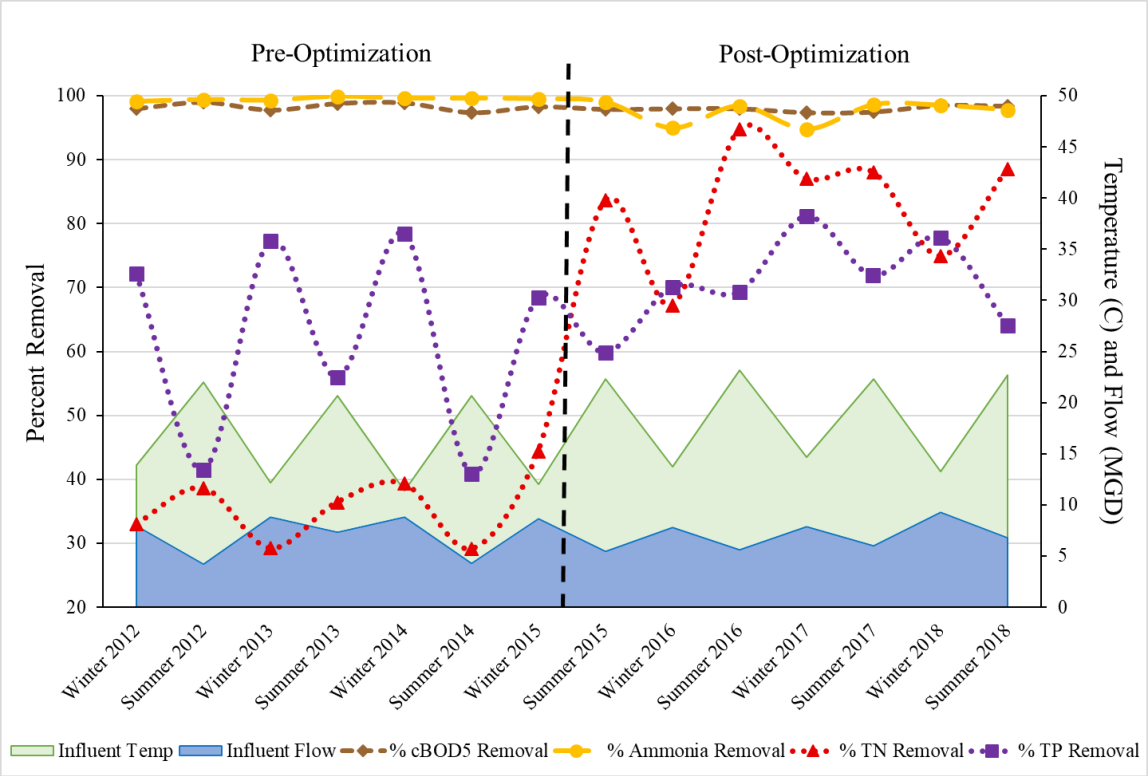


Figure 2-2: Influent characteristics and nutrient removal efficiency of Cookeville WRRF pre- and post-optimization. cBOD5 = five-day carbonaceous biochemical oxygen demand, TN = total nitrogen, TP = total phosphorus.

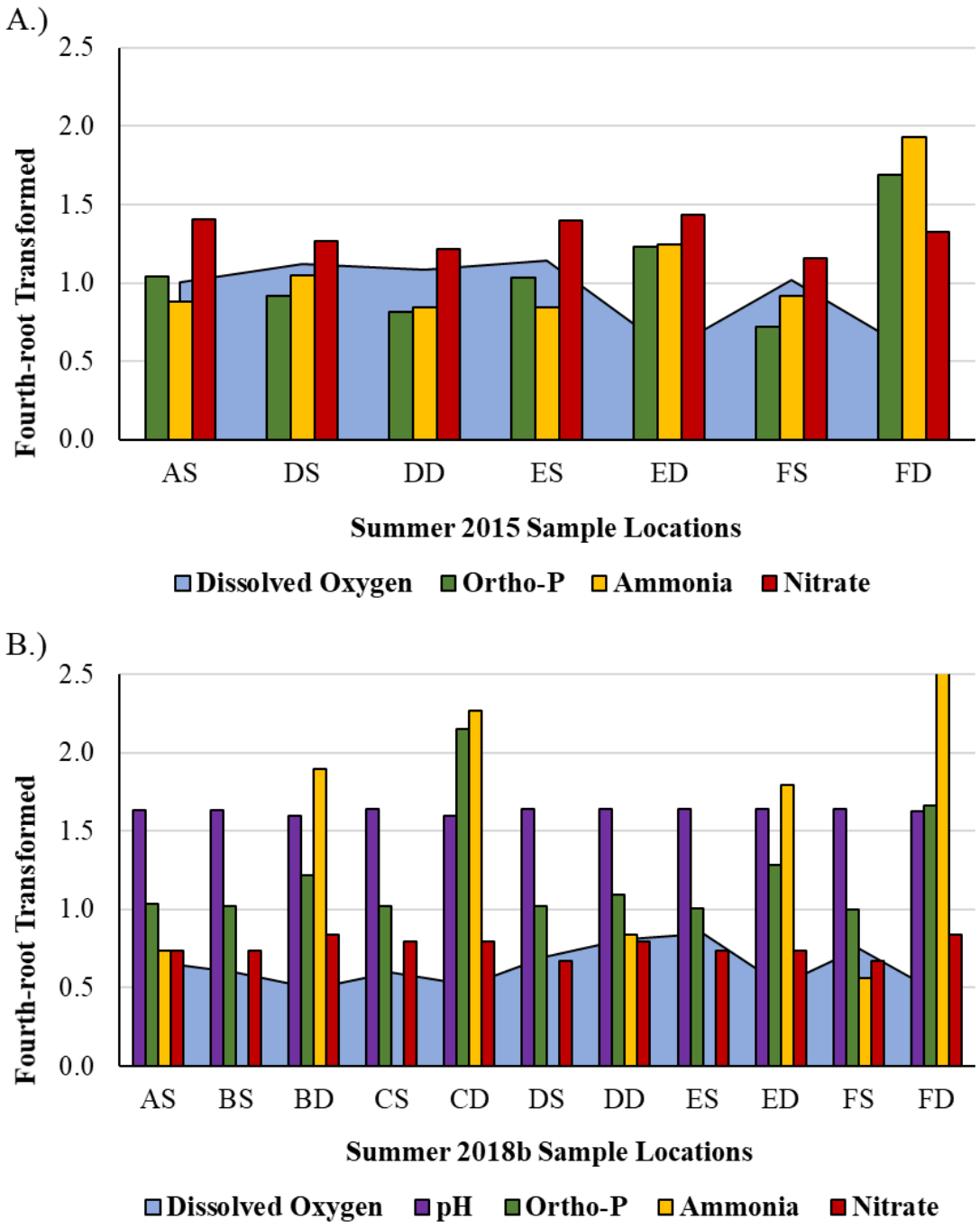
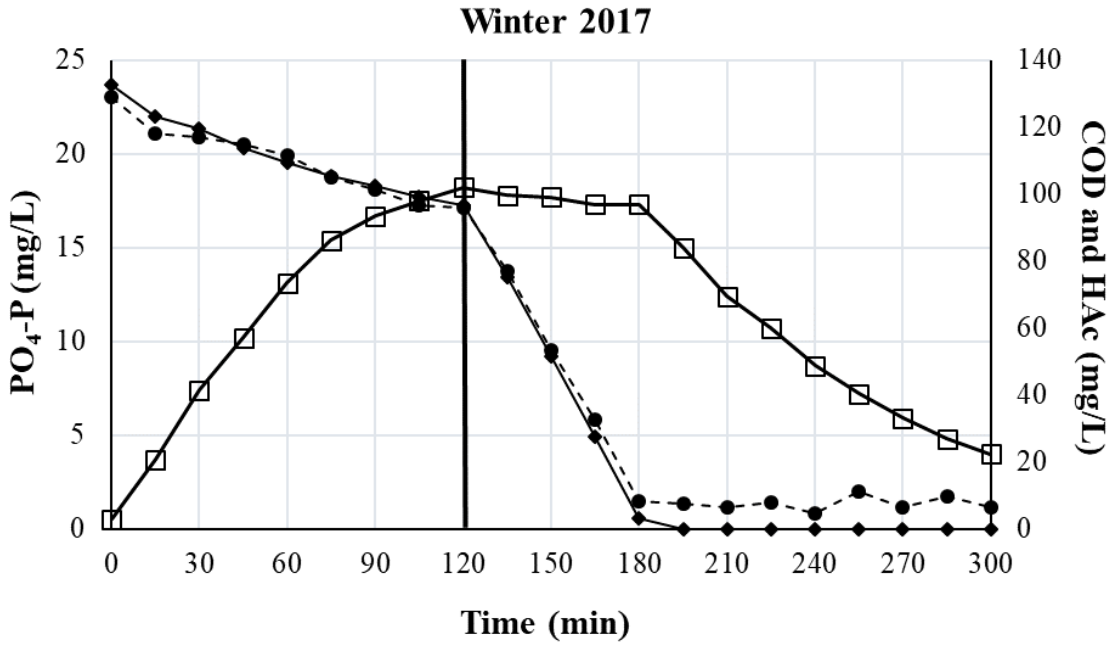
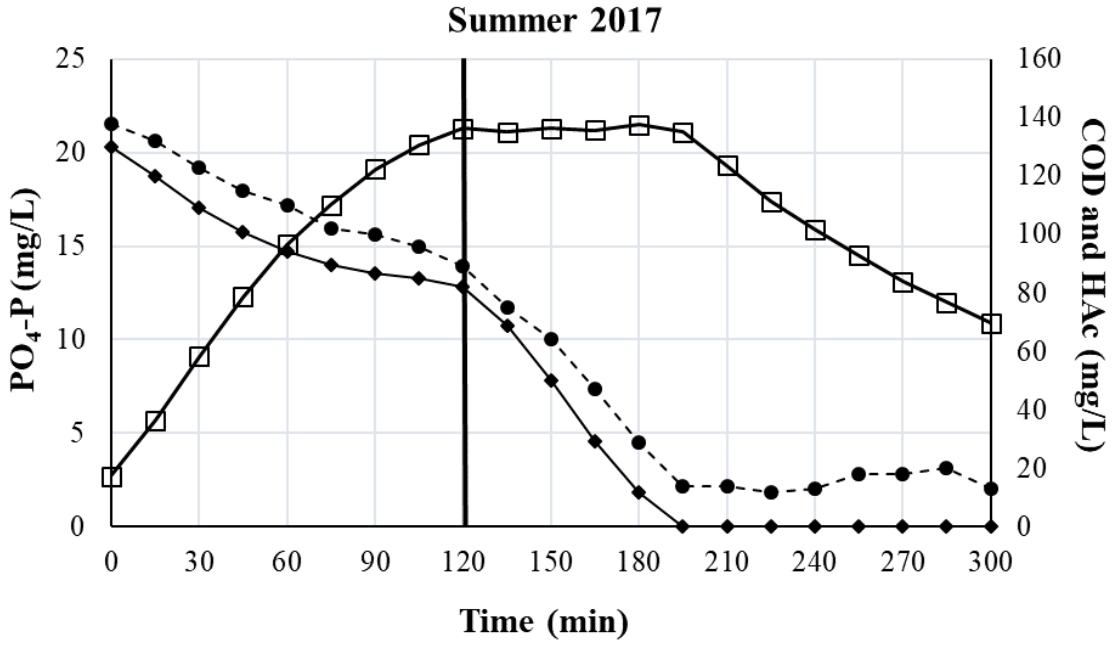
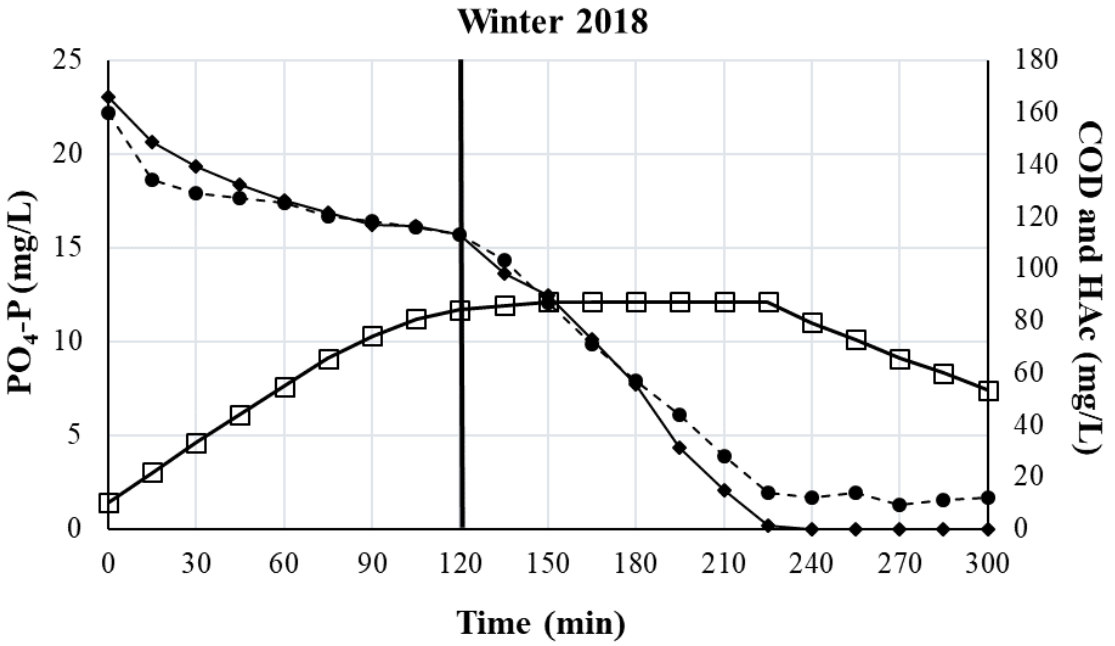
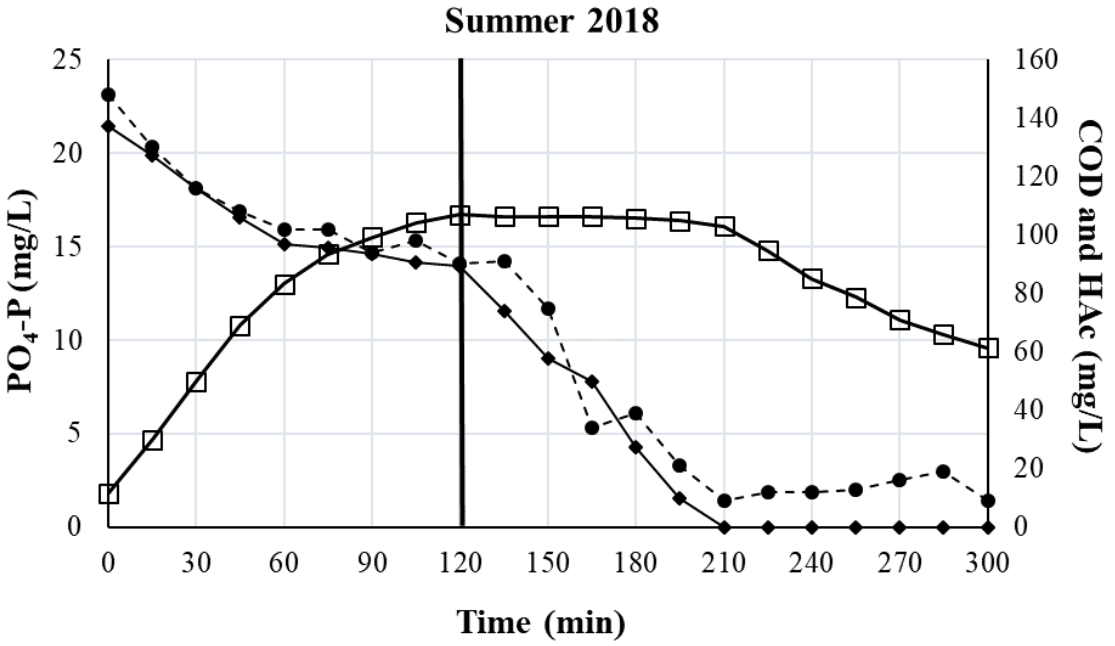


Figure 2-3: Trends of the wastewater characteristics in the oxidation ditch of Cookeville WRRF for the A.) first and B.) last sampling events. Sampling locations with an 'S' indicate a grab sample collected at the surface and locations with a 'D' indicate a sample collected at the bottom of the ditch.


Winter 2017
PO4-P (mg/L)
COD and HAc (mg/L)
Time (min)
0
5
10
15
20
25
0
20
40
60
80
100
120
140
0
30
60
90
120
150
180
210
240
270
300


Summer 2017
PO4-P (mg/L)
COD and HAc (mg/L)
Time (min)
0
5
10
15
20
25
0
20
40
60
80
100
120
140
160
0
30
60
90
120
150
180
210
240
270
300


Winter 2018
PO4-P (mg/L)
COD and HAc (mg/L)
Time (min)
0
5
10
15
20
25
0
20
40
60
80
100
120
140
160
180
0
30
60
90
120
150
180
210
240
270
300


Summer 2018
PO4-P (mg/L)
COD and HAc (mg/L)
Time (min)
0
5
10
15
20
25
0
20
40
60
80
100
120
140
160
0
30
60
90
120
150
180
210
240
270
300

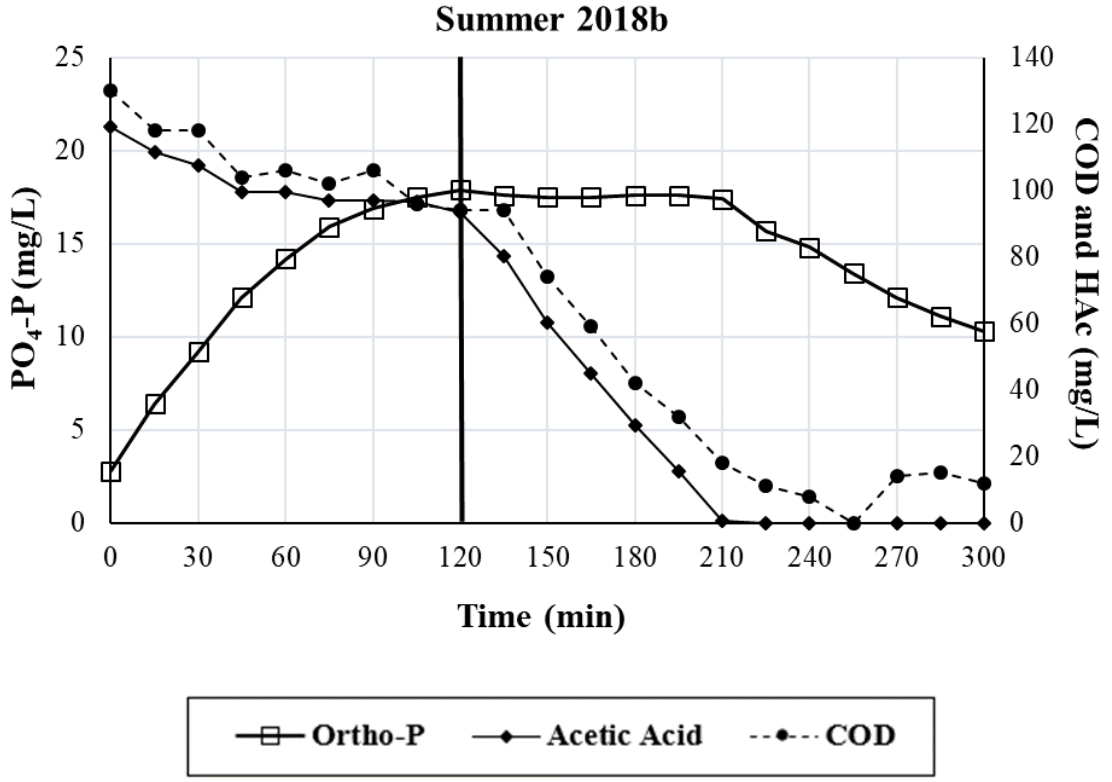


Figure 2-4: Graphical representations of biological phosphorus removal batch tests from Cookeville WRRF.

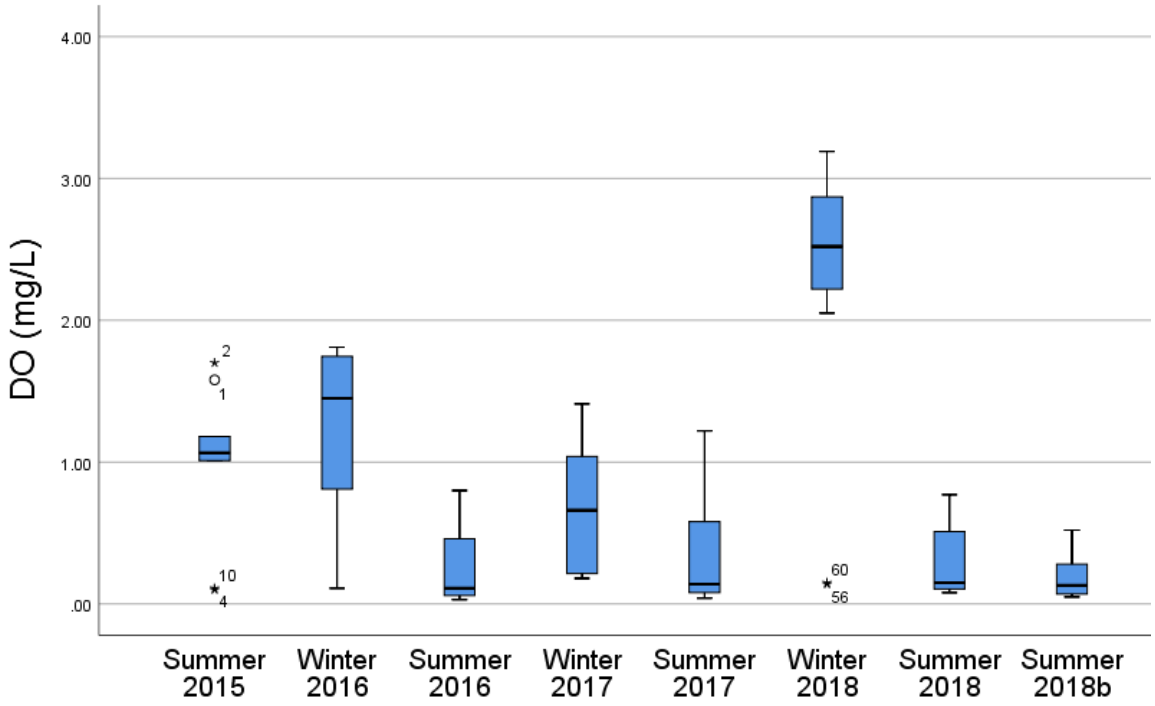


Figure 2-5: Dissolved oxygen concentrations for each sampling event measured in the oxidation ditch of Cookeville WRRF. Boxplots show the median value as a black bold bar, the upper and lower limits of the box being the third and first quartile of the data, the whiskers extend up to 1.5 times the interquartile range, and open circles are outlier points, with stars denoting extreme outliers.

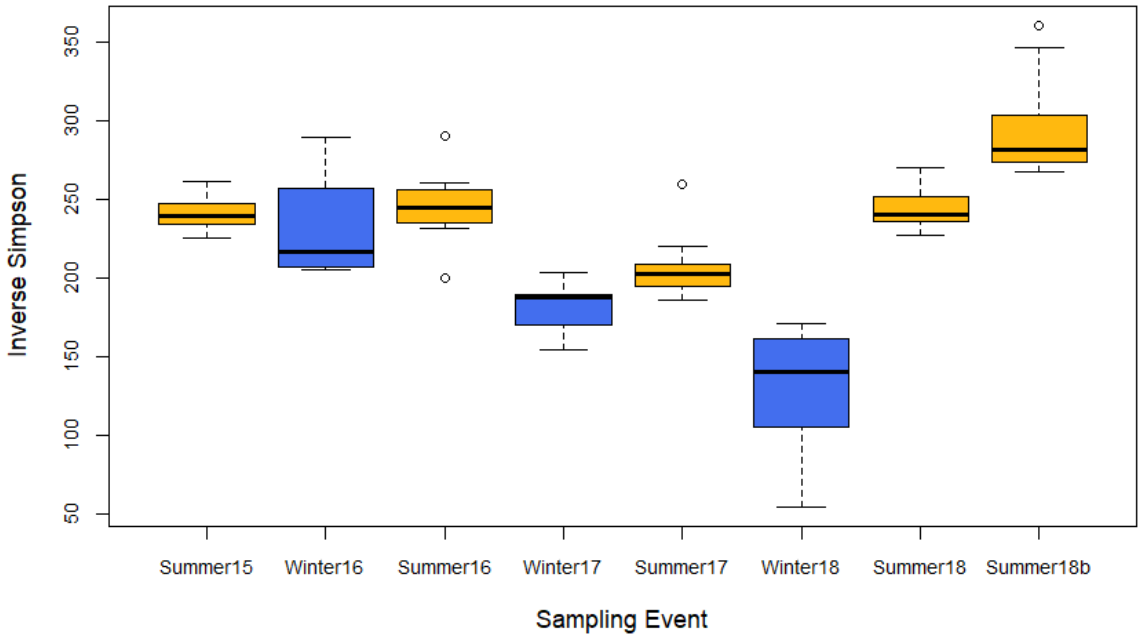


Figure 2-6: Alpha diversity across sampling events for the total microbial community for Cookeville WRRF. Boxplots show the median value as a black bold bar, the upper and lower limits of the box being the third and first quartile of the data, the whiskers extend up to 1.5 times the interquartile range, and open circles are outlier points.

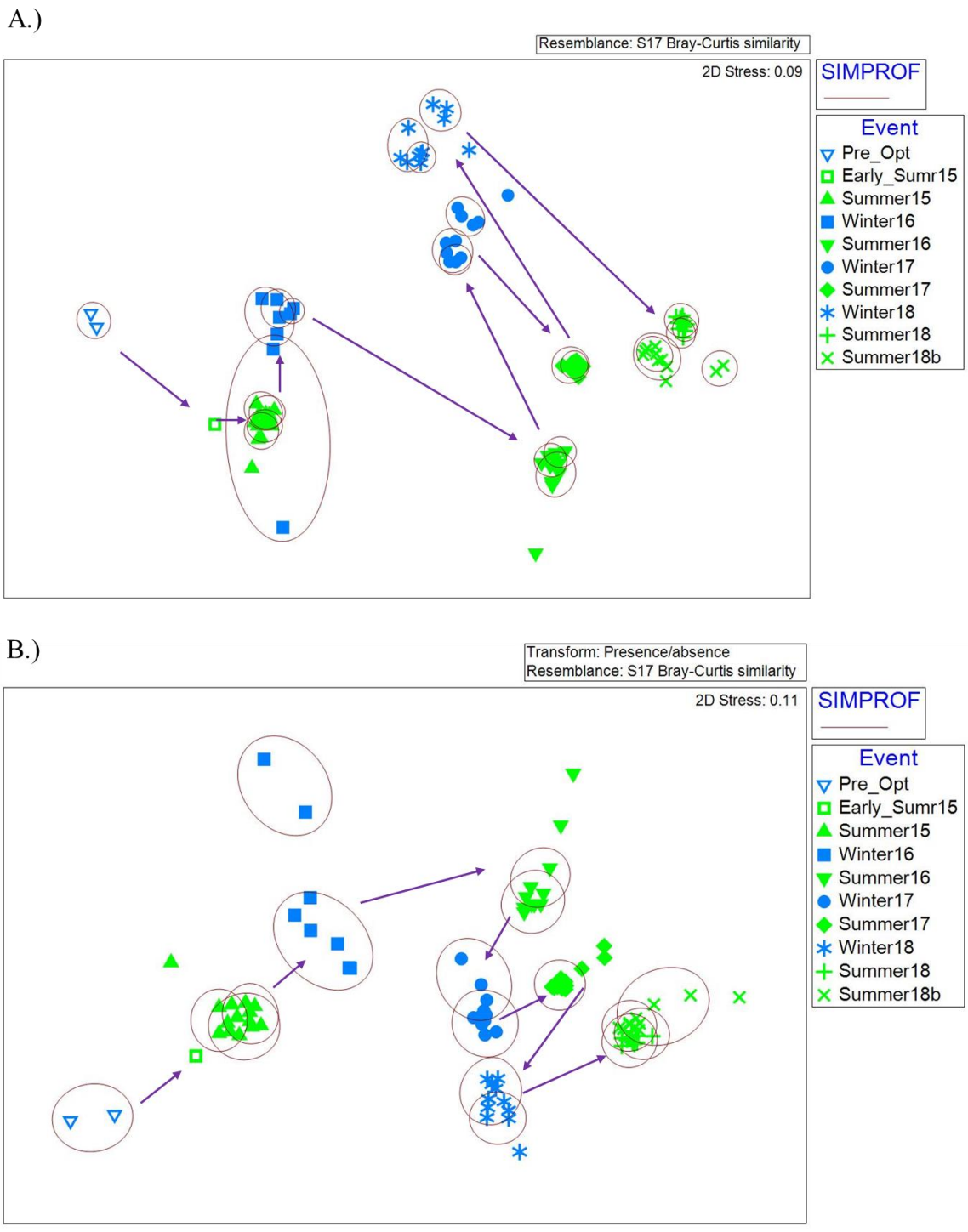


Figure 2-7: NMDS on species level for total microbial community from Cookeville WRRF based on A.) relative abundance and B.) presence/absence. Red circles indicate samples that are significantly similar ($p<0.05$) in regards to community composition. Purple arrows depict the order of sampling events.

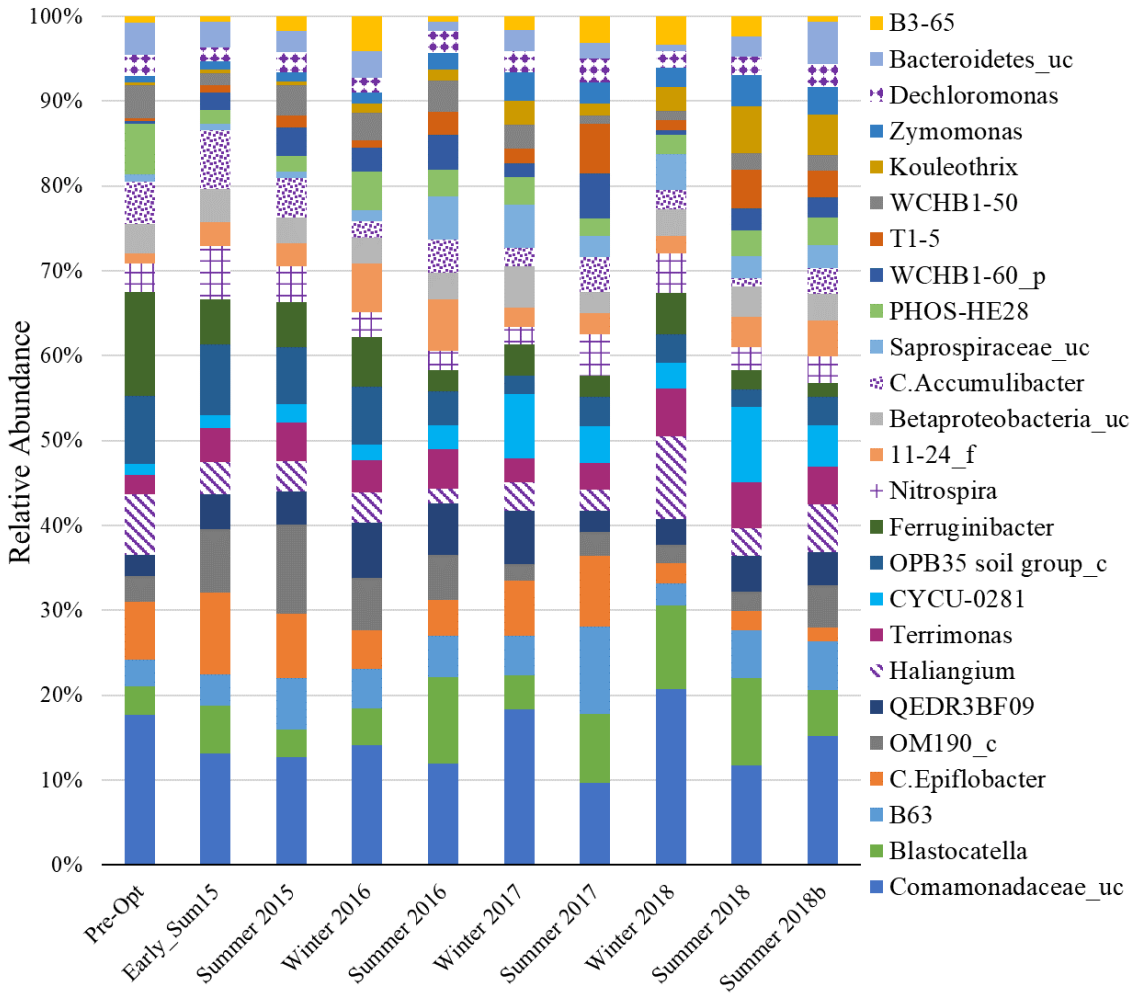


Figure 2-8: The total community compostion of the 25 most abundant genera observed across sampling events in Cookeville WRRF.

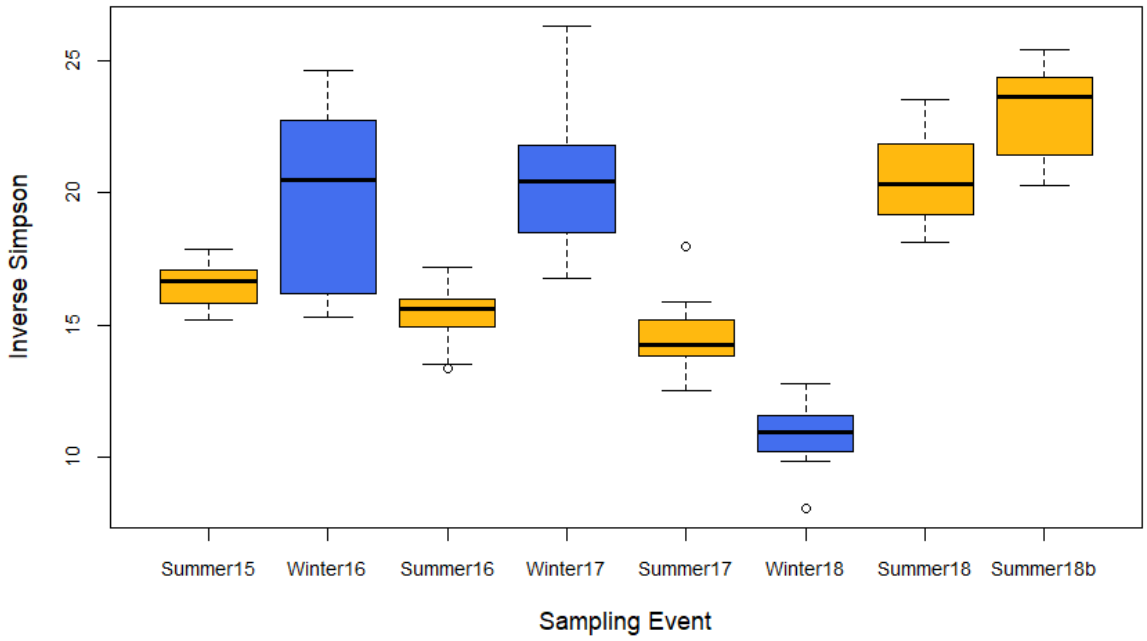


Figure 2-9: Alpha diversity across sampling events from Cookeville WRRF for the BNR-relevant microbial community. Boxplots show the median value as a black bold bar, the upper and lower limits of the box being the third and first quartile of the data, the whiskers extend up to 1.5 times the interquartile range, and open circles are outlier points.

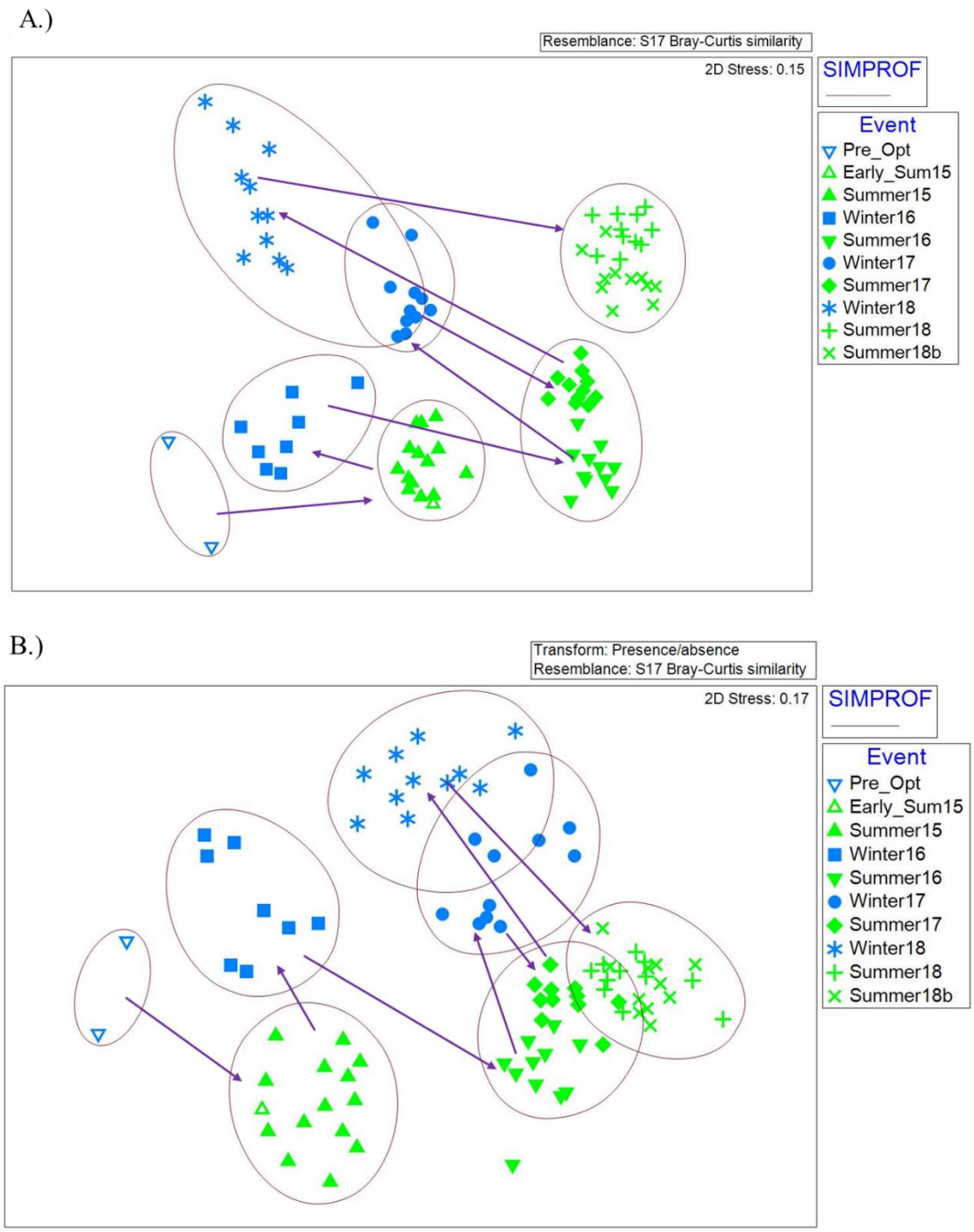


Figure 2-10: NMDS on species level for BNR-relevant microbial community from Cookeville WRRF based on A.) relative abundance and B.) presence/absence. Red circles indicate samples that are significantly similar ($p<0.05$) in regards to community composition. Purple arrows depict the order of sampling events.

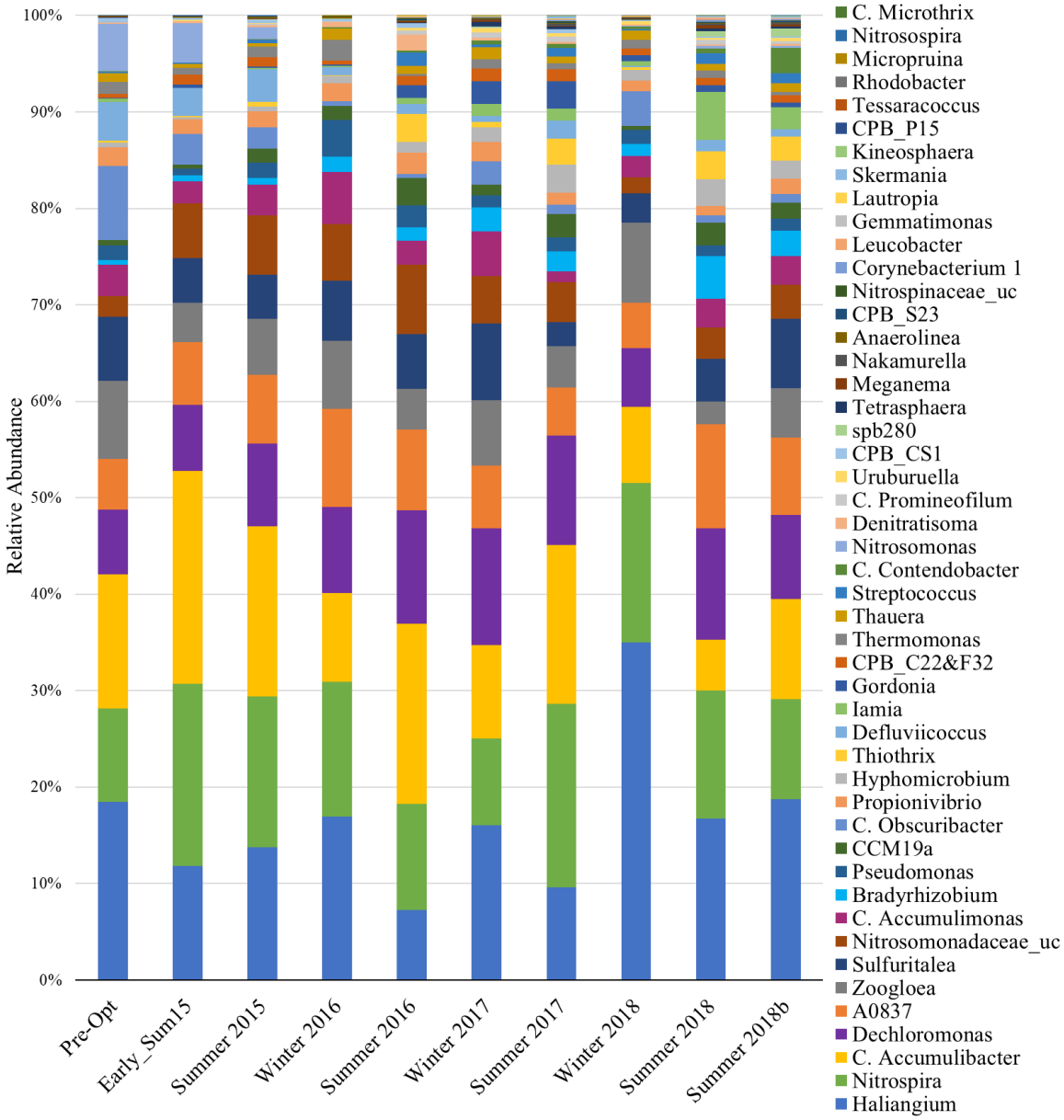


Figure 2-11: Genera-level BNR-relevant microbial community composition for each sampling event from Cookeville WRRF.

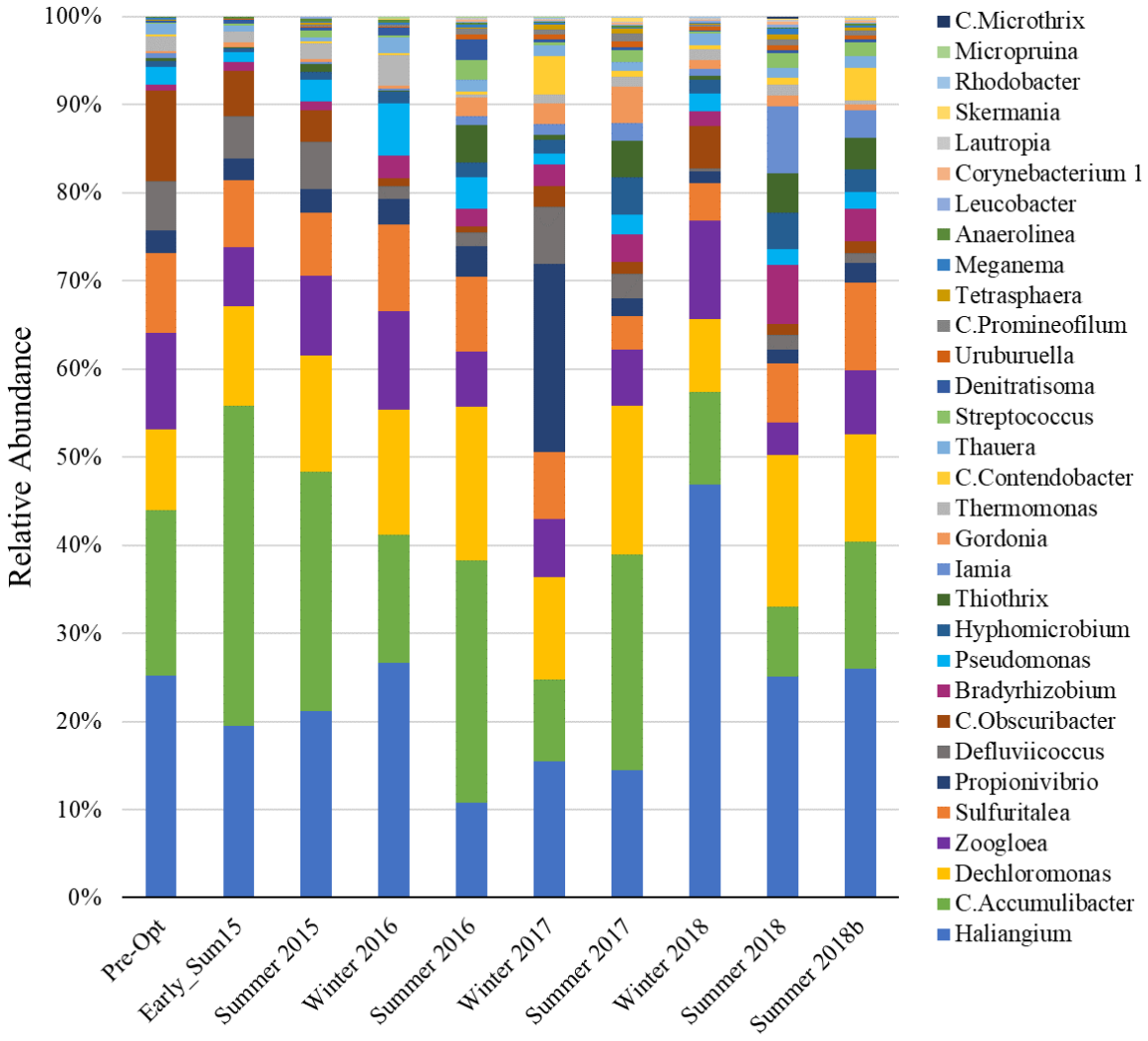


Figure 2-12: Genera with the potential to contribute to denitrification for each sampling event from Cookeville WRRF.

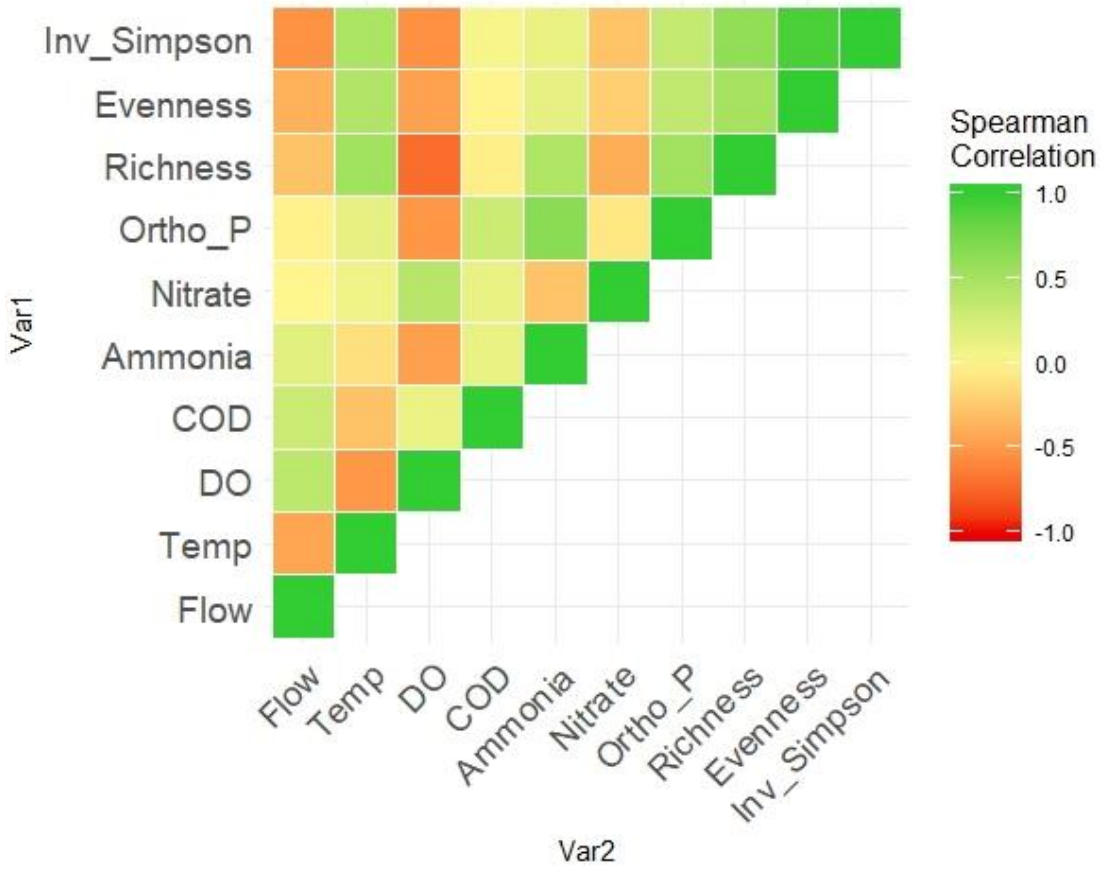


Figure 2-13: Correlation of Cookeville's wastewater characteristics to alpha diversity measurements of the total community.

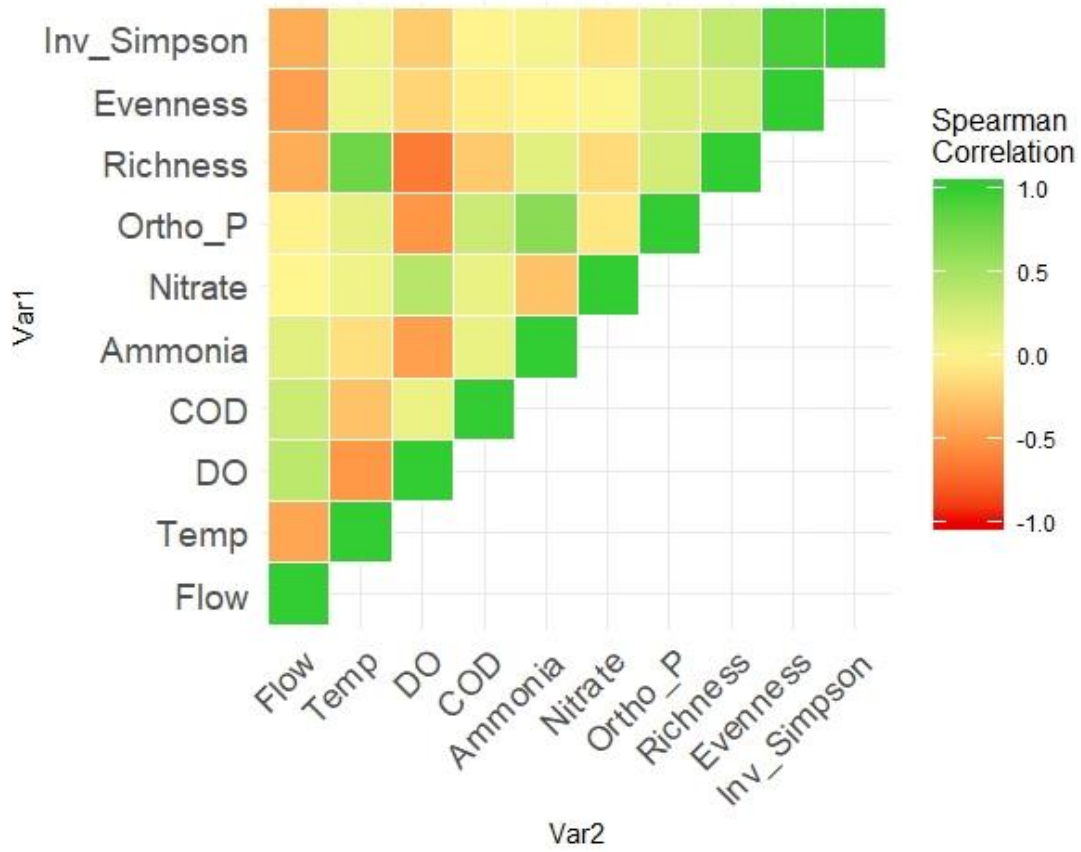


Figure 2-14: Correlation of Cookeville's wastewater characteristics to alpha diversity measurements of the BNR-relevant community.

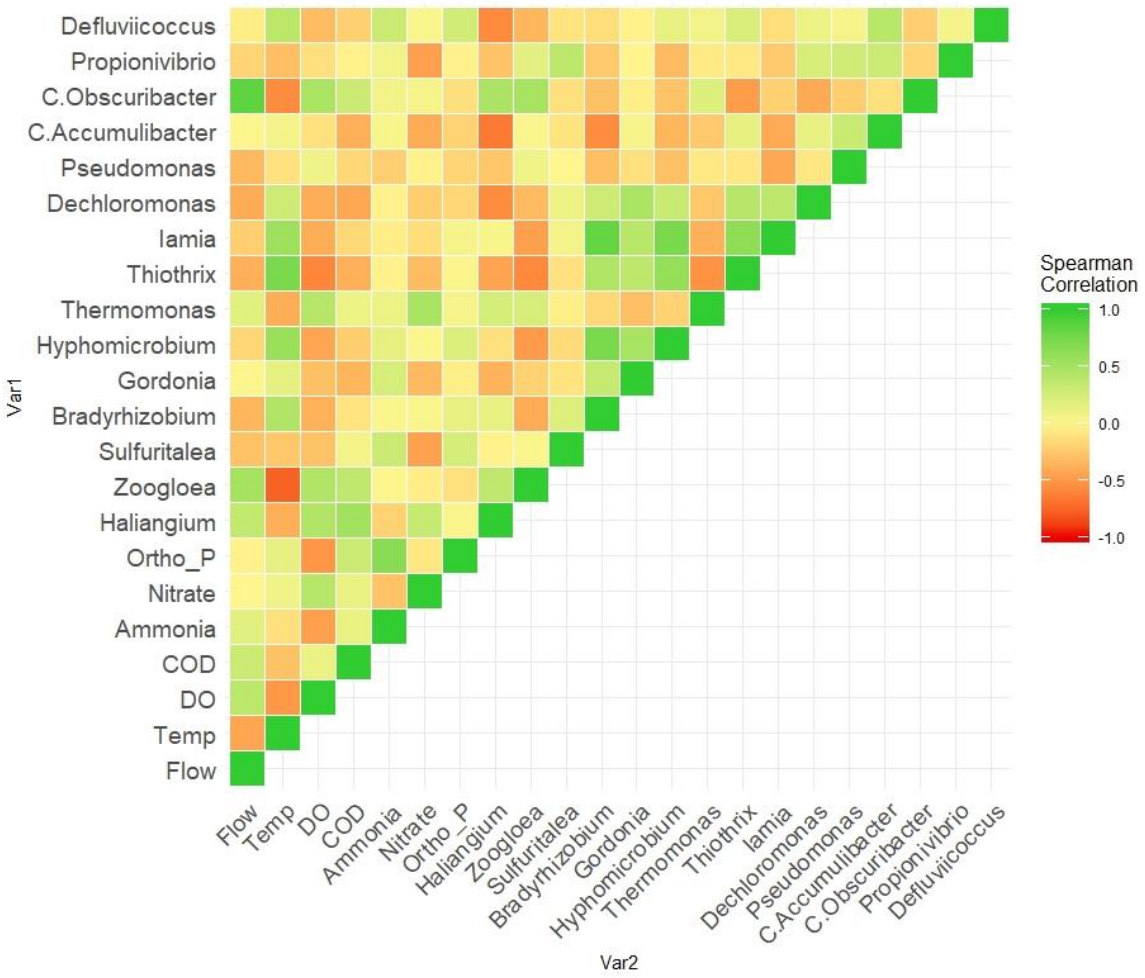


Figure 2-15: Correlation of Cookeville's wastewater characteristics to the most abundant genera with potential to contribute to denitrification.

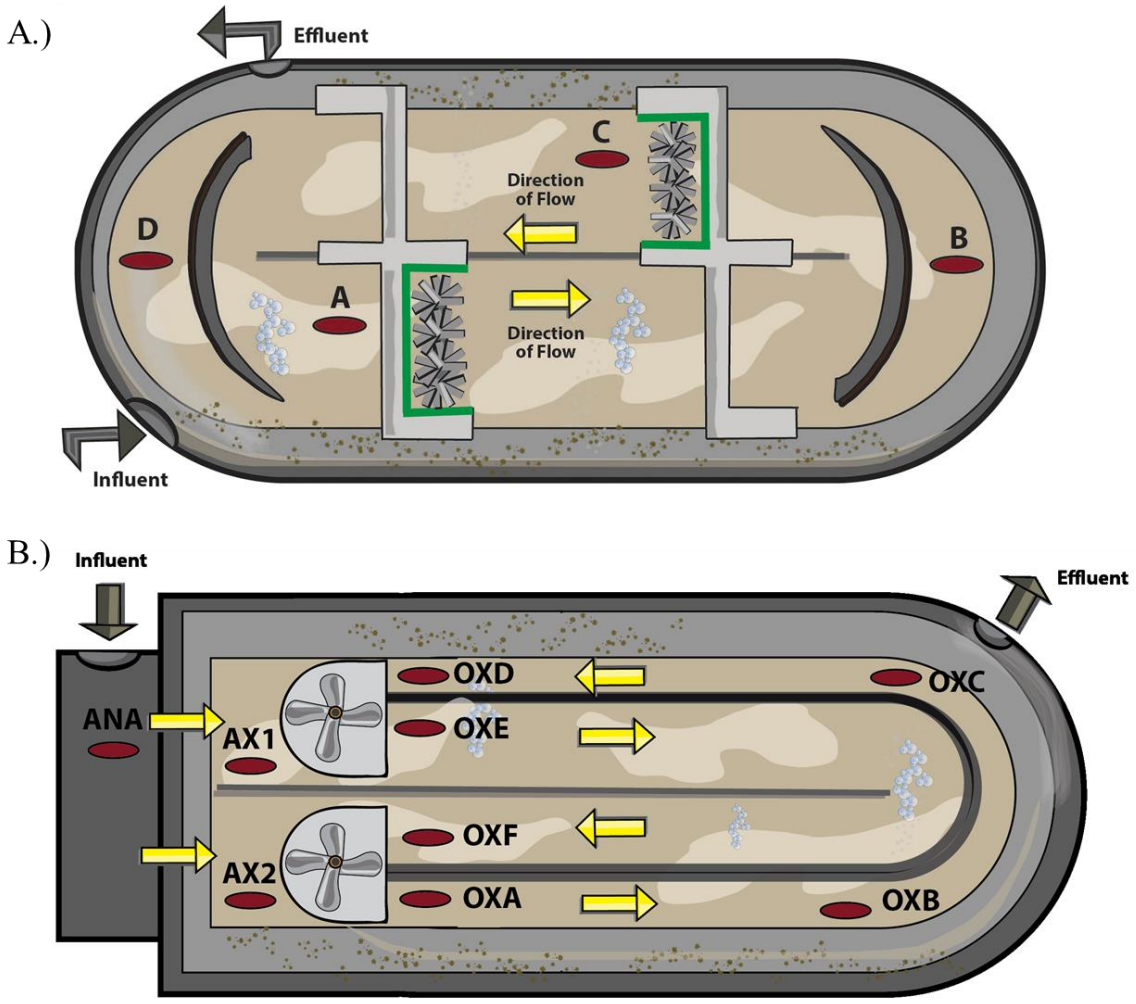


Figure 3-1: Aerial schematic of the secondary treatment processes for A.) Etowah WRRF and B.) Maryville WRRF during sampling events. Green indicates the pair of surface aerators in operation during sampling. Red dots (⬭) represent sampling locations.

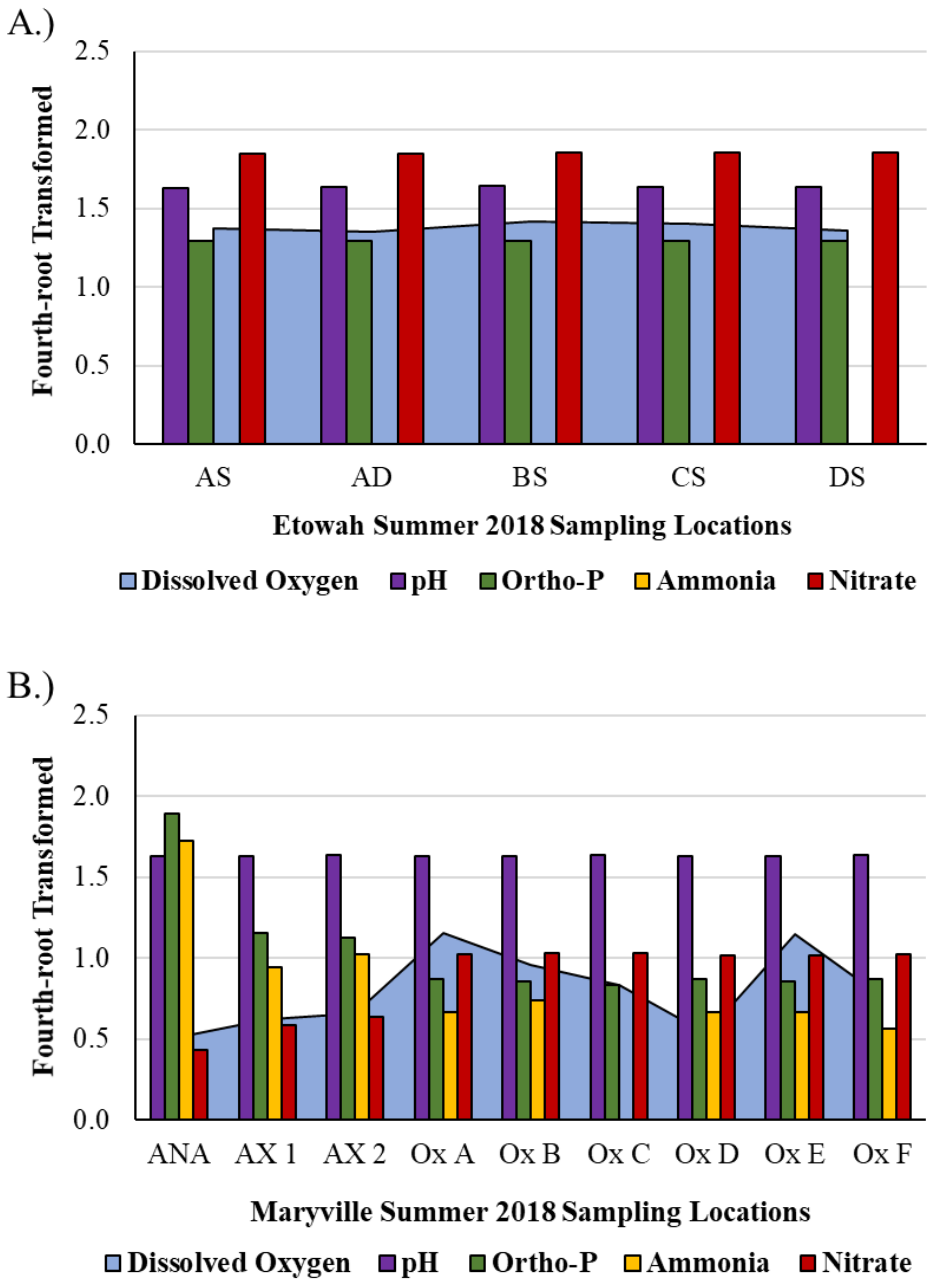


Figure 3-2: Typical trends of the wastewater characteristics in the secondary treatment processes for A.) Etowah and B.) Maryville. Sampling locations with an 'S' indicate a grab sample collected at the surface and locations with a 'D' indicate a sample collected at the bottom of the oxidation ditch; 'ANA' represents the anaerobic reactor and 'AX' represents the anoxic reactors.

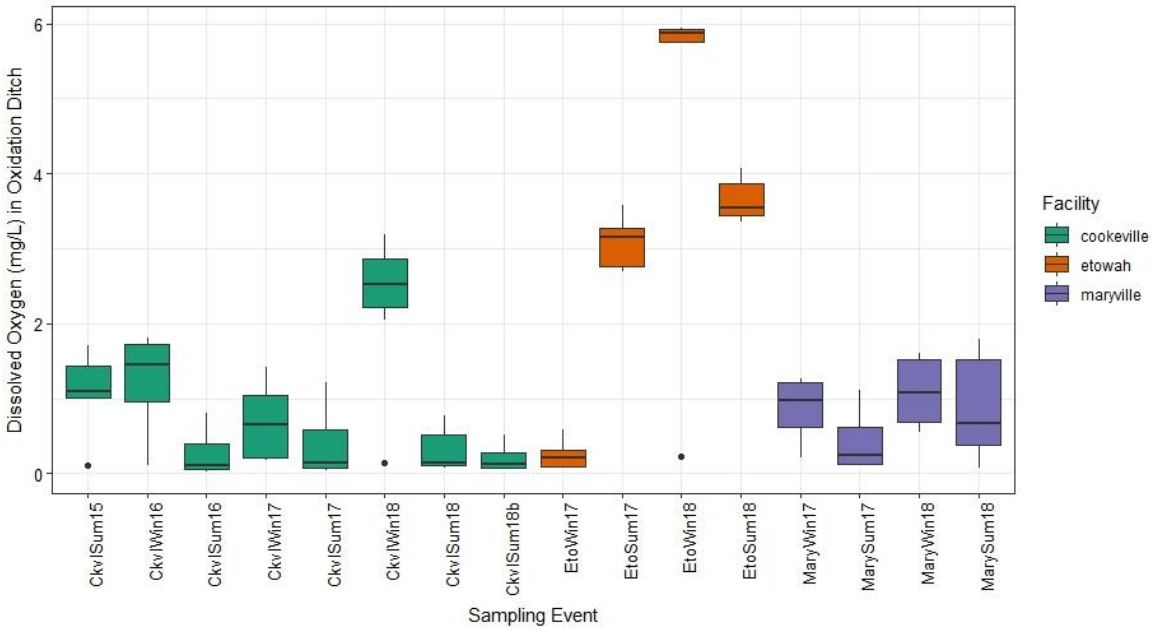
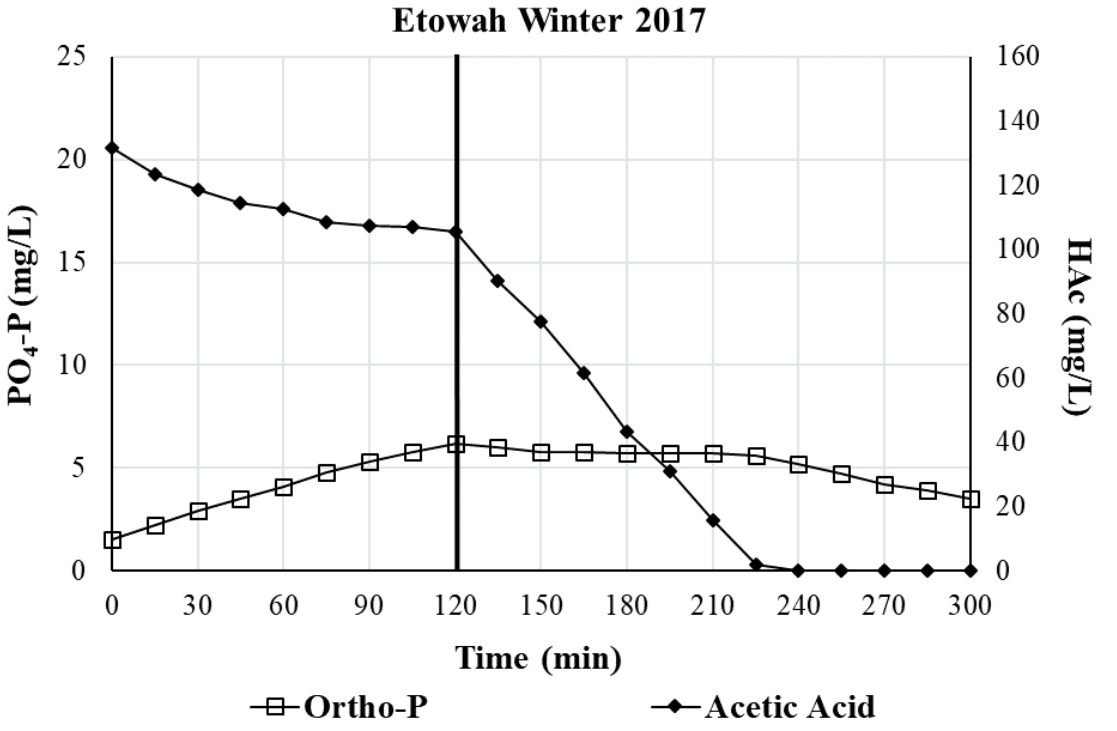
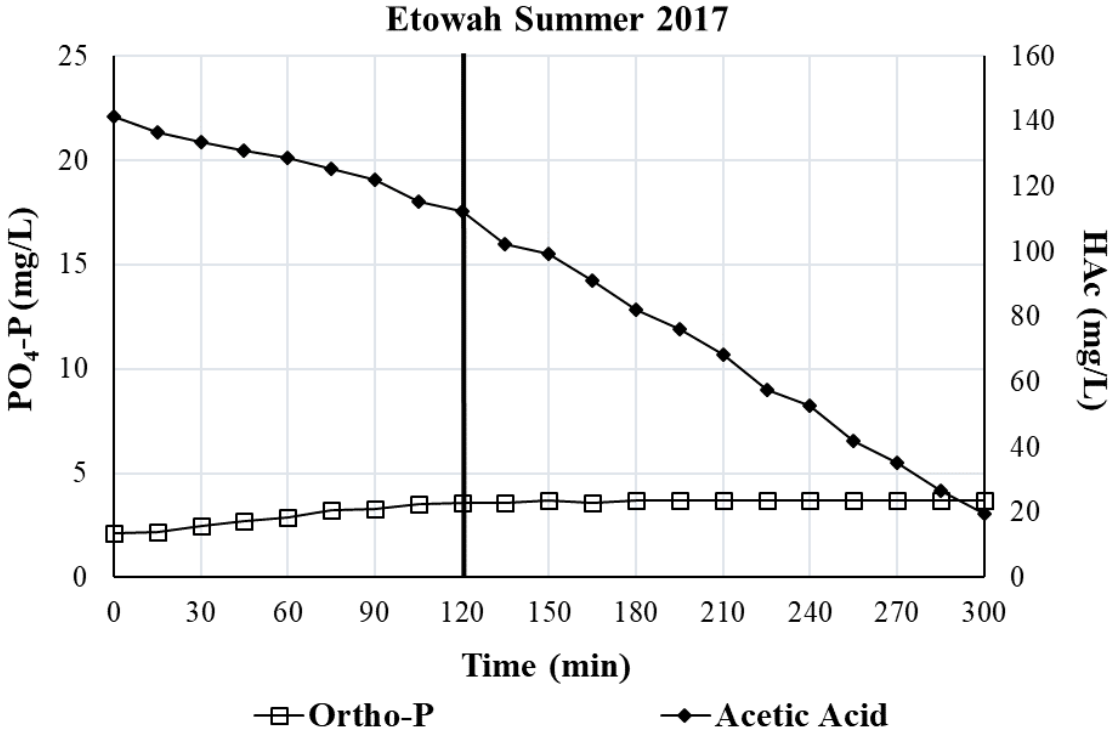
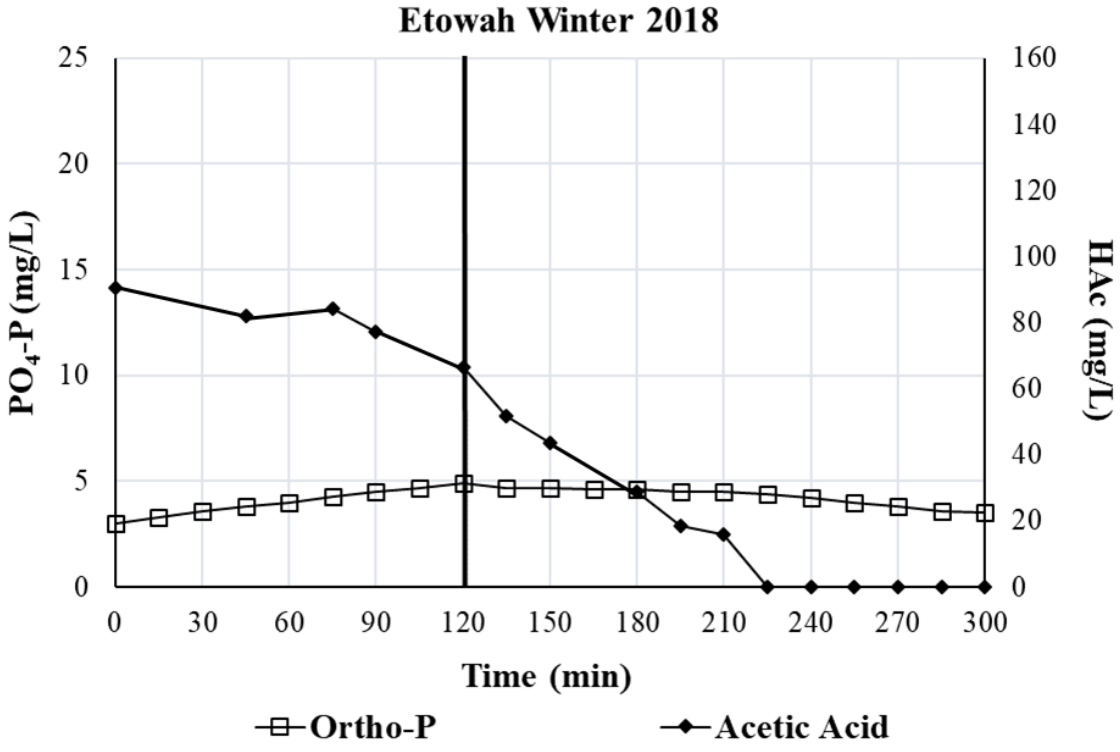
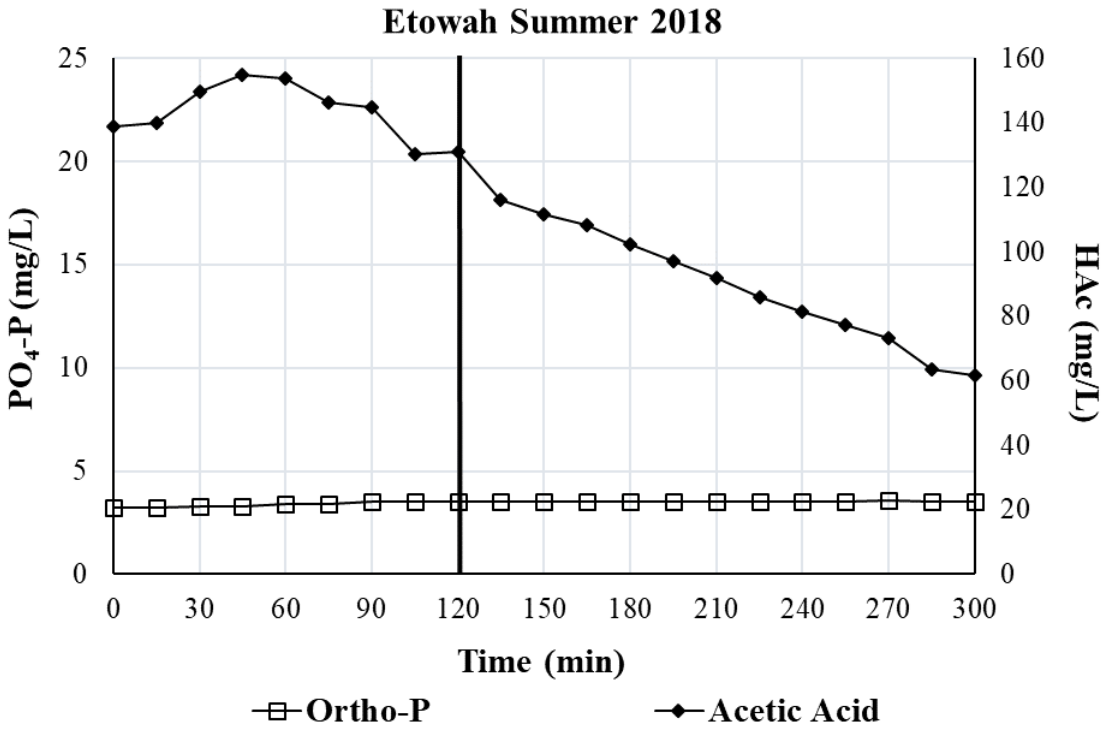


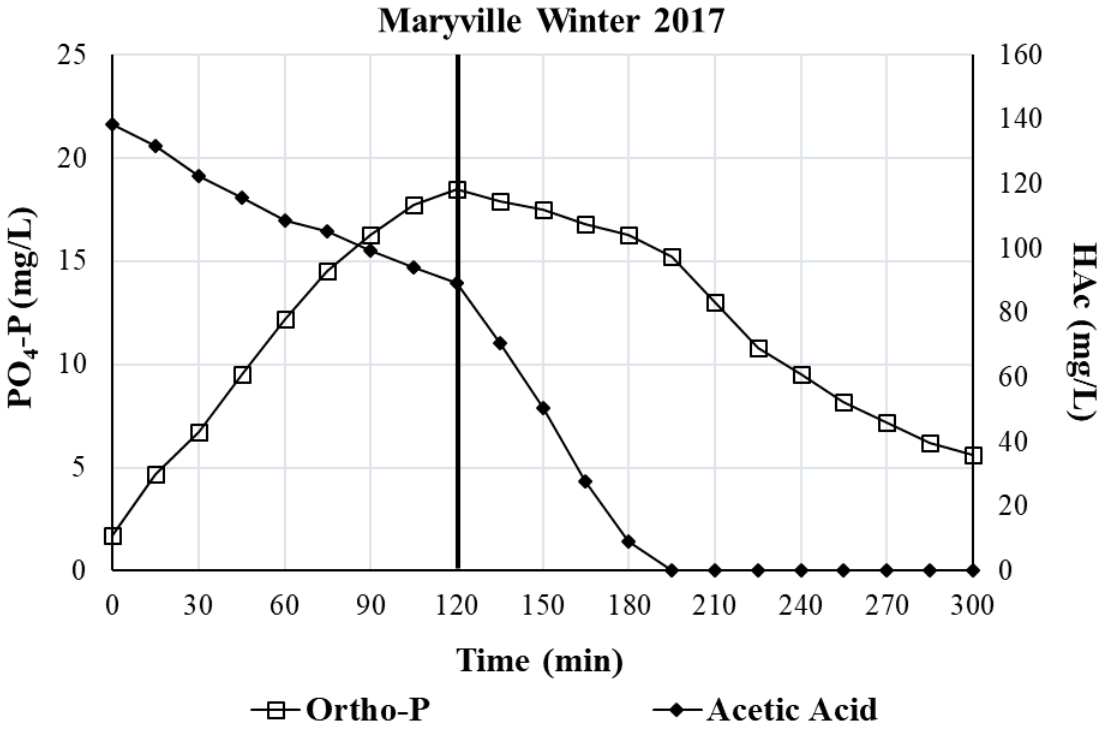
Figure 3-3: Dissolved oxygen concentrations for each sampling event measured in the oxidation ditches. Boxplots show the median value as a black bold bar, the upper and lower limits of the box being the third and first quartile of the data, the whiskers extend up to 1.5 times the interquartile range, and black circles are outlier points.

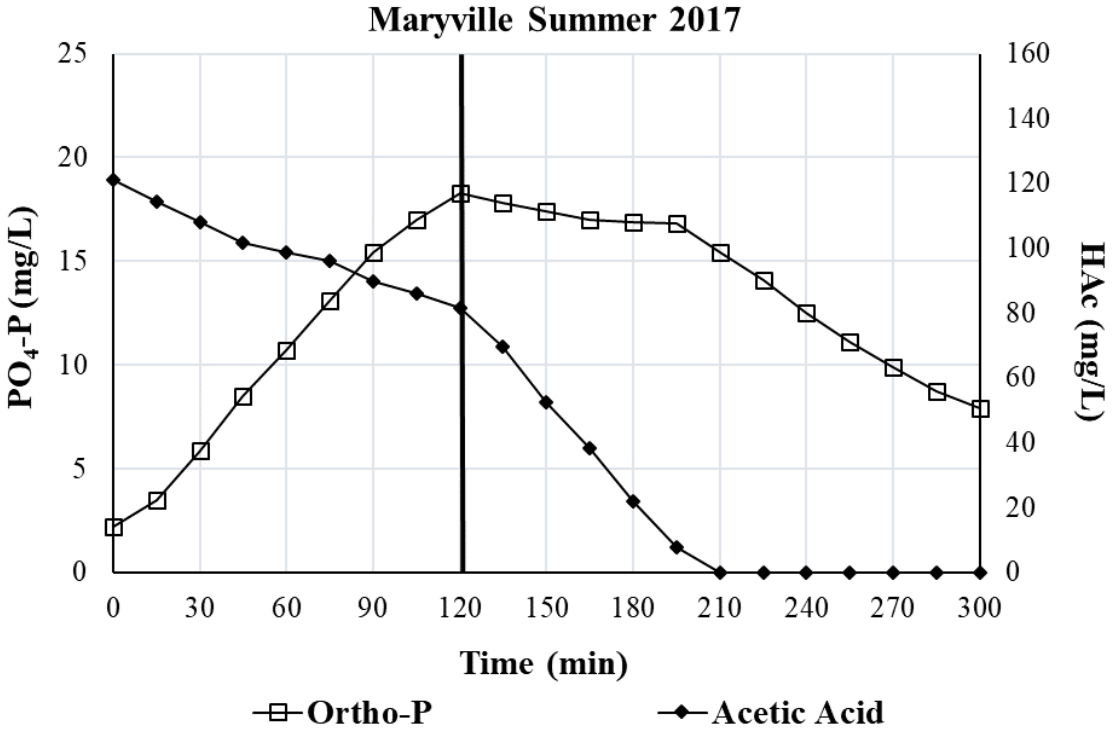

Etowah Winter 2017
PO4-P (mg/L)
HAc (mg/L)
Time (min)
Ortho-P
Acetic Acid


Etowah Summer 2017
PO4-P (mg/L)
HAc (mg/L)
Time (min)
Ortho-P
Acetic Acid


Etowah Winter 2018
PO4-P (mg/L)
HAc (mg/L)
Time (min)
Ortho-P
Acetic Acid


Etowah Summer 2018
PO4-P (mg/L)
HAc (mg/L)
Time (min)
Ortho-P
Acetic Acid


Maryville Winter 2017
PO4-P (mg/L)
HAc (mg/L)
Time (min)
Ortho-P
Acetic Acid


Maryville Summer 2017
PO4-P (mg/L)
HAc (mg/L)
Time (min)
Ortho-P
Acetic Acid

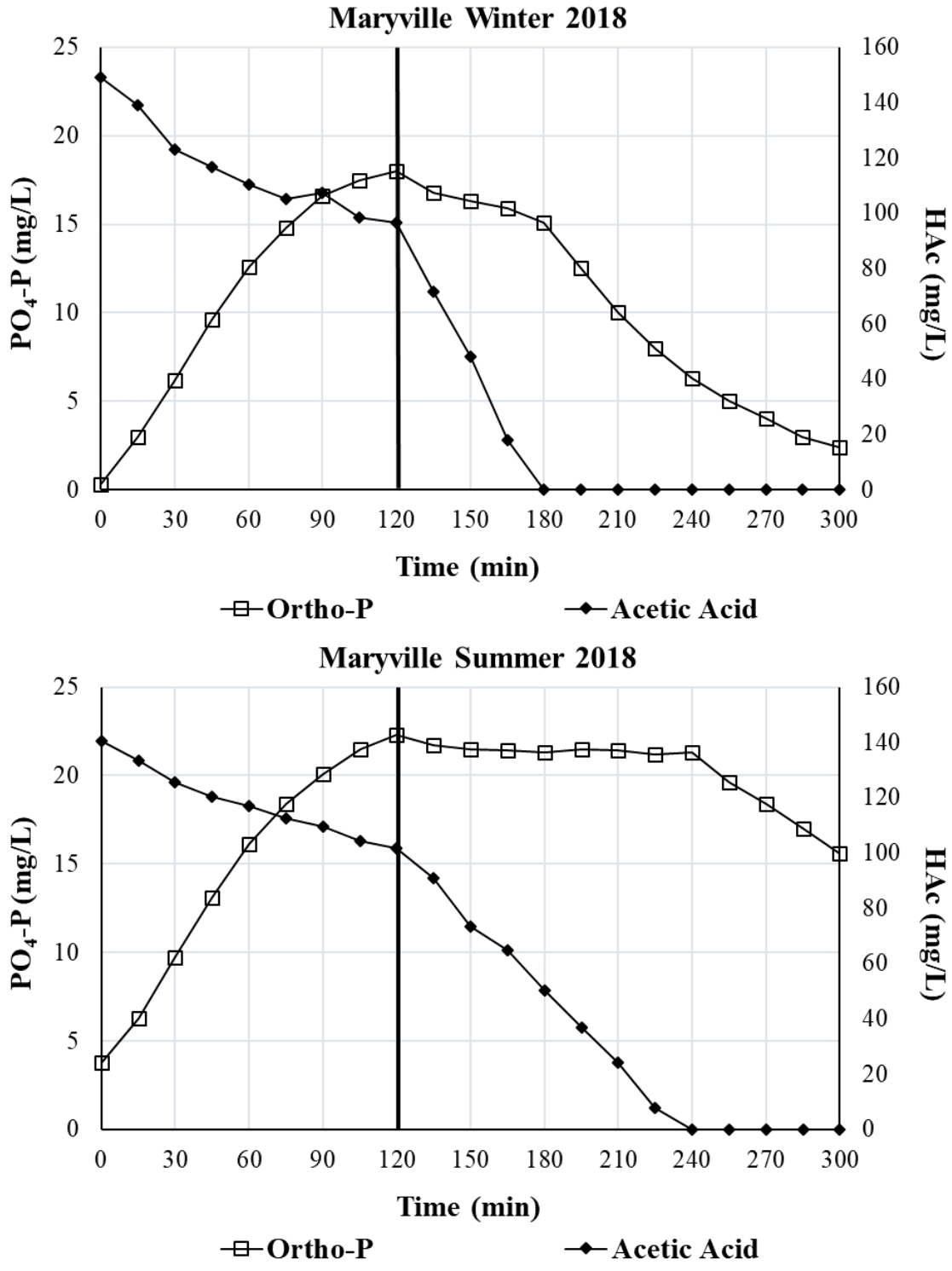


Figure 3-4: Graphical representations of biological phosphorus removal batch tests for Etowah and Maryville.

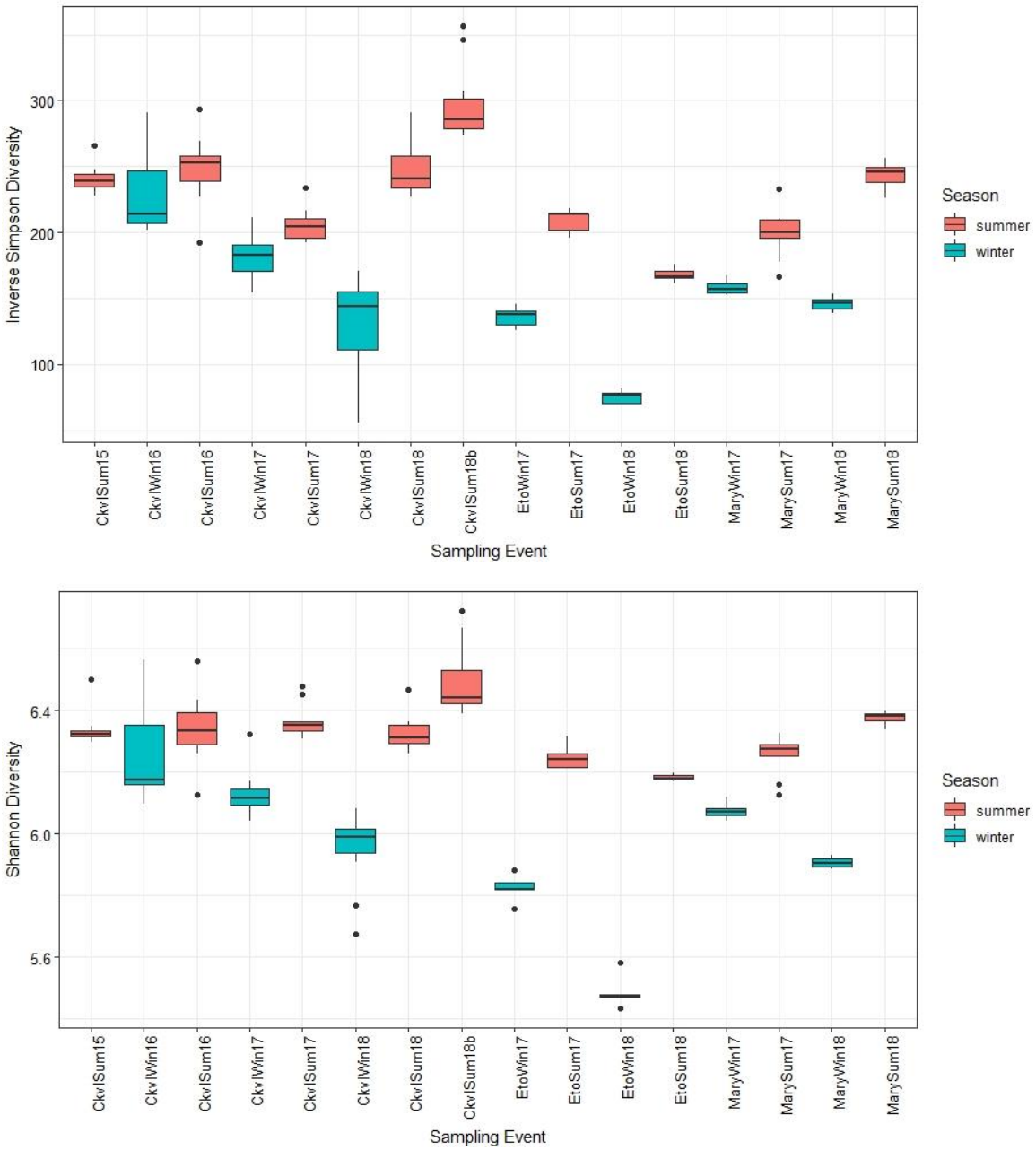


Figure 3-5: Alpha diversity across sampling events for the total microbial community. Boxplots show the median value as a black bold bar, the upper and lower limits of the box being the third and first quartile of the data, the whiskers extend up to 1.5 times the interquartile range, and black circles are outlier points.

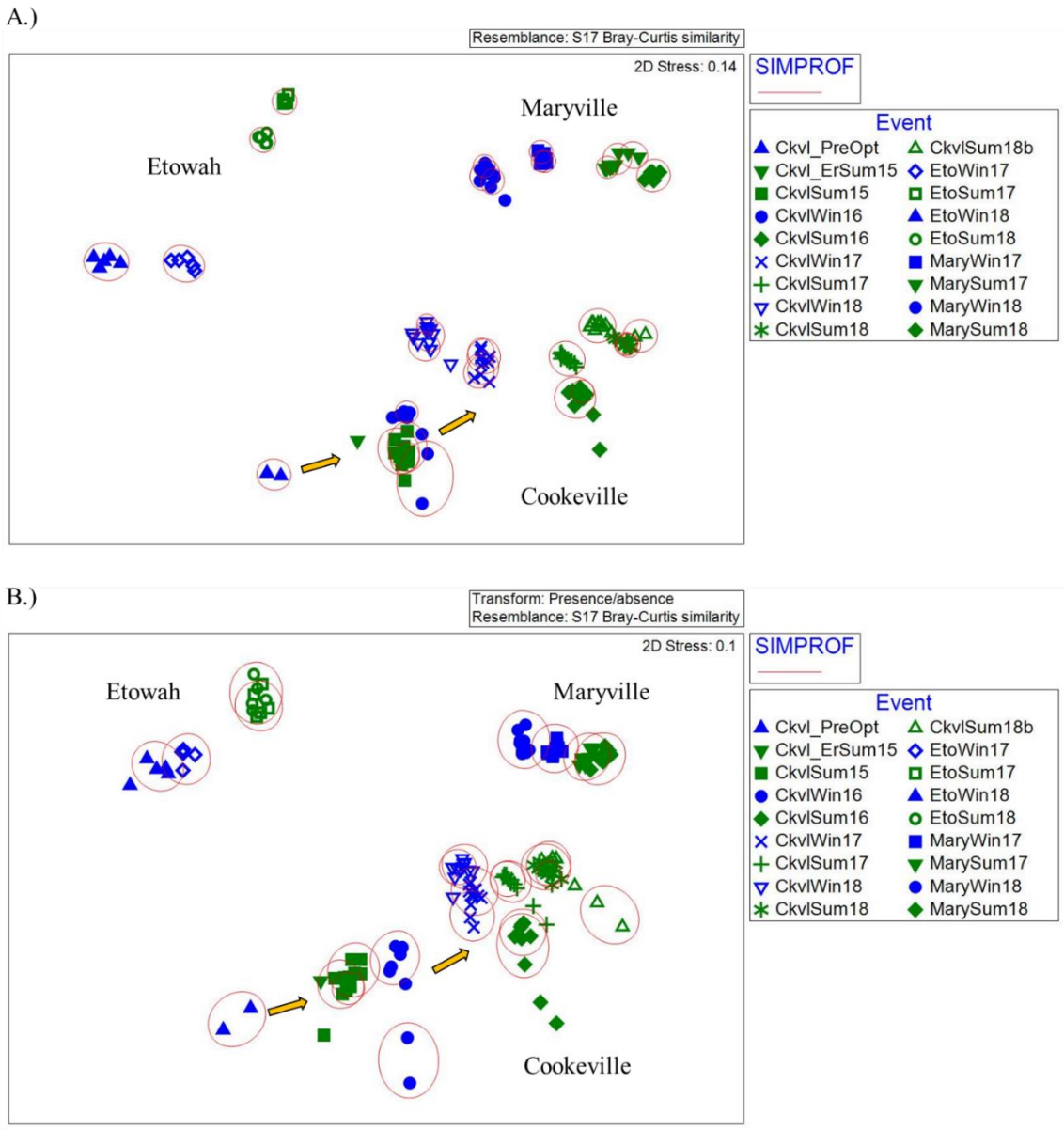


Figure 3-6: NMDS on species level for total microbial community based on A.) relative abundance and B.) presence/absence. Red circles indicate samples that are significantly similar ($p<0.05$) in regards to community composition. Yellow arrows depict the order of sampling events for Cookeville WRRF.

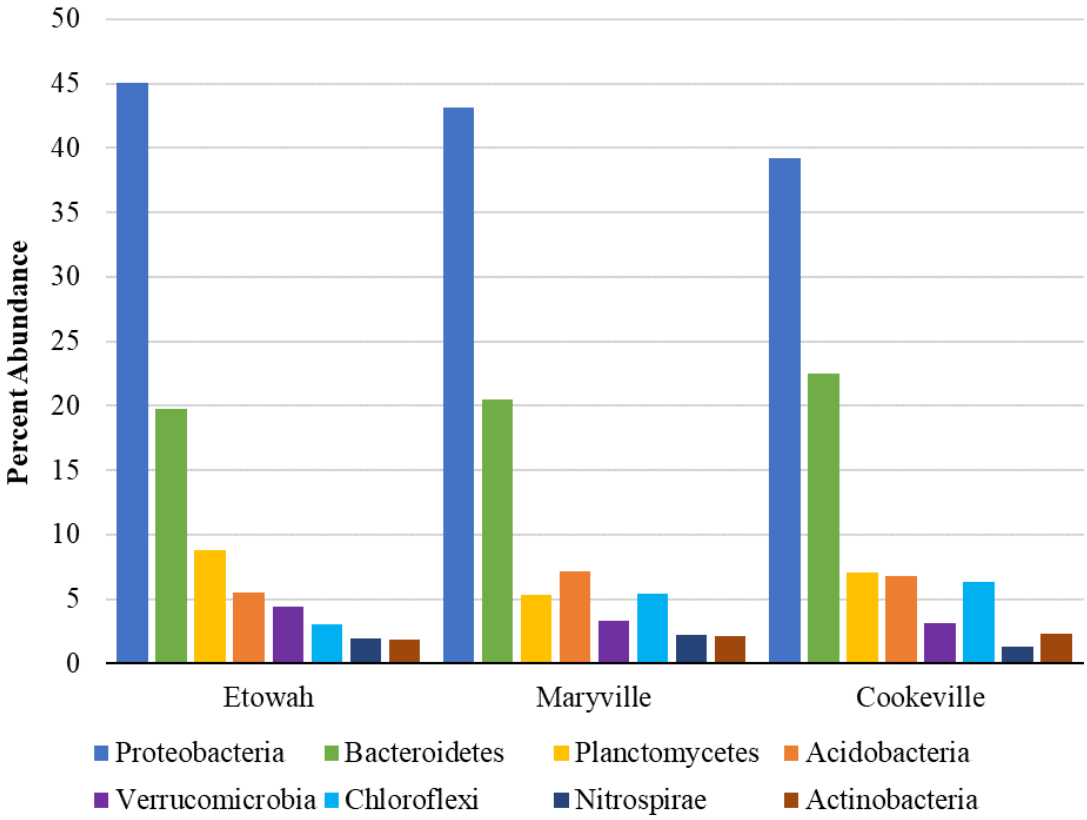
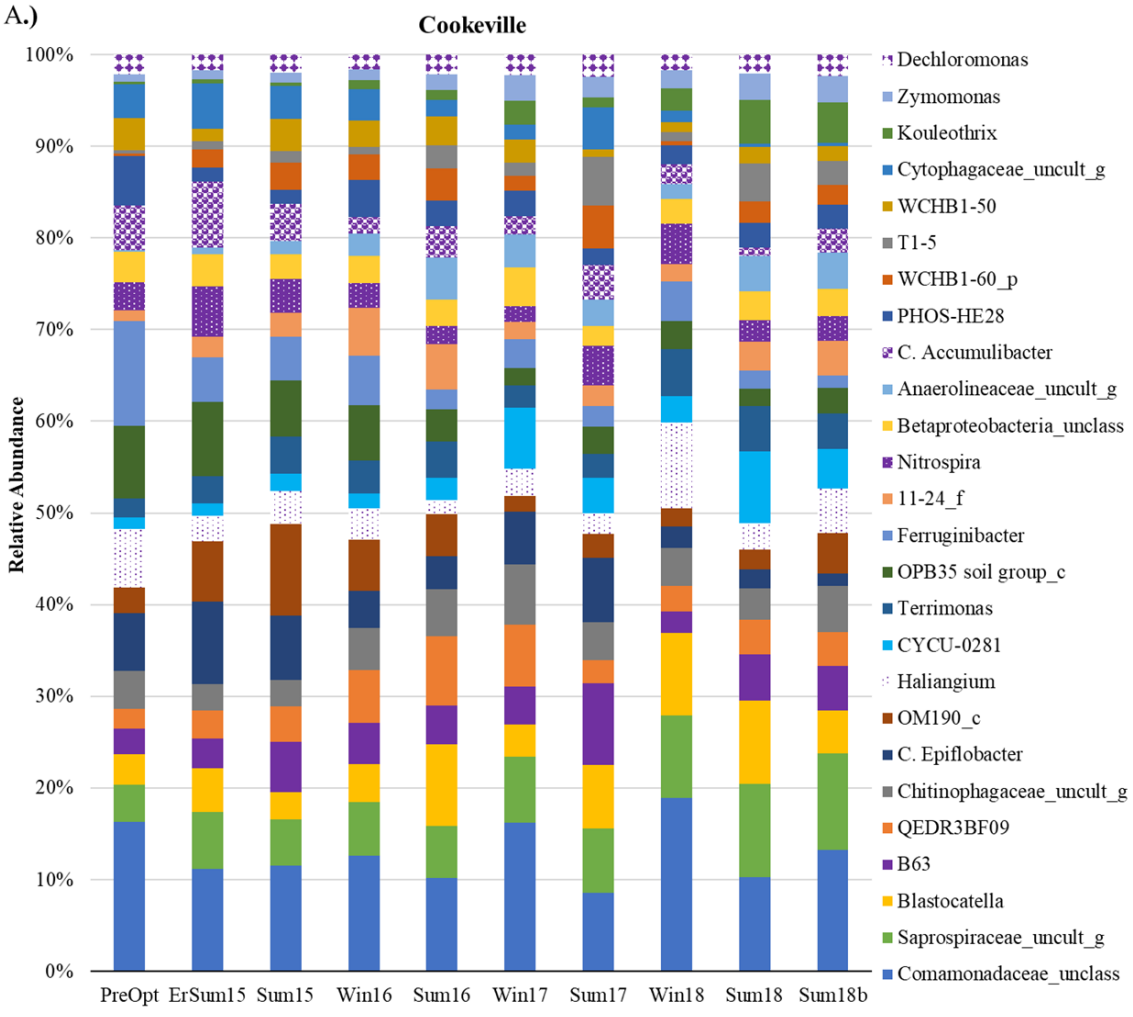
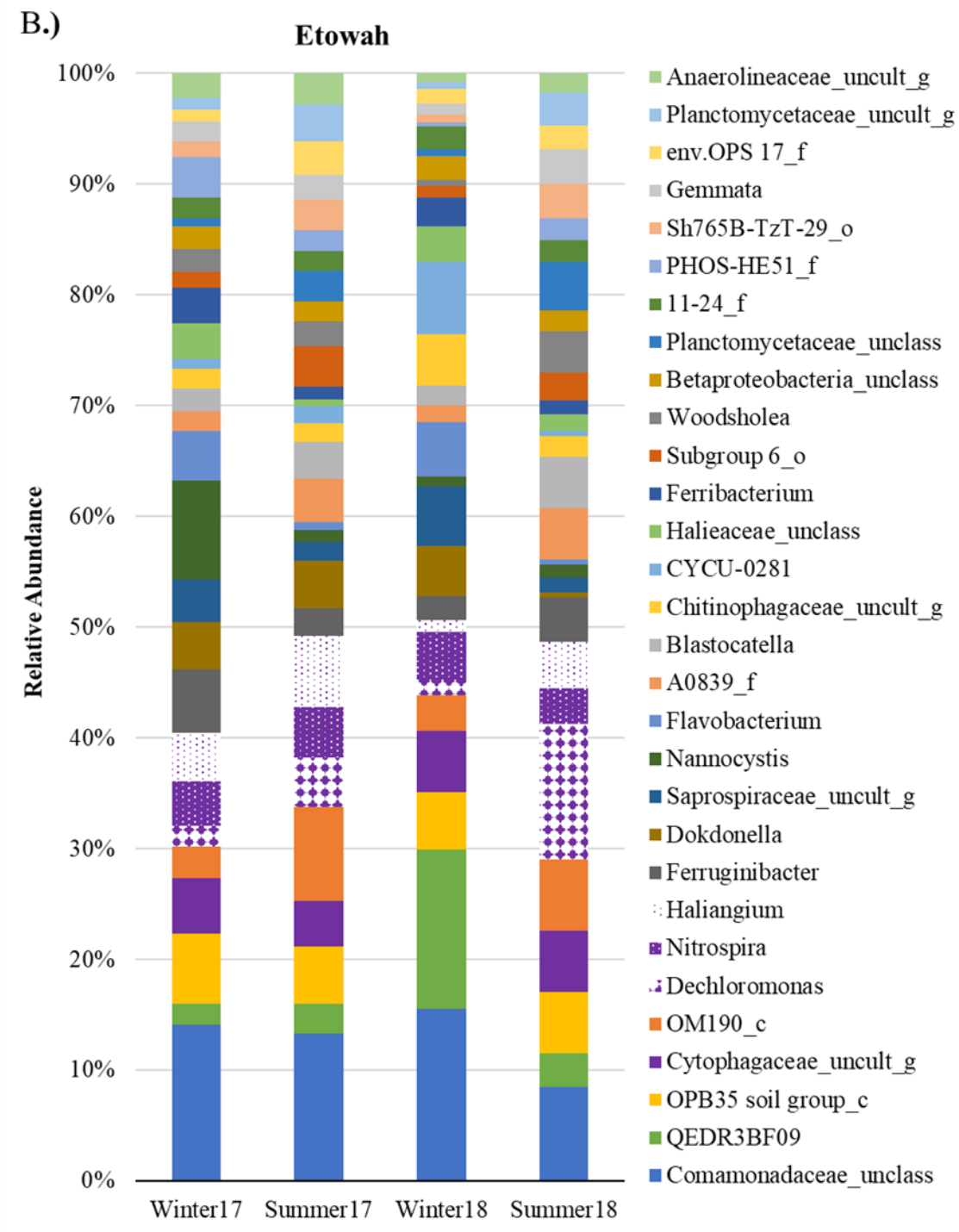


Figure 3-7: Top Phyla observed for each facility.

A.)


Cookeville
Relative Abundance
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
PreOpt
ErSum15
Sum15
Win16
Sum16
Win17
Sum17
Win18
Sum18
Sum18b
Dechloromonas
Zymomonas
Kouleothrix
Cytophagaceae_uncult_g
WCHB1-50
T1-5
WCHB1-60_p
PHOS-HE28
C. Accumulibacter
Anaerolineaceae_uncult_g
Betaproteobacteria_unclass
Nitrospira
11-24_f
Ferruginibacter
OPB35 soil group_c
Terrimonas
CYCU-0281
Haliangium
OM190_c
C. Epiflobacter
Chitinophagaceae_uncult_g
QEDR3BF09
B63
Blastocatella
Saprospiraceae_uncult_g
Comamonadaceae_unclass


B.)
Etowah
Relative Abundance
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Winter17
Summer17
Winter18
Summer18
Anaerolineaceae_uncult_g
Planctomycetaceae_uncult_g
env.OPS 17_f
Gemmata
Sh765B-TzT-29_o
PHOS-HE51_f
11-24_f
Planctomycetaceae_unclass
Betaproteobacteria_unclass
Woodsholea
Subgroup 6_o
Ferribacterium
Halieaceae_unclass
CYCU-0281
Chitinophagaceae_uncult_g
Blastocatella
A0839_f
Flavobacterium
Nannocystis
Saprospiraceae_uncult_g
Dokdonella
Ferruginibacter
Haliangium
Nitrospira
Dechloromonas
OM190_c
Cytophagaceae_uncult_g
OPB35 soil group_c
QEDR3BF09
Comamonadaceae_unclass

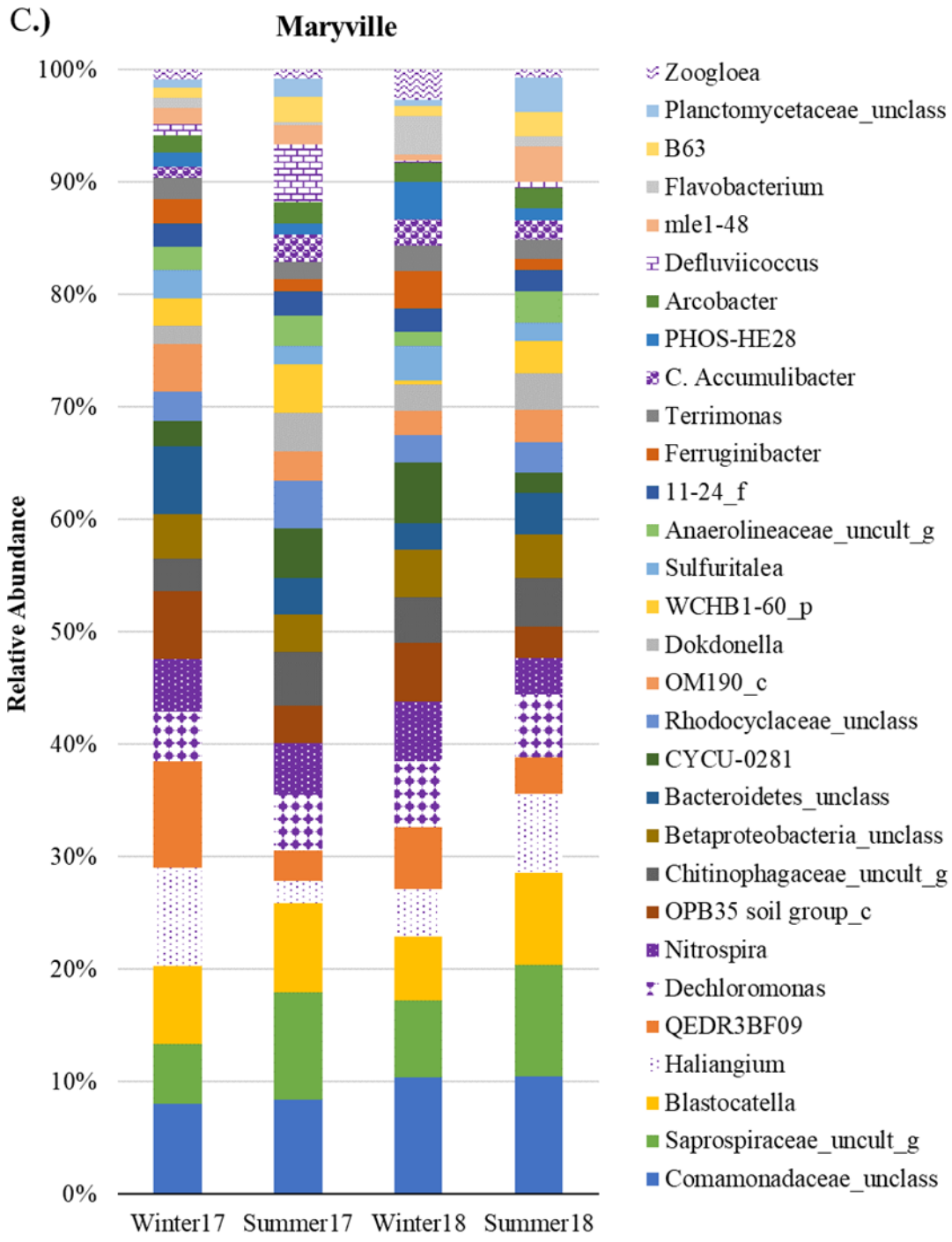


Figure 3-8: The total community compostion of the 30 most abundant genera observed across sampling events for A.) Cookeville, B.) Etowah, and C.) Maryville WRRF. Genera with hatch markings indicate BNR-relevant microorganisms.

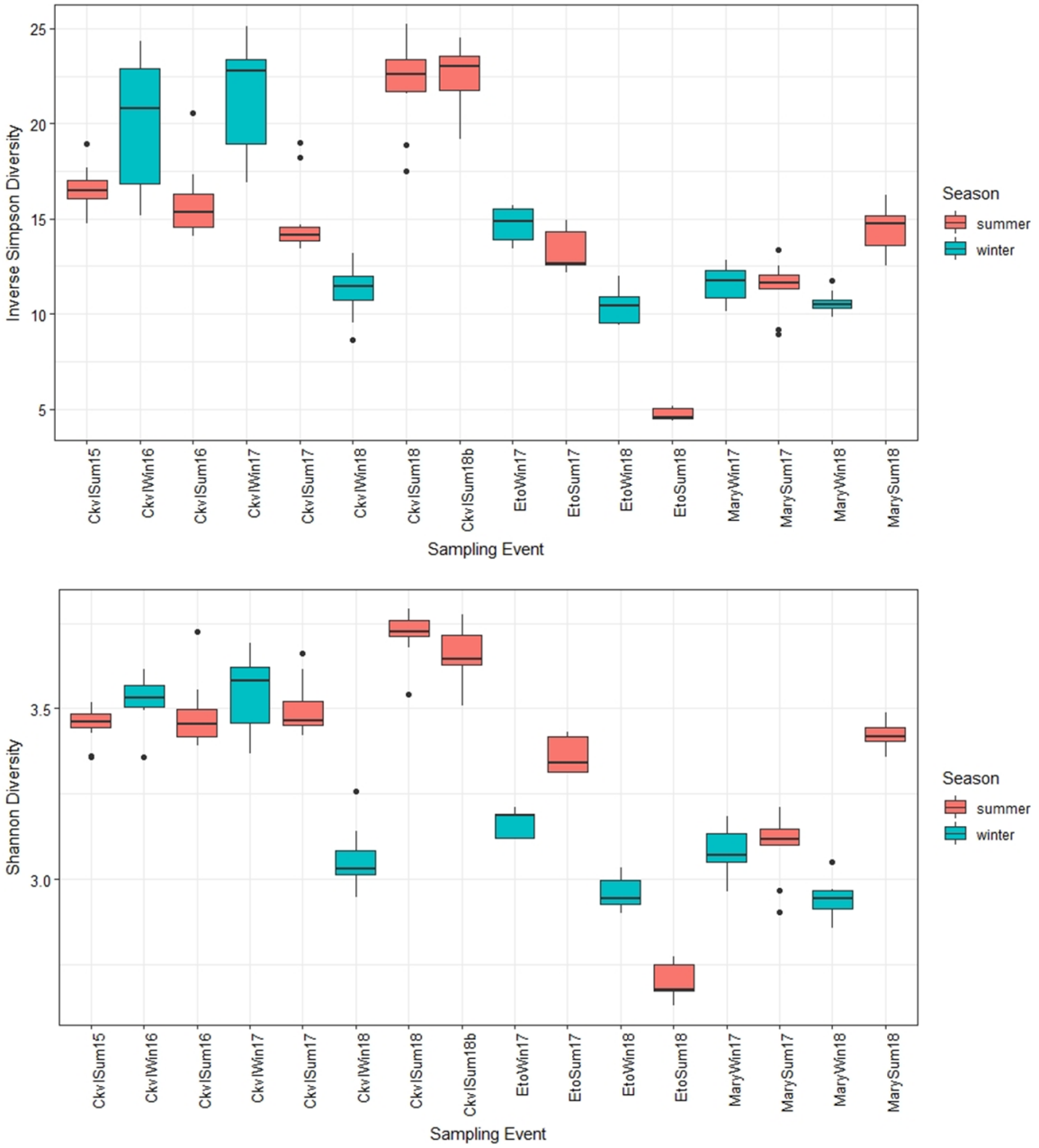


Figure 3-9: Alpha diversity across sampling events for the BNR-relevant microbial community. Boxplots show the median value as a black bold bar, the upper and lower limits of the box being the third and first quartile of the data, the whiskers extend up to 1.5 times the interquartile range, and black circles are outlier points.

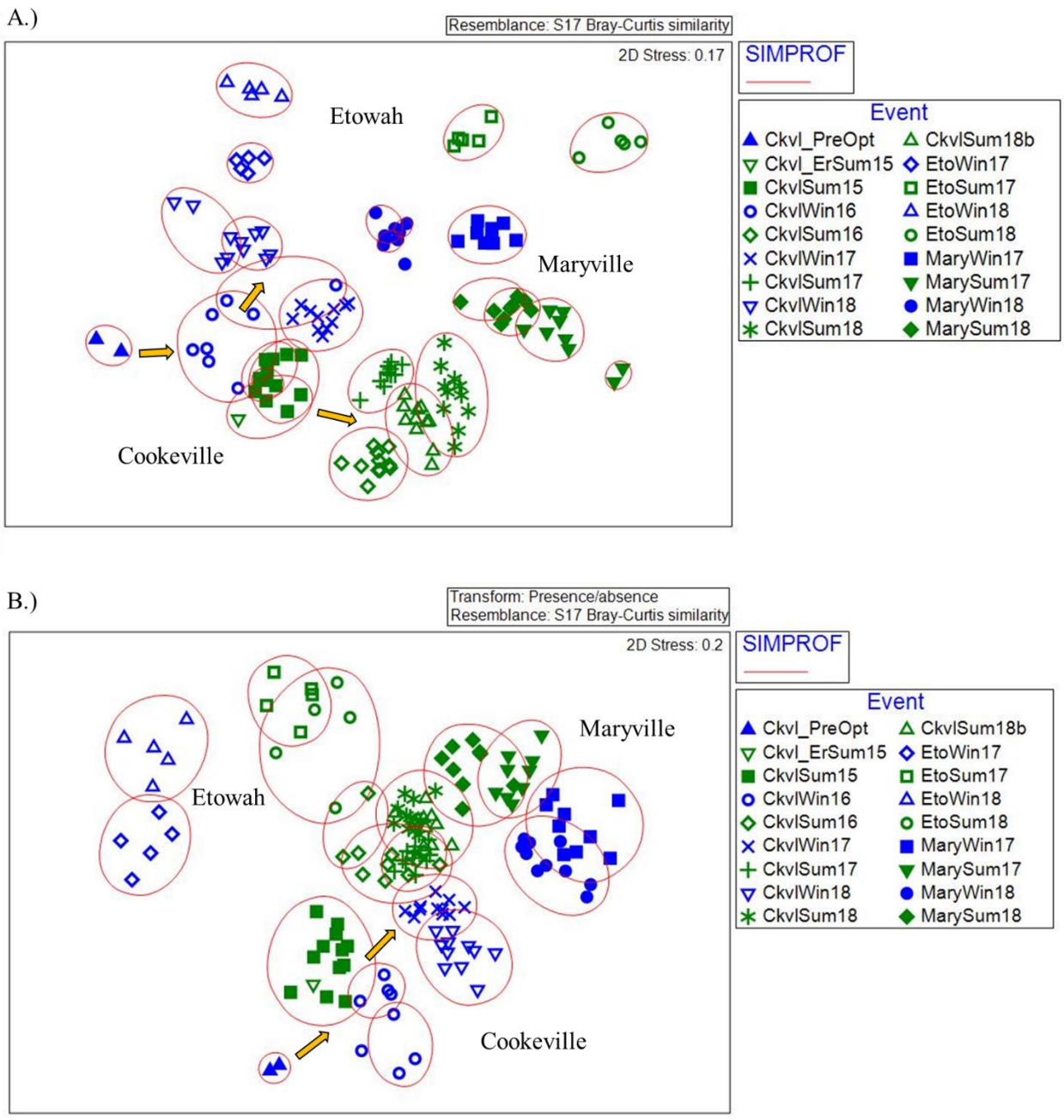
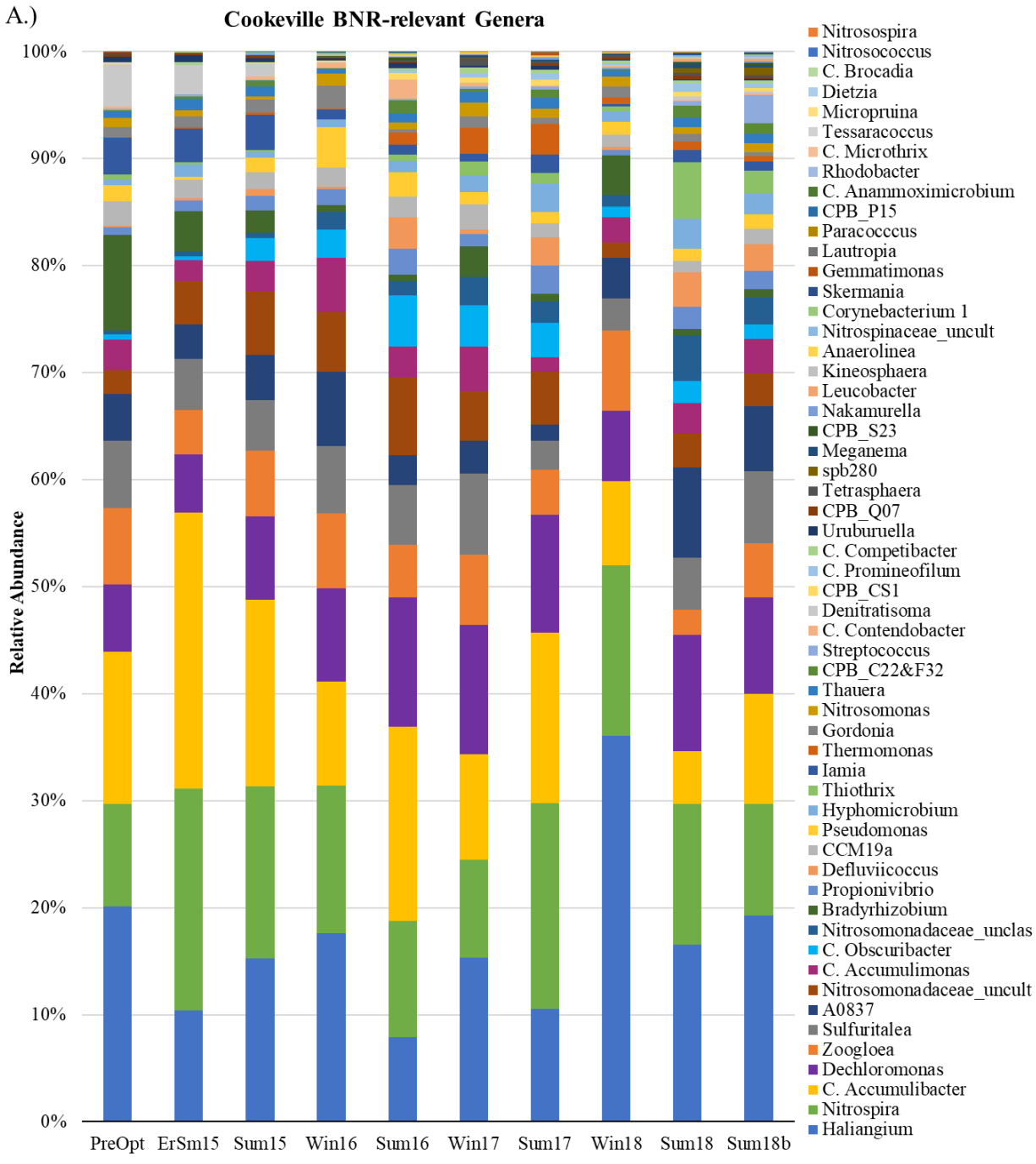
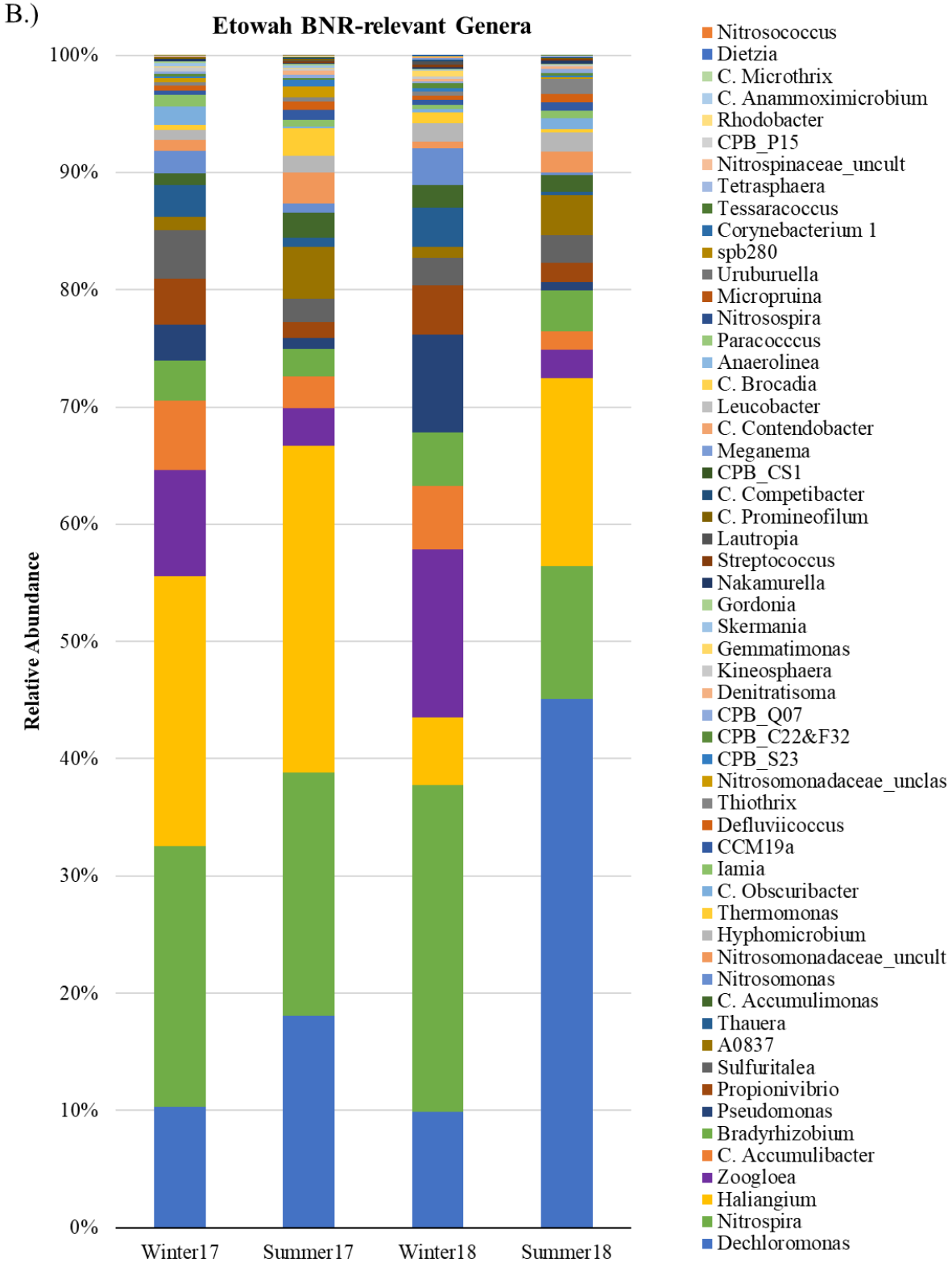


Figure 3-10: NMDS on species level for BNR-relevant microbial community based on A.) relative abundance and B.) presence/absence. Red circles indicate samples that are significantly similar ($p<0.05$) in regards to community composition. Yellow arrows depict the order of sampling events for Cookeville WRRF.


A.)
Cookeville BNR-relevant Genera
Relative Abundance
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
PreOpt
ErSm15
Sum15
Win16
Sum16
Win17
Sum17
Win18
Sum18
Sum18b
Nitrosospira
Nitrosococcus
C. Brocadia
Dietzia
Micropruina
Tessaracoccus
C. Microthrix
Rhodobacter
C. Anammoximicrobium
CPB_P15
Paracocccus
Lautropia
Gemmatimonas
Skermania
Corynebacterium 1
Nitrospinaceae_uncult
Anaerolinea
Kineosphaera
Leucobacter
Nakamurella
CPB_S23
Meganema
spb280
Tetrasphaera
CPB_Q07
Uruburuella
C. Competibacter
C. Promineofilum
CPB_CS1
Denitratisoma
C. Contendobacter
Streptococcus
CPB_C22&F32
Thauera
Nitrosomonas
Gordonia
Thermomonas
Iamia
Thiothrix
Hyphomicrobium
Pseudomonas
CCM19a
Defluviicoccus
Propionivibrio
Bradyrhizobium
Nitrosomonadaceae_unclas
C. Obscuribacter
C. Accumulimonas
Nitrosomonadaceae_uncult
A0837
Sulfuritalea
Zoogloea
Dechloromonas
C. Accumulibacter
Nitrospira
Haliangium

B.)


Etowah BNR-relevant Genera
Relative Abundance
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Winter17
Summer17
Winter18
Summer18
Nitrosococcus
Dietzia
C. Microthrix
C. Anammoximicrobium
Rhodobacter
CPB_P15
Nitrospinaceae_uncult
Tetrasphaera
Tessaracoccus
Corynebacterium 1
spb280
Uruburuella
Micropruina
Nitrosospira
Paracocccus
Anaerolinea
C. Brocadia
Leucobacter
C. Contendobacter
Meganema
CPB_CS1
C. Competibacter
C. Promineofilum
Lautropia
Streptococcus
Nakamurella
Gordonia
Skermania
Gemmatimonas
Kineosphaera
Denitratisoma
CPB_Q07
CPB_C22&F32
CPB_S23
Nitrosomonadaceae_unclas
Thiothrix
Defluviicoccus
CCM19a
Iamia
C. Obscuribacter
Thermomonas
Hyphomicrobium
Nitrosomonadaceae_uncult
Nitrosomonas
C. Accumulimonas
Thauera
A0837
Sulfuritalea
Propionivibrio
Pseudomonas
Bradyrhizobium
C. Accumulibacter
Zoogloea
Haliangium
Nitrospira
Dechloromonas

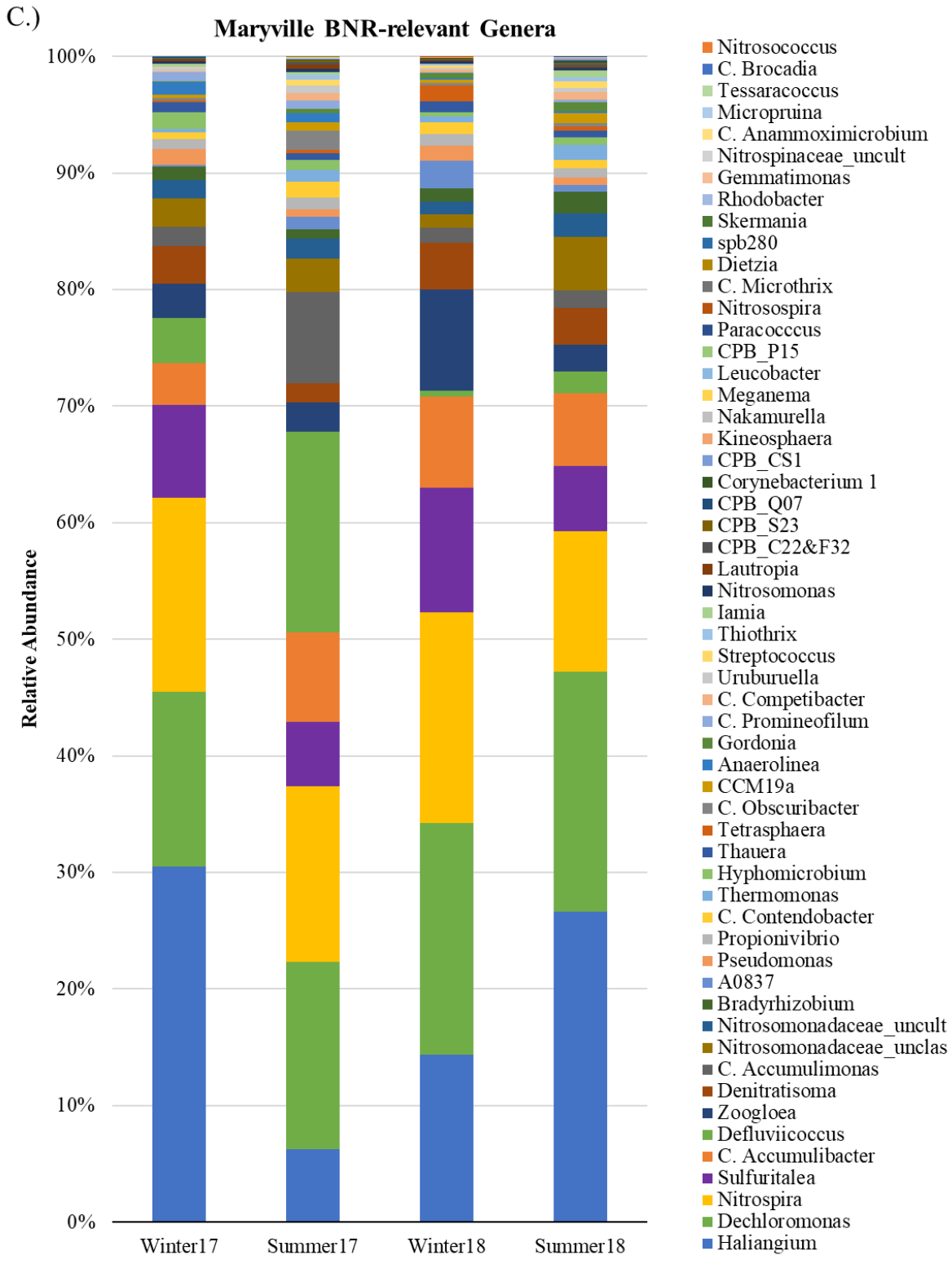


Figure 3-11: The BNR-relevant community composition observed across sampling events for A.) Cookeville, B.) Etowah, and C.) Maryville WRRF.

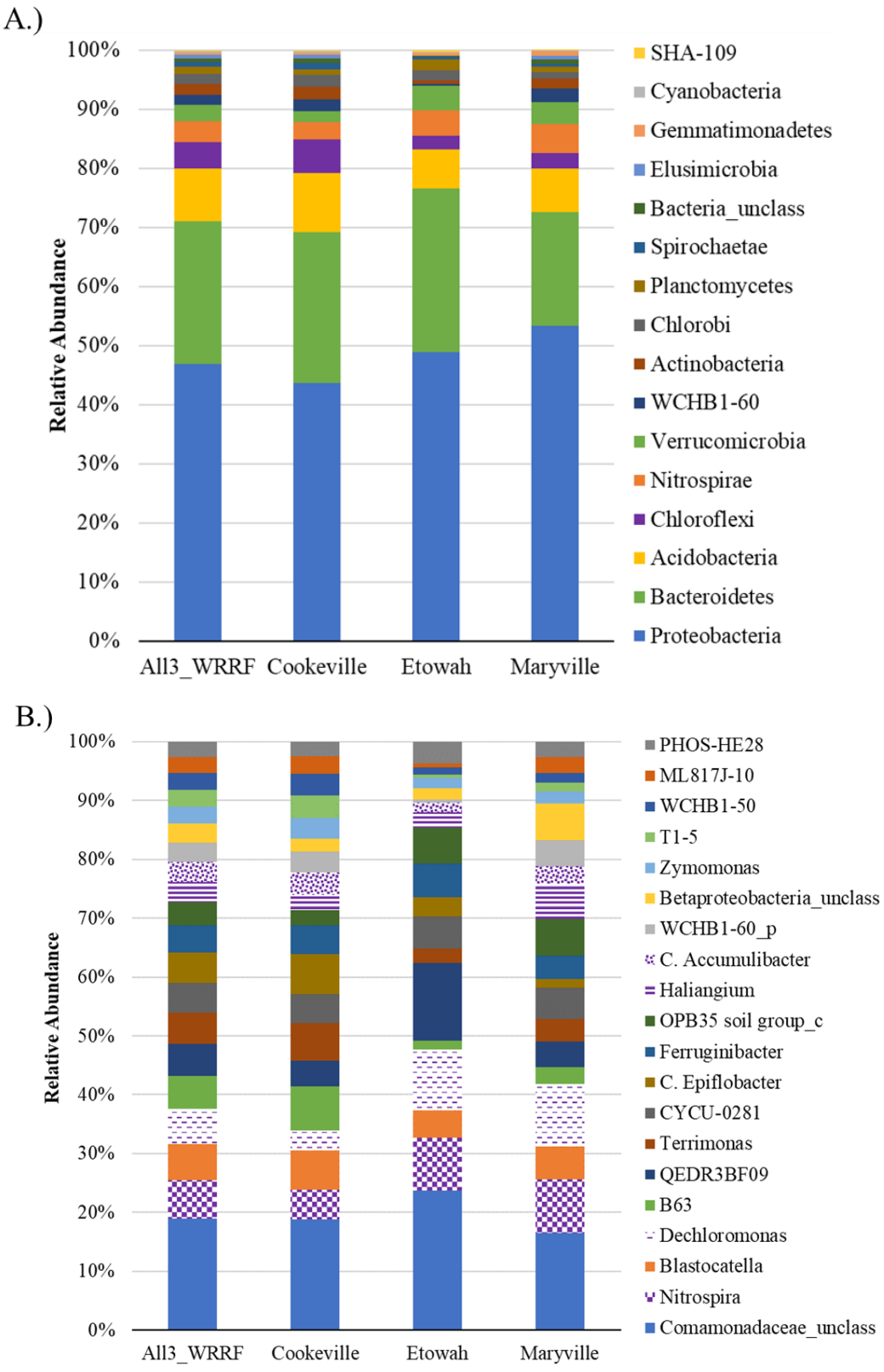
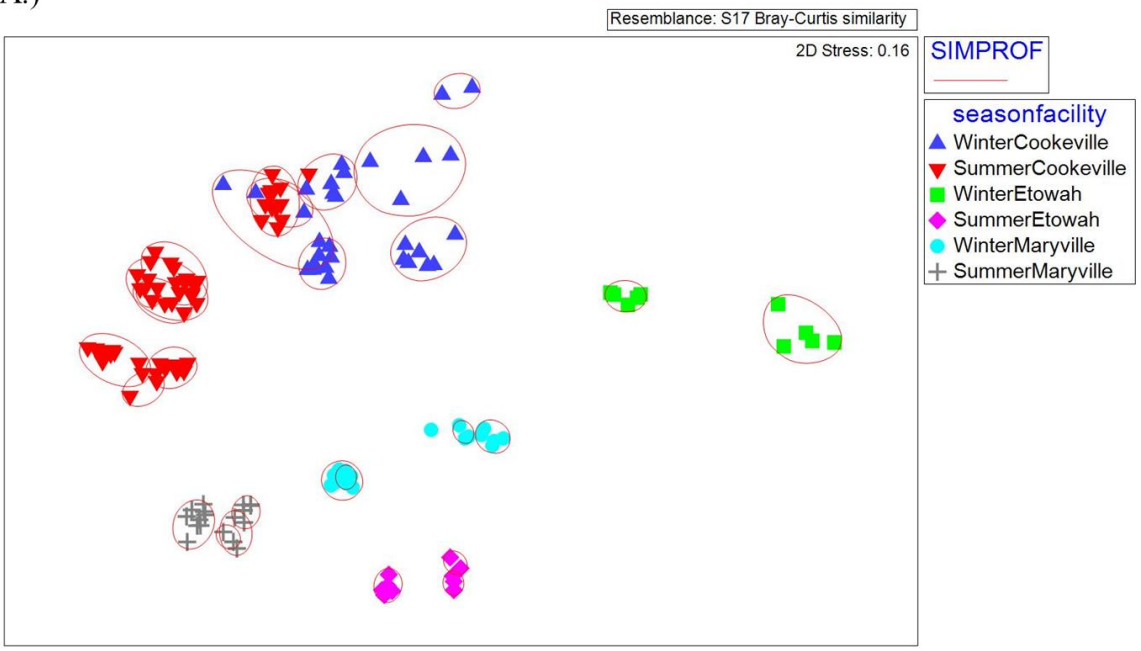


Figure 3-12: Core community among all three WRRFs on A.) the phylum level and B.) the top 20 core genera. Genera with hatch markings indicate BNR-relevant microorganisms.

A.)



B.)

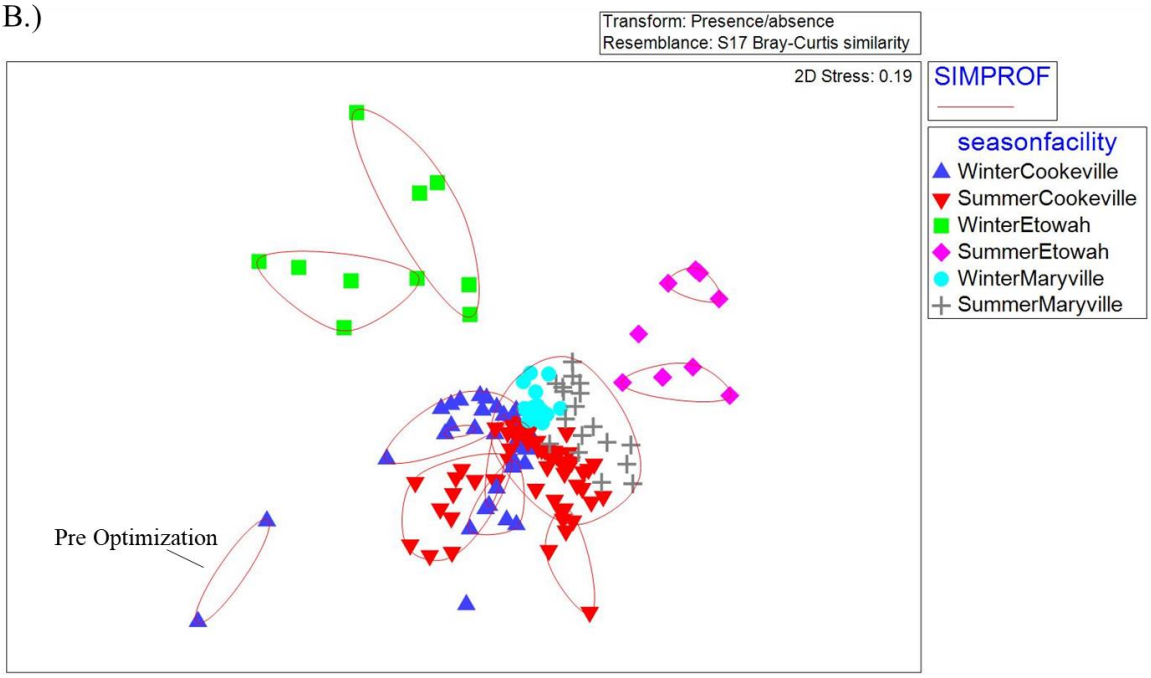


Figure 3-13: NMDS on species level for total core community across all facilities based on A.) relative abundance and B.) presence/absence.

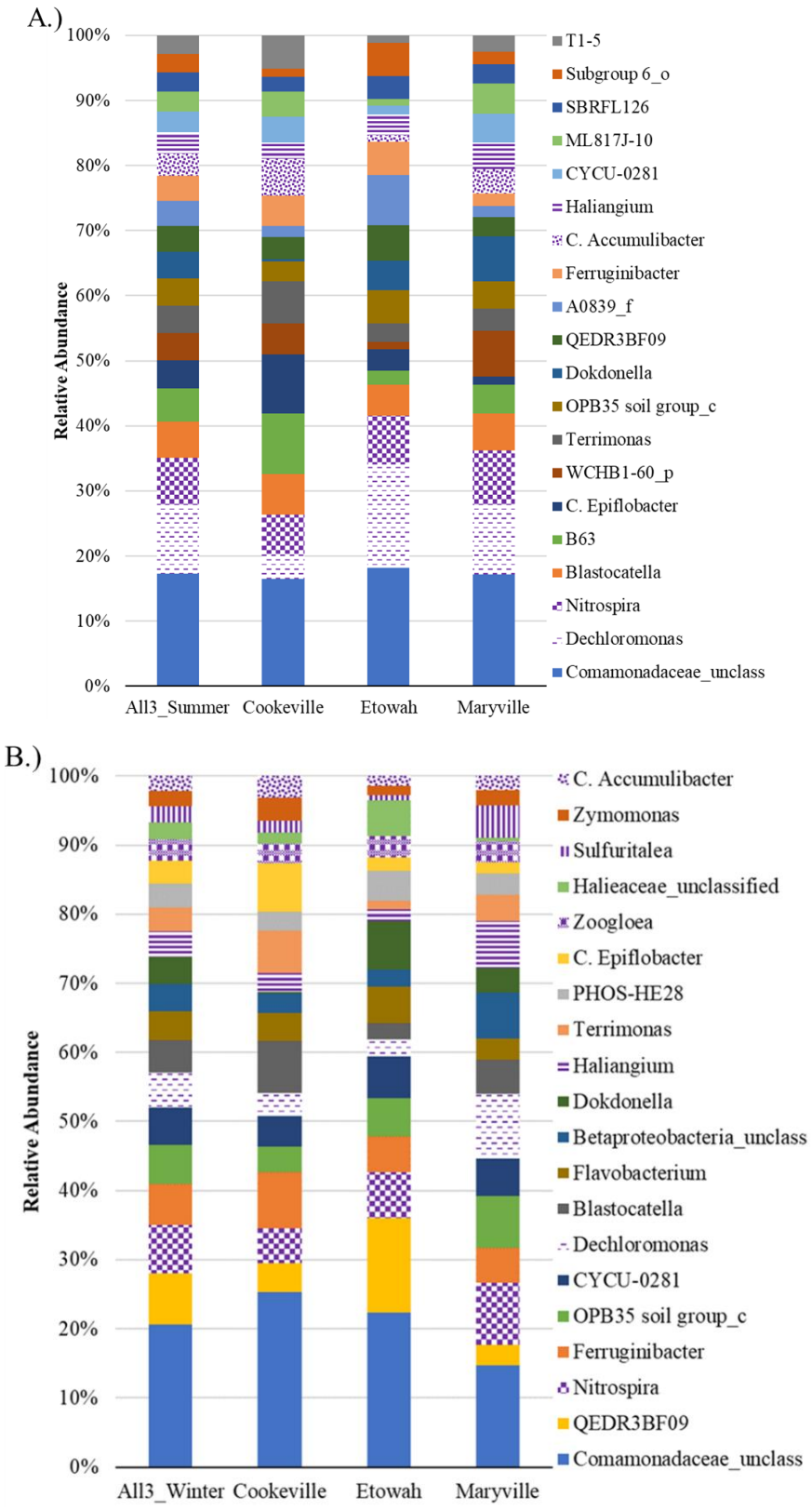


Figure 3-14: Top 20 core genera among all three facilities for A.) summer and B.) winter.

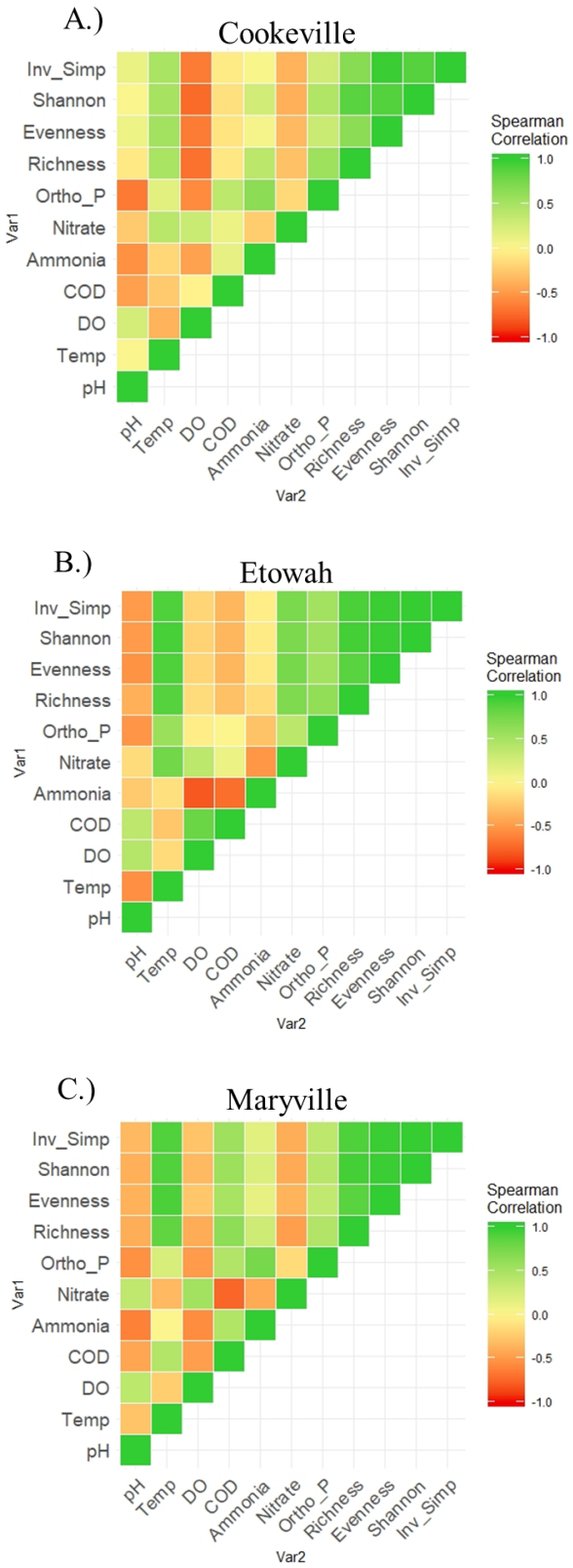


Figure 3-15: Correlation of wastewater characteristics to alpha diversity measurements of the total community for A.) Cookeville, B.) Etowah, and C.) Maryville.

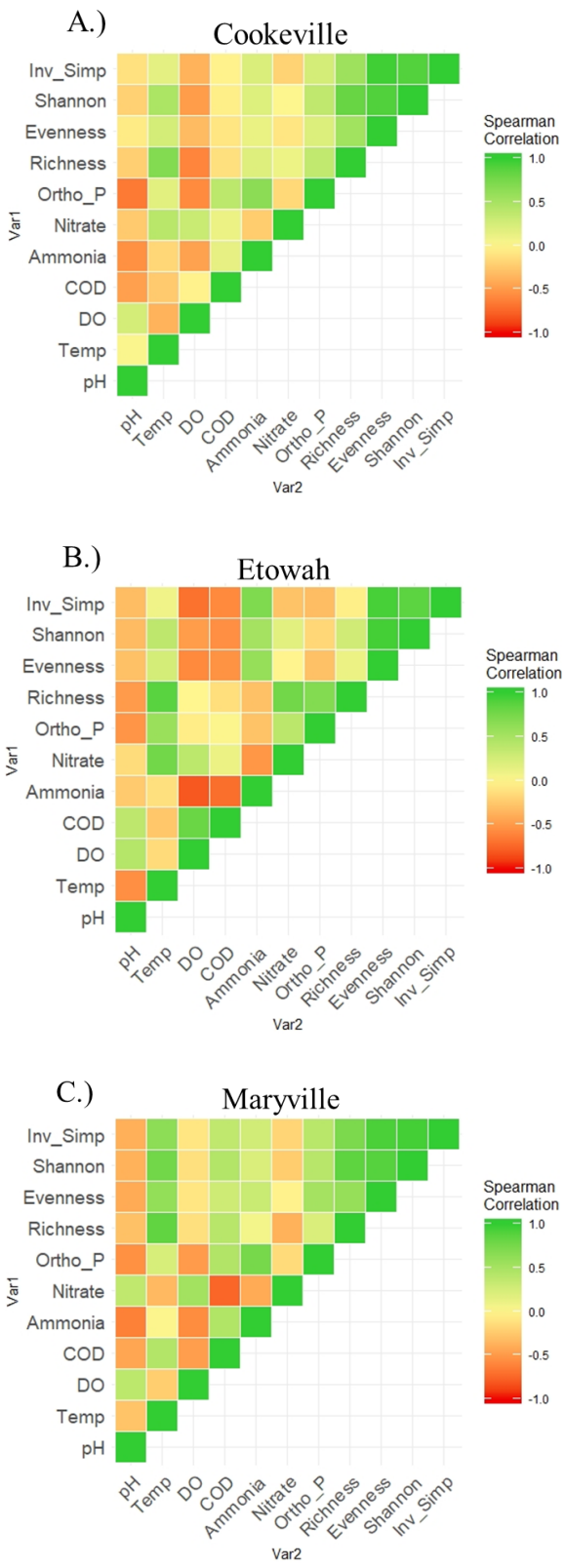
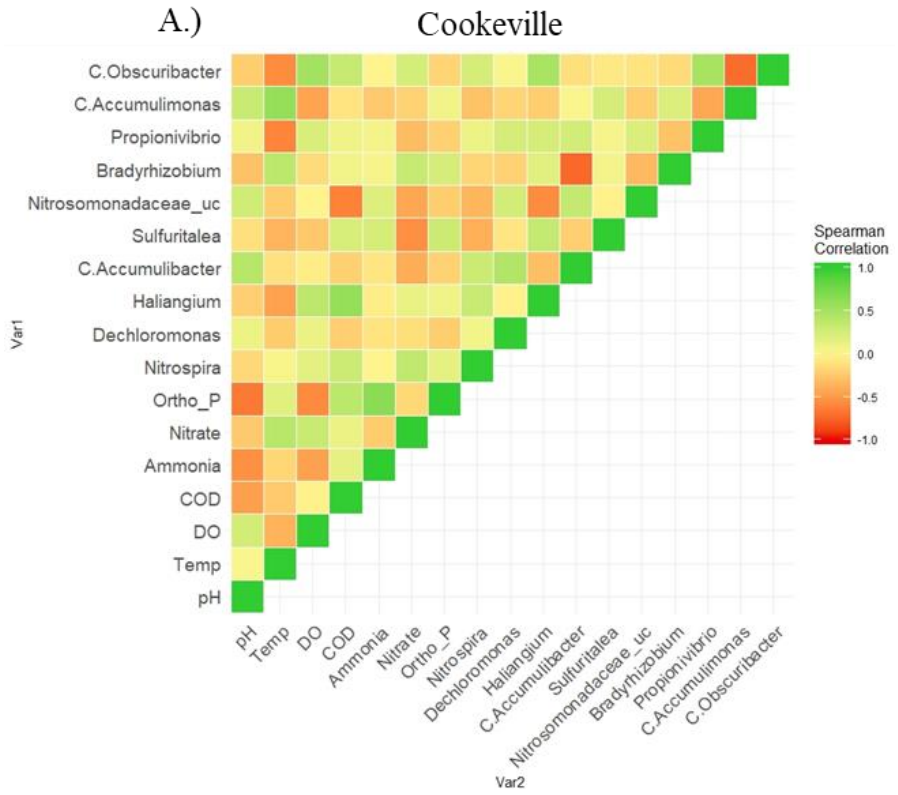
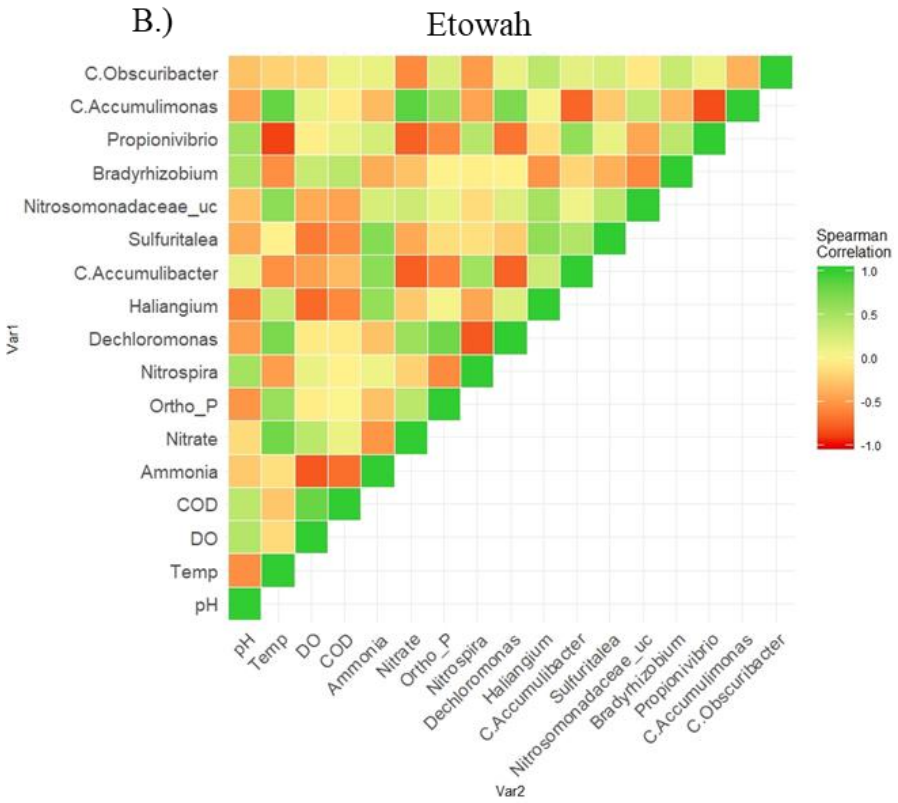


Figure 3-16: Correlation of wastewater characteristics to alpha diversity measurements of the BNR-relevant community A.) Cookeville, B.) Etowah, and C.) Maryville.


A.)
Cookeville
C.Obscuribacter
C.Accumulimonas
Propionivibrio
Bradyrhizobium
Nitrosomonadaceae_uc
Sulfuritalea
C.Accumulibacter
Haliangium
Dechloromonas
Nitrospira
Ortho_P
Nitrate
Ammonia
COD
DO
Temp
pH
Var1
Var2
Spearman Correlation
1.0
0.5
0.0
-0.5
-1.0


B.)
Etowah
C.Obscuribacter
C.Accumulimonas
Propionivibrio
Bradyrhizobium
Nitrosomonadaceae_uc
Sulfuritalea
C.Accumulibacter
Haliangium
Dechloromonas
Nitrospira
Ortho_P
Nitrate
Ammonia
COD
DO
Temp
pH
Var1
Var2
Spearman Correlation
1.0
0.5
0.0
-0.5
-1.0

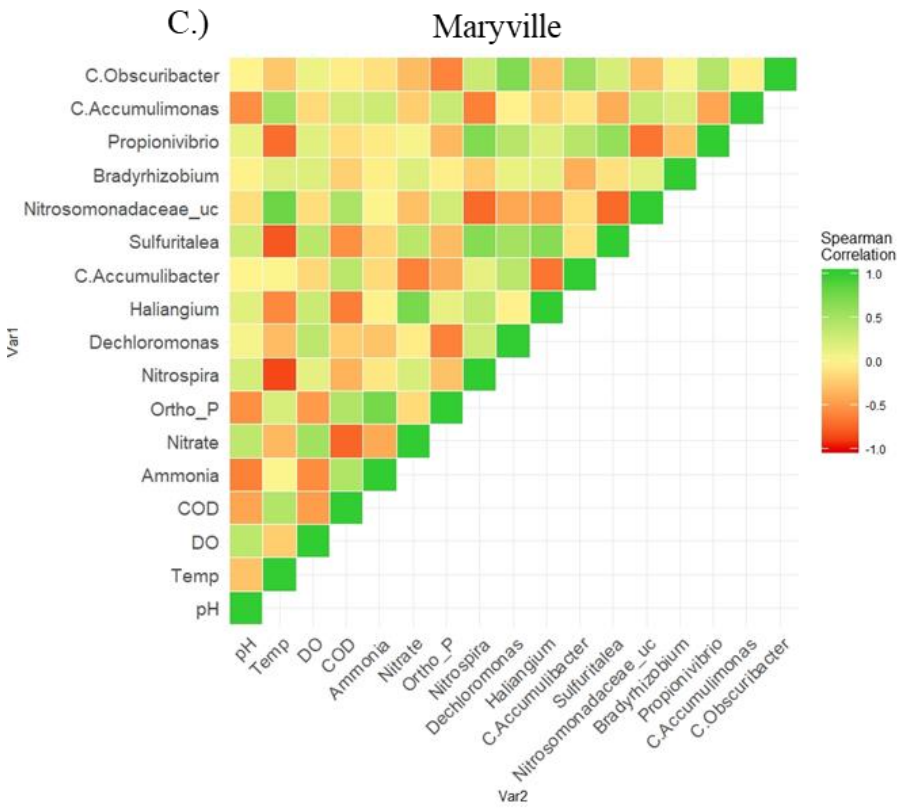


Figure 3-17: Correlation of wastewater characteristics to the BNR-relevant genera detected as part of the core community.

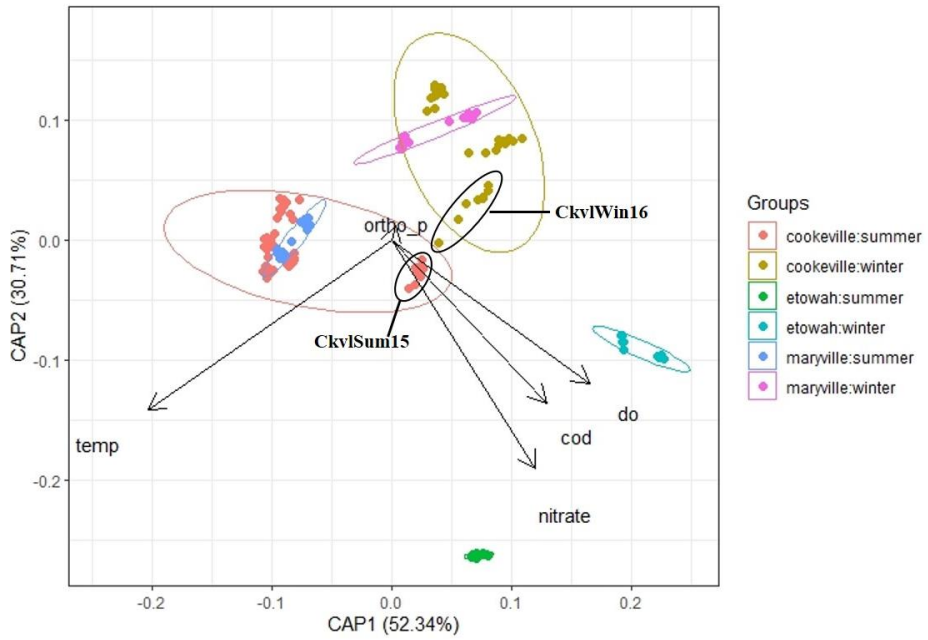


Figure 3-18: Capscale correlation of beta diversity with wastewater characteristics for the total microbial community.

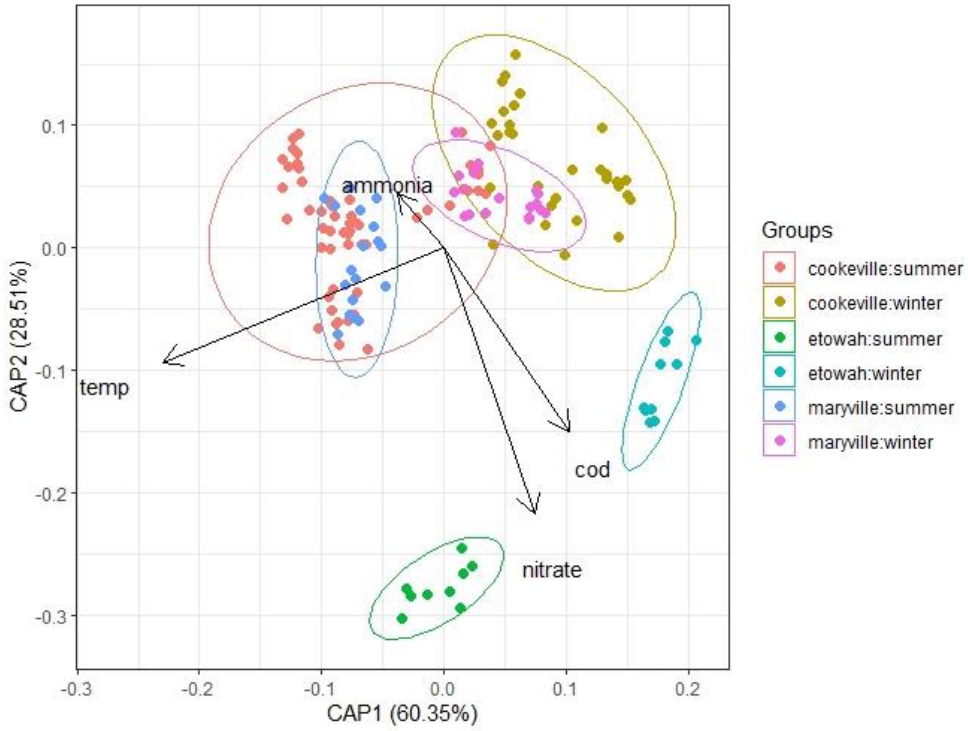


Figure 3-19: Capscale correlation of beta diversity with wastewater characteristics for the BNR-relevant microbial community.

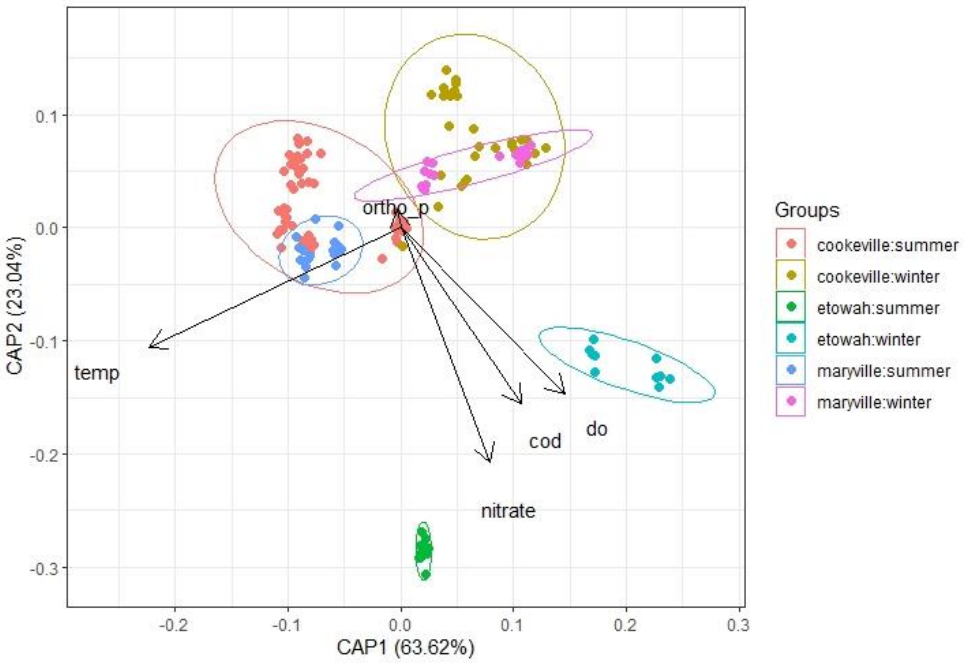


Figure 3-20: Capscale correlation of beta diversity with wastewater characteristics for the core microbial community.

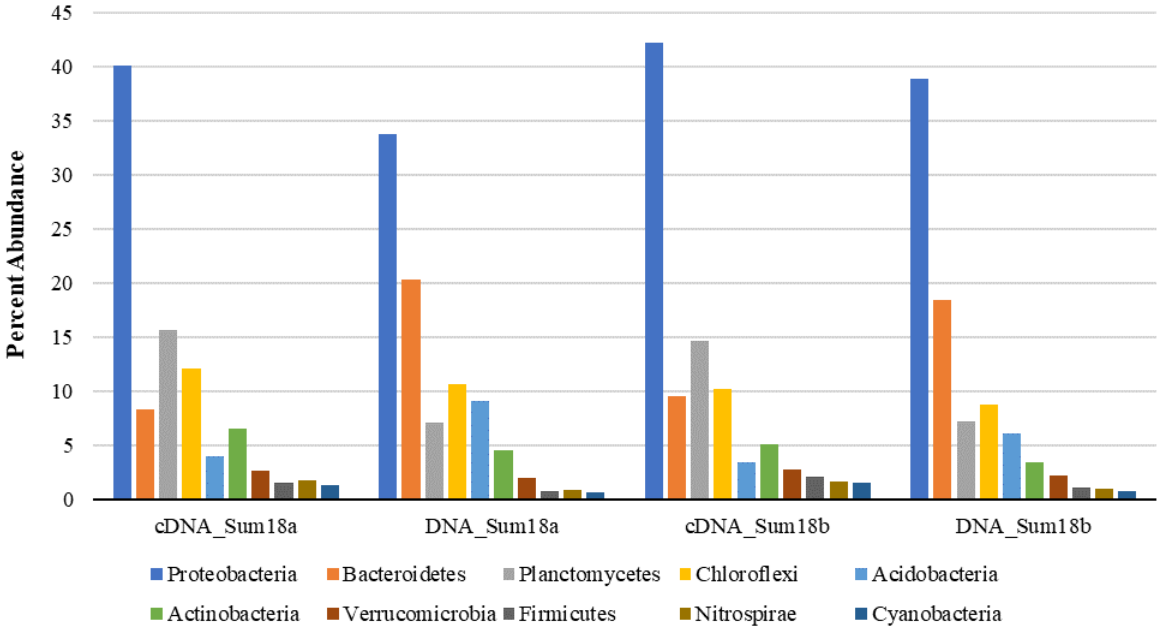


Figure 4-1: Top ten phyla detected in the total community for cDNA and DNA samples.

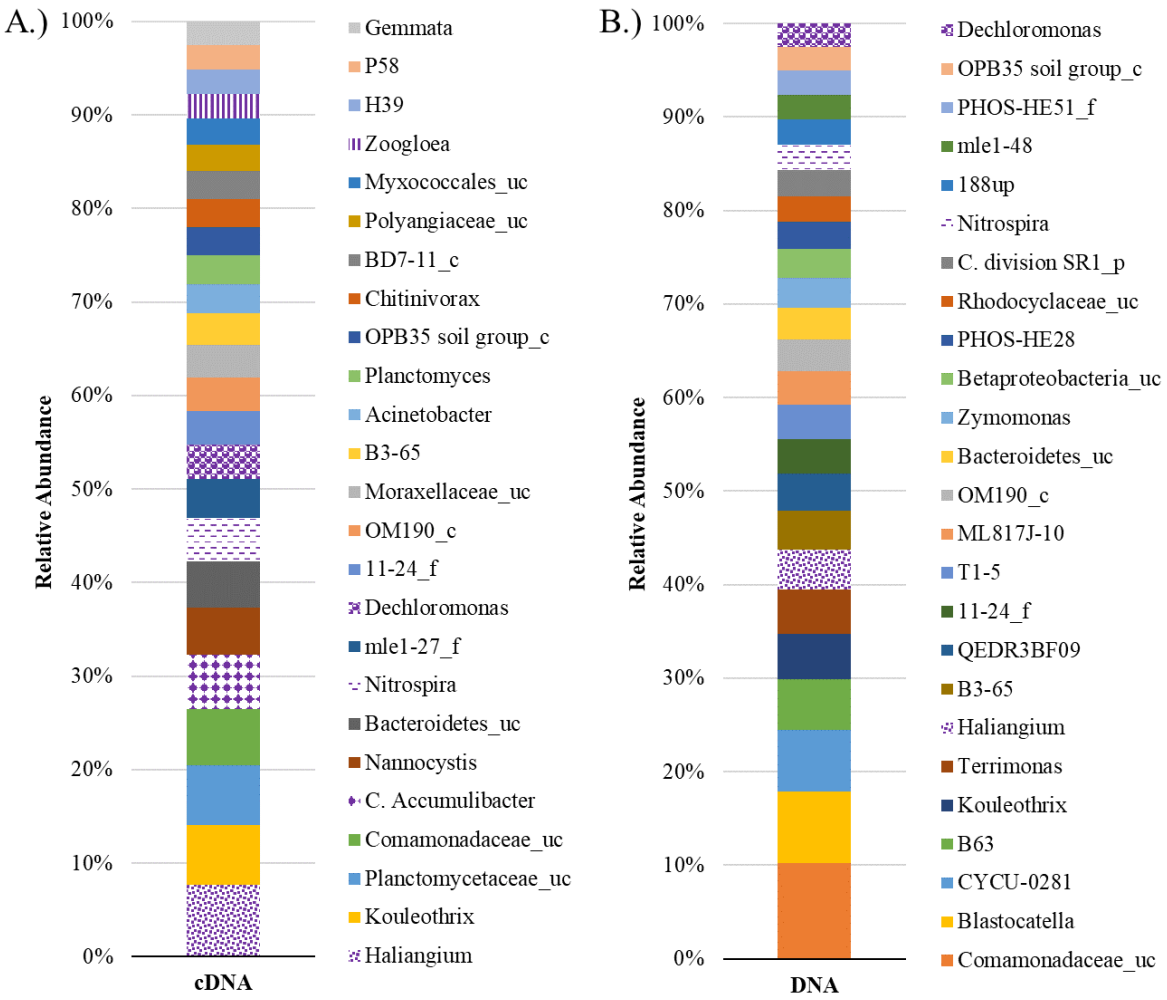


Figure 4-2: Top 25 genera detected in the total community from A.) cDNA and B.) DNA samples. Hatch markings indicate BNR-relevant genera.

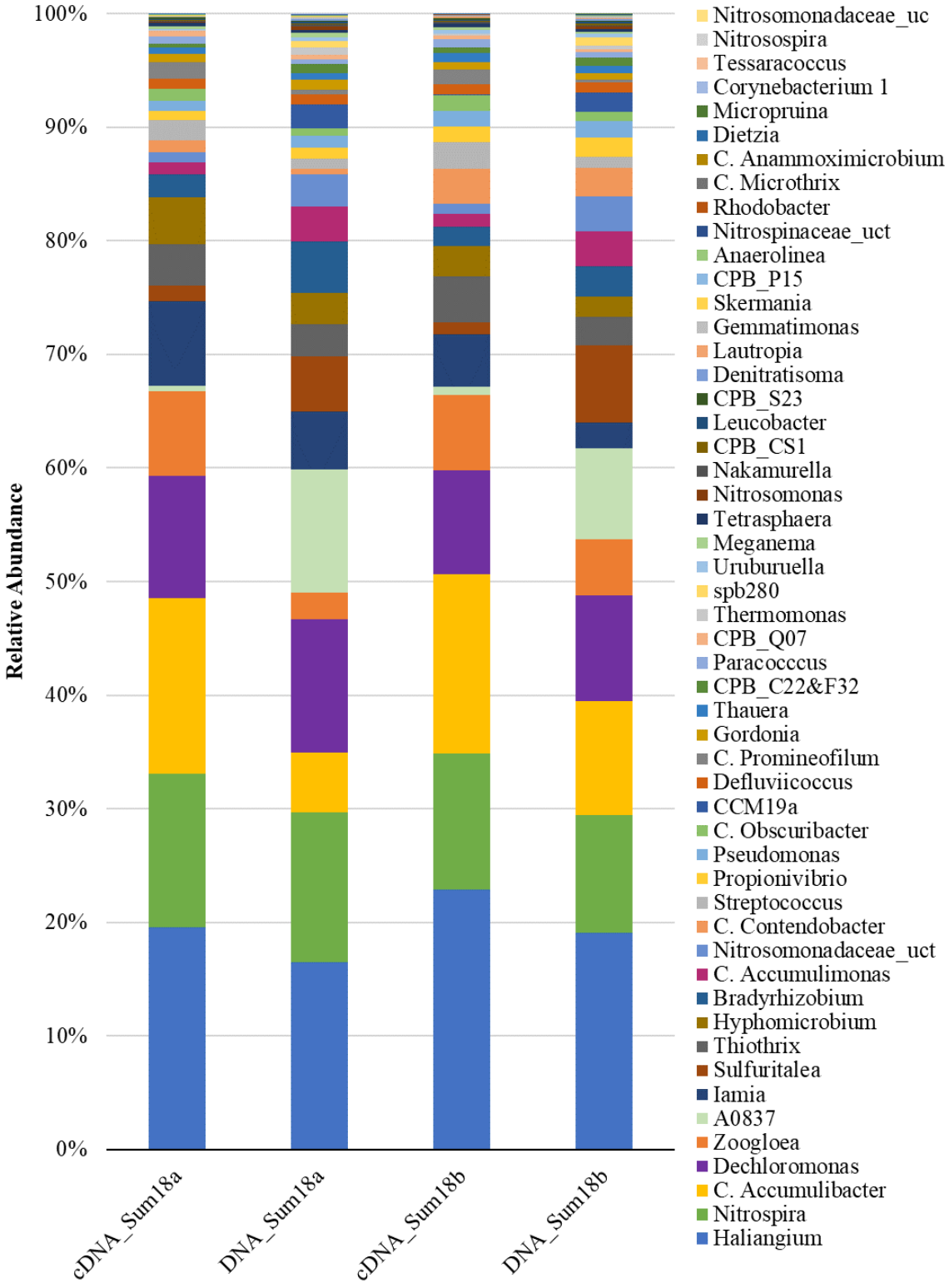


Figure 4-3: The BNR-relevant community composition based on genera.

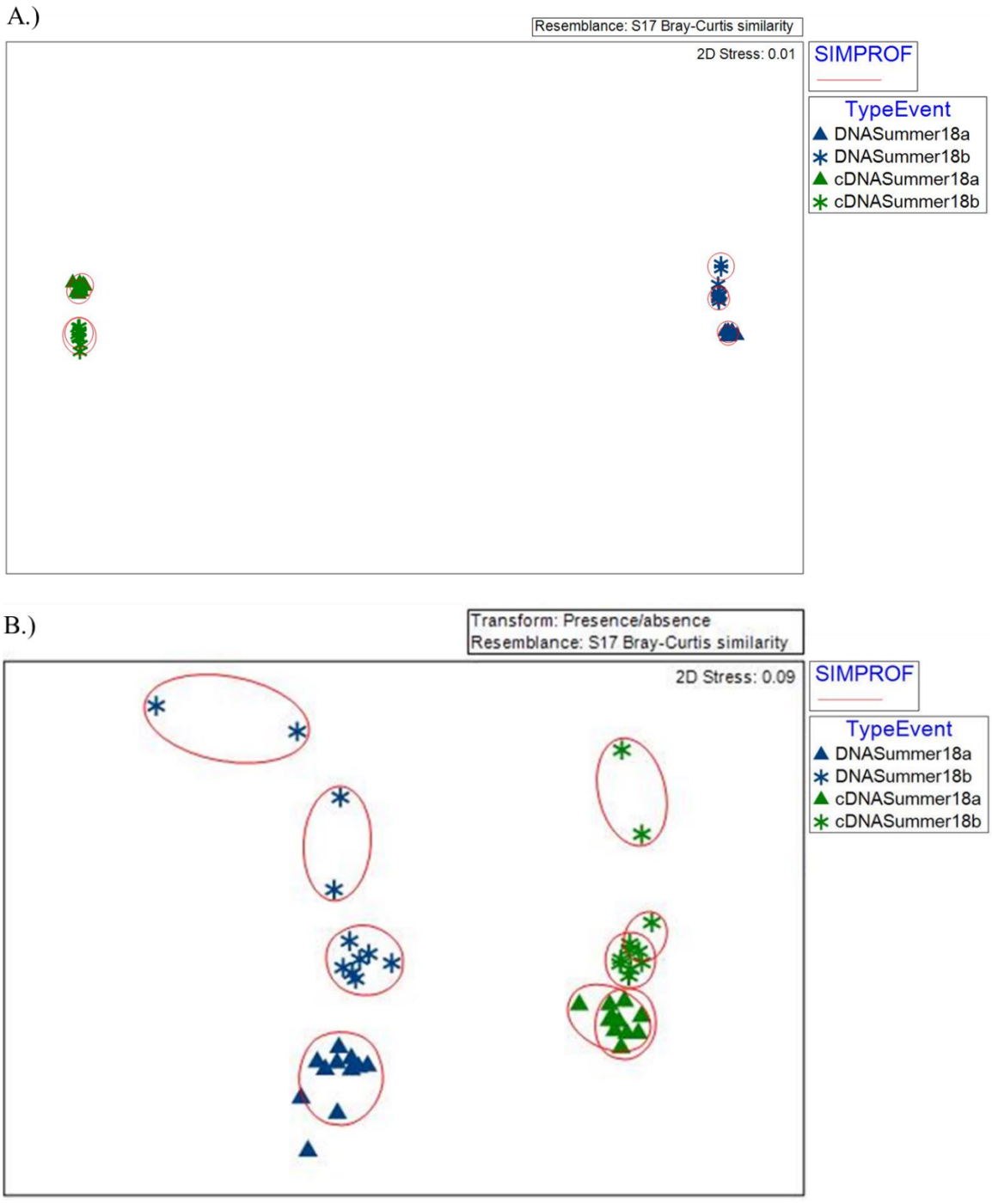


Figure 4-4: Samples collected from Cookeville WRRF during two sampling events in summer of 2018. NMDS on species level for total microbial community based on A.) relative abundance and B.) presence/absence. Red circles indicate samples that are significantly similar ($p<0.05$) in regards to community composition.

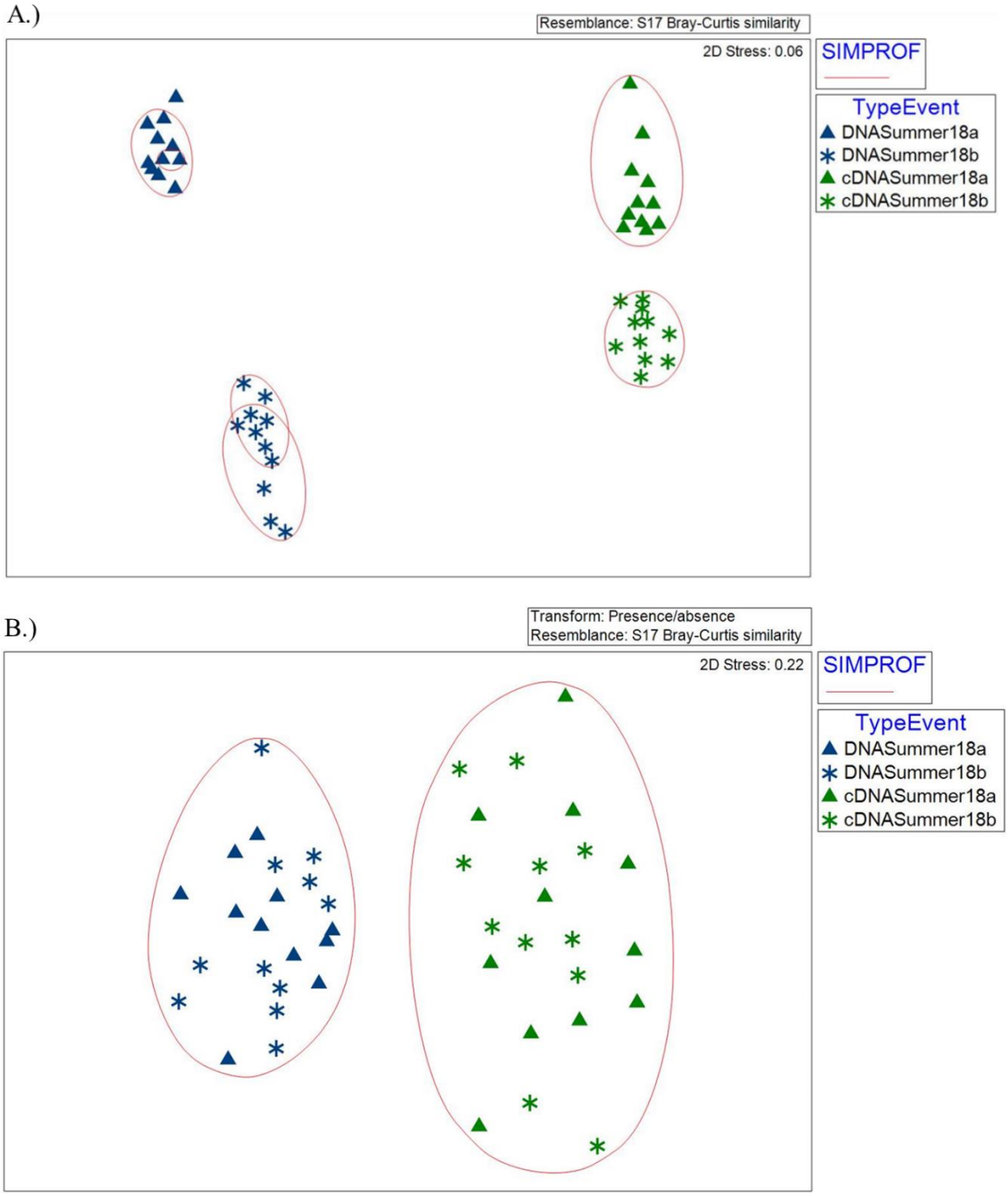


Figure 4-5: Samples collected from Cookeville WRRF during two sampling events in summer of 2018. NMDS on species level for BNR-relevant microbial community based on A.) relative abundance and B.) presence/absence. Red circles indicate samples that are significantly similar ($p<0.05$) in regards to community composition.

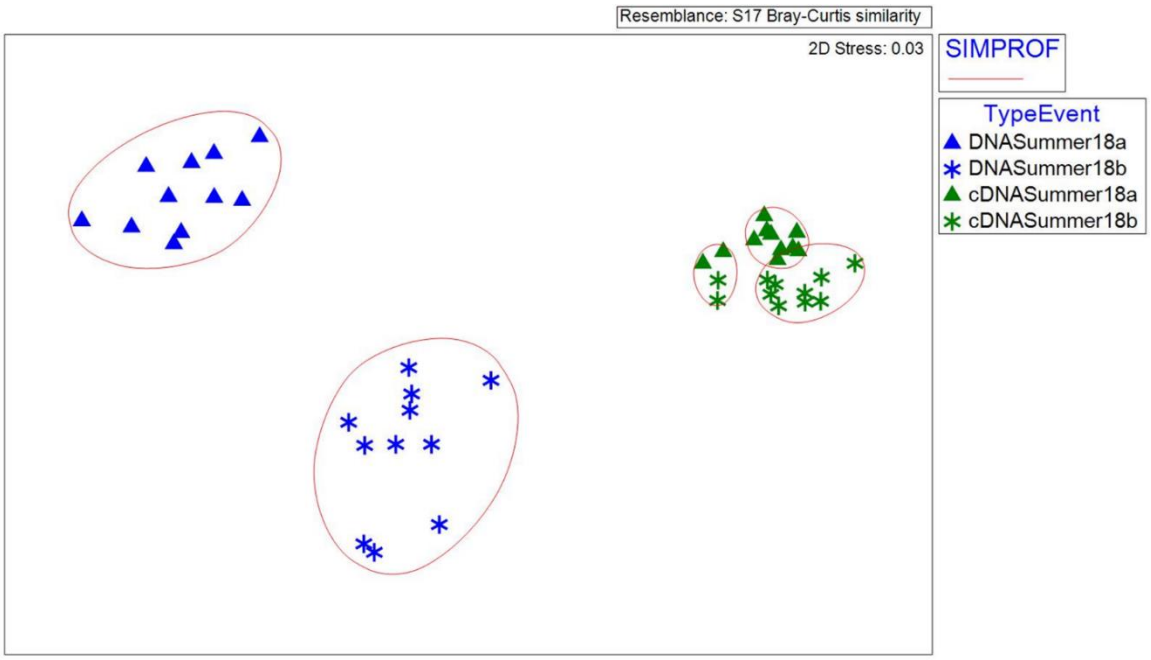


Figure 4-6: Samples collected from Cookeville WRRF during two sampling events in summer of 2018. NMDS on species level for putative PAO microbial community based on relative abundance. Red circles indicate samples that are significantly similar ($p<0.05$) in regards to community composition.

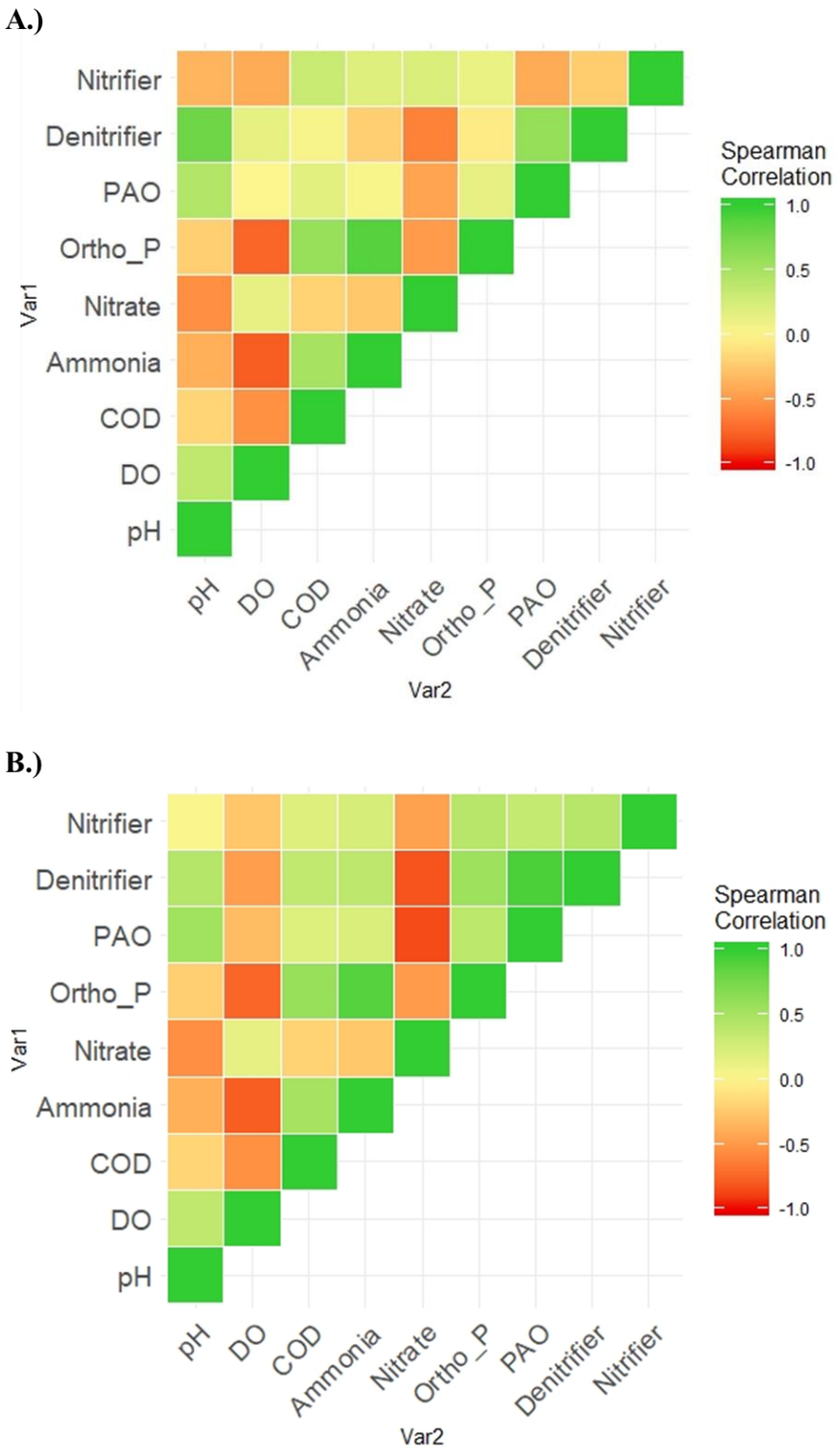
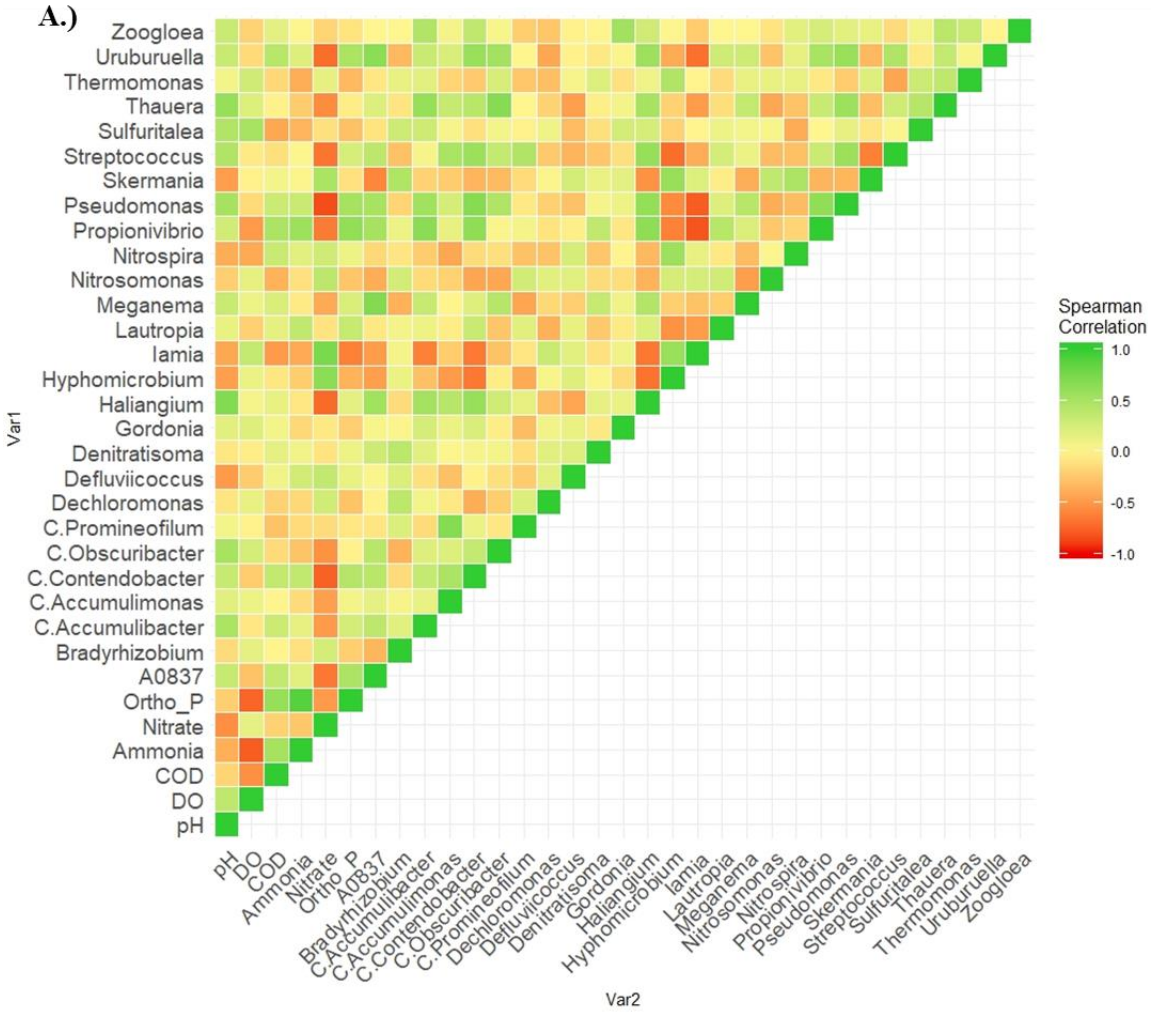


Figure 4-7: Correlation of wastewater characteristics to BNR-relevant groups based on a.) cDNA and b.) DNA datasets.


A.)
Zoogloea
Uruburuella
Thermomonas
Thauera
Sulfuritalea
Streptococcus
Skermania
Pseudomonas
Propionivibrio
Nitrospira
Nitrosomonas
Meganema
Lautropia
Iamia
Hyphomicrobium
Haliangium
Gordonia
Denitratisoma
Defluviicoccus
Dechloromonas
C.Promineofilum
C.Obscuribacter
C.Contendobacter
C.Accumulimonas
C.Accumulibacter
Bradyrhizobium
A0837
Ortho_P
Nitrate
Ammonia
COD
DO
pH
Var1
Var2
Spearman Correlation
1.0
0.5
0.0
-0.5
-1.0

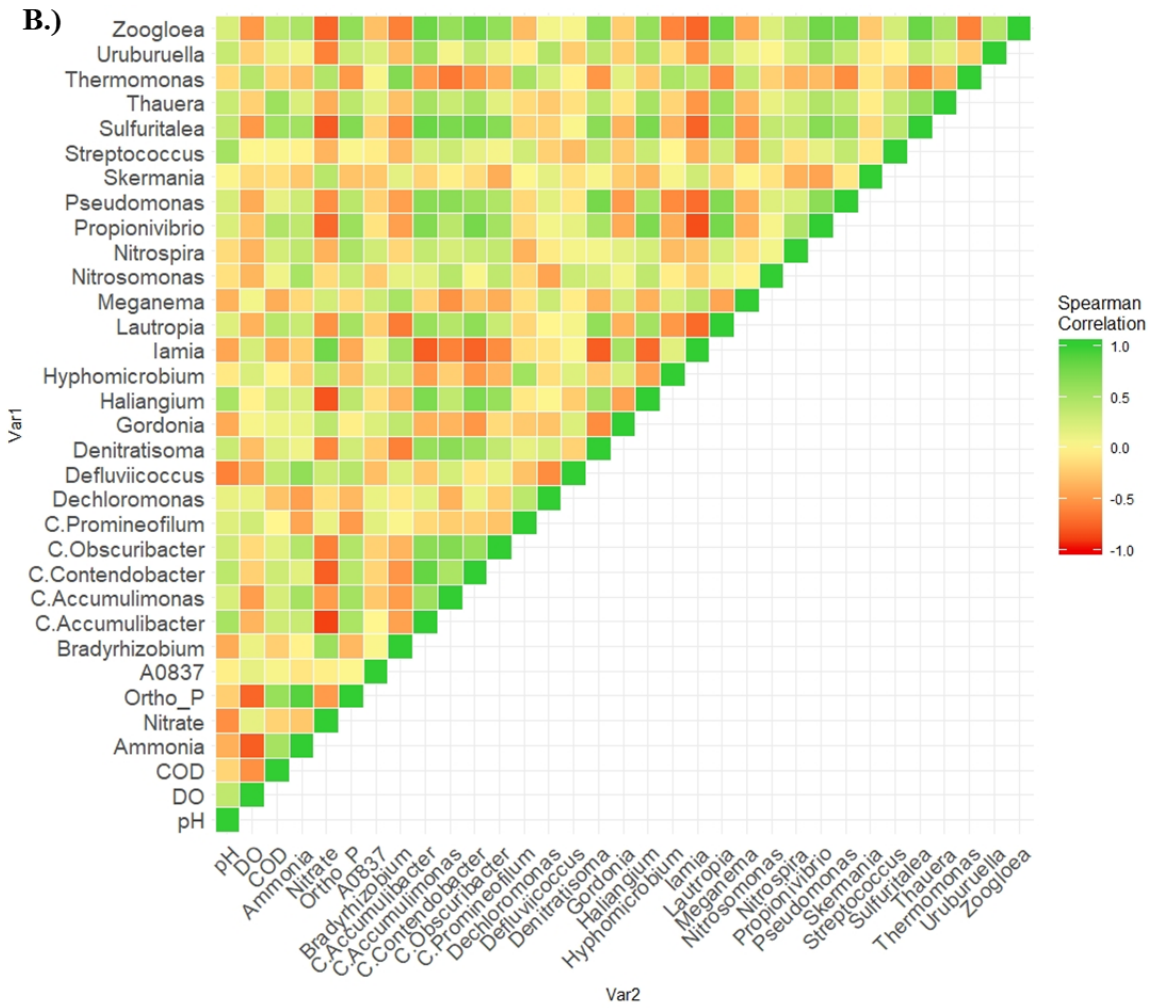
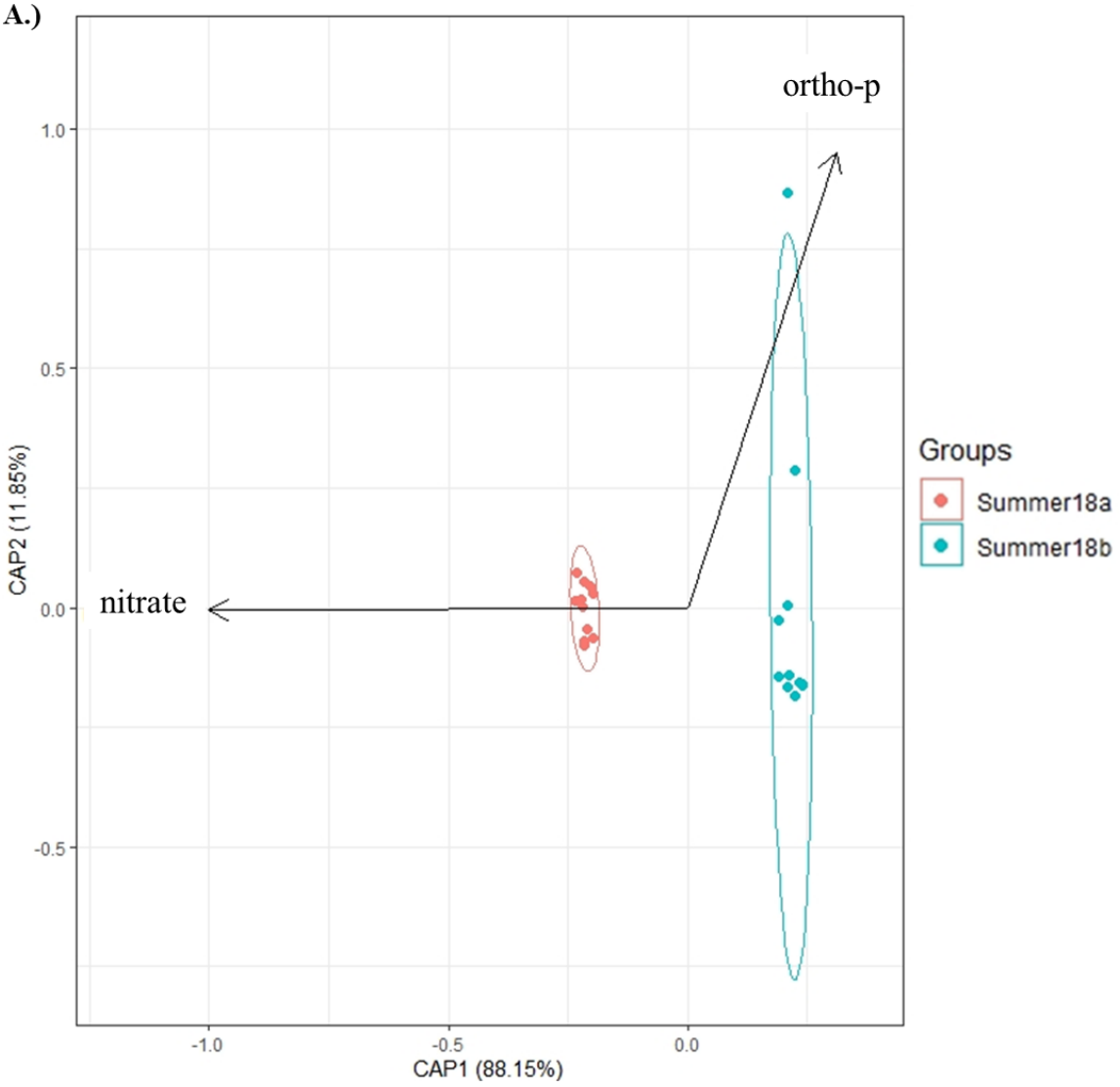


Figure 4-8: Correlation of wastewater characteristics to BNR-relevant genera based on a.) cDNA and b.) DNA datasets.

A.)


ortho-p
nitrate
1.0
0.5
0.0
-0.5
-1.0
-0.5
0.0
CAP2 (11.85%)
CAP1 (88.15%)
Groups
Summer18a
Summer18b

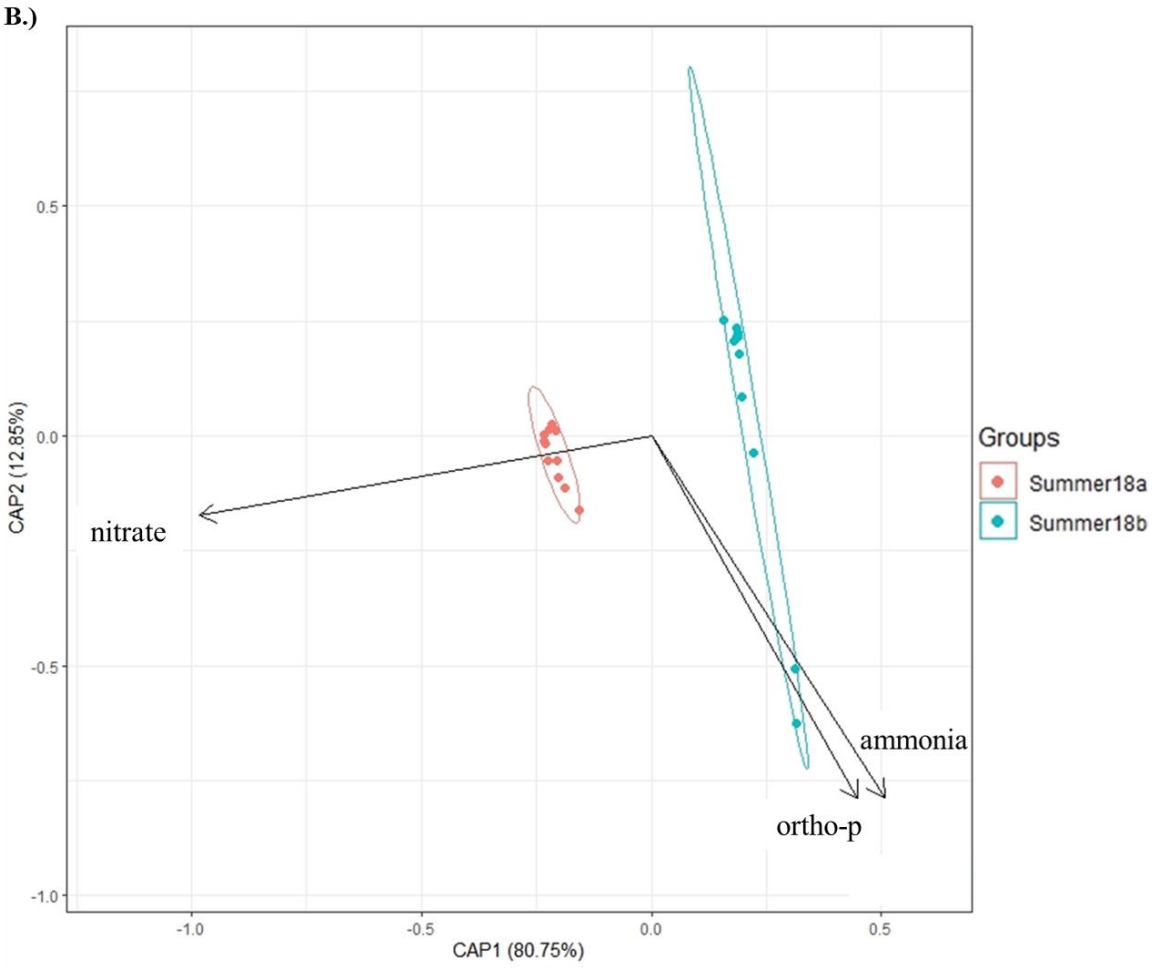
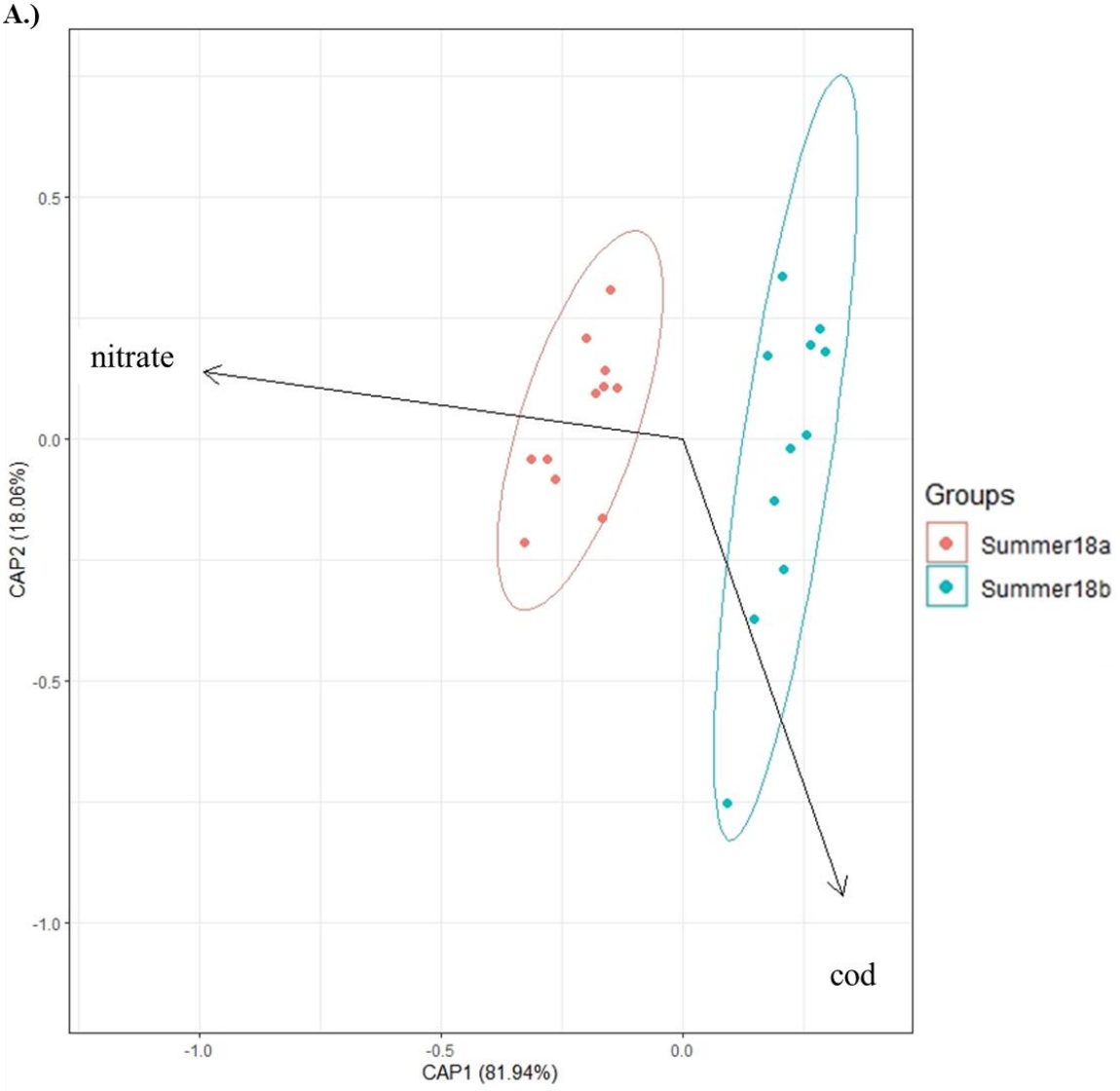


Figure 4-9: Samples collected from Cookeville WRRF during two sampling events in summer of 2018. Capscale correlation of beta diversity with wastewater characteristics for the total microbial community based on a.) cDNA and b.) DNA datasets.

A.)


nitrate
cod
0.5
0.0
-0.5
-1.0
CAP2 (18.06%)
-1.0
-0.5
0.0
CAP1 (81.94%)
Groups
Summer18a
Summer18b

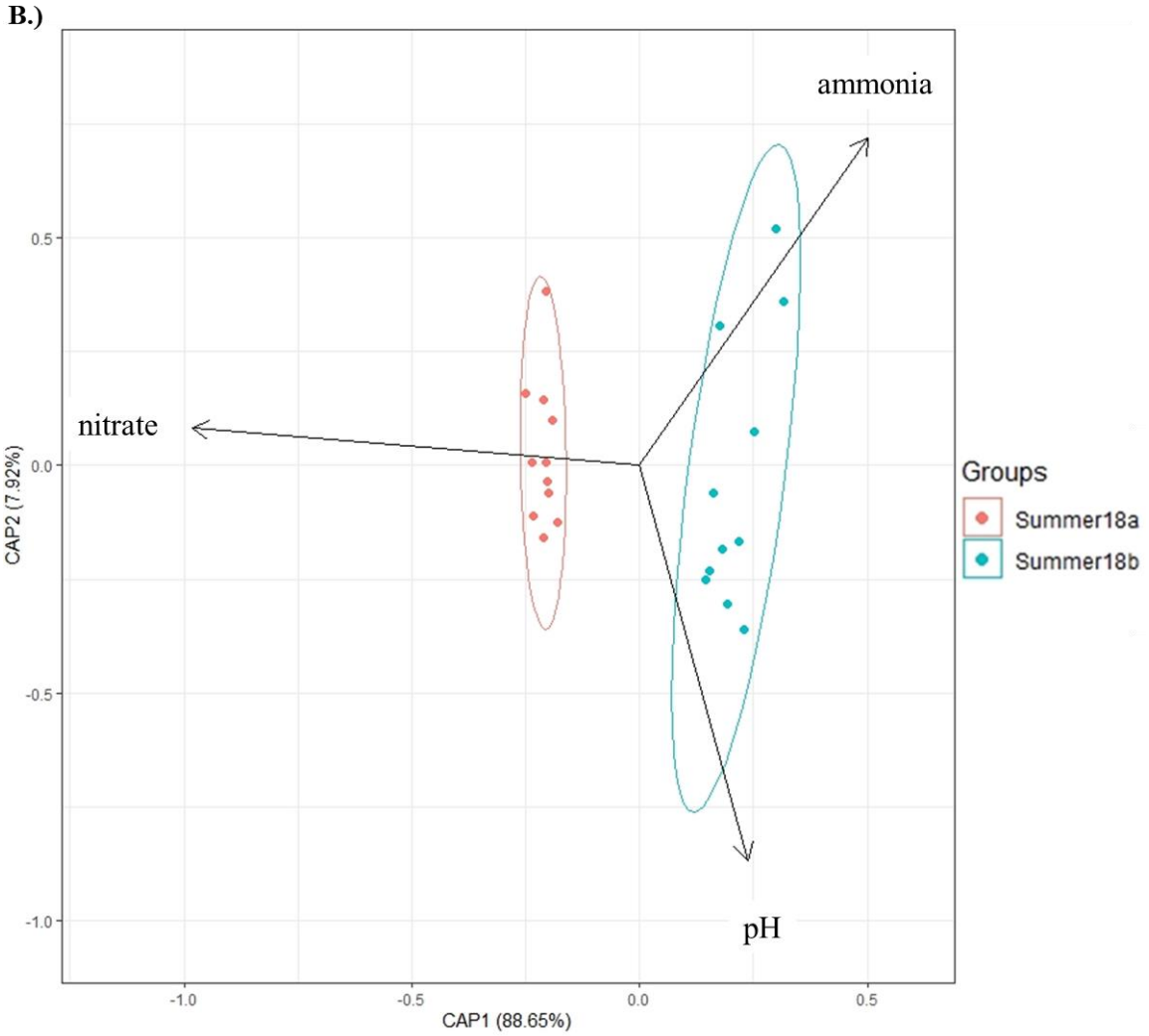


Figure 4-10: Samples collected from Cookeville WRRF during two sampling events in summer of 2018. Capscale correlation of beta diversity with wastewater characteristics for the BNR-relevant microbial community based on a.) cDNA and b.) DNA datasets.

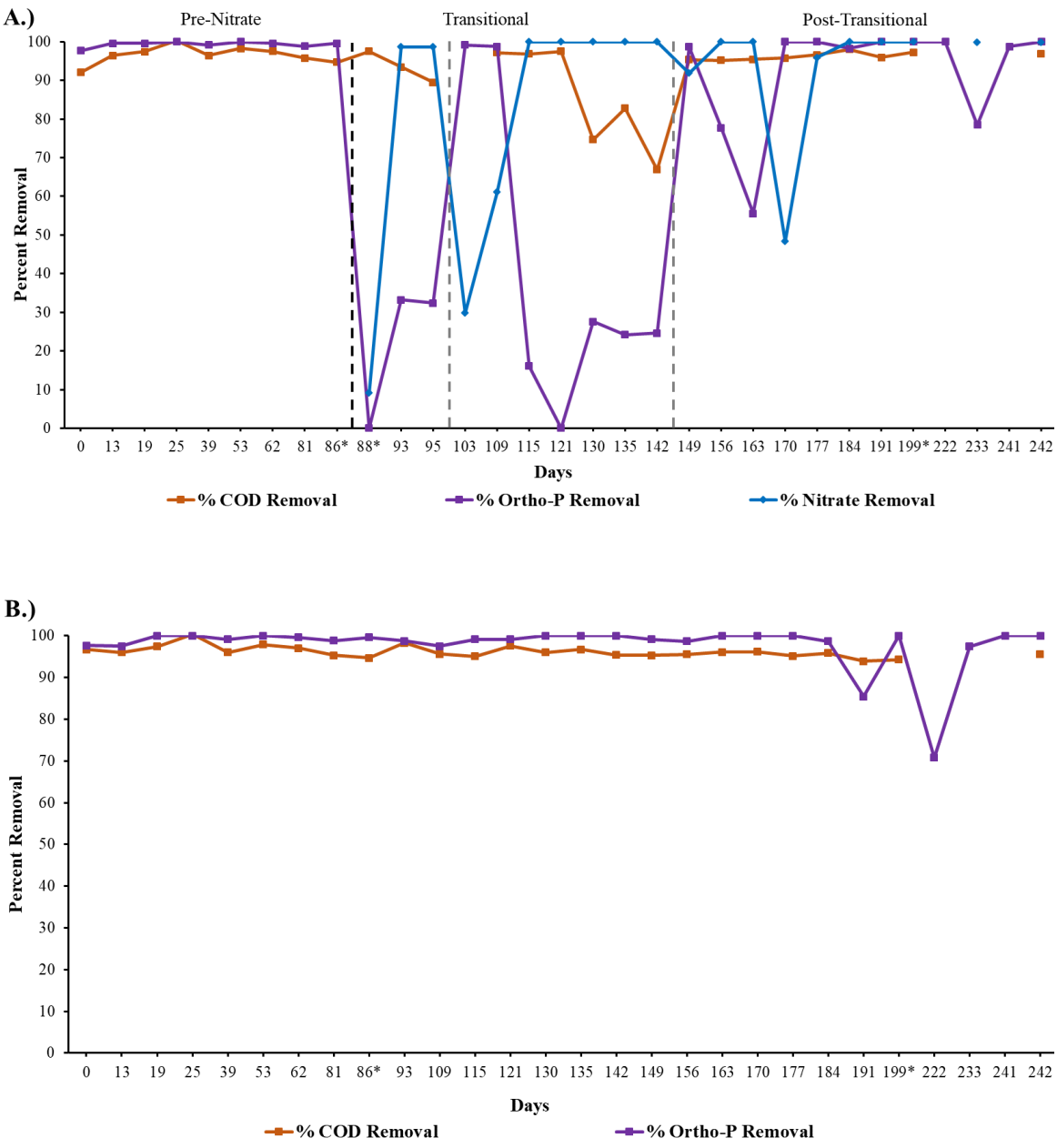


Figure 5-1: Monitoring data for COD and nutrient removal for the A.) Test and B.) Control reactors. The black dashed line indicates introduction of nitrate (20 mg/L NO_3-N in the reactor) and removal of DO pump. The first grey dashed line indicates the reduction of nitrate (10 mg/L NO_3-N in the reactor) and introduction of low DO (<0.25mg/L). The second grey dashed line indicates increased nitrate (20 mg/L NO_3-N in the reactor). (*) indicates days of kinetic studies.

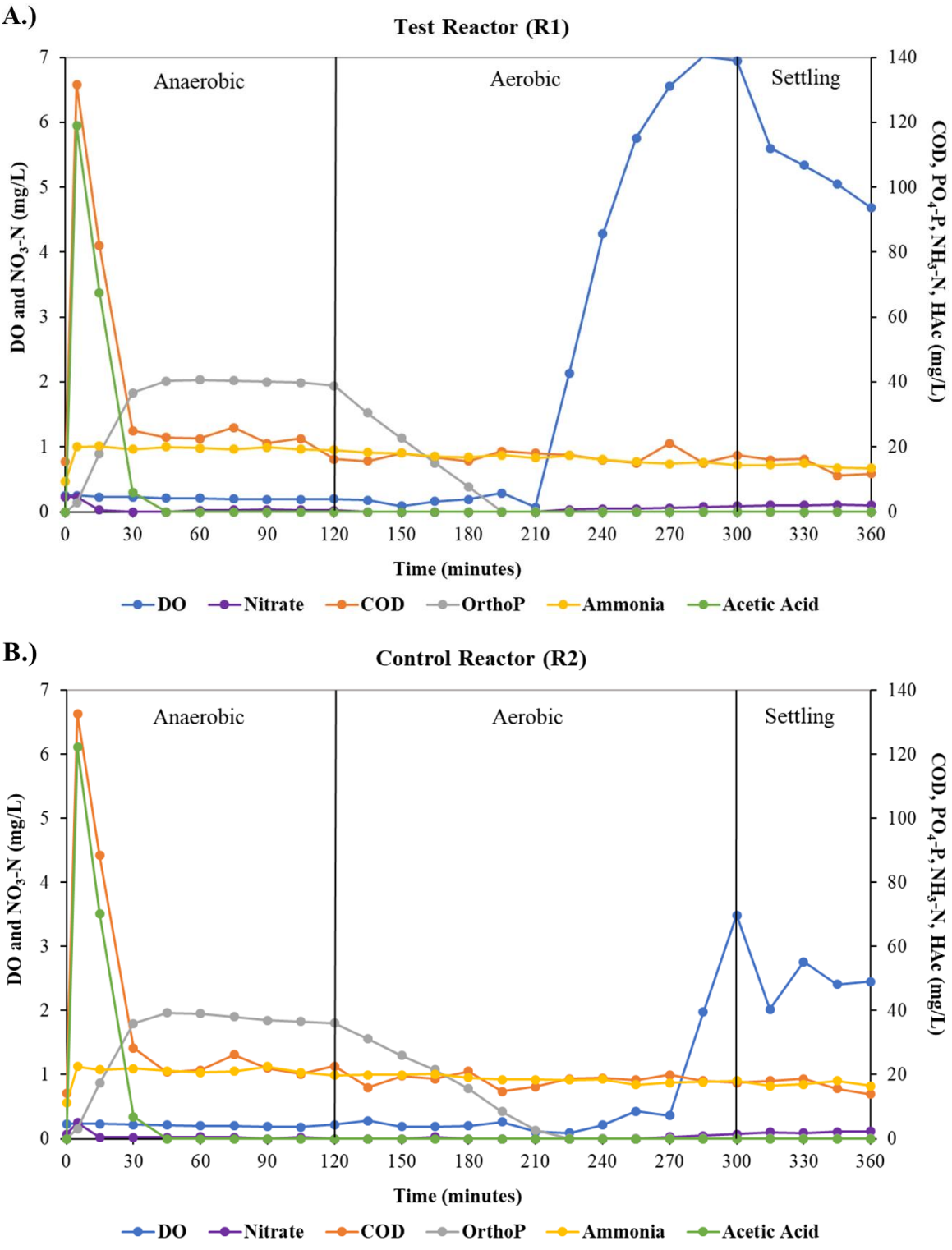


Figure 5-2: Kinetic study for the pre-nitrate/EBPR period for the A.) Test and B.) Control reactors.

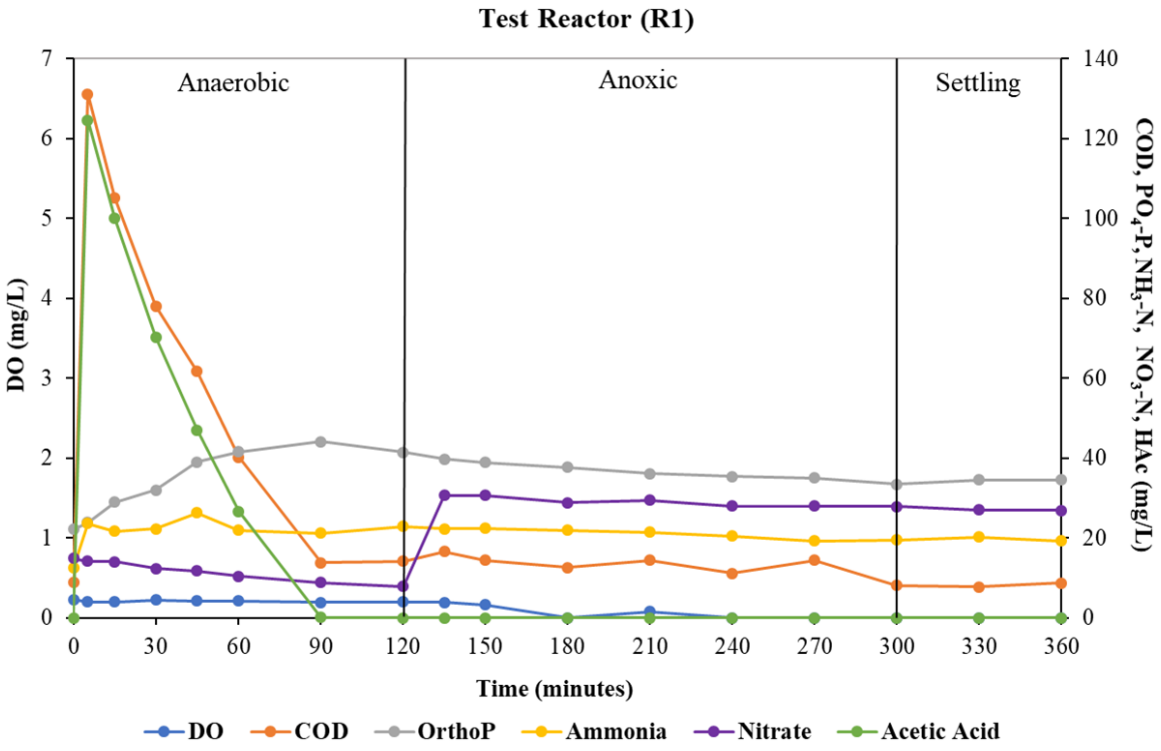


Figure 5-3: Kinetic study conducted the day after introduction of nitrate and removal of DO pump in the Test reactor.

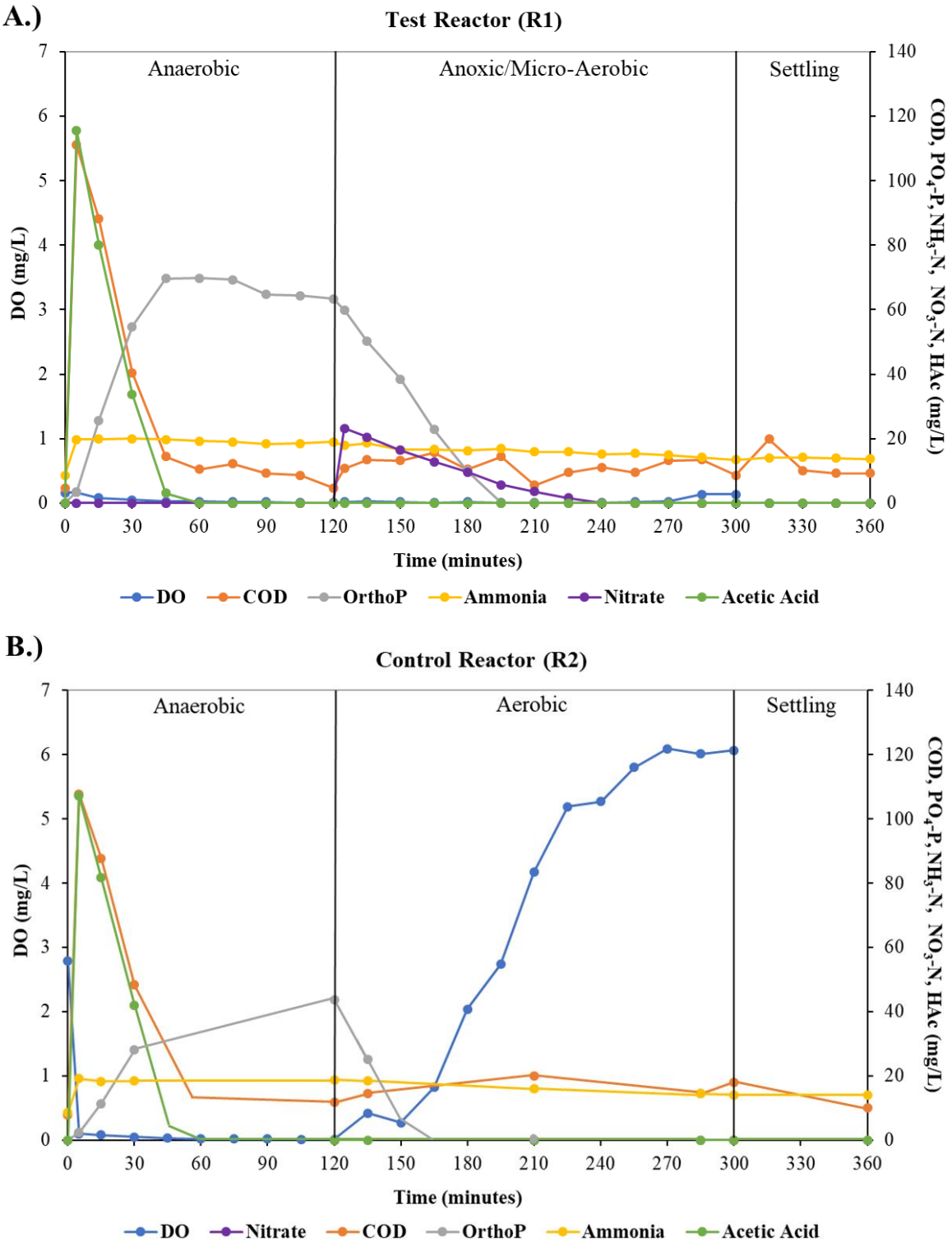


Figure 5-4: Kinetic study during productive dEBPR and EBPR periods for the A.) Test and B.) Control reactors, respectively.

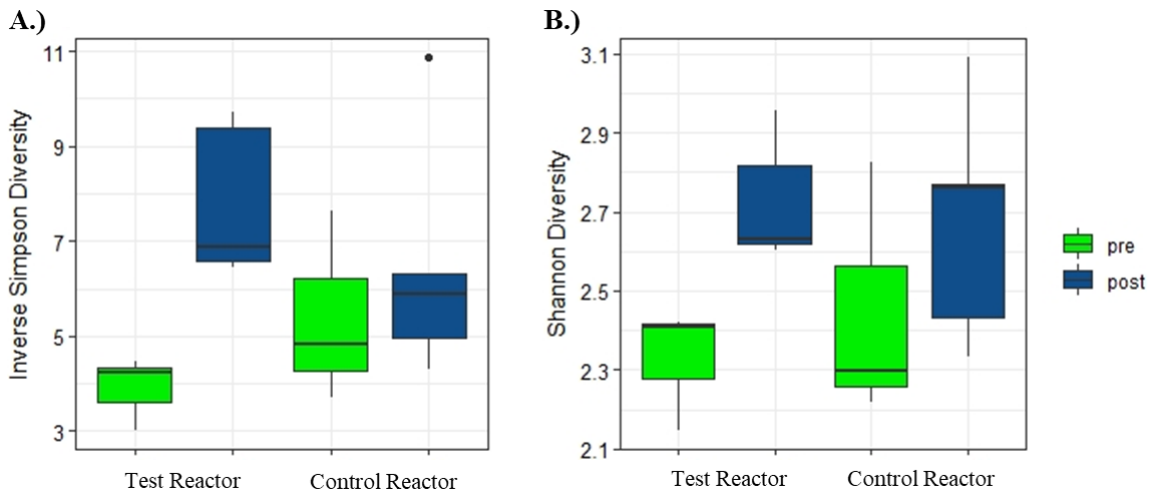


Figure 5-5: Alpha diversity of the total community in each reactor during productive EBPR period (pre) compared to productive dEBPR period (post) based on A.) inverse Simpson and B.) Shannon diversity metrics. Boxplots show the median value as a black bold bar, the upper and lower limits of the box being the third and first quartile of the data, the whiskers extend up to 1.5 times the interquartile range, and black circles are outlier points.

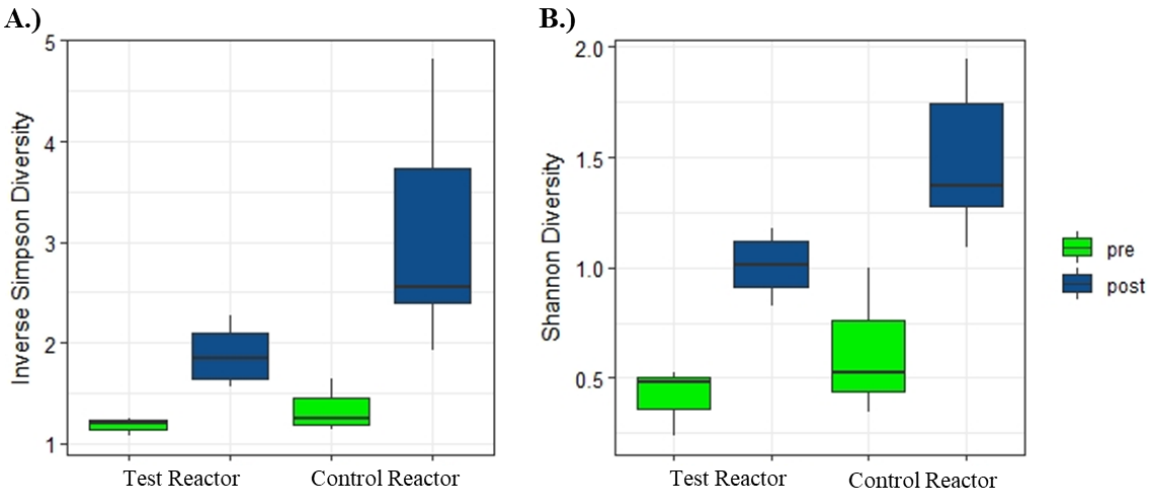


Figure 5-6: Alpha diversity of the BNR-relevant community in each reactor during productive EBPR period (pre) compared to productive dEBPR period (post) based on A.) inverse Simpson and B.) Shannon diversity metrics. Boxplots show the median value as a black bold bar, the upper and lower limits of the box being the third and first quartile of the data, and the whiskers extend up to 1.5 times the interquartile range.

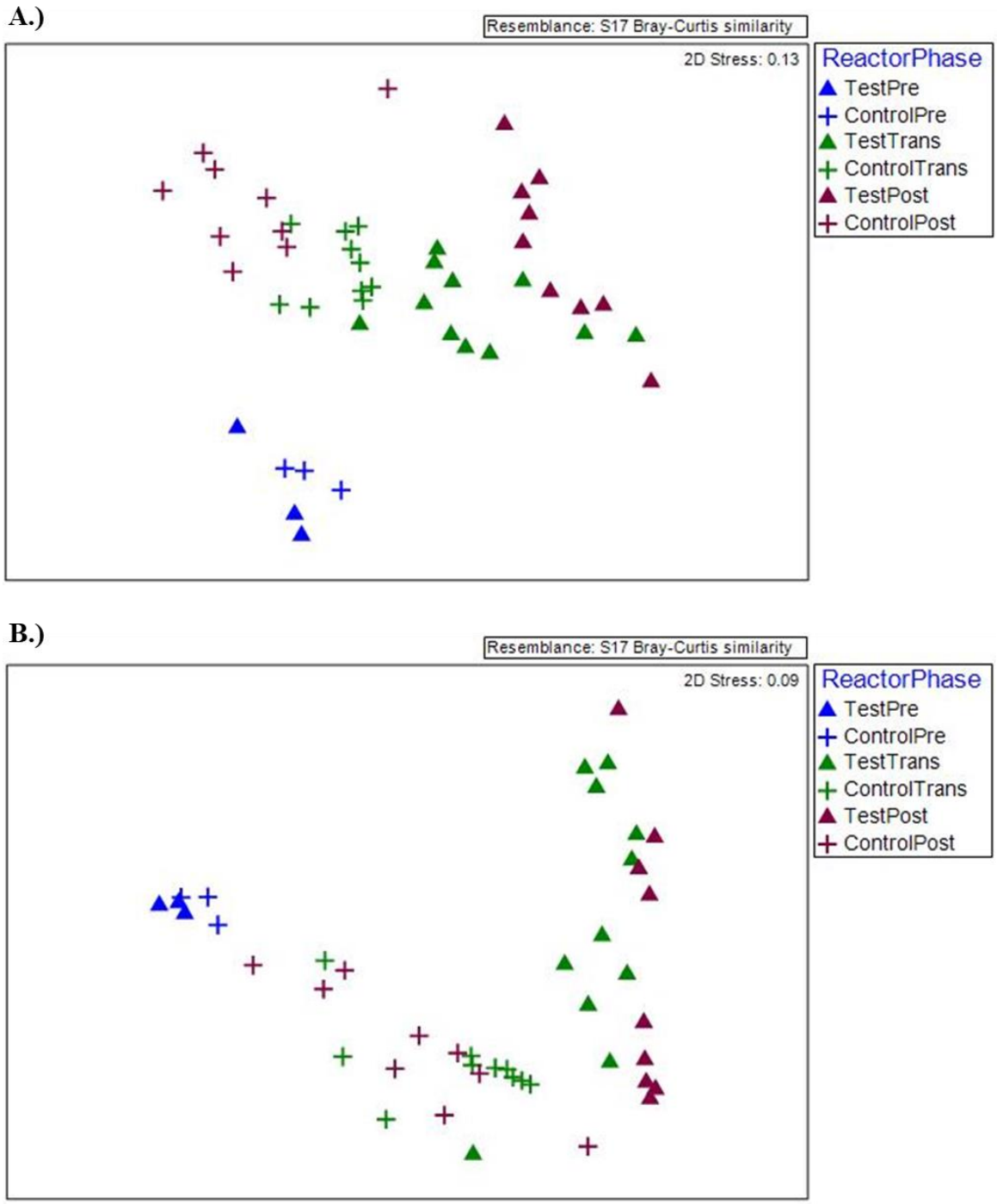


Figure 5-7: Samples collected from the laboratory-scale sequencing batch reactors. NMDS for DNA-based A.) total and B.) BNR-relevant communities generated from relative abundance data.

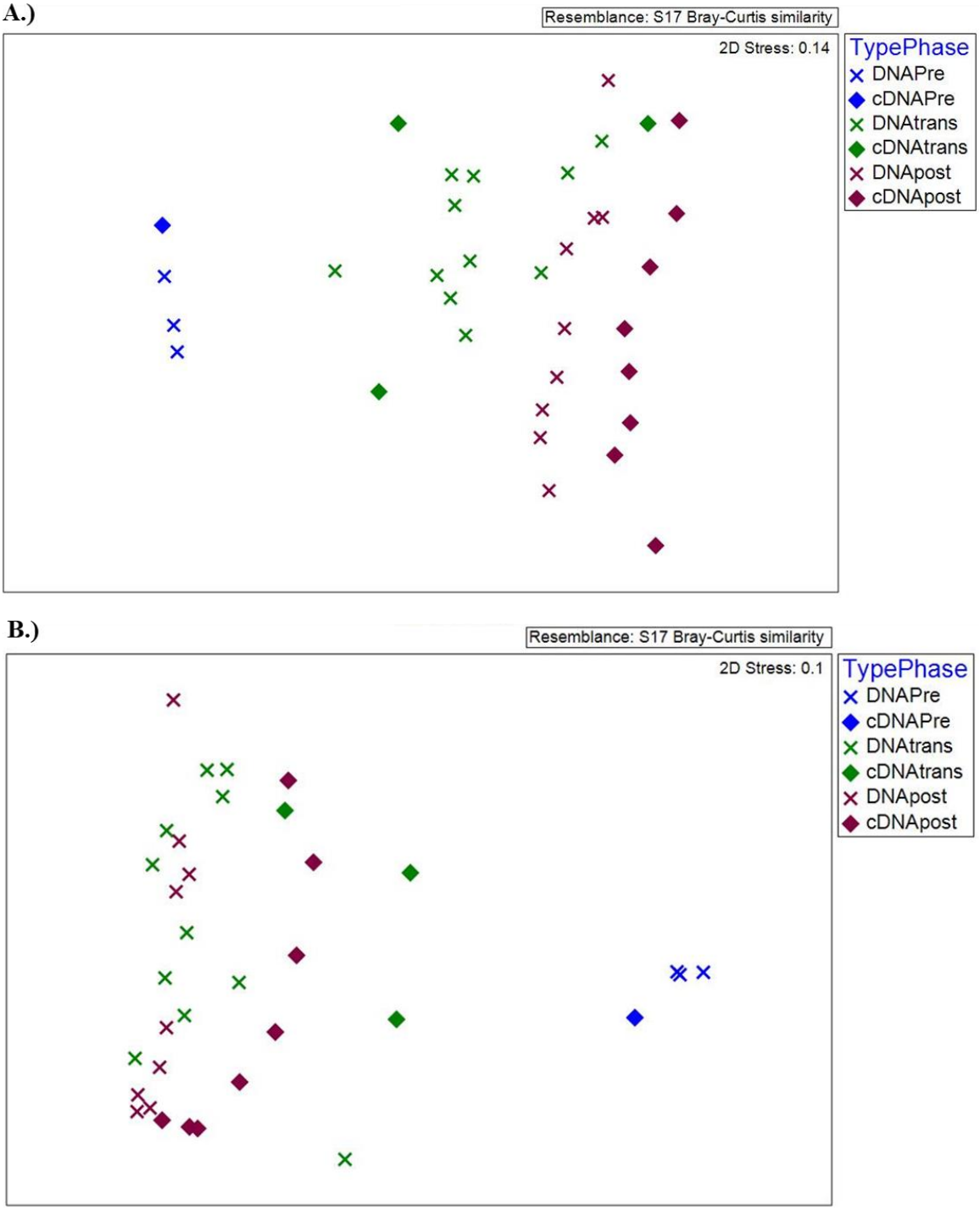
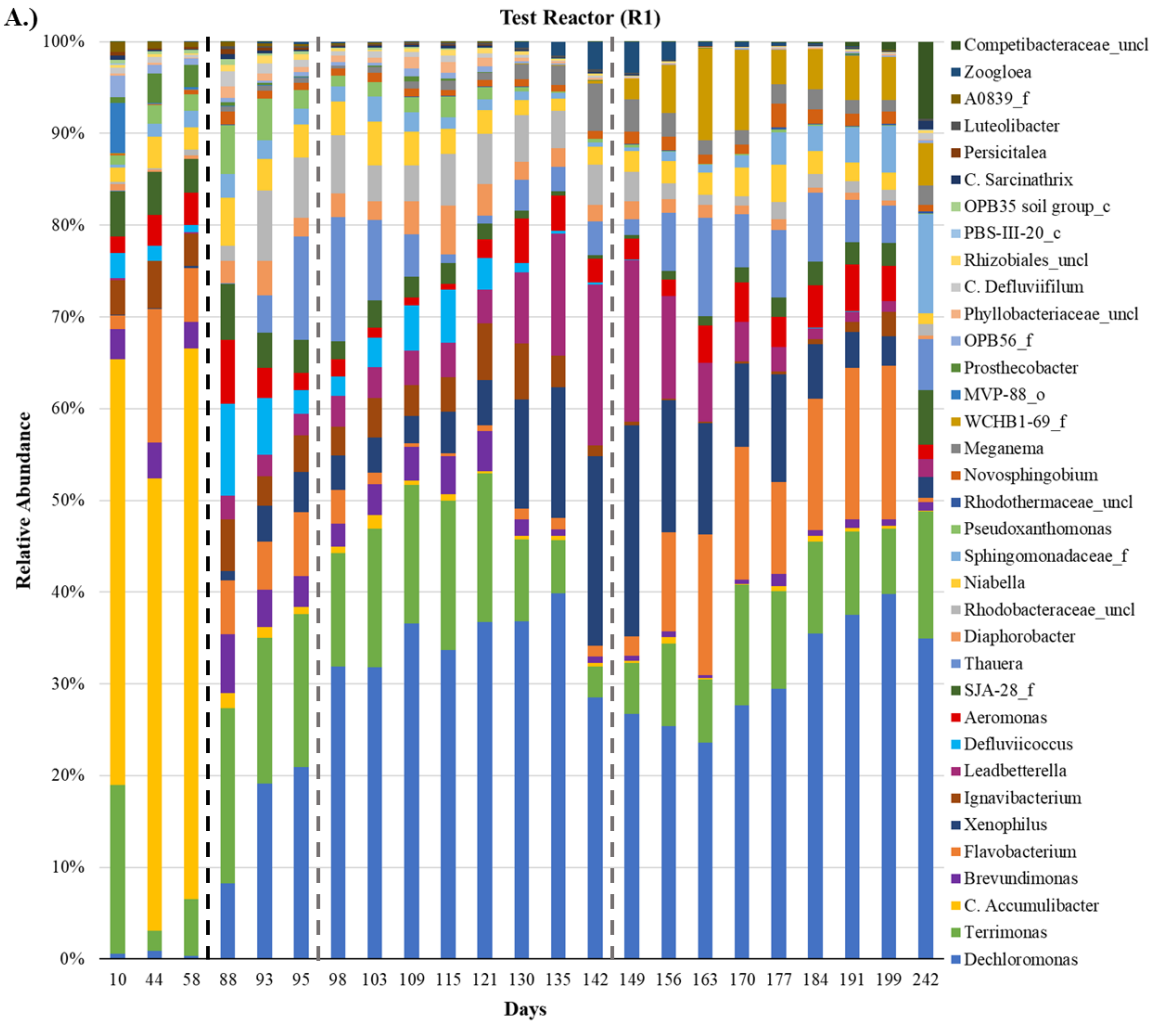


Figure 5-8: Samples collected from the laboratory-scale sequencing batch reactors. NMDS for cDNA-based A.) total and B.) BNR-relevant communities generated from relative abundance data.


A.)
Test Reactor (R1)
Relative Abundance
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
10 44 58 88 93 95 98 103 109 115 121 130 135 142 149 156 163 170 177 184 191 199 242
Days
Competibacteraceae_uncl
Zoogloea
A0839_f
Luteolibacter
Persicitalea
C. Sarcinathrix
OPB35 soil group_c
PBS-III-20_c
Rhizobiales_uncl
C. Defluviifilum
Phyllobacteriaceae_uncl
OPB56_f
Prosthecobacter
MVP-88_o
WCHB1-69_f
Meganema
Novosphingobium
Rhodothermaceae_uncl
Pseudoxanthomonas
Sphingomonadaceae_f
Niabella
Rhodobacteraceae_uncl
Diaphorobacter
Thauera
SJA-28_f
Aeromonas
Defluviicoccus
Leadbetterella
Ignavibacterium
Xenophilus
Flavobacterium
Brevundimonas
C. Accumulibacter
Terrimonas
Dechloromonas

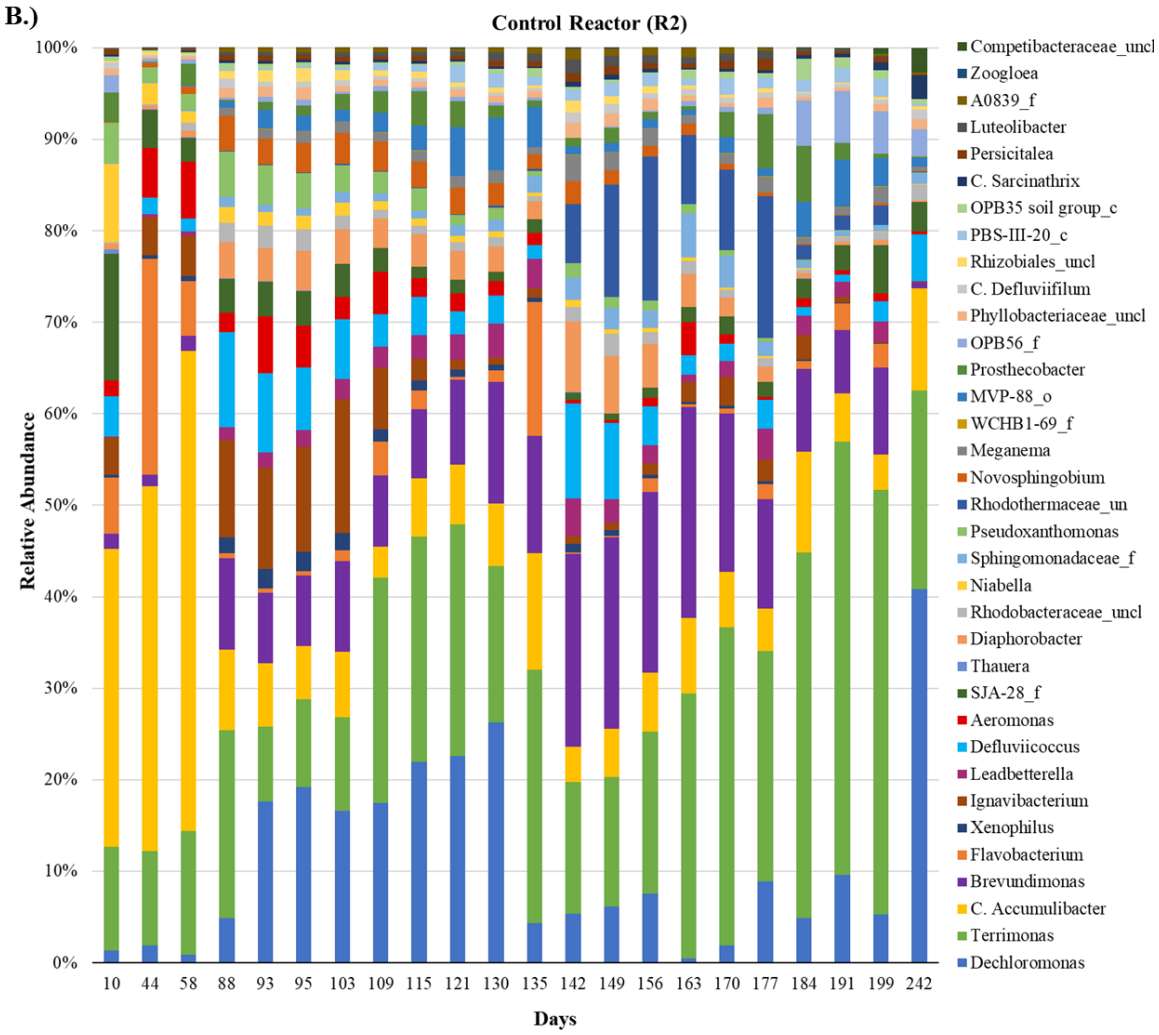
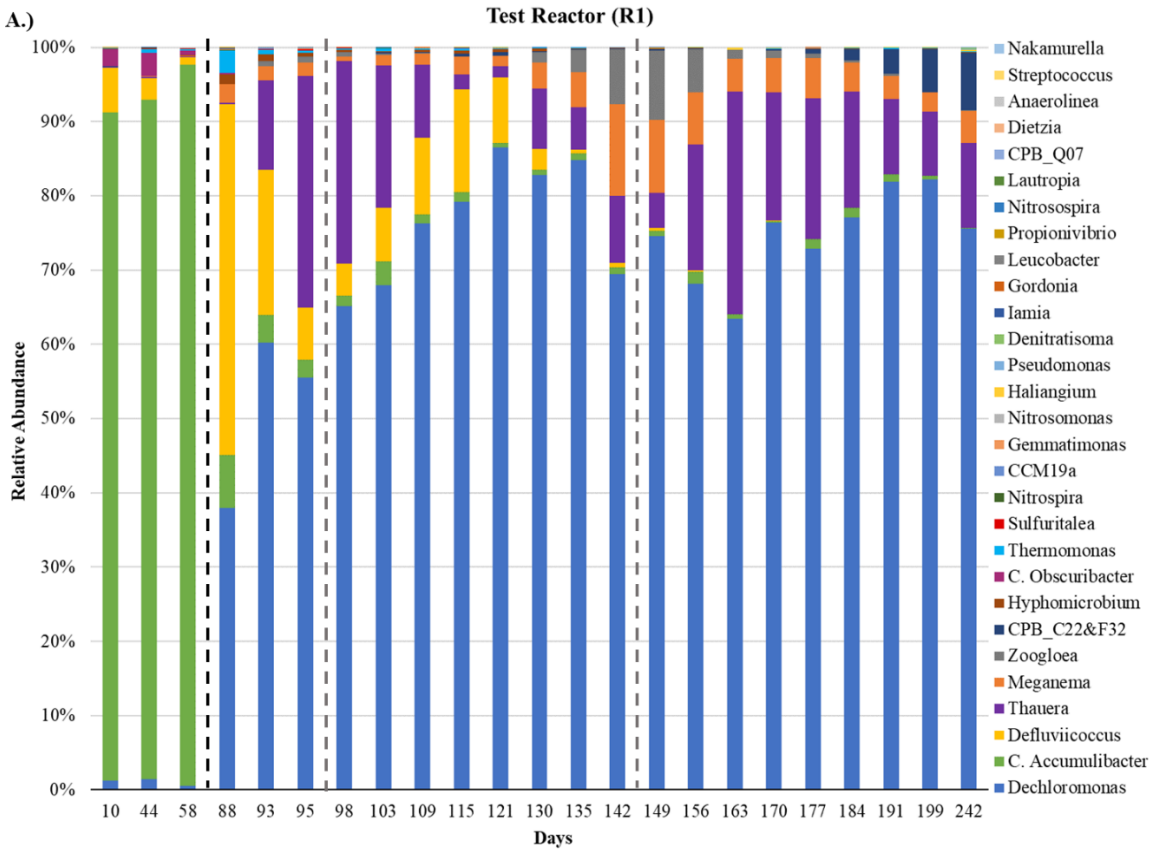


Figure 5-9: Top 35 most abundant genera for the DNA-based total community for A.) Test Reactor and B.) Control Reactor. The black dashed line indicates introduction of nitrate (20 mg/L NO_3-N in the reactor) and removal of DO pump. The first grey dashed line indicates the reduction of nitrate (10 mg/L NO_3-N in the reactor) and introduction of low DO (<0.25mg/L). The second grey dashed line indicates increased nitrate (20 mg/L NO_3-N in the reactor).


A.)
Test Reactor (R1)
Relative Abundance
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
10 44 58 88 93 95 98 103 109 115 121 130 135 142 149 156 163 170 177 184 191 199 242
Days
Nakamurella
Streptococcus
Anaerolinea
Dietzia
CPB_Q07
Lautropia
Nitrosospira
Propionivibrio
Leucobacter
Gordonia
Iamia
Denitratisoma
Pseudomonas
Haliangium
Nitrosomonas
Gemmatimonas
CCM19a
Nitrospira
Sulfuritalea
Thermomonas
C. Obscuribacter
Hyphomicrobium
CPB_C22&F32
Zoogloea
Meganema
Thauera
Defluviicoccus
C. Accumulibacter
Dechloromonas

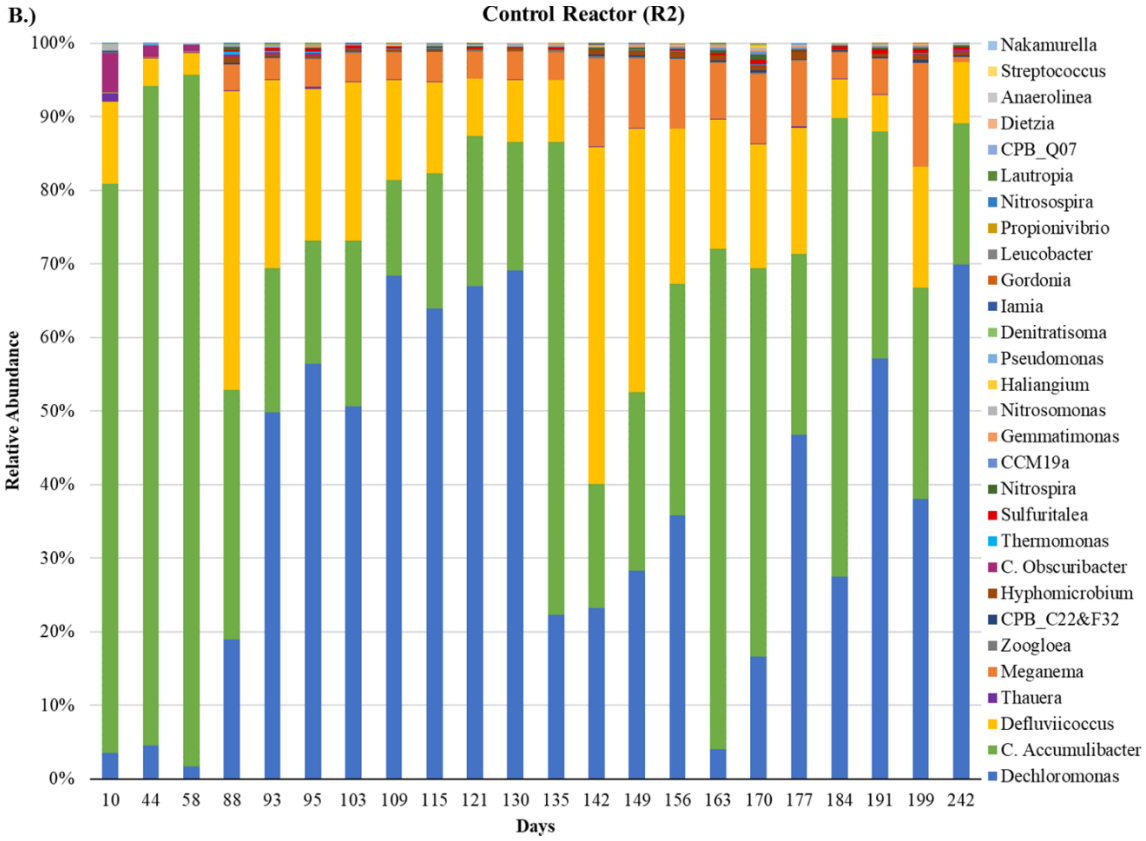
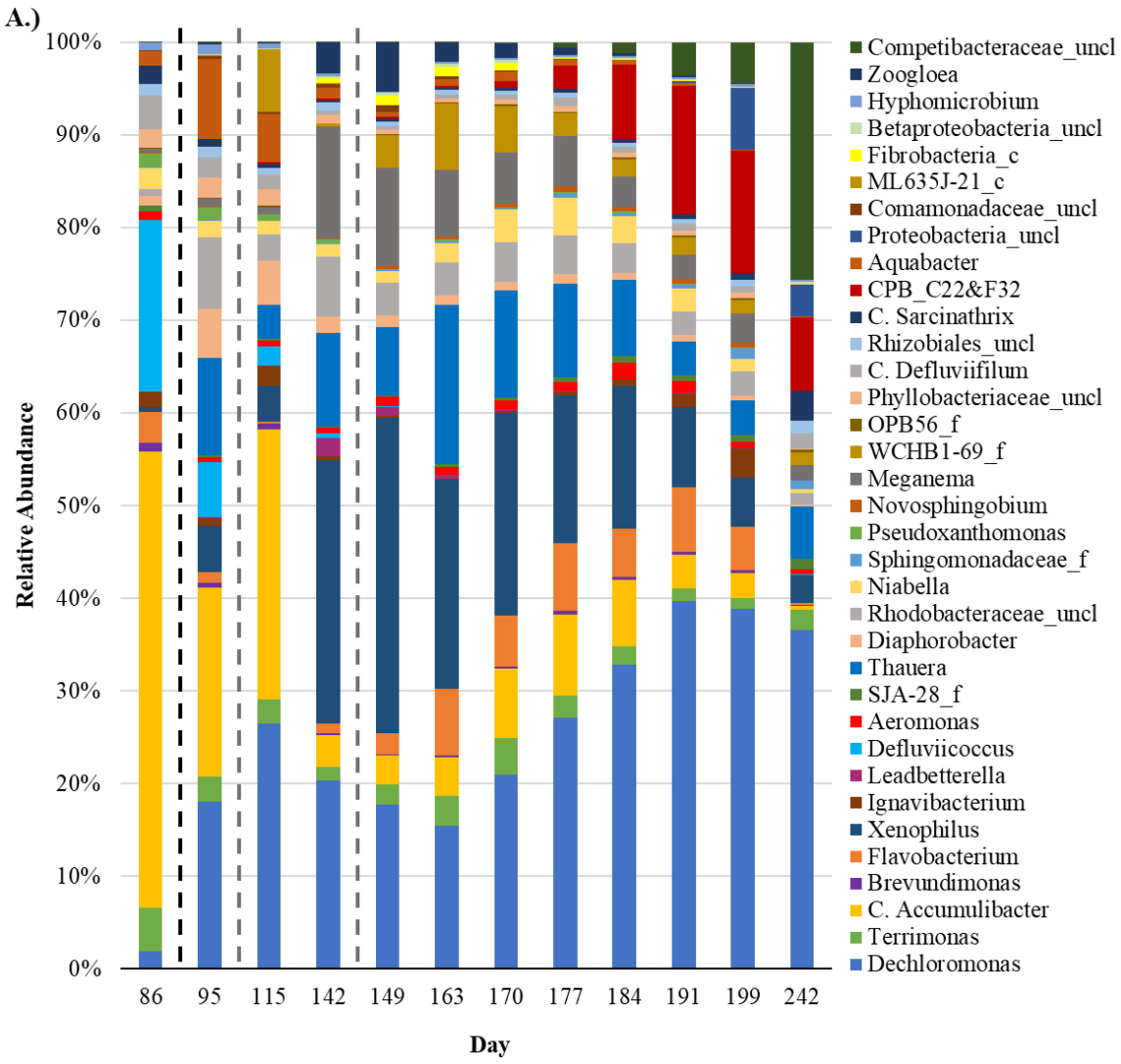


Figure 5-10: BNR-relevant genera community composition based on the DNA dataset for A.) Test Reactor and B.) Control Reactor. The black dashed line indicates introduction of nitrate (20 mg/L NO_3-N in the reactor) and removal of DO pump. The first grey dashed line indicates the reduction of nitrate (10 mg/L NO_3-N in the reactor) and introduction of low DO (<0.25mg/L). The second grey dashed line indicates increased nitrate (20 mg/L NO_3-N in the reactor).


A.)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative Abundance
86
95
115
142
149
163
170
177
184
191
199
242
Day
Competibacteraceae_uncl
Zoogloea
Hyphomicrobium
Betaproteobacteria_uncl
Fibrobacteria_c
ML635J-21_c
Comamonadaceae_uncl
Proteobacteria_uncl
Aquabacter
CPB_C22&F32
C. Sarcinathrix
Rhizobiales_uncl
C. Defluviifilum
Phyllobacteriaceae_uncl
OPB56_f
WCHB1-69_f
Meganema
Novosphingobium
Pseudoxanthomonas
Sphingomonadaceae_f
Niabella
Rhodobacteraceae_uncl
Diaphorobacter
Thauera
SJA-28_f
Aeromonas
Defluviicoccus
Leadbetterella
Ignavibacterium
Xenophilus
Flavobacterium
Brevundimonas
C. Accumulibacter
Terrimonas
Dechloromonas

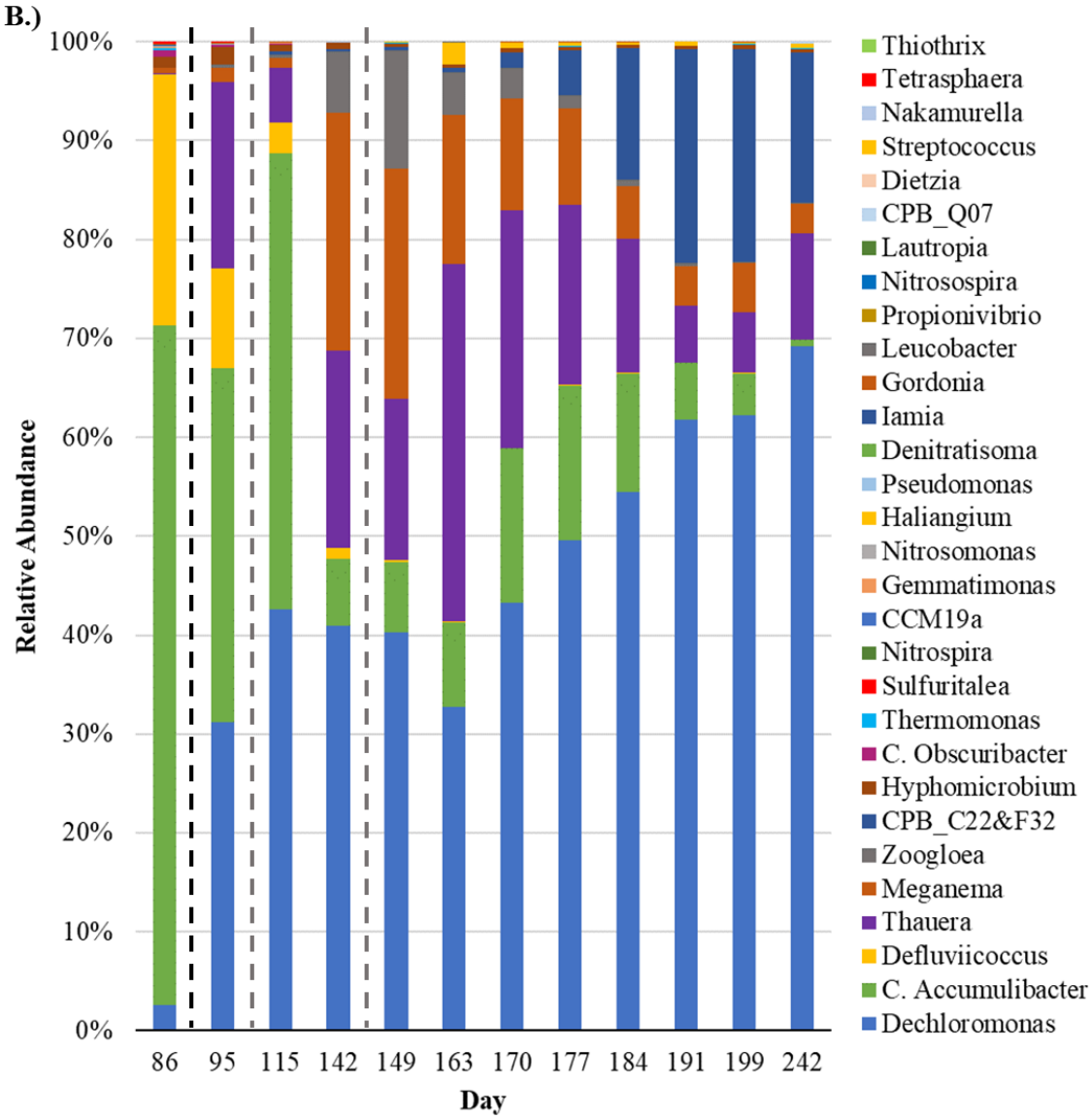


Figure 5-11: Active genera for the A.) total and B.) BNR-relevant communities for the Test reactor. The black dashed line indicates introduction of nitrate (20 mg/L NO_3-N in the reactor) and removal of DO pump. The first grey dashed line indicates the reduction of nitrate (10 mg/L NO_3-N in the reactor) and introduction of low DO (<0.25mg/L). The second grey dashed line indicates increased nitrate (20 mg/L NO_3-N in the reactor).

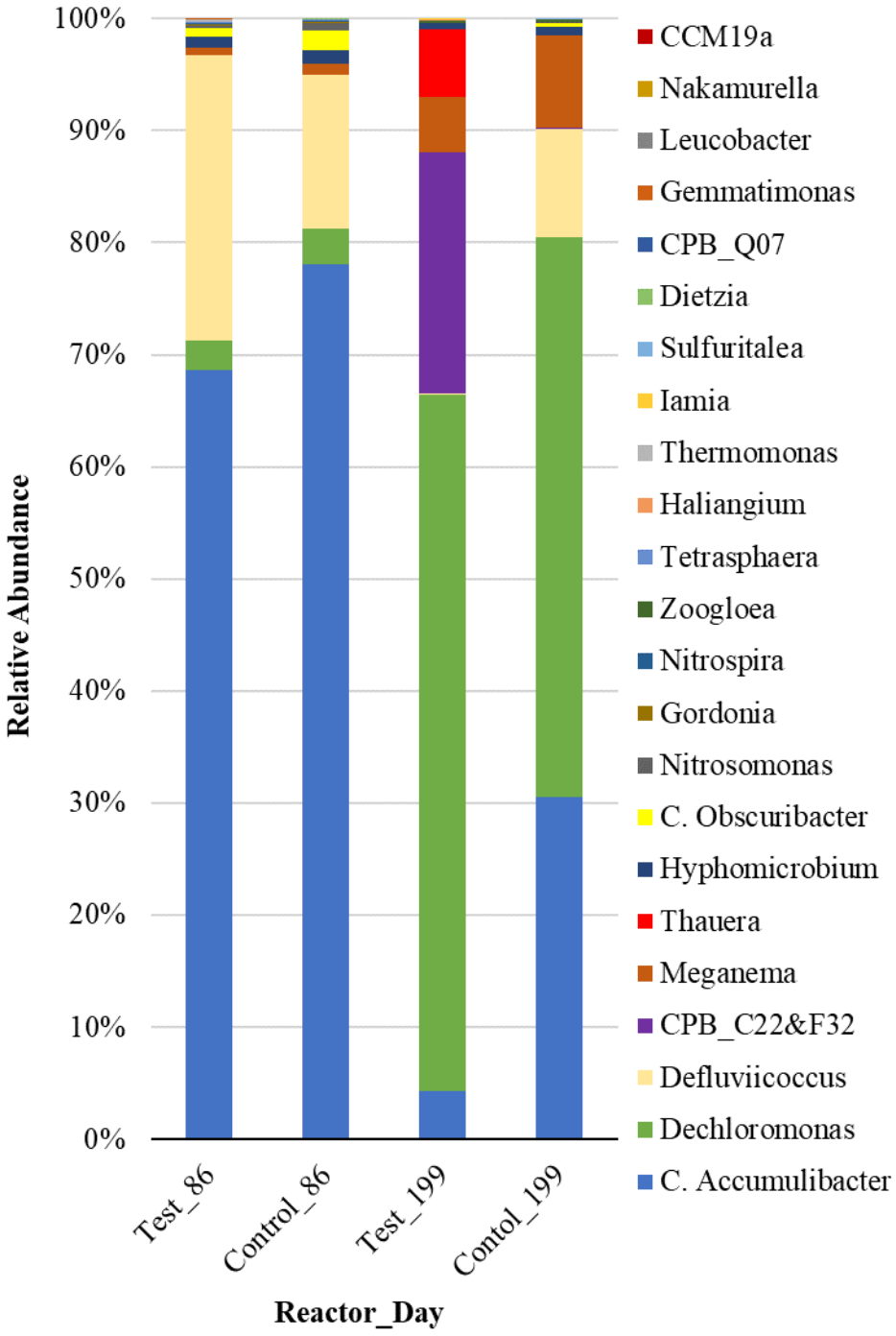
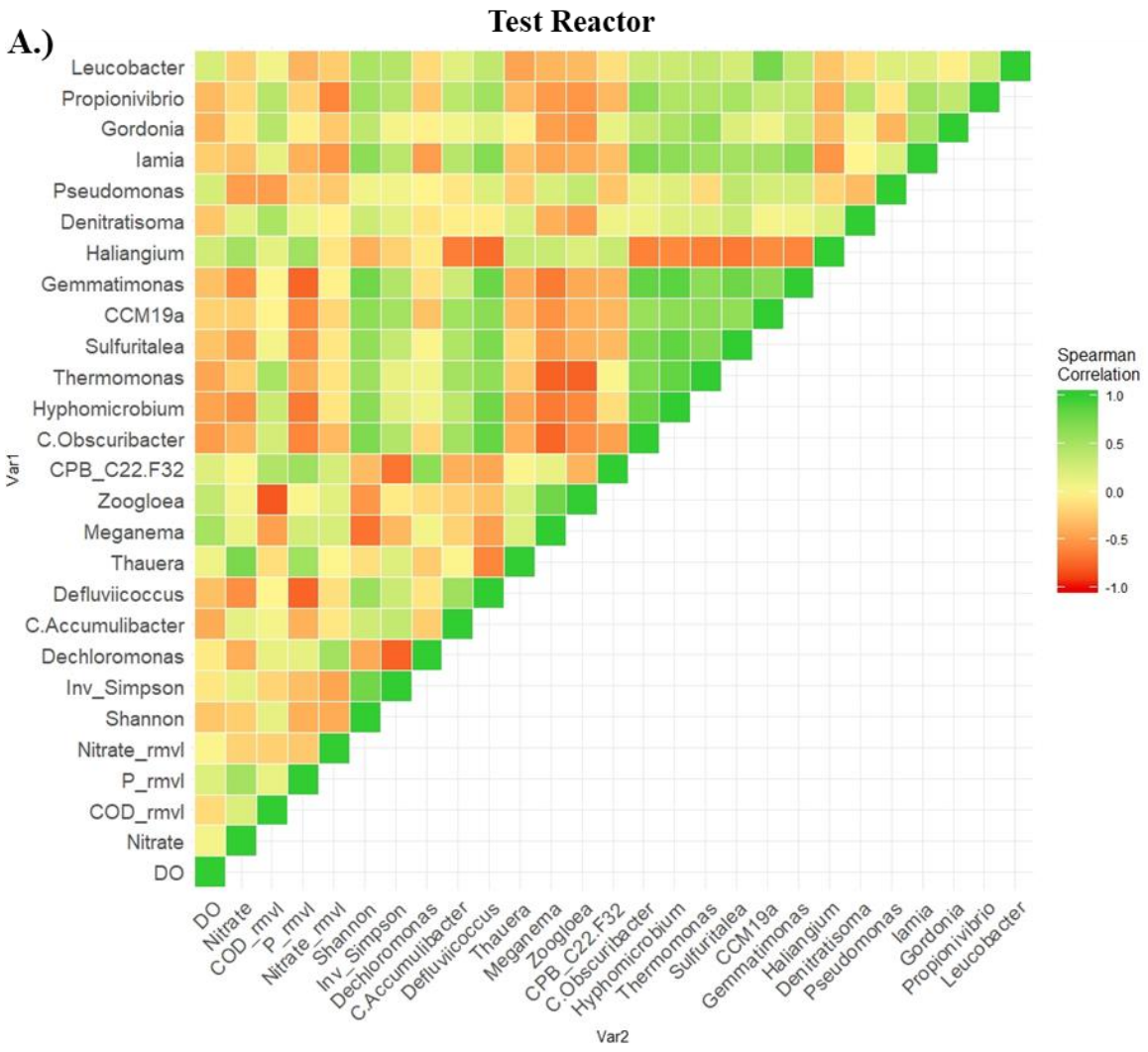
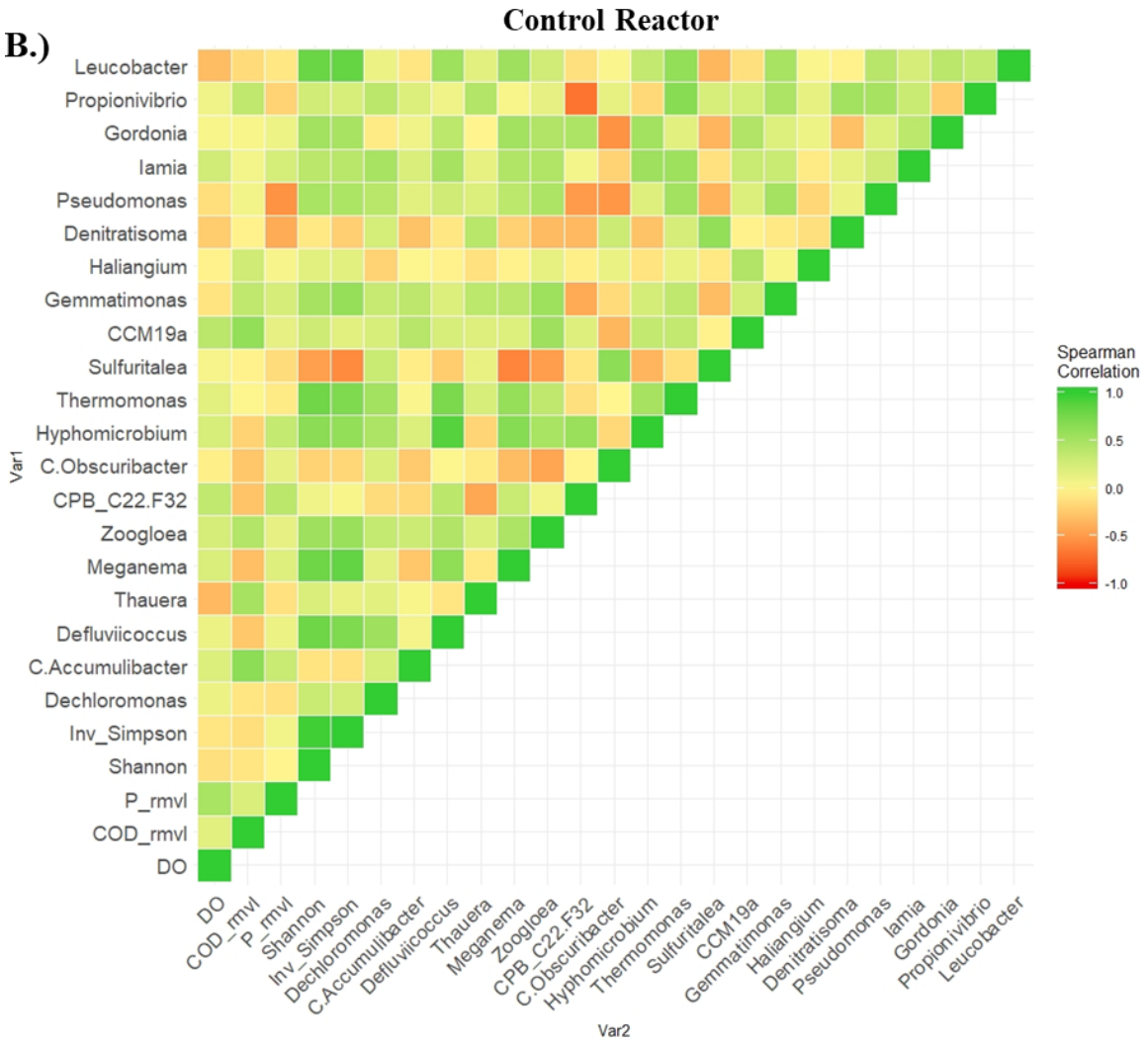


Figure 5-12: Active BNR-relevant community for Test and Control reactors during kinetic studies for EBPR on day 88 and dEBPR (test reactor) on day 199.

A.)


Test Reactor
Leucobacter
Propionivibrio
Gordonia
Iamia
Pseudomonas
Denitratisoma
Haliangium
Gemmatimonas
CCM19a
Sulfuritalea
Thermomonas
Hyphomicrobium
C.Obscuribacter
CPB_C22.F32
Zoogloea
Meganema
Thauera
Defluviicoccus
C.Accumulibacter
Dechloromonas
Inv_Simpson
Shannon
Nitrate_rmvl
P_rmvl
COD_rmvl
Nitrate
DO
Var1
Var2
Spearman Correlation
1.0
0.5
0.0
-0.5
-1.0

B.)


Control Reactor
Leucobacter
Propionivibrio
Gordonia
Iamia
Pseudomonas
Denitratisoma
Haliangium
Gemmatimonas
CCM19a
Sulfuritalea
Thermomonas
Hyphomicrobium
C.Obscuribacter
CPB_C22.F32
Zoogloea
Meganema
Thauera
Defluviicoccus
C.Accumulibacter
Dechloromonas
Inv_Simpson
Shannon
P_rmvl
COD_rmvl
DO
Var1
Var2
Spearman Correlation
1.0
0.5
0.0
-0.5
-1.0

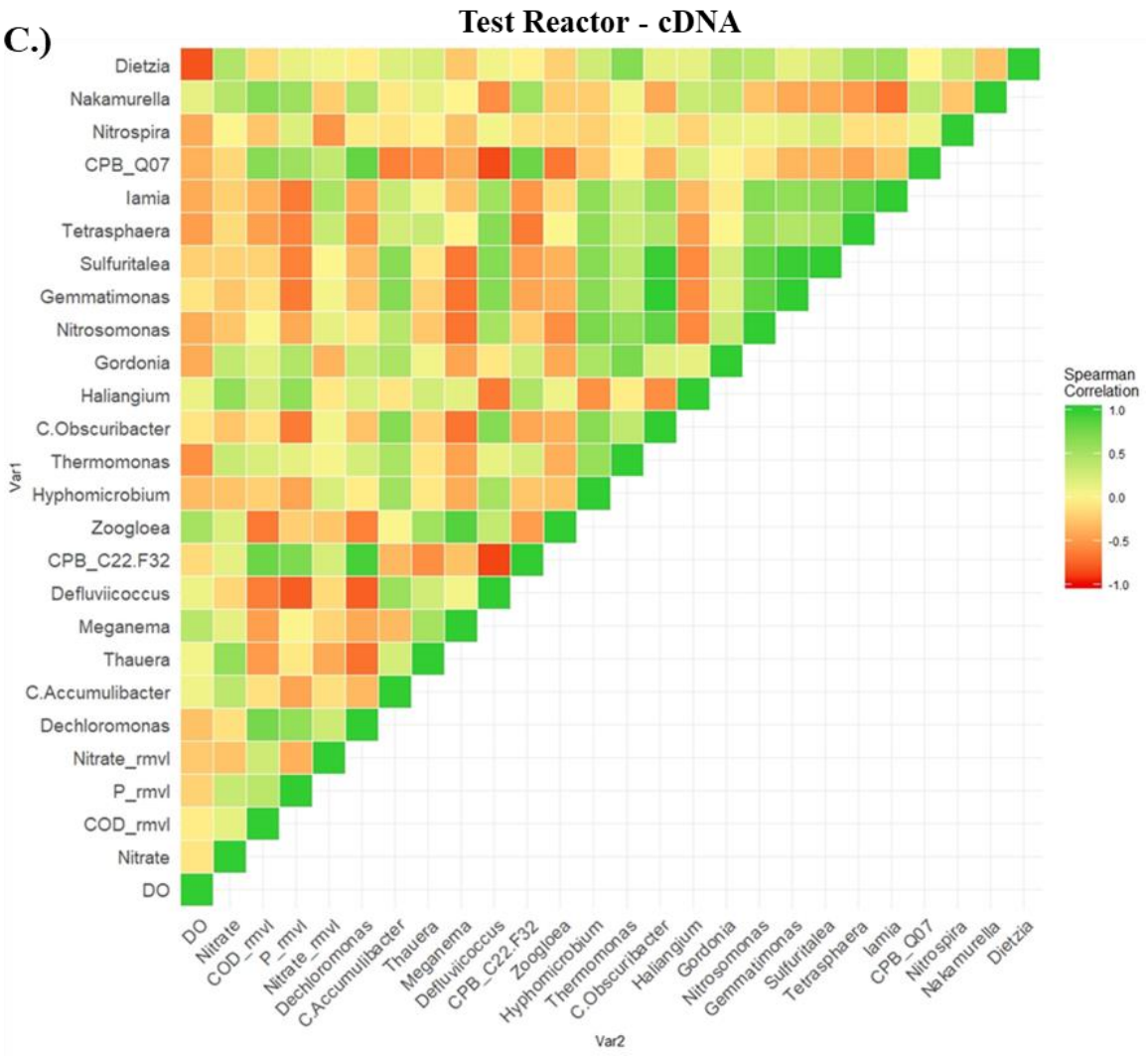


Figure 5-13: Spearman rank correlation for samples collected after the first introduction of nitrate (day 87) from the A.) Test and B.) Control reactors based on DNA datasets and from the C.) Test reactor based on the cDNA dataset. Dissolved oxygen (DO) concentrations are from the end of aerobic or anoxic/microaerobic stage, nitrate represents the concentration of nitrate in the reactor due to feed input, and removal-values are percentages.

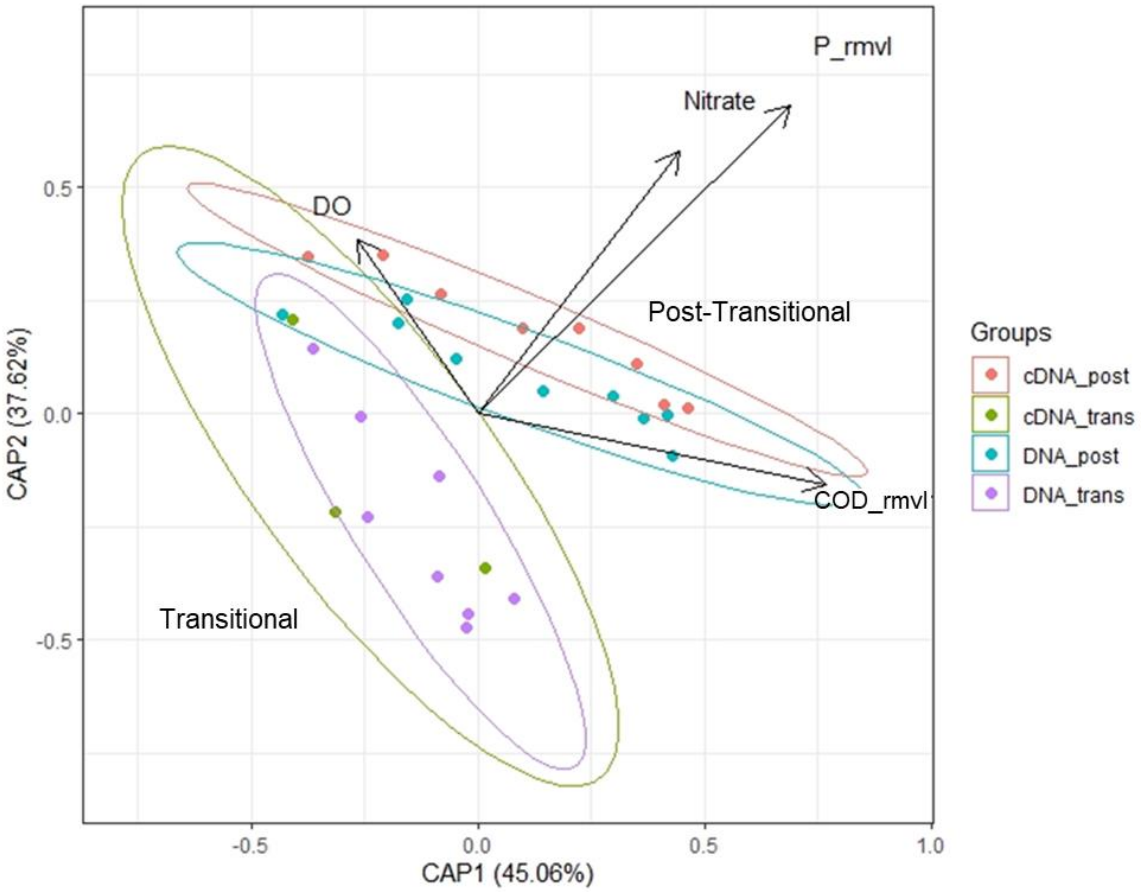


Figure 5-14: Samples collected from the laboratory-scale sequencing batch reactors. Capscale analysis for the total community for the test reactor based on the DNA and cDNA dataset. Nitrate represents the concentration of nitrate in the reactor due to feed input. DO is the concentration measured at the end of the aerobic or anoxic/microaerobic stage.

APPENDIX B: TABLES

Table 2-1: Influent characteristics and nutrient removal efficiency of Cookeville WRRF. Bold black line divides data collected before and after the start of optimization.

Season	Influent Flow (MGD)	Influent Temp (°C)	Influent BOD_5 (mg/L)	% BOD_5 Removal	Influent Ammonia (mg/L)	% Ammonia Removal	Influent TN (mg/L)	% TN Removal	Influent TP (mg/L)	% TP Removal
Winter 2012	8.0 ± 2.5	13.9 ± 1.3	75.6 ± 26.8	98.0 ± 1.5	9.1 ± 3.0	99.1 ± 0.4	16.4 ± 4.2	33.0 ± 17.0	2.7 ± 0.9	72.2 ± 11.3
Summer 2012	4.2 ± 1.8	22.0 ± 1.0	137.3 ± 43.5	99.0 ± 0.6	21.1 ± 8.6	99.4 ± 0.5	30.8 ± 8.2	38.6 ± 12.9	5.4 ± 2.3	41.5 ± 22.6
Winter 2013	8.8 ± 3.1	12.2 ± 1.3	78.9 ± 24.5	97.8 ± 1.8	11.6 ± 5.9	99.3 ± 1.6	17.0 ± 4.9	29.2 ± 19.3	3.5 ± 1.8	77.3 ± 12.7
Summer 2013	7.3 ± 4.0	20.7 ± 0.8	104.4 ± 47.0	98.8 ± 0.6	22.7 ± 14.2	99.9 ± 0.1	26.4 ± 13.5	36.4 ± 17.3	5.5 ± 2.7	55.9 ± 13.5
Winter 2014	8.8 ± 3.0	11.4 ± 1.0	138.8 ± 52.3	98.9 ± 0.4	10.1 ± 3.9	99.6 ± 0.4	20.1 ± 6.3	39.3 ± 23.6	3.3 ± 1.5	78.4 ± 14.3
Summer 2014	4.3 ± 1.6	20.7 ± 0.7	192.5 ± 60.6	97.4 ± 0.8	13.0 ± 3.9	99.6 ± 0.4	28.7 ± 4.9	29.1 ± 6.5	5.8 ± 1.2	40.9 ± 12.8
Winter 2015	8.7 ± 4.0	12.0 ± 1.3	123.4 ± 38.7	98.3 ± 0.6	8.5 ± 2.6	99.5 ± 0.2	20.2 ± 4.6	44.4 ± 8.1	2.8 ± 1.0	68.4 ± 45.1
Summer 2015	5.5 ± 3.4	22.3 ± 0.7	152.6 ± 50.7	97.9 ± 0.9	11.3 ± 5.0	99.0 ± 1.9	25.9 ± 7.3	83.6 ± 6.6	5.0 ± 1.4	59.8 ± 19.0
Winter 2016	7.8 ± 3.3	13.7 ± 1.2	125.5 ± 48.7	98.0 ± 1.5	10.2 ± 3.9	95.0 ± 5.9	19.3 ± 6.1	67.2 ± 19.7	3.2 ± 1.2	70.1 ± 17.7
Summer 2016	5.6 ± 2.2	23.2 ± 0.9	152.5 ± 46.2	98.0 ± 1.1	16.5 ± 5.2	98.4 ± 2.5	25.6 ± 4.0	94.7 ± 2.5	4.7 ± 1.1	69.3 ± 15.9
Winter 2017	7.9 ± 2.5	14.7 ± 0.6	135.1 ± 52.3	97.4 ± 1.8	12.6 ± 4.1	94.8 ± 9.2	21.8 ± 6.9	87.0 ± 9.2	3.2 ± 1.0	81.1 ± 9.8
Summer 2017	6.0 ± 2.0	22.3 ± 0.7	152.0 ± 30.5	97.5 ± 0.7	15.5 ± 3.9	98.6 ± 1.6	28.2 ± 6.9	88.0 ± 5.4	4.6 ± 1.4	71.9 ± 15.8
Winter 2018	9.3 ± 4.3	13.3 ± 0.9	127.1 ± 35.1	98.5 ± 0.8	13.2 ± 4.5	98.5 ± 1.3	22.8 ± 7.6	74.9 ± 11.2	3.9 ± 1.2	77.8 ± 5.8
Summer 2018	6.8 ± 3.3	22.7 ± 0.8	149.7 ± 46.0	98.4 ± 1.0	16.3 ± 5.2	97.8 ± 2.9	26.7 ± 7.4	88.5 ± 11.9	5.3 ± 1.8	64.1 ± 19.1

Table 2-2: Date and location for samples collected from oxidation ditch for DNA extraction. Samples were collected from the surface (S), middle (M), and deep (D) of the oxidation ditch.

Sample ID	**Sampling Event**	**Date**	**Location in OxDitch**
CkvlPreOpt1	Pre_Opt	4/27/2015	BS
CkvlPreOpt2	Pre_Opt	4/27/2015	BS
Ckvl2015	Early Summer 2015	6/19/2015	BS
Ckvl15S2	Summer 2015	7/10/2015	BS
Ckvl15S3	Summer 2015	7/10/2015	BM
Ckvl15S4	Summer 2015	7/10/2015	BD
Ckvl15S5	Summer 2015	7/10/2015	CS
Ckvl15S6	Summer 2015	7/10/2015	CM
Ckvl15S7	Summer 2015	7/10/2015	CD
Ckvl15S8	Summer 2015	7/10/2015	DS
Ckvl15S9	Summer 2015	7/10/2015	DM
Ckvl15S10	Summer 2015	7/10/2015	DD
Ckvl15S11	Summer 2015	7/10/2015	ES
Ckvl15S12	Summer 2015	7/10/2015	EM
Ckvl15S13	Summer 2015	7/10/2015	ED
Ckvl16W1	Winter 2016	3/9/2016	CD
Ckvl16W2	Winter 2016	3/9/2016	CS
Ckvl16W3	Winter 2016	3/9/2016	ED
Ckvl16W4	Winter 2016	3/9/2016	ES
Ckvl16W5	Winter 2016	3/9/2016	AD
Ckvl16W6	Winter 2016	3/9/2016	AS
Ckvl16W7	Winter 2016	3/9/2016	BD
Ckvl16W8	Winter 2016	3/9/2016	BS
Ckvl16S1	Summer 2016	9/22/2016	AS
Ckvl16S2	Summer 2016	9/22/2016	BS
Ckvl16S3	Summer 2016	9/22/2016	BD
Ckvl16S4	Summer 2016	9/22/2016	CS
Ckvl16S5	Summer 2016	9/22/2016	CD
Ckvl16S6	Summer 2016	9/22/2016	DS

Table 2-2 (Continued)			
Sample ID	**Sampling Event**	**Date**	**Location in OxDitch**
Ckvl16S7	Summer 2016	9/22/2016	DD
Ckvl16S8	Summer 2016	9/22/2016	ES
Ckvl16S9	Summer 2016	9/22/2016	ED
Ckvl16S10	Summer 2016	9/22/2016	FS
Ckvl16S11	Summer 2016	9/22/2016	FD
Ckvl17W1	Winter 2017	3/16/2017	AS
Ckvl17W2	Winter 2017	3/16/2017	BS
Ckvl17W3	Winter 2017	3/16/2017	BD
Ckvl17W4	Winter 2017	3/16/2017	CS
Ckvl17W5	Winter 2017	3/16/2017	CD
Ckvl17W6	Winter 2017	3/16/2017	DS
Ckvl17W7	Winter 2017	3/16/2017	DD
Ckvl17W8	Winter 2017	3/16/2017	ES
Ckvl17W9	Winter 2017	3/16/2017	ED
Ckvl17W10	Winter 2017	3/16/2017	FS
Ckvl17W11	Winter 2017	3/16/2017	FD
Ckvl17S1	Summer 2017	8/22/2017	AS
Ckvl17S2	Summer 2017	8/22/2017	BS
Ckvl17S3	Summer 2017	8/22/2017	BD
Ckvl17S4	Summer 2017	8/22/2017	CS
Ckvl17S5	Summer 2017	8/22/2017	CD
Ckvl17S6	Summer 2017	8/22/2017	DS
Ckvl17S7	Summer 2017	8/22/2017	DD
Ckvl17S8	Summer 2017	8/22/2017	ES
Ckvl17S9	Summer 2017	8/22/2017	ED
Ckvl17S10	Summer 2017	8/22/2017	FS
Ckvl17S11	Summer 2017	8/22/2017	FD
Ckvl18W1	Winter 2018	3/6/2018	AS
Ckvl18W2	Winter 2018	3/6/2018	BS
Ckvl18W3	Winter 2018	3/6/2018	BD
Ckvl18W4	Winter 2018	3/6/2018	CS
Ckvl18W5	Winter 2018	3/6/2018	CD
Ckvl18W6	Winter 2018	3/6/2018	DS

Table 2-2 (Continued)			
Sample ID	**Sampling Event**	**Date**	**Location in OxDitch**
Ckvl18W7	Winter 2018	3/6/2018	DD
Ckvl18W8	Winter 2018	3/6/2018	ES
Ckvl18W9	Winter 2018	3/6/2018	ED
Ckvl18W10	Winter 2018	3/6/2018	FS
Ckvl18W11	Winter 2018	3/6/2018	FD
Ckvl18S1	Summer 2018	8/30/2018	AS
Ckvl18S2	Summer 2018	8/30/2018	BS
Ckvl18S3	Summer 2018	8/30/2018	BD
Ckvl18S4	Summer 2018	8/30/2018	CS
Ckvl18S5	Summer 2018	8/30/2018	CD
Ckvl18S6	Summer 2018	8/30/2018	DS
Ckvl18S7	Summer 2018	8/30/2018	DD
Ckvl18S8	Summer 2018	8/30/2018	ES
Ckvl18S9	Summer 2018	8/30/2018	ED
Ckvl18S10	Summer 2018	8/30/2018	FS
Ckvl18S11	Summer 2018	8/30/2018	FD
Ckvl18Sb1	Summer2 2018	9/13/2018	AS
Ckvl18Sb2	Summer2 2018	9/13/2018	BS
Ckvl18Sb3	Summer2 2018	9/13/2018	BD
Ckvl18Sb4	Summer2 2018	9/13/2018	CS
Ckvl18Sb5	Summer2 2018	9/13/2018	CD
Ckvl18Sb6	Summer2 2018	9/13/2018	DS
Ckvl18Sb7	Summer2 2018	9/13/2018	DD
Ckvl18Sb8	Summer2 2018	9/13/2018	ES
Ckvl18Sb9	Summer2 2018	9/13/2018	ED
Ckvl18Sb10	Summer2 2018	9/13/2018	FS
Ckvl18Sb11	Summer2 2018	9/13/2018	FD

Table 2-3: Cookeville WRRF influent and effluent (mg/L) composite samples collected during each sampling event. Summer 2018 sampling event had unusually high influent nutrient concentrations due to maintenance on waste reactor.

	Summer 2015			Winter 2016			Summer 2016			Winter 2017		
	Influent	Effluent	% Removal	Influent	Effluent	% Removal	Influent	Effluent	% Removal	Influent	Effluent	% Removal
Total COD	-	-	-	485	24	95	-	-	-	150	10	93
Soluble COD	47.7	7.2	85	-	-	-	60	9	85	55	5	91
Total P	1.5	0.2	89	-	-	-	3.2	1.4	56	2.8	0.8	71
Ortho P	1.7	0.8	50	1.7	0.9	46	2.5	1.4	44	1.0	0.0	100
NH_3-N	6.1	0.2	97	10.9	0.0	100	9.7	0.3	97	7.8	1.5	81
NO_3-N	0.6	1.7	0	0.0	6.5	0	0.2	0.5	0	1.4	0.5	64
Total N	-	-	-	21.3	9.6	55	15.2	1.2	92	18.2	8.7	52

	Summer 2017			Winter 2018			Summer 2018			Summer 2018b		
	Influent	Effluent	% Removal	Influent	Effluent	% Removal	Influent	Effluent	% Removal	Influent	Effluent	% Removal
Total COD	807	28	97	782	39	95	1706	24	99	261	21	92
Soluble COD	67	21	69	68	33	51	84	23	73	54	20	63
Total P	5.7	1.2	79	3.1	0.7	78	49.0	0.6	99	4.3	1.8	58
Ortho P	1.7	1.1	34	0.9	0.5	50	15.6	0.5	97	3.2	1.7	47
NH_3-N	11.4	0.5	96	4.8	0.0	100	13.8	0.0	100	8.7	0.3	97
NO_3-N	0.8	4.9	0	0.8	4.2	0	16.4	6.7	59	3.2	0.5	84
Total N	31.0	3.3	89	19.0	4.4	77	122.0	6.6	95	28.0	1.3	95

Table 2-4: Oxidation ditch sample measurements (raw data) from Cookeville WRRF.

Summer 2015 7/10/2015						
Location	**Dissolved Oxygen (mg/L)**	**Temp, C**	**Soluble COD (mg/L)**	**Ortho P (PO_4^{3-}-P) (mg/L)**	**Ammonia (NH_3-N) (mg/L)**	**Nitrate (NO_3-N) (mg/L)**
OX Influent	-	-	11	1.2	2.3	0.9
AS	1.02	22.9	20	1.2	0.6	3.9
DS	1.57	22.5	22	0.7	1.2	2.6
DD	1.40	22.7	27	0.4	0.5	2.2
ES	1.70	22.6	17	1.1	0.5	3.8
ED	0.11	22.9	0	2.3	2.4	4.2
FS	1.08	22.6	17	0.3	0.7	1.8
FD	0.10	22.6	21	8.2	13.9	3.1
OX Effluent	-	-	19	0.9	0.6	3.9

Winter 2016 3/9/2016						
Location	**Dissolved Oxygen (mg/L)**	**Temp, C**	**Soluble COD (mg/L)**	**Ortho P (PO_4^{3-}-P) (mg/L)**	**Ammonia (NH_3-N) (mg/L)**	**Nitrate (NO_3-N) (mg/L)**
OX Influent	-	-	32	1.4	7.2	3.0
AS	1.81	14.5	25	0.8	0.0	8.8
BS	1.48	14.0	13	0.9	0.0	8.6
BD	1.11	14.0	16	1.6	1.2	8.5
CS	1.42	14.5	20	0.9	0.0	8.7
CD	0.51	14.5	16	3.4	3.7	6.2
ES	1.72	14.5	23	0.9	0.0	8.6
ED	0.11	14.5	45	22.9	5.0	4.2
OX Effluent	-	-	22	0.8	0.0	8.8

Table 2-4 (Continued)

Summer 2016 9/22/2016							
Location	**Dissolved Oxygen (mg/L)**	**Temp, C**	**pH**	**Soluble COD (mg/L)**	**Ortho P (PO_4^{3-}-P) (mg/L)**	**Ammonia (NH_3-N) (mg/L)**	**Nitrate (NO_3-N) (mg/L)**
OX Influent	-	-	-	73	3.3	15.3	0.2
AS	0.33	24.0	7.20	12	0.1	0.1	0.2
BS	0.08	24.3	7.33	18	0.1	0.0	0.3
BD	0.03	24.3	6.74	16	1.4	1.0	0.2
CS	0.11	24.3	7.37	17	0.2	0.0	0.3
CD	0.05	24.3	6.75	22	4.2	7.0	0.0
DS	0.37	24.6	7.38	6	0.2	0.0	0.4
DD	0.55	24.4	7.36	7	0.3	1.7	0.4
ES	0.80	24.5	7.39	9	0.2	0.0	0.5
ED	0.06	24.4	6.87	2	7.2	9.8	0.3
FS	0.64	24.7	7.42	5	0.4	0.1	0.3
FD	0.06	24.6	7.32	12	0.7	0.5	0.5
OX Effluent	-	-	-	13	0.2	0.2	0.3

Winter 2017 3/16/2017							
Location	**Dissolved Oxygen (mg/L)**	**Temp, C**	**pH**	**Soluble COD (mg/L)**	**Ortho P (PO_4^{3-}-P) (mg/L)**	**Ammonia (NH_3-N) (mg/L)**	**Nitrate (NO_3-N) (mg/L)**
OX Influent	-	-	-	23	0.6	5.9	0.8
AS	0.97	12.9	6.86	10	0.0	1.0	0.5
BS	0.66	12.7	7.21	11	0.0	0.5	0.7
BD	0.18	13.5	6.47	19	7.7	4.1	0.4
CS	0.30	12.8	7.18	5	0.0	1.1	0.7
CD	0.20	13.4	6.45	13	9.9	15.9	0.3
DS	1.21	12.5	7.22	3	0.0	0.8	0.7
DD	0.96	13.1	7.16	8	0.0	1.1	0.8
ES	1.41	12.7	7.20	6	0.0	0.9	0.8
ED	0.22	13.4	6.51	14	9.0	16.0	0.5
FS	1.11	12.6	7.26	9	0.7	0.9	0.8
FD	0.21	13.1	6.33	17	24.5	10.4	0.2
OX Effluent	-	-	-	7	0.0	1.0	0.4

Table 2-4 (Continued)

Summer 2017 8/22/2017							
Location	**Dissolved Oxygen (mg/L)**	**Temp, C**	**pH**	**Soluble COD (mg/L)**	**Ortho P (PO_4^{3-}-P) (mg/L)**	**Ammonia (NH_3-N) (mg/L)**	**Nitrate (NO_3-N) (mg/L)**
OX Influent	-	-	-	15	1.5	7.5	0.9
AS	0.54	24.7	7.06	4	0.9	0.6	3.1
BS	0.14	24.7	7.18	13	0.9	0.4	3.3
BD	0.06	24.6	6.79	11	1.4	1.2	3.1
CS	0.14	24.8	7.21	6	0.9	0.8	3.2
CD	0.10	24.8	6.89	15	4.1	11.3	3.0
DS	0.62	24.7	7.21	9	1.0	0.5	3.2
DD	0.64	24.7	7.14	9	1.0	0.7	3.3
ES	1.22	24.9	7.23	15	1.0	0.8	3.4
ED	0.06	24.8	6.97	9	1.6	2.7	3.6
FS	0.54	24.7	7.20	5	1.0	0.6	3.4
FD	0.04	24.7	6.93	33	6.7	33.6	2.8
OX Effluent	-	-	-	12	1.0	0.5	3.4

Winter 2018 3/6/2018							
Location	**Dissolved Oxygen (mg/L)**	**Temp, C**	**pH**	**Soluble COD (mg/L)**	**Ortho P (PO_4^{3-}-P) (mg/L)**	**Ammonia (NH_3-N) (mg/L)**	**Nitrate (NO_3-N) (mg/L)**
OX Influent	-	-	-	43	0.9	3.6	1.1
AS	2.98	14.2	7.07	30	0.4	0.0	4.4
BS	2.76	14.2	7.00	34	0.4	0.0	4.3
BD	2.52	14.1	6.51	33	1.9	1.9	4.2
CS	2.39	14.3	7.10	33	0.4	0.1	4.4
CD	0.15	14.4	6.22	39	13.8	2.6	3.1
DS	2.73	14.2	7.10	29	0.4	0.0	4.4
DD	2.46	14.3	7.02	29	0.4	0.0	4.6
ES	3.19	14.2	7.14	30	0.4	0.0	4.7
ED	0.14	14.4	6.29	51	18.1	4.4	3.4
FS	3.12	14.4	7.17	26	0.4	0.3	4.8
FD	2.05	14.4	6.66	37	3.7	4.5	3.1
OX Effluent	-	-	-	31	0.4	0.1	4.6

Table 2-4 (Continued)

Summer 2018 8/30/2018							
Location	**Dissolved Oxygen (mg/L)**	**Temp, C**	**pH**	**Soluble COD (mg/L)**	**Ortho P (PO_4^{3-}-P) (mg/L)**	**Ammonia (NH_3-N) (mg/L)**	**Nitrate (NO_3-N) (mg/L)**
OX Influent	-	-	-	25	3	6.8	3.8
AS	0.34	25.0	6.98	15	0.5	0.0	8.1
BS	0.15	25.1	7.03	21	0.6	0.0	7.9
BD	0.09	25.1	6.71	16	1.3	4.4	7.2
CS	0.15	25.1	7.15	12	0.7	0.0	7.7
CD	0.10	25.1	6.52	19	2.7	2.0	5.0
DS	0.77	25.2	7.03	18	0.7	0.0	7.2
DD	0.68	25.2	6.99	15	0.9	0.0	7.4
ES	0.71	25.2	7.07	11	0.9	0.0	7.5
ED	0.11	25.2	6.99	18	1.3	3.0	7.3
FS	0.23	25.2	7.02	16	1.0	0.0	7.9
FD	0.08	25.2	6.78	15	1.2	0.8	7.2
OX Effluent	-	-	-	12	0.7	0.0	7.8

Summer 2018b 9/13/2018							
Location	**Dissolved Oxygen (mg/L)**	**Temp, C**	**pH**	**Soluble COD (mg/L)**	**Ortho P (PO_4^{3-}-P) (mg/L)**	**Ammonia (NH_3-N) (mg/L)**	**Nitrate (NO_3-N) (mg/L)**
OX Influent	-	-	-	22	2.6	5.1	1.9
AS	0.19	24.5	7.08	14	1.1	0.3	0.3
BS	0.13	24.3	7.17	13	1.1	0.0	0.3
BD	0.06	24.4	6.50	24	2.2	13.0	0.5
CS	0.13	24.4	7.22	41	1.1	0.0	0.4
CD	0.07	24.4	6.55	50	21.5	26.3	0.4
DS	0.24	24.4	7.20	22	1.1	0.0	0.2
DD	0.43	24.4	7.20	19	1.4	0.5	0.4
ES	0.52	24.4	7.29	15	1.0	0.0	0.3
ED	0.07	24.4	7.26	26	2.7	10.4	0.3
FS	0.32	24.5	7.30	14	1.0	0.1	0.2
FD	0.05	24.5	7.04	45	7.7	44.7	0.5
OX Effluent	-	-	-	14	1.1	0.0	0.3

Table 2-5: Batch testing data representing biological P-release and uptake performance of the mixed liquor collected from oxidation ditch of Cookeville WRRF pre and post optimization.

	Units	Pre-Optimization	Winter 2017	Summer 2017
HAc uptake rate	mg HAc/g VSS*hr	5.21	6.23	8.13
P release rate	mg P/g VSS*hr	2.14	3.06	3.16
P uptake rate	mg P/g VSS*hr	1.66	2.30	2.06
P rel/HAc up	mg P/mg HAc	0.42	0.49	0.39
	Units	**Winter 2018**	**Summer 2018**	**Summer 2018b**
HAc uptake rate	mg HAc/g VSS*hr	12.34	10.99	6.01
P release rate	mg P/g VSS*hr	2.41	3.43	3.54
P uptake rate	mg P/g VSS*hr	1.76	2.00	2.22
P rel/HAc up	mg P/mg HAc	0.20	0.31	0.59

Table 3-1: Date and location for samples collected from the activated sludge of Etowah and Maryville WRRFs for DNA extraction. Samples were collected from the surface (S) and deep (D) of Etowah's oxidation ditch.

Sample ID	Sampling Event	Date	Location
Eto17W1	Winter 2017	3/9/2017	AS
Eto17W2	Winter 2017	3/9/2017	AD
Eto17W3	Winter 2017	3/9/2017	BS
Eto17W4	Winter 2017	3/9/2017	CS
Eto17W6	Winter 2017	3/9/2017	DS
Eto17S1	Summer 2017	8/15/2017	AS
Eto17S2	Summer 2017	8/15/2017	AD
Eto17S3	Summer 2017	8/15/2017	BS
Eto17S4	Summer 2017	8/15/2017	CS
Eto17S6	Summer 2017	8/15/2017	DS
Eto18W1	Winter 2018	3/8/2018	AS
Eto18W2	Winter 2018	3/8/2018	AD
Eto18W3	Winter 2018	3/8/2018	BS
Eto18W4	Winter 2018	3/8/2018	CS
Eto18W6	Winter 2018	3/8/2018	DS
Eto18S1	Summer 2018	8/22/2018	AS
Eto18S2	Summer 2018	8/22/2018	AD
Eto18S3	Summer 2018	8/22/2018	BS
Eto18S4	Summer 2018	8/22/2018	CS
Eto18S6	Summer 2018	8/22/2018	DS
Mary17W1	Winter 2017	2/18/2017	ANA
Mary17W2	Winter 2017	2/18/2017	AX1
Mary17W3	Winter 2017	2/18/2017	AX2
Mary17W4	Winter 2017	2/18/2017	OX A
Mary17W5	Winter 2017	2/18/2017	OX B
Mary17W6	Winter 2017	2/18/2017	OX C
Mary17W7	Winter 2017	2/18/2017	OX D
Mary17W8	Winter 2017	2/18/2017	OX E
Mary17W9	Winter 2017	2/18/2017	OX F
Mary17S1	Summer 2017	8/1/2017	ANA

Table 3-1 (Continued)			
Sample ID	**Sampling Event**	**Date**	**Location**
Mary17S2	Summer 2017	8/1/2017	AX1
Mary17S3	Summer 2017	8/1/2017	AX2
Mary17S4	Summer 2017	8/1/2017	OX A
Mary17S5	Summer 2017	8/1/2017	OX B
Mary17S6	Summer 2017	8/1/2017	OX C
Mary17S7	Summer 2017	8/1/2017	OX D
Mary17S8	Summer 2017	8/1/2017	OX E
Mary17S9	Summer 2017	8/1/2017	OX F
Mary18W1	Winter 2018	3/9/2018	ANA
Mary18W3	Winter 2018	3/9/2018	AX1
Mary18W4	Winter 2018	3/9/2018	AX2
Mary18W5	Winter 2018	3/9/2018	OX A
Mary18W6	Winter 2018	3/9/2018	OX B
Mary18W7	Winter 2018	3/9/2018	OX C
Mary18W8	Winter 2018	3/9/2018	OX D
Mary18W9	Winter 2018	3/9/2018	OX E
Mary18W10	Winter 2018	3/9/2018	OX F
Mary18S1	Summer 2018	9/5/2018	ANA
Mary18S2	Summer 2018	9/5/2018	AX1
Mary18S3	Summer 2018	9/5/2018	AX2
Mary18S4	Summer 2018	9/5/2018	OX A
Mary18S5	Summer 2018	9/5/2018	OX B
Mary18S6	Summer 2018	9/5/2018	OX C
Mary18S7	Summer 2018	9/5/2018	OX D
Mary18S8	Summer 2018	9/5/2018	OX E
Mary18S9	Summer 2018	9/5/2018	OX F

Table 3-2: Facility influent and effluent (mg/L) composite samples collected during sampling events for A.) Etowah and B.) Maryville WRRF.

A.)

Etowah												
	Winter 2017			Summer 2017			Winter 2018			Summer 2018		
	Influent	Effluent	% Removal	Influent	Effluent	% Removal	Influent	Effluent	% Removal	Influent	Effluent	% Removal
Total COD	132	48	64	106	42	61	195	65	67	132	47	64
Soluble COD	71	35	51	72	39	46	79	54	32	80	45	44
Total P	3.7	1.9	49	2.8	1.9	32	1.5	0.0	100	2.0	1.1	45
Ortho P	2.0	0.9	55	2.3	1.9	19	2.5	1.3	47	3.1	2.8	10
NH_3-N	7.9	1.0	87	8.3	0.1	99	7.8	0.0	100	10.9	0.0	100
NO_3-N	0.8	3.7	0	0.6	14.8	0	3.3	10.8	0	0.3	11.9	0
Total N	28.0	9.0	68	23.0	24.0	0	33.0	22.0	33	27.0	19.2	29

B.)

Maryville												
	Winter 2017			Summer 2017			Winter 2018			Summer 2018		
	Influent	Effluent	% Removal	Influent	Effluent	% Removal	Influent	Effluent	% Removal	Influent	Effluent	% Removal
Total COD	98.1	14.0	86	440	21	95	126	6	95	410	20	95
Soluble COD	50	11	79	102	27	74	51	4	92	95	19	80
Total P	2.3	1.6	30	3.5	0.2	94	5.6	0.0	100	3.3	0.7	78
Ortho P	1.7	1.4	18	0.7	0.2	74	1.6	0.0	100	2.5	0.6	75
NH_3-N	11.8	0.1	99	17.3	0.1	99	11.1	0.1	99	17.5	0.3	98
NO_3-N	0.7	4.5	0	0.9	0.7	22	0.2	2.2	0	0.0	0.9	0
Total N	14.6	4.5	69	32.0	1.0	97	33.0	2.5	92	32.0	1.1	97

Table 3-3: Measurements (raw data) for samples collected from the secondary treatment processes of Etowah and Maryville WRRFs.

Winter 2017 3/9/2017	Etowah						
Location	**Dissolved Oxygen (mg/L)**	**Temp, C**	**pH**	**Soluble COD (mg/L)**	**Ortho P (PO_4^{3-}-P) (mg/L)**	**Ammonia (NH_3-N) (mg/L)**	**Nitrate (NO_3-N) (mg/L)**
OX Influent	-	-	-	35	1.7	1.6	3.5
AS	0.58	14.5	7.16	34	1.6	0.8	3.2
AD	0.08	14.7	6.61	36	4.1	1.6	2.9
BS	0.09	14.6	7.22	36	1.2	1.5	3.2
CS	0.32	14.8	7.14	34	1.1	1.0	3.4
DS	0.21	14.7	7.11	38	1.7	1.6	3.2
OX Effluent	-	-	-	32	0.9	1.6	3.4

Summer 2017 8/15/2017	Etowah						
Location	**Dissolved Oxygen (mg/L)**	**Temp, C**	**pH**	**Soluble COD (mg/L)**	**Ortho P (PO_4^{3-}-P) (mg/L)**	**Ammonia (NH_3-N) (mg/L)**	**Nitrate (NO_3-N) (mg/L)**
OX Influent	-	-	-	50	2.2	5.8	5.1
AS	3.57	25.8	7.06	35	2.1	0.2	15.9
AD	3.28	25.6	7.14	38	2.0	0.4	15.6
BS	3.16	26.1	7.17	37	2.1	0.2	15.7
CS	2.76	25.7	7.17	35	2.1	0.1	15.7
DS	2.69	26.1	7.13	38	2.2	0	15.7
OX Effluent	-	-	-	37	2.1	0.2	15.7

Winter 2018 3/8/2018	Etowah						
Location	**Dissolved Oxygen (mg/L)**	**Temp, C**	**pH**	**Soluble COD (mg/L)**	**Ortho P (PO_4^{3-}-P) (mg/L)**	**Ammonia (NH_3-N) (mg/L)**	**Nitrate (NO_3-N) (mg/L)**
OX Influent	-	-	-	70	2.0	3.9	6.2
AS	5.92	12.5	7.32	50	1.4	0.0	10.6
AD	0.22	12.7	7.36	21	1.6	1.0	10.3
BS	5.75	12.7	7.35	53	1.4	0.1	10.6
CS	5.87	12.8	7.32	47	1.4	0.0	10.5
DS	5.94	12.7	7.27	55	1.4	0.0	10.6
OX Effluent	-	-	-	50	1.5	0.2	10.6

Table 3-3 (Continued)

Summer 2018 8/22/2018	Etowah						
Location	**Dissolved Oxygen (mg/L)**	**Temp, C**	**pH**	**Soluble COD (mg/L)**	**Ortho P (PO_4^{3-}-P) (mg/L)**	**Ammonia (NH_3-N) (mg/L)**	**Nitrate (NO_3-N) (mg/L)**
OX Influent	-	-	-	59	2.9	5.3	4.8
AS	3.55	25.2	7.06	42	2.8	0	11.8
AD	3.36	25.2	7.15	44	2.8	0	11.7
BS	4.07	25.4	7.26	46	2.8	0	11.8
CS	3.86	25.3	7.21	45	2.8	0	12.0
DS	3.44	25.3	7.14	45	2.8	0	11.9
OX Effluent	-	-	-	48	2.9	0	11.9

Winter 2017 2/18/2017	Maryville						
Location	**Dissolved Oxygen (mg/L)**	**Temp, C**	**pH**	**Soluble COD (mg/L)**	**Ortho P (PO_4^{3-}-P) (mg/L)**	**Ammonia (NH_3-N) (mg/L)**	**Nitrate (NO_3-N) (mg/L)**
Influent + RAS	-	-	-	17	5.2	5.2	1.0
Anaerobic	0.24	15.1	6.93	18	7.6	5.9	0.5
Anoxic 1	0.11	15.6	7.10	11	2.0	0.9	3.3
Anoxic 2	0.14	15.4	7.14	10	2.4	1.4	2.6
Ox A	1.26	15.2	7.25	11	1.2	0.1	5.0
Ox B	1.18	15.1	7.23	10	1.1	0.2	4.8
Ox C	0.78	15.3	7.19	12	1.1	0.0	4.8
Ox D	0.21	15.6	7.20	10	1.1	0.3	4.9
Ox E	1.23	15.4	7.31	5	1.2	0.0	5.0
Ox F	0.56	15.4	7.15	12	1.1	0.1	4.9
OX Effluent	-	-	-	10	1.1	0.1	4.0

Summer 2017 8/1/2017	Maryville						
Location	**Dissolved Oxygen (mg/L)**	**Temp, C**	**pH**	**Soluble COD (mg/L)**	**Ortho P (PO_4^{3-}-P) (mg/L)**	**Ammonia (NH_3-N) (mg/L)**	**Nitrate (NO_3-N) (mg/L)**
Influent + RAS	-	-	-	32	6.7	7.3	0.4
Anaerobic	0.18	23.4	7.04	45	12.3	11.1	0.4
Anoxic 1	0.08	24.2	7.06	27	4.8	3.1	0.4
Anoxic 2	0.14	24.2	6.97	28	5.1	2.9	0.4
Ox A	1.11	24.6	7.27	20	0.2	0	0.5
Ox B	0.33	24.6	7.19	20	0.2	0	0.4
Ox C	0.15	24.6	7.21	20	0.2	0	0.4
Ox D	0.11	24.4	7.19	20	0.3	0	0.3
Ox E	0.71	24.5	7.19	18	0.2	0	0.4
Ox F	0.10	24.5	7.19	20	0.3	0.3	0.3
OX Effluent	-	-	-	17	0.2	0	0.4

Table 3-3 (Continued)

Winter 2018 3/9/2018	Maryville						
Location	**Dissolved Oxygen (mg/L)**	**Temp, C**	**pH**	**Soluble COD (mg/L)**	**Ortho P (PO_4^{3-}-P) (mg/L)**	**Ammonia (NH_3-N) (mg/L)**	**Nitrate (NO_3-N) (mg/L)**
Influent + RAS	-	-	-	23	1.9	4.7	0.1
Anaerobic	0.22	14.9	7.02	21	1.7	5.9	0.1
Anoxic 1	0.23	14.4	7.17	6	0.2	1.1	0.9
Anoxic 2	0.22	13.5	7.26	22	0.5	2.0	0.1
Ox A	1.61	14.6	7.14	11	0.0	0.0	1.8
Ox B	1.30	14.6	7.28	12	0.0	0.0	2.0
Ox C	0.85	14.5	7.21	14	0.0	0.0	2.0
Ox D	0.55	14.4	7.19	10	0.0	0.1	1.8
Ox E	1.59	14.5	7.17	7	0.0	0.0	1.9
Ox F	0.64	14.6	7.13	10	0.0	0.0	1.8
OX Effluent	-	-	-	11	0.0	0.0	2.0

Summer 2018 9/5/2018	Maryville						
Location	**Dissolved Oxygen (mg/L)**	**Temp, C**	**pH**	**Soluble COD (mg/L)**	**Ortho P (PO_4^{3-}-P) (mg/L)**	**Ammonia (NH_3-N) (mg/L)**	**Nitrate (NO_3-N) (mg/L)**
Influent + RAS	-	-	-	40	6.6	6.9	0.0
Anaerobic	0.07	24.0	7.03	38	12.9	8.9	0.0
Anoxic 1	0.15	25.2	7.08	19	1.8	0.8	0.1
Anoxic 2	0.19	25.6	7.15	16	1.6	1.1	0.2
Ox A	1.79	25.1	7.08	16	0.6	0.2	1.1
Ox B	0.84	26.0	7.12	18	0.5	0.3	1.1
Ox C	0.49	25.7	7.15	18	0.5	0.0	1.1
Ox D	0.08	25.1	7.04	16	0.6	0.2	1.1
Ox E	1.74	25.1	7.11	20	0.5	0.2	1.1
Ox F	0.34	25.3	7.14	21	0.6	0.1	1.1
OX Effluent	-	-	-	19	0.4	0.1	1.2

Table 3-4: Batch testing data representing biological P-release and uptake performance of the mixed liquor collected from the oxidation ditches of A.) Etowah and B.) Maryville WRRFs.

A.)

Etowah					
	Units	**Winter 2017**	**Summer 2017**	**Winter 2018**	**Summer 2018**
HAc uptake rate	mg HAc/g VSS*hr	5.39	6.64	6.21	8.29
P release rate	mg P/g VSS*hr	0.97	0.35	0.49	0.10
P uptake rate	mg P/g VSS*hr	0.87	X	0.37	X
P rel/HAc up	mg P/mg HAc	0.18	0.05	0.08	0.01

B.)

Maryville					
	Units	**Winter 2017**	**Summer 2017**	**Winter 2018**	**Summer 2018**
HAc uptake rate	mg HAc/g VSS*hr	8.23	9.41	8.23	7.32
P release rate	mg P/g VSS*hr	2.81	3.82	2.77	3.49
P uptake rate	mg P/g VSS*hr	1.83	2.37	1.99	2.15
P rel/HAc up	mg P/mg HAc	0.34	0.41	0.34	0.48

Table 4-1: Data representing readily biodegradable COD and phosphorus removal due to biomass consumption from samples collected from Cookeville WRRF.

A.) May

Composite Samples	Total COD (mg/L)	Soluble COD (mg/L)	Treated and Filtered (mg/L)	TP as P (mg/L)	Ortho-P as P (mg/L)	rbCOD/ TP
Influent	328	65	37	3.3	2.5	7.6
Effluent	11	13	12	0.81	0.76	
		rbCOD =	25			

Before RAS Pump Shutdown	TP as P (mg/L)	Ortho-P as P (mg/L)	MLVSS (mg/L)	% P in Biomass (mg P/mg VSS)	Soluble COD (mg/L)
Clarifier 3	105	2	6120	1.68	14
Clarifier 4	160	4	7800	2.00	14

24hrs after RAS pump shutdown	TP as P (mg/L)	Ortho-P as P (mg/L)	MLVSS (mg/L)	% P in Biomass (mg P/mg VSS)	Soluble COD (mg/L)
Clarifier 3	22	1	1820	1.15	15
Clarifier 4	412	68	17930	1.92	56

Table 4-1 (Continued)

B.) November

Composite Samples	Total COD (mg/L)	Soluble COD (mg/L)	Treated and Filtered (mg/L)	TP as P (mg/L)	Ortho-P as P (mg/L)	rbCOD/ TP
Influent	446	87	39	4.8	3.5	7.9
Effluent	1	3	1	2.35	2.30	
		rbCOD =	38			

Before RAS Pump Shutdown	TP as P (mg/L)	Ortho-P as P (mg/L)	MLVSS (mg/L)	% P in Biomass (mg P/mg VSS)	Soluble COD (mg/L)
Clarifier 3	50	3.1	4.1	1.75	11
Clarifier 4	84	5.1	5.1	1.83	18

24hrs after RAS pump shutdown	TP as P (mg/L)	Ortho-P as P (mg/L)	MLVSS (mg/L)	% P in Biomass (mg P/mg VSS)	Soluble COD (mg/L)
Clarifier 3	52	4.7	3090	1.53	7
Clarifier 4	440	57	17925	2.15	33

Table 5-1: Descriptions for samples collected from reactors for DNA and RNA extractions.

Sample ID DNA	Reactor	Date	Day
Plab22	R1	11/12/2018	58
Plab23	R2	11/12/2018	58
Plab24	R1	9/25/2018	10
Plab25	R2	9/25/2018	10
Plab26	R1	10/29/2018	44
Plab27	R2	10/29/2018	44
Plab30	R1	12/12/2018	88
Plab31	R2	12/12/2018	88
Plab32	R1	12/17/2018	93
Plab33	R2	12/17/2018	93
Plab34	R1	12/19/2018	95
Plab35	R2	12/19/2018	95
Plab36	R1	12/22/2018	98
Plab37	R1	12/27/2018	103
Plab38	R2	12/27/2018	103
Plab39	R1	1/2/2019	109
Plab40	R2	1/2/2019	109
Plab41	R1	1/8/2019	115
Plab42	R2	1/8/2019	115
Plab43	R1	1/14/2019	121
Plab44	R2	1/14/2019	121
Plab45	R1	1/23/2019	130
Plab46	R2	1/23/2019	130
Plab47	R1	1/28/2019	135
Plab48	R2	1/28/2019	135
Plab49	R1	2/4/2019	142
Plab50	R2	2/4/2019	142
Plab51	R1	2/11/2019	149
Plab52	R2	2/11/2019	149
Plab53	R1	2/18/2019	156
Plab54	R2	2/18/2019	156
Plab55	R1	2/25/2019	163
Plab56	R2	2/25/2019	163
Plab57	R1	3/4/2019	170
Plab58	R2	3/4/2019	170
Plab59	R1	3/11/2019	177
Plab60	R2	3/11/2019	177

Table 5-1 (Continued)			
Sample ID DNA	**Reactor**	**Date**	**Day**
Plab61	R1	3/18/2019	184
Plab62	R2	3/18/2019	184
Plab63	R1	3/25/2019	191
Plab64	R2	3/25/2019	191
Plab65	R1	4/2/2019	199
Plab66	R2	4/2/2019	199
Plab67	R1	5/15/2019	242
Plab68	R2	5/15/2019	242
Sample ID RNA	**Reactor/Phase**	**Date**	**Day**
PlabRNA1	R1 Anaerobic	12/10/2018	86
PlabRNA2	R1 Aerobic	12/10/2018	86
PlabRNA3	R2 Anaerobic	12/10/2018	86
PlabRNA4	R2 Aerobic	12/10/2018	86
PlabRNA5	R1 Anaerobic	12/19/2018	95
PlabRNA6	R1 Aerobic	12/19/2018	95
PlabRNA7	R1 Anaerobic	1/8/2019	115
PlabRNA8	R1 Aerobic	1/8/2019	115
PlabRNA9	R1 Anaerobic	2/4/2019	142
PlabRNA10	R1 Aerobic	2/4/2019	142
PlabRNA11	R1 Anaerobic	2/11/2019	149
PlabRNA12	R1 Aerobic	2/11/2019	149
PlabRNA13	R1 Anaerobic	2/25/2019	163
PlabRNA14	R1 Aerobic	2/25/2019	163
PlabRNA15	R1 Anaerobic	3/4/2019	170
PlabRNA16	R1 Aerobic	3/4/2019	170
PlabRNA17	R1 Anaerobic	3/11/2019	177
PlabRNA18	R1 Aerobic	3/11/2019	177
PlabRNA19	R1 Anaerobic	3/18/2019	184
PlabRNA20	R1 Aerobic	3/18/2019	184
PlabRNA21	R1 Anaerobic	3/25/2019	191
PlabRNA22	R1 Aerobic	3/25/2019	191
PlabRNA23	R1 Anaerobic	4/2/2019	199
PlabRNA24	R1 Aerobic	4/2/2019	199
PlabRNA25	R2 Anaerobic	4/2/2019	199
PlabRNA26	R2 Aerobic	4/2/2019	199
PlabRNA27	R1 Anaerobic	5/15/2019	242
PlabRNA28	R1 Aerobic	5/15/2019	242

Table 5-2: Rates measured from kinetic studies during EBPR and dEBPR periods for the A.) Test and B.) Control reactors.

A.)

Test Reactor (R1)					
	Units	**11/7/2016**	**Day 86**	**Day 88**	**Day 199**
HAc uptake rate	mg HAc/g VSS*hr	80.05	88.29	30.47	83.13
COD uptake rate	mg COD/g VSS*hr	33.29	83.29	28.71	71.52
P release rate	mg P/g VSS*hr	30.34	26.48	4.95	48.97
P uptake rate	mg P/g VSS*hr	10.64	10.20	0.92	26.39
NO_3-N uptake rate	mg N/g VSS*hr	x	x	0.35	6.39
P rel/HAc up	mg P/mg HAc	0.38	0.30	0.16	0.59

B.)

Control Reactor (R2)				
	Units	**11/7/2016**	**Day 86**	**Day 199**
HAc uptake rate	mg HAc/g VSS*hr	106.60	102.82	81.88
COD uptake rate	mg COD/g VSS*hr	37.01	92.92	74.71
P release rate	mg P/g VSS*hr	19.64	29.35	32.20
P uptake rate	mg P/g VSS*hr	15.16	8.37	30.78
P rel/HAc up	mg P/mg HAc	0.18	0.29	0.39

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VITA

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