2019

Microbial community dynamics of attached biofilm BioCord technology in wastewater treatment

Adam Skoyles
University of Windsor

Microbial community dynamics of attached biofilm BioCord technology in wastewater treatment

By

Adam Skoyles

A Thesis
Submitted to the Faculty of Graduate Studies
through the Great Lakes Institute for Environmental Research
in Partial Fulfillment of the Requirements for
the Degree of Master of Science
at the University of Windsor

Windsor, Ontario, Canada

2019

Microbial community dynamics of attached biofilm BioCord technology in wastewater treatment

by

Adam Skoyles

APPROVED BY:

_____

S. Mundle
Departmental of Chemistry and Biochemistry

_____

K. Drouillard
Great Lakes Institute for Environmental Research

_____

S. Chaganti, Co-Advisor
Great Lakes Institute for Environmental Research

_____

C. Weisener, Co-Advisor
Great Lakes Institute for Environmental Research

June 5, 2019

DECLARATION OF CO-AUTHORSHIP / PREVIOUS PUBLICATION

i. Co-Authorship

I hereby declare that this thesis incorporates material that is result of joint research, as follows: Chapters 2 and 3 of the thesis were coauthored with Dr. Scott Mundle, under the supervision of Dr. Subba Rao Chaganti and Dr. Christopher G. Weisener. The primary contributions, data analysis, interpretation, and writing were performed by the author. Co-authors contributed by contributing to editing of manuscript, statistical analysis, and project conceptualization.

I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my thesis and have obtained written permission from each of the co-author(s) to include the above material(s) in my thesis.

I certify that, with the above qualification, this thesis, and the research to which it refers, is the product of my own work.

ii. Previous Publication

This thesis includes one original paper that have been previously published/submitted for publication in peer reviewed journals, as follows:

Thesis Chapter	Publication title/full citation	Publication status*
Chapters 2 and 3	*Skoyles, A., Chaganti, SR., Mundle, SOC., Wesener, CG. (2019) Nitrification kinetics and microbial community dynamics of attached biofilm in wastewater treatment.*	*Submitted*

I certify that I have obtained a written permission from the copyright owner(s) to include the above published material(s) in my thesis. I certify that the above material describes work completed during my registration as a graduate student at the University of Windsor.

iii. General

I declare that, to the best of my knowledge, my thesis does not infringe upon anyone's copyright nor violate any proprietary rights and that any ideas, techniques, quotations, or any other material from the work of other people included in my thesis, published or otherwise, are fully acknowledged in accordance with the standard referencing practices. Furthermore, to the extent that I have included copyrighted material that surpasses the bounds of fair dealing within the meaning of the Canada Copyright Act, I certify that I have obtained a written permission from the copyright owner(s) to include such material(s) in my thesis.

I declare that this is a true copy of my thesis, including any final revisions, as approved by my thesis committee and the Graduate Studies office, and that this thesis has not been submitted for a higher degree to any other University or Institution.

ABSTRACT

Nutrient pollution is a global environmental problem that has led to increasingly stricter regulations in wastewater treatment. As a result, many systems require upgrades. A comparative bench-scale and field site analysis of BioCord, a biotechnology that improves nutrient removal, was conducted to investigate seasonal microbial dynamics and its impact on nitrogen removal. This was assessed using metabolite (NO_3^-) stable isotope analysis, high-throughput sequencing of the 16S rRNA gene, and RT-qPCR of key genes in biological treatment representing nitrification, anammox, and denitrification. Bench-scale experiments using BioCord showed an increase in nitrifiers with increasing ammonia concentrations, resulting in an ammonia removal efficiency up to 98% in the BioCord system with only up to 25% observed in the control system. Compound specific stable isotope analysis showed that $^{15}\varepsilon$ and $\delta^{18}O_{NO3}$ could be used in monitoring the efficiency of the enhanced biological nitrification. In the lagoon field trials, an increase in nitrogen promoted three principle nitrifying genera (*Nitrosomonas, Nitrospira, Candidatus Nitrotoga*) and enhanced the expression of denitrification genes (nirK, norB, and nosZ). Further, anaerobic ammonia oxidizers were active within biofilm of BioCord. Even at lower temperatures (2 to 6 ^oC) the nitrifying bacteria were active on the BioCord. Future research should focus on using metatranscripotomics to identify syntrophic metabolic relationships in BioCord to better understand and predict treatment.

DEDICATION

I dedicate this thesis to my parents, Rosanne and Craig Skoyles, for providing me with endless encouragement and support throughout my studies.

ACKNOWLEDGEMENTS

The funding for the research in this thesis was provided by MITACS (1T08131), Bishop Water Technologies (33062), Ontario Genomics (33336), and the NSERC ENGAGE plus program.

I would like to thank my co-supervisors, Dr. Christopher Weisener and Dr. Subba Rao Chaganti, for providing me with the opportunity to pursue this research project. I appreciate all the discussions and advice that they have provided me throughout my time at GLIER which has been instrumental not only to my growth as a researcher but also as an individual. I would also like to thank my committee member and co-author, Dr. Scott Mundle, for providing guidance and pushing me to set higher expectations for myself. Additionally, I would like to thank my committee member Dr. Kenneth Drouillard for always providing excellent feedback and support. I am also thankful to the staff at Bishop Water Technologies for all the assistance they have provided, which was critical for my research to go smoothly. Additional thanks go out to all the faculty, staff, and students at GLIER that helped make this experience enjoyable and productive, with special thanks to everyone in the Weisener lab group. Lastly, I would like to thank all of my family and friends for the encouragement and support throughout my studies at the University of Windsor.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF APPENDICES

Appendix Tables

Appendix Figures

LIST OF ABBREVIATIONS/SYMBOLS

AMX- Anaerobic Ammonia-Oxidizing Bacteria

ANOSIM- Analysis of Similarity

ANOVA- Analysis of Variance

AOB- Ammonia-Oxidizing Bacteria

CCA- Canonical Correspondence Analysis

HTS- High-Throughput Sequencing

L#- Lagoon#

NMDS- Non-metric multidimensional scaling

NNR- Net Nitrification Rate

NOB- Nitrite-Oxidizing Bacteria

OTU(s)- Operational Taxonomic Unit(s)

PCoA- Principal Coordinates Analysis

RT-qPCR- Reverse Transcription Quantitative Polymerase Chain Reaction

WWTP(s)- Wastewater Treatment Plant(s)

CHAPTER 1

Introduction to Nutrient Pollution and Biological Wastewater Treatment

1.1 Environmental concern of nutrient pollution

Every year, approximately 150 billion litres of ineffectively treated wastewater is released into Canadian water bodies (Environment Canada, 2012). Wastewater is defined as water received by water treatment plants and water discharged into drains, sewers, and directly into the environment (Statistics Canada, 2012). Excess nutrient loading from wastewater, specifically nitrogen and phosphorus, is a serious environmental concern and is contributing to the eutrophication of watersheds, including the Great Lakes (Michalak, et al., 2013). According to data from the National Pollutant Release Inventory, nitrate, ammonia, and phosphorus are the three most abundant substances released by industry into water, representing approximately 95% of total released substances (Statistics Canada, 2012). Both phosphorus and nitrogen are essential nutrients to sustain life and are often considered to be crucial limiting factors in aquatic and terrestrial ecosystems (Conley, et al., 2009). Elevated nitrogen levels in released wastewater have been shown to cause significant environmental damage leading to habitat destruction, hypoxic zones, harmful algal blooms, and eutrophication (Conley et al., 2009; Diaz and Rosenberg, 2009; Howarth, 2008). The global biogeochemical cycling of nitrogen is almost entirely controlled through oxidation-reduction reactions by microbes (Falkowski, 1997). Since microorganisms play such a significant role in nutrient cycling they are commonly used by municipalities in biological wastewater treatment to reduce effluent nitrogen concentrations.

1.2 Lagoon wastewater treatment

Nutrient pollution is a global environmental problem that has led to increasingly stricter regulations in wastewater treatment processes (Lyu et al., 2016)**.** With new legislation enforcing stricter regulations, such as the Environmental Protection Act (2016), there are over 100 lagoon wastewater treatment plants (WWTPs) in Ontario alone that are now considered ill-equipped to properly treat water to current standards (National Guide to Sustainable Municipal Infrastructure, 2004). Lagoon systems are a commonly used treatment option for rural communities because they are less expensive than mechanical plants with substantially more land requirements. Upgrading these systems to traditional mechanical treatment options is not financially feasible for many rural municipalities. As a result, nitrogen removal is less reliable in temperate regions due to the colder seasonal temperatures inhibiting microbial activity, which lagoon treatment systems rely on (Hurse and Connor, 1999). Improvements to lagoon treatment systems in temperate environments are therefore required to effectively remove nitrogen, most importantly ammonia, year round. Enhanced biological treatment upgrades are becoming more widely used as relatively cheaper options. Traditional biological treatments commonly used include membrane bioreactors, alternating anoxic and oxic conditions, and wetlands construction, with many other novel treatments in development (EPA, 2013; Wu, et al., 2015). A recent study determined that one of the most effective improvements for increasing the nitrogen removal capacity of a wastewater stabilization pond was an attached biofilm technology known as BioCord (Gan et al., 2018).

1.3 BioCord in wastewater treatment

BioCord was designed by Bishop Water Technologies, a water technology company based in Ontario, Canada. It is a cost-effective and sustainable biotechnology

for wastewater treatment that provides a high surface area ring of polymer threads to encourage natural biofilm development (Gan et al., 2018). In previously conducted bench-scale experiments, air scouring was shown to increase the ammonia treatment capacity of BioCord with variable ammonia loading rates leading to a maximum observed removal efficiency of 97.0 ± 0.6% (Tian et al., 2017). BioCord has been shown to successfully sustain a microbial community which effectively reduced ammonia and total nitrogen in polluted river water by 55.2–74.0% and 46.2–55.9%, respectively (Yuan et al., 2012). Another study determined that BioCord effectively promotes a similar attached microbial community to that of submerged macrophytes, which are commonly used for agricultural drainage ditch treatment, making it a sustainable alternative (Zhou et al., 2018). However, to the best of my knowledge there has not been any published research into using BioCord bioreactors to upgrade a full size lagoon WWTP. Previous studies have also not characterized the active BioCord microbial community, which is particularly important to understand and predict its role in wastewater treatment.

1.4 Metabolic nitrogen removal in wastewater treatment

It is widely known that nitrogen removal by microbes in wastewater has been accomplished through several metabolic processes including nitrification, denitrification, and anaerobic ammonium oxidation (anammox) (Munch et al., 1996; Schmidt et al., 2003; Strous et al., 1997). Nitrification is a two-step process where ammonium (NH_4^+) is first oxidized to nitrite (NO_2^-) which is then oxidized into nitrate (NO_3^-). The first step is completed by ammonia-oxidizing bacteria (AOB) or ammonia-oxidizing archaea and the second step is finished by nitrite-oxidizing bacteria (NOB). The exception is the recent discovery of some species from the *Nitrospira* genus that contain the genes for both steps

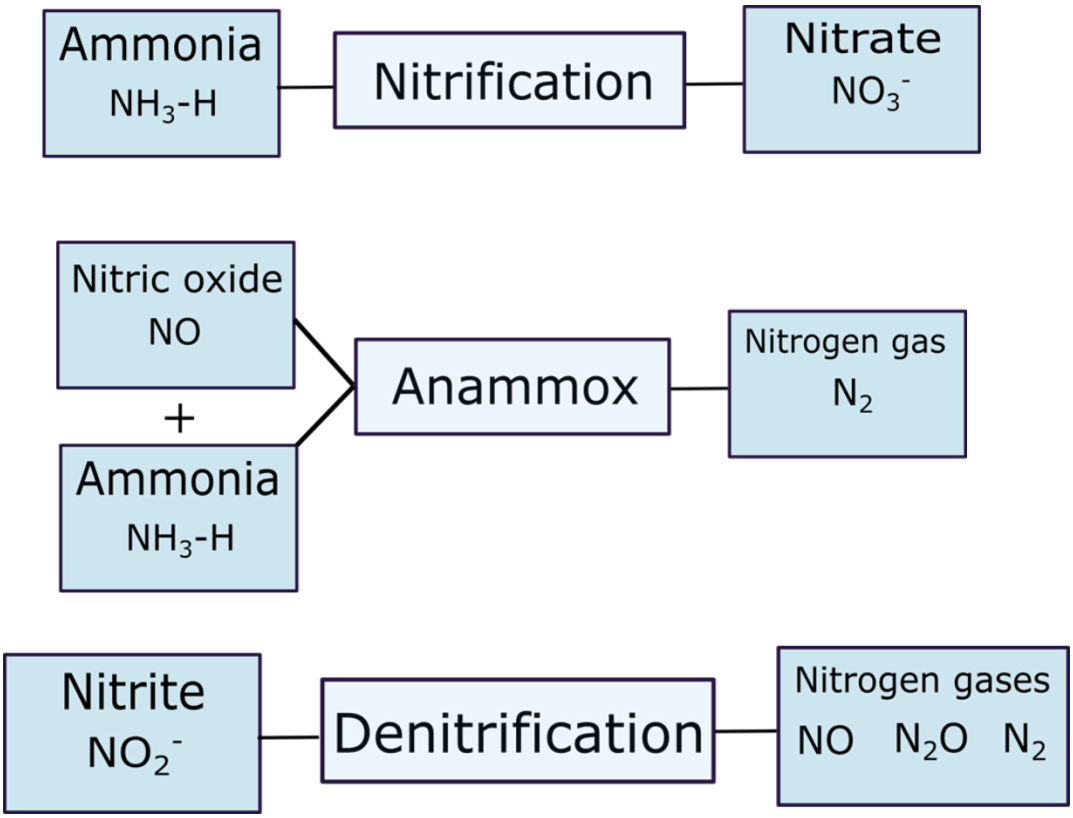

Figure 1.1. Overview of important nitrogen removal metabolic processes in wastewater treatment.

and are known as complete ammonia oxidizing (commamox) bacteria (Daims et al., 2015). The more commonly found AOB in WWTPs are the genera *Nitrosomonas* and *Nitrosospira* while the more common NOB are the genera *Nitrospira*, *Nitrobacter*, and the more recently discovered *Candidatus Nitrotoga* (Cydzik-Kwiatkowska and Zielińska, 2016; Lucker et al., 2015; Siripong and Rittmann, 2007). Denitrification is the process of reducing NO_3^- to molecular gaseous nitrogen (N_2) with intermediates of NO_2^-, nitric oxide (NO), and nitrous oxide (N_2O). In order of reduction from NO_3^-, these microbial processes are controlled by the enzymes nar, nir, nor, and nos respectively. In WWTPs, the denitrification process can be controlled by many different phylogenetic groups

including *Rhodoferax, Dechloromonas, and Thermomonas* (Mcilroy et al., 2016). Anammox, the most recently discovered pathway in which nitrogen can be removed from wastewater, is the reaction of NH_4^+ and NO_2^- to produce N_2 through a multistep process by a unique subgroup of *Planctomycetes* bacteria (Kuenen, 2008; Mulder et al., 1995). To identify the specific metabolic processes of and track the dynamics of nitrogen treatment in a system, stable isotope techniques can be used.

Compound specific isotope approach of NO_3^- ($\delta^{15}N$ and $\delta^{18}O$) has been used to investigate nitrogen cycling by measuring relative changes in isotopic fractionation caused by the increase in energy required to break the bond between heavy isotopes (Buchwald and Casciotti, 2010). A dual tracer approach is crucial to isolate specific effects due to metabolic transformation from nitrification, where $\delta^{15}N$ is influenced by the ammonia input and $\delta^{18}O$ is influenced by surrounding water and oxygen (Boshers et al., 2019; Botrel et al., 2017). This approach has been important in quantifying kinetic effects of a changing physicochemical environment on nitrification to optimize treatment strategies (Yun and Ro, 2014). The isotopic fractionation effect of nitrification is relatively large but has been observed to vary significantly depending on the microbial community (Casciotti et al., 2003). Combining NO_3^- stable isotope analysis with a microbial community assessment will help to determine which microbes are responsible for the observed kinetic differences due to enhanced biological treatment.

1.5 Research scope

In order to determine the biological treatment efficiency of BioCord technology under real world conditions (e.g munciple sewage lagoons) it is improtant to understand how the biofilm will respond to both changes in chemical (e.g. nutrient load) and

environmental gradient (e.g. temperature). There is still a lack of understanding with respect to how this technology can be optimised for lagoon WWTPs for removal of ammonia during colder months and whether BioCord promotes the attachment of nitrifiers throughout all seasons. To address these gaps in knowledge, this thesis will identify the active BioCord microbial community and determine its overall metabolic function in nitrogen removal. A bench-scale experiment was designed to develop an understanding of the potential chemical and biological mechanisms of nitrogen removal with increasing ammonia concentrations in a controlled system using BioCord; this is described in chapter two. An assessment of the active community was combined with stable isotope analysis to track the specific metabolic processes and their kinetics, which is critical to predict accurate functionality of the system. In chapter three, the microbial community of BioCord was assessed in a full-scale lagoon WWTP. This research offers the first novel insight into the microbial community development of BioCord by describing the seasonal variability within the attached biofilm and comparing it to the surrounding microbes in water and sediment. Within the lagoon system the influence of physicochemical changes on the attachec biofilm community was described.

1.6 Research objective

The objective of this thesis was to assess the microbial community dynamics of BioCord to identify key members of that community and determine the metabolic function of nitrogen removal. In order to characterize the active community, which can often be different from the total community, the extracted RNA was used in this study. Tracking the specific metabolic processes and their kinetics in addition to active microbial dynamics is critical to predict accurate functionality of the system. In doing so,

chemical and biological signatures can be identified to monitor the effectiveness of BioCord in wastewater treatment.

In chapter two, the primary hypothesis was that specific microbes would respond to the increasing influent ammonia concentration in a controlled system. To support this hypothesis, the microbial community was assessed using targeted amplicon sequencing of the 16S rRNA gene and quantitative polymerase chain reaction (qPCR), a more effective tool for quantifying specific pathway or bacterial group. The secondary hypothesis was the isotopic fractionation of NO_3^- would change due to kinetic effects of BioCord on nitrification; this will be tested using compound specific isotope fractionation of NO_3^-. The third hypothesis was the kinetics and microbial data would show strong relationships for specific nitrifiers that are responsible for kinetic differences, which will be tested using regression analysis.

In chapter three, the first novel insight into the microbial community of BioCord in a full-scale lagoon WWTP was observed. The first hypothesis for this chapter was the overall active BioCord community will shift with specific changing physicochemical conditions (e.g. temperature) in the lagoon system throughout the year. This will be tested through correlating water chemistry and temperature with observed major community differences. The second hypothesis of this chapter was the BioCord community would be most similar with the lagoon water community, when compared with water and sediment. It is also predicted for the BioCord to contain the highest abundance of nitrifiers. This was tested by sequencing the bacterial communities of all three samples and will be useful in predicting biofilm development once BioCord is installed into new systems. The third hypothesis was that the identified nitrifiers from chapter two would increase with an

increasing ammonia gradient in the lagoons. This was tested by moving the bioreactors closer to the wastewater inflow, which increased the chemical gradient. Seasonal effects will also be compared between both of the installed bioreactor locations which will be used to test the last hypothesis of this chapter: the reduction in temperature would reduce the abundance of microbes that are capable of removing ammonia. This will be tested by collecting samples at throughout the year to capture colder temperature effects. This is important to consider because the magnitude of change will determine whether the implemented Biocord treatment could remain effective throughout the winter months.

In chapter four, the results from both chapters are summarized to compare how the BioCord community scales from bench-scale to field trials. Suggestions for future research is also presented in this chapter.

CHAPTER 2

Nitrification kinetics and microbial community dynamics of attached biofilm in wastewater treatment

2.1 Introduction

Nutrient pollution is a global environmental problem that has led to increasingly stricter regulations in wastewater treatment processes (Lyu et al., 2016)**.** Elevated levels of nitrogen and phosphorus in released wastewater can lead to significant environmental damage including habitat destruction, hypoxic zones, harmful algal blooms, and eutrophication (Conley et al., 2009; Diaz and Rosenberg, 2009; Howarth, 2008). The global biogeochemical cycling of nitrogen is almost entirely controlled through oxidation-reduction reactions by microbes (Falkowski, 1997). Since microorganisms play such a significant role in nutrient cycling they are commonly used by municipalities in biological wastewater treatment to reduce effluent nitrogen concentrations. One proposed improvement to lagoon systems for effective nitrogen removal is an attached biofilm technology known as BioCord (Gan et al., 2018). BioCord technology provides a high surface area ring of polymer threads that encourages natural biofilm development. It has been successfully used to sustain a microbial community, which reduces nutrient levels of effluent waters in various settings including laboratory, river, and drainage ditch environments (Tian et al., 2017; Yuan et al., 2012; Zhou et al., 2018). Previous studies have also not characterized the active BioCord microbial community, which is particularly important for predicting and developing treatment.

It is widely known that nitrogen removal by microbes in wastewater has been accomplished through several metabolic processes including nitrification, denitrification, and anaerobic ammonium oxidation (anammox) (Munch et al., 1996; Schmidt et al.,

2003; Strous et al., 1997). Nitrification is a two-step process where ammonium (NH_4^+) is first oxidized to nitrite (NO_2^-) which is then oxidized into nitrate (NO_3^-). The first step is completed by ammonia-oxidizing bacteria (AOB) or ammonia-oxidizing archaea and the second step is finished by nitrite-oxidizing bacteria (NOB). The exception is the recent discovery of some species from the *Nitrospira* genus that contain the genes for both steps and are known as complete ammonia oxidizing (commamox) bacteria (Daims et al., 2015). The more commonly found AOB in WWTPs are the genera *Nitrosomonas* and *Nitrosospira* while the more common NOB are the genera *Nitrospira*, *Nitrobacter,* and the more recently discovered *Candidatus Nitrotoga* (Cydzik-Kwiatkowska and Zielińska, 2016; Lucker et al., 2015; Siripong and Rittmann, 2007). Denitrification is the process of reducing NO_3^- to molecular gaseous nitrogen (N_2) with intermediates of NO_2^-, nitric oxide (NO), and nitrous oxide (N_2O). In order of reduction from NO_3^-, these microbial processes are controlled by the enzymes nar, nir, nor, and nos respectively. In WWTPs, the denitrification process can be controlled by many different phylogenetic groups including *Rhodoferax, Dechloromonas, and Thermomonas* (Mcilroy et al., 2016). Anammox, the most recently discovered pathway in which nitrogen can be removed from wastewater, is the reaction of NH_4^+ and NO_2^- to produce N_2 through a multistep process by a unique subgroup of *Planctomycetes* bacteria (Kuenen, 2008; Mulder et al., 1995). To identify the specific metabolic processes of and track the dynamics of nitrogen treatment in a system, stable isotope techniques can be used.

Compound specific isotope analysis of NO_3^- ($\delta^{15}N$ and $\delta^{18}O$) has been used to investigate nitrogen cycling by measuring relative changes in isotopic fractionation caused by the increase in energy required to break the bond between heavy isotopes

(Buchwald and Casciotti, 2010). This approach has been important in quantifying kinetic effects of a changing environment on nitrification to optimize treatment strategies (Boshers et al., 2019). The isotopic fractionation effect of nitrification is relatively large but has been observed to vary significantly depending on the microbial community (Casciotti et al., 2003). Combining NO_3^- stable isotope analysis with a microbial community assessment will help to determine which microbes are responsible for the observed kinetic differences due to enhanced biological treatment.

Molecular methods used to assess microbial communities in wastewater treatment include targeted amplicon sequencing of the 16S rRNA gene and qPCR (De Sotto et al., 2018; Harms et al., 2003; Yapsakli et al., 2011). High-throughput sequencing (HTS) of the amplified 16S rRNA gene is one of the most widely used tools to assess the bacterial population and recently it has also been shown to have a good representation of nitrifying microbial guilds (Diwan et al., 2018). In comparison, qPCR is a more effective tool for quantifying a specific pathway or bacterial group (e.g. AOB). The total microbial community can often be significantly different from the active community (Yu and Zhang, 2012). In order to represent the active community, the extracted RNA was used in this study.

Previous studies have not characterized the active BioCord community or combined it with metabolite stable isotope analysis, which is important for understanding and developing its use in treatment. The goal of this study was to assess key members of the microbial community and their possible function in the attached biofilm present on BioCord in ammonia removal treatment. The bench-scale experiment was designed to develop a greater understanding of the potential chemical and biological mechanisms of

nitrogen removal using BioCord. This study offers novel insight into the microbial community dynamics of BioCord with increasing ammonia concentrations and will be assessed using water chemistry, stable isotopes, targeted amplicon sequencing of the 16S rRNA region, and RT-qPCR.

2.2 Methods

2.2.1 Sampling and bench-scale design

The bench-scale experiments consisted of a simple flow-through system designed to measure the chemical effect of BioCord treatment and the microbial community response. The flow-through system used two parallel sets of 3.5 L volume receiving vessels: one containing a 30 cm length suspended BioCord and one without any BioCord (**Figure 2.1**). The influent water used was obtained from the fourth lagoon of the lagoon WWTP in Dundalk, Ontario, Canada, in October 2017. The influent water was preserved at 4 ^{o}C to slow down biological activity but reached room temperature during aerated treatment (e.g. 20 ^{o}C) upon entry into the vessels. The hydraulic retention time (HRT) of the system was maintained at six days. The system was set up in duplicate and the experiment was run over a four-week period. At the end of a seven- day period, ammonium chloride (NH_4Cl) was added to the influent water thus increasing the chemical gradient each week (4.01, 10.3, and 28.2 mg/L NH_3-H). During each incremental seven-day cycle, 50 mL of water was collected from the effluent for chemical analysis. Replicate samples of BioCord were also collected at each time point in duplicate from each system (2 x 2 = 4 total) and preserved at -80 ^{o}C until RNA extraction.

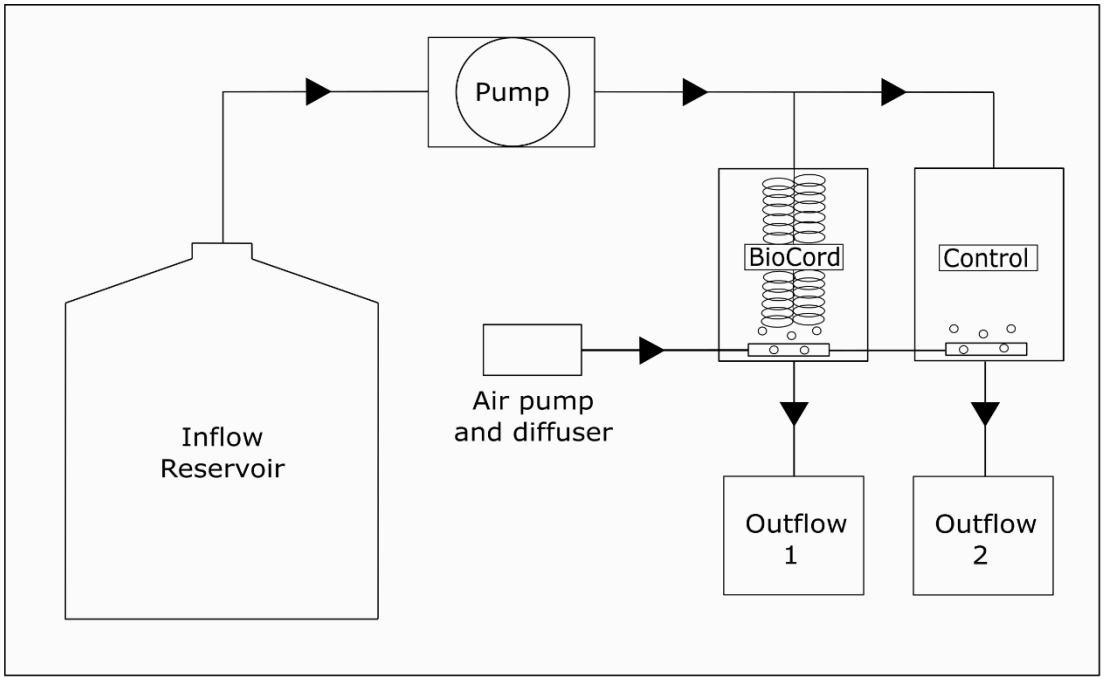

Figure 2.1. Bench-scale design overview.

2.2.2 Water Chemistry and Isotope Analysis

In the bench-scale experiment the collected outflow was used to measure the concentrations of nitrate, ammonia as nitrogen, and the pH. The pH was measured using a pH meter and the nitrate and ammonia as nitrogen were measured using ORION Aquafast II AC2007 and AC2012 respectively with an ORION AQ4000 spectrophotometer. Using 0.2 µ nylon membrane filters, 25 mL of filtered water was collected from each of the two systems at the beginning of the bench-scale experiment and every following seven days for the remainder of the study. The samples were kept frozen at -20 ^{o}C before stable isotope analysis. The $\delta^{15}N$ and $\delta^{18}O$ of NO_3^- in the outflow were measured as the inflow concentration of NH_3-H was increased to determine potential fractionation effects of BioCord. The net nitrification rate (NNR) was calculated using the following equation (k = rate constant):

$$k = \frac{(C_\circ - C)}{t}$$

Where C and Co represent the concentrations of ammonia at time t and initial respectively. The $^{15}N/^{14}N$ ratio of the NH_4Cl that was added to the inflow was analyzed in triplicate with an elemental analyzer (Costech Analytical Technologies Inc., Valencia, CA USA) coupled with a continuous-flow isotope ratio mass spectrometer (ThermoFinnigan, San Jose, CA USA). The ratios for $^{15}N/^{14}N$ and $^{18}O/^{16}O$ of dissolved NO_3^- in the effluent were analyzed using the denitrifier method which measures the resulting N_2O from denitrification of NO_3^- using an HP Agilent 6890 Gas Chromatograph with a PreCon® device interfaced to a Finnigan Mat DELTAplus XL mass spectrometer, with a precision of 0.5‰ for $\delta^{15}N$ and 1.0‰ for $\delta^{18}O$ (Casciotti et al., 2002; Sigman et al., 2001). The isotopic ratios were then calculated using:

$$\delta^{15}N \text{ or } \delta^{18}O\ (‰) = \left[\left(\frac{R_{sample}}{R_{standard}}\right) - 1\right] x\ 1000$$

Where R = $^{15}N/^{14}N$ or $^{18}O/^{16}O$. The standards used for N and O were atmospheric N_2 and standard mean ocean water (SMOW), respectively. Estimates for the $\delta^{15}N$ isotope enrichment effect ($^{15}\varepsilon$) were calculated based on the following relationship where f $=[NH_4^+]_{final}/[NH_4^+]_{initial}$ (Mariotti et al., 1981):

$$\delta^{15}N - NO_{3\,final}^- = \delta^{15}N - NH_{4\,initial}^+ - {}^{15}\varepsilon \left[\frac{f\ x\ \ln(f)}{(1-f)}\right]$$

2.2.3 RNA extraction, High-Throughput Sequencing, and RT-qPCR

The total RNA was extracted from all samples (two BioCord loops in each extraction) using RNeasy Powersoil Total RNA isolation kits following the instructions

of the manufacturer. The Applied Biosystems High Capacity cDNA Reverse Transcription Kit was then used on the extracted RNA. The synthesized cDNA from all the samples was amplified targeting the V5-V6 region of the 16S rRNA gene (all primers shown in **Table A2**. The $PCR_{(1)}$ reaction contained 1 µL cDNA, 0.5 µL (10 µM) forward primer, 0.5 µL (10 µM) reverse primer, 2.5 µL 10X Taq buffer, 1 µL $MgCl_2$ (25 mM), 0.5 µL DMSO, 0.5 µL BSA (50 mg/mL), 0.5 µL dNTPs (10 mM each), 0.1 µL Taq DNA polymerase, and 17.9 µL ddH_2O with a final volume of 25 mL. The thermocycler profile for $PCR_{(1)}$ consisted of initial denaturation for at 94 °C for 5 min followed by 25 cycles of 94 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s with a final extension at 72 °C for 1 min. The $PCR_{(1)}$ products were then purified using Agencourt AMPure XP bead purification, per the manufacturer's protocol. The second $PCR_{(2)}$ for barcoding had a reaction volume of 20 µL and contained 2.5 µL 10X Taq Buffer, 1 µL $MgCl_2$ (25 mM), 0.5 µL DMSO, 0.5 µL BSA (50 mg/mL), 0.5 µL dNTPs (10 mM each), 0.1 µL Taq, 3.9uL ddH_2O, 0.5 µL (10 µM) reverse primer, 0.5 µL (10 µM) of a specific barcode primer to each sample, and 10 µL of AMPure purification product. Thermocycler conditions for $PCR_{(2)}$ followed initial denaturation at 95 ^oC for 5 min followed by 7 cycles at 94 ^oC for 15 s, 60 ^oC for 15 s, 72 ^oC for 30 s with a final extension at 72 ^oC for 1 min. The $PCR_{(2)}$ products showed similar concentrations in agarose gel images and were all pooled together as a result. The pooled samples were run on a 1% agarose gel in triplicate then excised and extracted using QIAquick Gel Extraction Kit. The Agilent 2100 Bioanalyzer was used to check the final sample library to test the quality and determine appropriate dilution for sequencing. Samples were then sequenced using the

Ion Torrent PGM Next Gen Sequencer (Environmental Genomics Facility, University of Windsor).

To take a more quantitative approach in community assessment, seven different targets were selected for quantitative amplification shown in **Table A2**. The targets were chosen to measure the amount of active AOB, active NOB, active anammox bacteria (AMX), and the expression of denitrification activity (nirK, nosZ, and norB). The amplification of the 16S rRNA gene was used as a reference gene for all targets to more accurately compare amplification between samples. The RT-qPCR reactions had a 10 µL reaction volume containing 5 µL Applied Biosystems PowerUp SYBR Green master mix, 0.4 µL F primer (10uM), 0.4 µL R primer (10uM), 1 µL cDNA Sample, and 3.2 µL ddH_2O. All samples were run on the Quantstudio 12K Flex Real-Time PCR System (Environmental Genomics Facility, University of Windsor). The thermocycler profile used followed 2 min at 50 ^oC, 2 min at 95 ^oC, followed by 40 cycles of 95 ^oC for 15 s and 60 ^oC for 1 min.

2.2.4 Data Analysis

The outflow $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values were compared with NO_3^- concentrations using regression analysis to determine the fractionation effects of nitrification. The inclusion of baseline NO_3^- values from week one is crucially included to assess the fractionation due to nitrification of the new source of amended nitrogen (NH_4Cl). For logarithmic and linear regression analysis the highest R^2 value was used to determine best fit.

The obtained 16S rRNA amplified dataset was quality filtered (Q20), barcodes trimmed, and demultiplexed using QIIME 1.9 (Caporaso et al., 2010). Samples containing read totals <5000 were filtered out from downstream analysis. Chimera sequences were identified and filtered using VSEARCH (Rognes et al., 2016). Sequences were clustered into Operational Taxonomic Units (OTUs) with open reference picking using the uclust algorithm with a 97% similarity threshold (Edgar, 2010). Samples were then filtered to remove singleton and doubleton OTUs followed by taxonomy assignment using uclust with SILVA as the reference database (Quast et al., 2013). The OTUs were normalized using cumulative sum-scaling (CSS) for taxonomy analysis (Paulson, JN et al., 2013). The unnormalized OTU table was normalized using the average of 10 rarefactions with a minimum of 5000 sequences per sample to calculate alpha-diversity**.** To test the similarity of the microbial community between treatments in the bench-scale study, ANOSIM of the top 500 OTUs based on Bray-Curtis with 9999 permutations was used with sequential Bonferroni correction. One-way ANOVA of square root transformed relative abundance data was used to determine if there are any statistical differences of specific taxa between samples followed by Tukey post-hoc test to determine which samples are specifically different ($p < 0.05$). Statistical analysis was completed using Past version 3 (Hammer et al., 2001).

The RT-qPCR efficiencies were determined and corrected for using LinRegPCR and the RT-qPCR data was analyzed looking at the starting concentration (N_o) ratios (Ruijter et al., 2009). The N_o ratios were calculated by dividing the target N_o value by the N_o value for the 16S rRNA reference gene and averaging all individual sample values for that timepoint. One-way ANOVA of logarithmic transformed gene abundance data was

used to determine if there are any statistically significant differences between samples for each gene followed by Tukey post-hoc test to determine which samples are specifically different ($p < 0.05$).

2.3 Results and Discussion

2.3.1 Water Chemistry, Nitrification Kinetics, and Stable Isotope Analysis

The influent and effluent pH remained moderately alkaline (7.8-8.4) over the course of the experiment. The initial influent NH_3-H concentration was 0.07 mg/L and was followed by three successive treatments. After seven days, in the first treatment 4.01 mg/L NH_3-H was added to followed by 10.3 mg/L after 14 days and 28.2 mg/L after 21 days. In the BioCord system, the NH_3-H concentrations significantly decreased after each treatment to 0.13-0.15 mg/L after 14 days, 0.12-0.15 mg/L after 21 days, and 15.4-17.0 mg/L after 28 days. Compared to the control system which contained no biocord, the NH_3-H concentration in the effluent was 4.0 mg/L, 8.0-8.8 mg/L, and 21.1-23.3 mg/L after 14, 21 and 28 days of treatment, respectively. The resulting NH_3-H removal efficiencies ranged from 40% to 98% in the BioCord system and 0% to 25% in the control system. The NO_3^- concentrations entering the systems showed a gradual increase after 21 days from <0.1 mg/L to 0.87 mg/L. The effluent from the BioCord system after 14, 21, and 28 days of treatment contained 5.0-5.7 mg/L, 12.6-12.7 mg/L, and 16.7-19.0 mg/L of NO_3^-, respectively. In comparison, the control system effluent NO_3^- concentrations were 0.3 mg/L, 5.8-6.2 mg/L, and 15.6-17.0 mg/L, respectively. The contrasting increase in NO_3^- and decrease in NH_3-H suggests that nitrification occurred in both systems with BioCord resulting in significantly enhanced nitrification. The calculated NNR was significantly higher in the BioCord system and increased as NH_3-H

concentration increased in the influent. As the NH_3-H increased, the NNR ranged from 0.55 to 1.71 mg/L/day and 0 to 0.85 mg/L/day in the BioCord and control systems, respectively. This study demonstrated a maximum potential NH_3-H removal efficiency of 98% compared to previous BioCord flow-through studies which reported maximum NH_3-H removal efficiencies ranging from 55 to 97 % (Tian et al., 2017; Yang et al., 2014; Yuan et al., 2012). The biofilm used in this study was perhaps limited with respect to surface area and was likely unable to effectively reduce NH_3-H in the final treatment. Additionally, the bench-scale system may have required a longer residence time for the higher concentration gradient or required more oxygen stimulus (Ødegaard, 2006). Although the NO_3^- concentrations were similar in both systems after 28 days, the NH_3-H removal efficiency was double in the BioCord system suggesting anaerobic nitrogen removal processes (e.g. anammox) were likely occurring within the biofilm. This was suggested in a previous study where anaerobic denitrifier DNA was observed in the BioCord community but the activity was not confirmed (Yuan et al., 2012).

Baseline NO_3^- concentrations were < 0.1 mg/L for the control and 0.81-1.1 mg/L for the BioCord system. The corresponding baseline isotope values for $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ were 20.6‰, 32.8‰, and 23.4‰, -3.5‰ for the control and BioCord, respectively. The increase in concentration of NO_3^- and distinct $\delta^{18}O_{NO3}$ values for the control and BioCord suggest that the biofilm associated with the BioCord stimulated NO_3^- production within the baseline system prior to amendments with NH_3-H. The change in $\delta^{18}O_{NO3}$ values suggests that the BioCord pathway catalyzes oxidation with the oxygen sourced from the water, where the oxygen isotope value reflects projected values for the inflow source water (Yang et al., 1996). The system treated with 4.01 mg/L NH_3-

H, resulting in an increased concentration of NO_3^- to 5.0-5.7 mg/L in the BioCord system, showed a $\delta^{15}N_{NO3}$ value of 16.6‰, and $\delta^{18}O_{NO3}$ value of -8.5‰. In comparison, the control system NO_3^- concentration was 0.3 mg/L with $\delta^{15}N_{NO3}$ value 12.2‰, and $\delta^{18}O_{NO3}$ value -22.4‰. The change in $\delta^{15}N_{NO3}$ values were consistent with the ^{15}N-depletion anticipated for isotopic fractionation from the NH_3-H amendment to the inflow ($\delta^{15}N_{NH4}$ value = 9.9 ± 0.1 ‰). As the NH_3-H amendments were increased in the bench-scale study with the addition of NH_4Cl, the BioCord and control systems showed different relationships for $\delta^{15}N$ and $\delta^{18}O$ values. The $\delta^{15}N_{NO3}$ values decreased in both systems as NO_3^- concentrations increased but showed a linear relationship (R^2= 0.9904, p=0.0048) and logarithmic relationship (R^2=0.9635, p=0.0184) in the BioCord and control systems, respectively (**Figure 2.2**). The logarithmic relationship in the control system is likely a result of lag time in nitrifier growth, which has been observed in other nitrification studies, and was the reason why negligible nitrification was observed in the first treatment of the control system (Boshers et al., 2019). The enhanced nitrification in the BioCord system did not show a lag time and resulted in relatively less fractionation of the $\delta^{15}N_{NO3}$ values. A linear regression of $\delta^{15}N_{NO3}$ values versus [-f x lnf/(1-f)] for all samples in the BioCord and control where nitrification was observed resulted in an enrichment factor of $^{15}\varepsilon$ = -17.8 ± 4.1 ‰ (R^2=0.8621, p=0.0227, ± based on 95% CI), which is comparable to nitrification enrichment factors reported in other experiments (Casciotti et al., 2003). The $\delta^{18}O_{NO3}$ values for the control system followed a similar logarithmic relationship (R^2=0.9495, p=0.0256) as the $\delta^{15}N_{NO3}$ values but the BioCord system only showed a slight change in $\delta^{18}O_{NO3}$ values with increasing nitrate

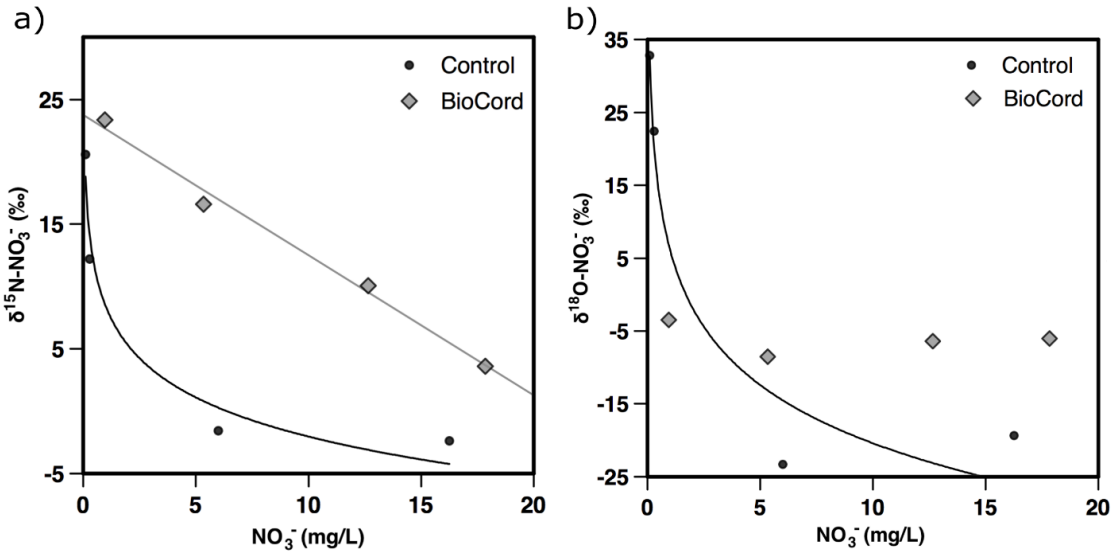

Figure 2.2. Nitrate isotopic data in the bench-scale system throughout all treatments in the BioCord and control experiments. a) The $\delta^{15}N_{NO3}$ (‰) values versus the outflow NO_3^- concentrations (BioCord: R^2= 0.9904, p=0.0048 and control: R^2=0.9635, p=0.0184); b) $\delta^{18}O_{NO3}$ (‰) values versus the outflow NO_3^- concentrations (control: R^2=0.9495, p=0.0256).

concentrations and remained close to the proposed $\delta^{18}O_{H2O}$ values. The $\delta^{18}O_{NO3}$ values have been shown to approach the $\delta^{18}O_{H2O}$ value during nitrification. The deviation observed in the control system is attributed to the lag phase of growth and reduced NNR which increases both the NO_2^- equilibrium isotopic exchange of O with H_2O and the effect of kinetic O isotope fractionation (Boshers et al., 2019; Buchwald and Casciotti, 2010). These observations suggest that the similarity of $\delta^{18}O_{NO3}$ values to $\delta^{18}O_{H2O}$ values could be a useful proxy for determining whether BioCord is impacting nitrification rates and thus could be used for optimization and efficiency monitoring. If the $\delta^{18}O_{H2O}$ and

$\delta^{18}O_{NO3}$ are analyzed before implementation of the treatment, then the nitrification rate changes could be approximated independently from concentration in a WWTP based on the change in isotope value.

Although the NNR continued to increase in the last treatment with the BioCord (1.45 to 1.71 mg/L/day), and the $\delta^{18}O_{NO3}$ did not change, the efficiency decreased significantly from 98% to 40-45%. This suggests the system was still working normally but a longer residence time or an increase in surface area was required to successfully treat the high NH_3-H levels. Based on the isotopic data, the BioCord was shown to accelerate oxidation by augmenting nitrification pathways in the system rather than alter the mechanism. The increasing NNR in the BioCord system suggests there could be substantial attachment and active growth of nitrifying organisms which was confirmed and shown below using HTS and RT-qPCR.

2.3.2 Variations in the bacterial community from HTS

The microbial community dynamics of BioCord were investigated in throughout the duration of the bench-scale experiment. A total of 7 140 OTUs were generated from 596 787 filtered sequence reads. To understand the effect of changing nutrient concentrations on species richness of the attached biofilm the chao1 alpha-diversity metric was compared using ANOVA followed by Tukey post-hoc test. The chao1 values showed no significant differences within the bench-scale samples. The similarity of microbial communities within the bench-scale experiment was assessed using ANOSIM. Results demonstrate there was no significant differences between the overall initial microbial community and the BioCord after each successive treatment in the bench-scale experiment. Therefore, the OTUs obtained from all samples were compiled to assess the

representative BioCord community structure. The average relative abundances of bacterial phyla that had a 1% or greater relative abundance in the microbial community was shown in **Figure 2.3**. The most dominant phylum in the BioCord bacterial community was *Proteobacteria* followed by *Bacteroidetes*, with the most dominant *Proteobacteria* taxonomic classes being *Alphaproteobacteria* and *Betaproteobacteria.* *Deltaproteobacteria* and *Gammaproteobacteria* were still both present but significantly less abundant in comparison**.** *Actinobacteria* and *Acidobacteria*, were also dominant phyla throughout the BioCord community**.** *Cyanobacteria*, *Planctomycetes*, and

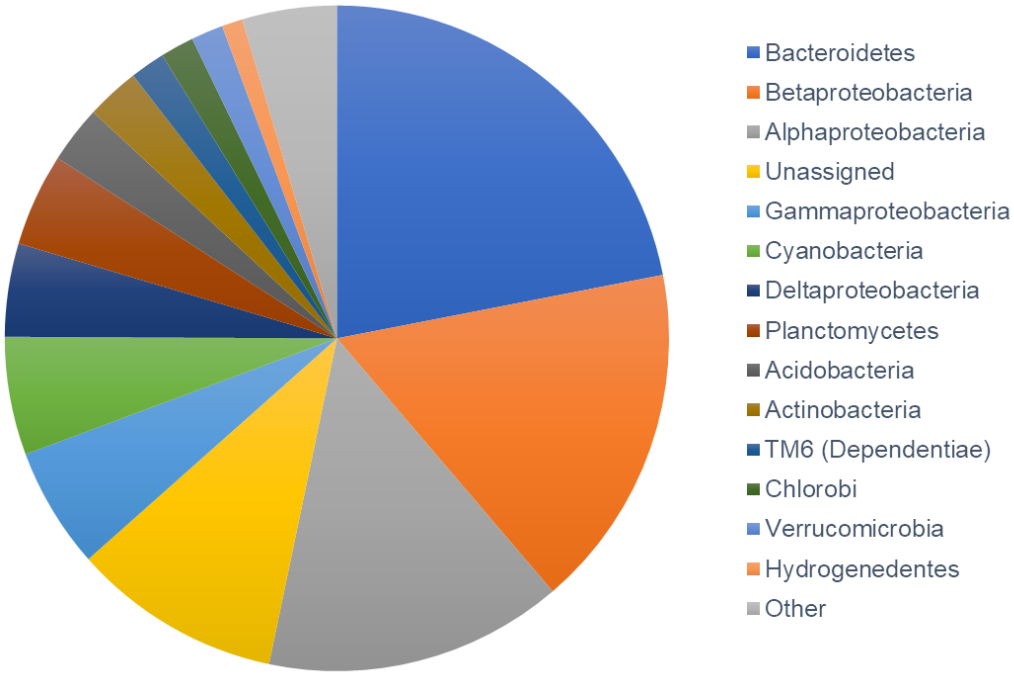

Figure 2.3. Relative abundances greater than 1% within the bacterial phyla for the averages of the bench-scale experiments. The total relative abundance for proteobacteria phyla is subdivided into taxonomic class.

Verrucomicrobia were relatively less dominant but still important phyla in the community throughout the bench-scale experiment. *Nitrospirae*, an important nitrifying containing phylum in wastewater treatment, was a minor community member representing 0.6% of OTUs. Previous microbial characterizations of BioCord using extracted DNA found similar total community structures of bacteria phyla with the exception of *Planctomycetes*, which was not detected in bench-scale, and *Verrucomicrobia* which was determined to be significantly more dominant in an eco-ditch environment (Yuan et al., 2012; Zhou et al., 2018). Furthermore, this study found the *Proteobacterial* class to be largely dominated by *Alpha* and *Betaproteobacteria* while the previously mentioned studies determined *Betaproteobacteria* or *Alpha/Gamma-proteobacteria* to dominate.

2.3.3 Characterization and trends of nitrifying bacteria in the bench-scale experiment

Within the observed *Proteobacteria* and *Nitrospirae* phyla, the primary nitrifying genera in the bench-scale samples were determined to be *Candidatus Nitrotoga, Nitrosomonas, Nitrospira,* and an uncultured genus of *Nitrosomonadaceae*, which have all been widely found in wastewater treatment (Cydzik-Kwiatkowska and Zielińska, 2016). *Nitrosospira* and *Nitrosomonas* were detected in the previous bench-scale BioCord community study but none of the other nitrifiers detected in this study were observed (Yuan et al., 2012). Although each identified nitrifying genus followed different relational patterns throughout the experiment, there was a generally consistent increase in the total relative abundance of nitrifiers in the bacterial community as ammonia inflow concentrations increased (**Figure 2.4**). Although both NOB, *Candidatus Nitrotoga* and *Nitrospira*, are present across all samples, *Nitrospira* was consistently the more dominant

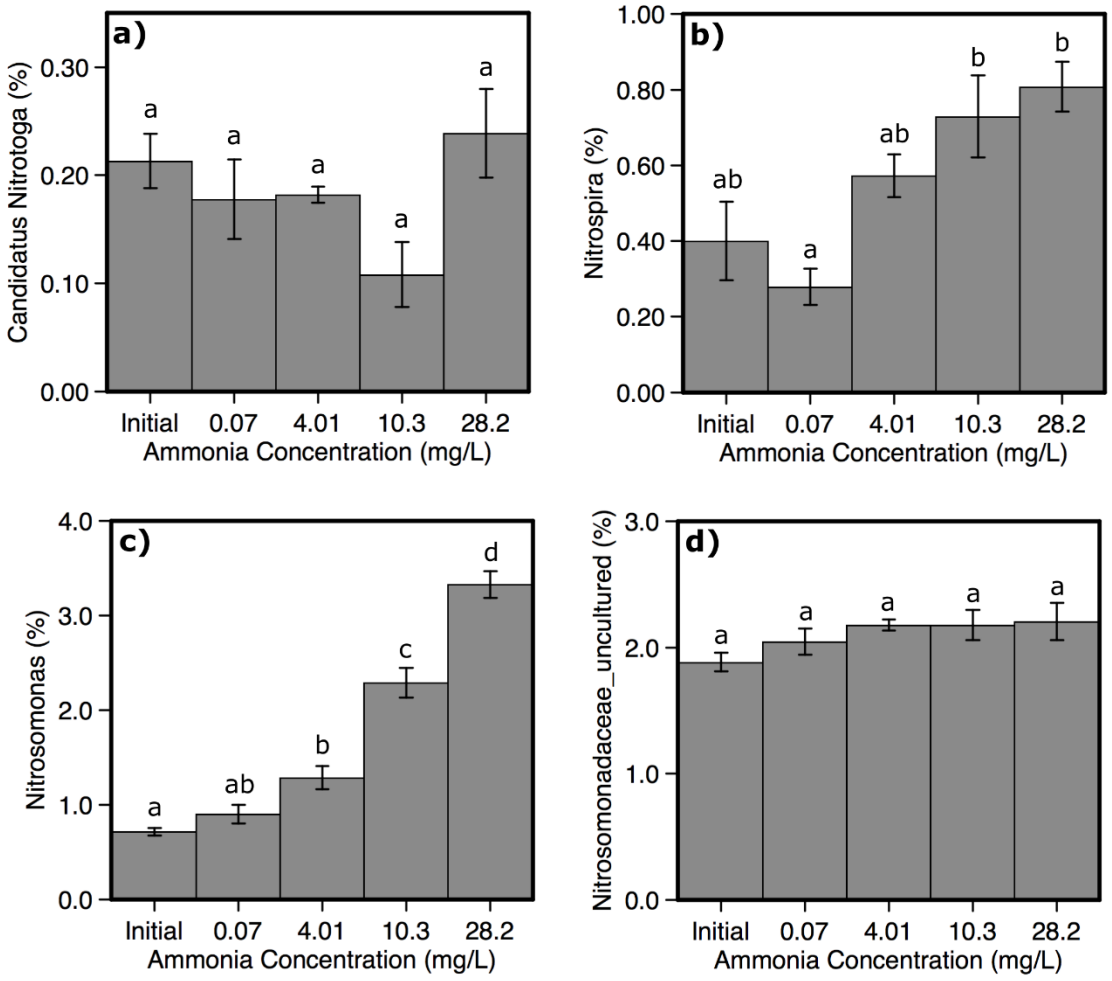

Figure 2.4. Relative abundance of the identified nitrifying genera (mean ± SE) in the bench-scale samples with increasing ammonia concentrations (mg/L) in the influent on the x-axis. Letters above bars represent significant difference based on Tukey's post-hoc ($p < 0.05$).

nitrite-oxidizer being 1.6 to 6.7 times more abundant. The relative abundance of *Nitrospira* did not increase after the first 4.01 mg/L NH_3-H treatment but showed a significant increase after 10.3 and 28.2 mg/L NH_3-H was added in the system (p=0.021

and p=0.013). Additionally, the identified *Nitrosmonas* genus gradually increased to be the dominant AOB from 0.4 to 1.5 times abundance compared to the uncultured genus of *Nitrosomonadaceae*. Similar to *Nitrospira, Nitrosomonas* only increased significantly after the 10.3 mg/L NH_3-H amendment (p=0.001). However, it also continued to increase after 28.2 mg/L NH_3-H was added and represented 3.3% of OTUs making it the second most abundant genus in the community (p=0.012). Overall, the bench-scale conditions were relatively more favourable for the growth of *Nitrospira* and *Nitrosomonas* on the BioCord substrate. The ratio of total AOB/total NOB ranged from 4.2 to 6.5. The optimal AOB/NOB ratio for nitrification was previously theoretically determined to be 2.0 with increased values representing potential anaerobic nitrogen removal (e.g. anammox) (Winkler et al., 2012). When the system was at its highest NH_3-H removal efficiency of 98% with an inflow of 10.3 mg/L, the AOB/NOB ratio was 5.3 suggesting anaerobic nitrogen removal was an important metabolic process in treatment. This ratio did not significantly change when the removal efficiency dropped to 40-45% suggesting the reduction in removal efficiency was not related to a specific microbial shift and that the treatment was potentially limited by residence time (Ødegaard, 2006).

2.3.3 Variability in the of abundance and expression of key genes representing nitrogen metabolism from RT-qPCR

Based on the results of the HTS data from the bench-scale study, primers were selected to quantify *Nitrospira*, *Nitrosomonas*, and anammox bacteria denoted by NOB, AOB, and AMX respectively. All primers were selected through literature review and shown in **Table A2**. Representative genes responsible for denitrification metabolism did not show any gene expression throughout the bench-scale experiment. The *Planctomycetes* bacteria targeted by AMX was not specifically determined with

confidence at the genus level within the HTS data. However, there was a significant amount of uncultured OTUs from the *Planctomycetes* phylum seen in the taxonomy results throughout all samples. Therefore, the AMX primer was chosen to determine if the underdefined bacterial groups contained an active anammox population. A recognized limitation of 16S based primers are that they cannot conclude with certainty the specific metabolic activity of the organism, but the primary objective of this study was to better understand how the community structure changes with increasing NH_3-H concentrations.

The gene abundances for AMX, AOB, and NOB of the BioCord were all determined to be highly abundant throughout the bench-scale experiments (**Figure 2.5**). Although evidence for denitrification metabolism was not found in the bench-scale samples, the AMX presence suggests that anaerobic nitrogen removal processes still play a significant role. The average gene abundances for both AMX and AOB increased gradually but did not show a significant difference due to the inherent variability between replicates. This signifies that the biofilm community was variable throughout its location on the BioCord which has been a feature identified with biofilm in previous studies (Yuan et al., 2012). In contrast, NOB showed a particularly sensitive response by statistically significantly increasing in average abundance with each increase of NH_3-H ($p<0.05$). This resulted in a 12-fold NOB increase from lowest to highest NH_3-H influent concentration. When the influent NH_3-H increased from 4.01 to 10.3 mg/L the removal efficiency remained elevated at 98% even though there was only an observed increase in gene abundance for NOB. It is therefore likely that the increasing *Nitrospira* gene abundance contained

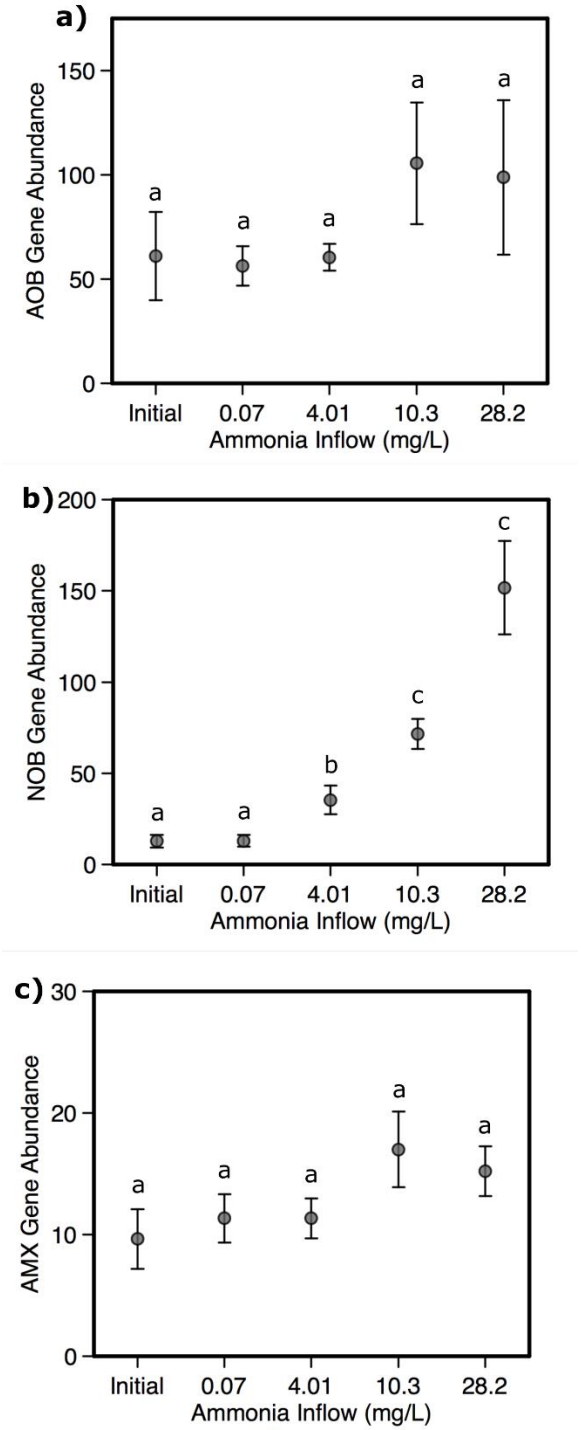

Figure 2.5. Comparing gene abundance (=N_o Target/N_o Control) for key genes (mean ± SE) in bench-scale samples with increasing influent concentrations of ammonia (mg/L). Letters above bars represent significant difference based on Tukey's post-hoc ($p < 0.05$).

significant comammox species. A similar trend was observed when NH_3-H inflow was increased from 10.3 to 28.2 mg/L where NOB gene abundance increased two-fold.

The RT-qPCR gene abundance data for AOB, NOB, and AMX were compared with NNR to quantitatively determine which microbial groups were the strongest predictors of NH_3-H removal in the BioCord system (**Figure 2.6**). The gene abundance of AMX did not show any significant trends with NNR suggesting that most of the ammonia removed was due to nitrification, which was expected in the well oxygenated system. In

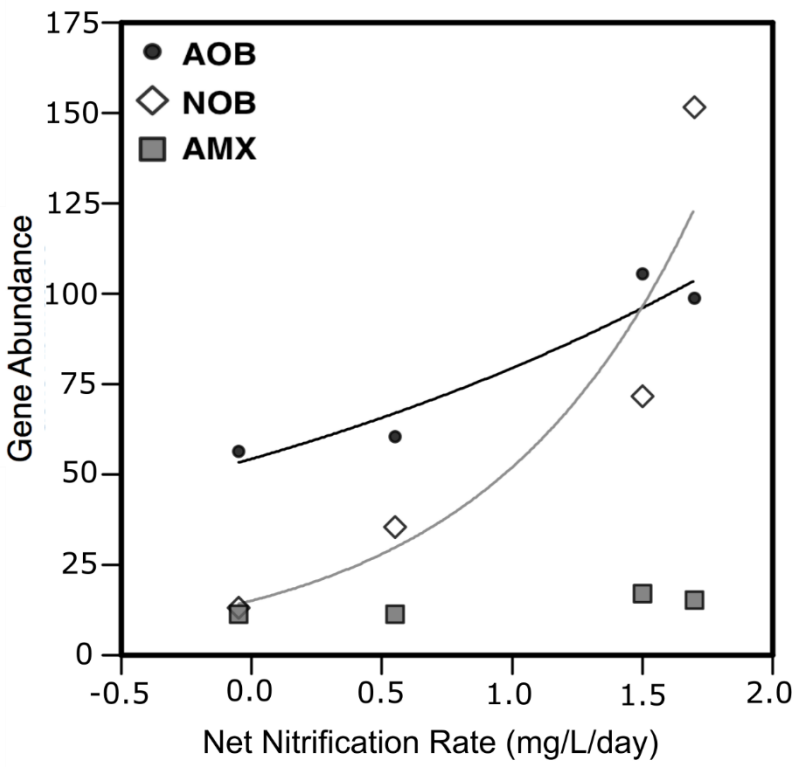

Figure 2.6. AOB, NOB, and AMX from the BioCord versus nitrification rates (mg/L/day) in the BioCord bench-scale system (AOB: R^2=0.9088, p=0.0467 and NOB: R^2=0.9609, p=0.0197).

and NOB (R^2=0.9609, p=0.0197). This demonstrates that an increase in *Nitrosomonas* (AOB) and *Nitrospira* (NOB) significantly increased the NNR, with the latter showing the most significant relationship. This suggests that an increase in NH_3-H concentration results in an exponential increase of NOB and AOB gene abundance in the BioCord, which results in an increase of NNR.

2.4 Conclusion

The abundance of nitrifiers significantly increased with an increasing NH_3-H gradient resulting in increasing net nitrification rates. The control system only had a maximum NH_3-H removal efficiency of 25% while the BioCord system showed up to 98% removal. The BioCord system did not show a lag time in enhanced nitrification which resulted in relatively less fractionation of the $\delta^{15}N_{NO3}$ values. Based on stable isotope analysis, $^{15}\varepsilon$ and $\delta^{18}O_{NO3}$ values were effective regulatory monitors of enhanced biological nitrification rates. The resulting nitrification enrichment factor for the observed nitrifiers was $^{15}\varepsilon$ = -17.8 ± 4.1 ‰ and the change in $\delta^{18}O_{NO3}$ values suggests that the BioCord pathway catalyzed oxidation with the oxygen sourced from the water. The nitrifying community was dominated by the genera *Nitrosomonas* and *Nitrospira*, with *Nitrospira* being the strongest predictor of increased nitrification. Results from RT-qPCR showed anammox bacteria were active community members, but denitrification was not observed. Future work should focus on further RT-qPCR targets or metatranscriptomics in combination with stable isotope analysis to better understand the biofilm mechanisms to improve its effectiveness in different environmental conditions.

CHAPTER 3

Microbial community dynamics of BioCord in lagoon wastewater treatment

3.1 Introduction

Nutrient pollution is a global environmental problem that has led to increasingly stricter regulations in wastewater treatment processes (Lyu et al., 2016)**.** Elevated levels of nitrogen and phosphorus in released wastewater can lead to significant environmental damage including habitat destruction, hypoxic zones, harmful algal blooms, and eutrophication (Conley et al., 2009; Diaz and Rosenberg, 2009; Howarth, 2008). The global biogeochemical cycling of nitrogen is almost entirely controlled through oxidation-reduction reactions by microbes (Falkowski, 1997). Since microorganisms play such a significant role in nutrient cycling they are commonly used by municipalities in biological wastewater treatment to reduce effluent nitrogen concentrations. Lagoon wastewater treatment plants (WWTP) are a commonly used treatment option for rural communities because they are less expensive and require more land than mechanical plants. However, nitrogen removal is less reliable in temperate regions due to the colder seasonal temperatures inhibiting microbial activity, which lagoon treatment systems rely on (Hurse and Connor, 1999). Improvements to lagoon treatment systems in temperate environments are therefore required to effectively remove nitrogen, most importantly ammonia, year round.

One proposed improvement to lagoon systems for effective nitrogen removal is an attached biofilm technology known as BioCord (Gan et al., 2018). BioCord provides a high surface area ring of polymer threads that encourages natural biofilm development. It has been successfully used to sustain a microbial community which reduces nutrient levels of effluent waters in various settings including laboratory, river, and drainage ditch

environments (Tian et al., 2017; Yuan et al., 2012; Zhou et al., 2018). However, there has not been any published research into using BioCord bioreactors to upgrade a full size lagoon WWTP. Previous studies have also not characterized the active BioCord microbial community, which is particularly important for predicting and developing treatment.

It is widely known that nitrogen removal by microbes in wastewater has been accomplished through several metabolic processes including nitrification, denitrification, and anaerobic ammonium oxidation (anammox) (Munch et al., 1996; Schmidt et al., 2003; Strous et al., 1997). Nitrification is a two-step process where ammonium (NH_4^+) is first oxidized to nitrite (NO_2^-) which is then oxidized into nitrate (NO_3^-). The first step is completed by ammonia-oxidizing bacteria (AOB) or ammonia-oxidizing archaea and the second step is finished by nitrite-oxidizing bacteria (NOB). The exception is the recent discovery of some species from the *Nitrospira* genus that contain the genes for both steps and are known as complete ammonia oxidizing (commamox) bacteria (Daims et al., 2015). The more commonly found AOB in WWTPs are the genera *Nitrosomonas* and *Nitrosospira* while the more common NOB are the genera *Nitrospira*, *Nitrobacter,* and the more recently discovered *Candidatus Nitrotoga* (Cydzik-Kwiatkowska and Zielińska, 2016; Lucker et al., 2015; Siripong and Rittmann, 2007). Denitrification is the process of reducing NO_3^- to molecular gaseous nitrogen (N_2) with intermediates of NO_2^-, nitric oxide (NO), and nitrous oxide (N_2O). In order of reduction from NO_3^-, these microbial processes are controlled by the enzymes nar, nir, nor, and nos respectively. In WWTPs, the denitrification process can be controlled by many different phylogenetic groups including *Rhodoferax, Dechloromonas, and Thermomonas* (Mcilroy et al., 2016).

Anammox, the most recently discovered pathway in which nitrogen can be removed from wastewater, is the reaction of NH_4^+ and NO_2^- to produce N_2 through a multistep process by a unique subgroup of *Planctomycetes* bacteria (Kuenen, 2008; Mulder et al., 1995). To identify if the microbes that are useful in nitrification and nitrogen removal are present, molecular methods can be used.

Molecular methods used to assess microbial communities in wastewater treatment include targeted amplicon sequencing of the 16S rRNA gene and quantitative polymerase chain reaction (qPCR) (De Sotto et al., 2018; Harms et al., 2003; Yapsakli et al., 2011). High throughput sequencing (HTS) of the amplified 16S rRNA gene is one of the most widely used tools to assess the bacterial population and recently it has also been shown to have a good representation of nitrifying microbial guilds (Diwan et al., 2018). In comparison, qPCR is a more effective tool for quantifying a specific pathway or bacterial group (e.g. AOB). The total microbial community can often be significantly different from the active community (Yu and Zhang, 2012). In order to represent the active community, the extracted RNA was the primary focus in this study.

The goal of this study was to assess the microbial community to identify key members of that community and determine the metabolic function of nitrogen removal within the BioCord in a lagoon WWTP. This study offers the first novel insight into the microbial community development of BioCord by describing the seasonal variability within the attached biofilm community and comparing it to the surrounding water and sediment. The influence of physicochemical changes on BioCord described in this study are important to better predict and implement the use of the technology in wastewater

treatment. This will be assessed using water chemistry, targeted amplicon sequencing of the 16S rRNA gene, and RT-qPCR.

3.2 Methods

3.2.1 Field site description and sampling

The wastewater treatment lagoons are a municipal treatment system in Dundalk, Ontario, Canada (44°09'12.0"N 80°23'07.0"W) and consist of five lagoons in series shown in **Figure 3.1**. Wastewater enters directly into lagoon one and is released from

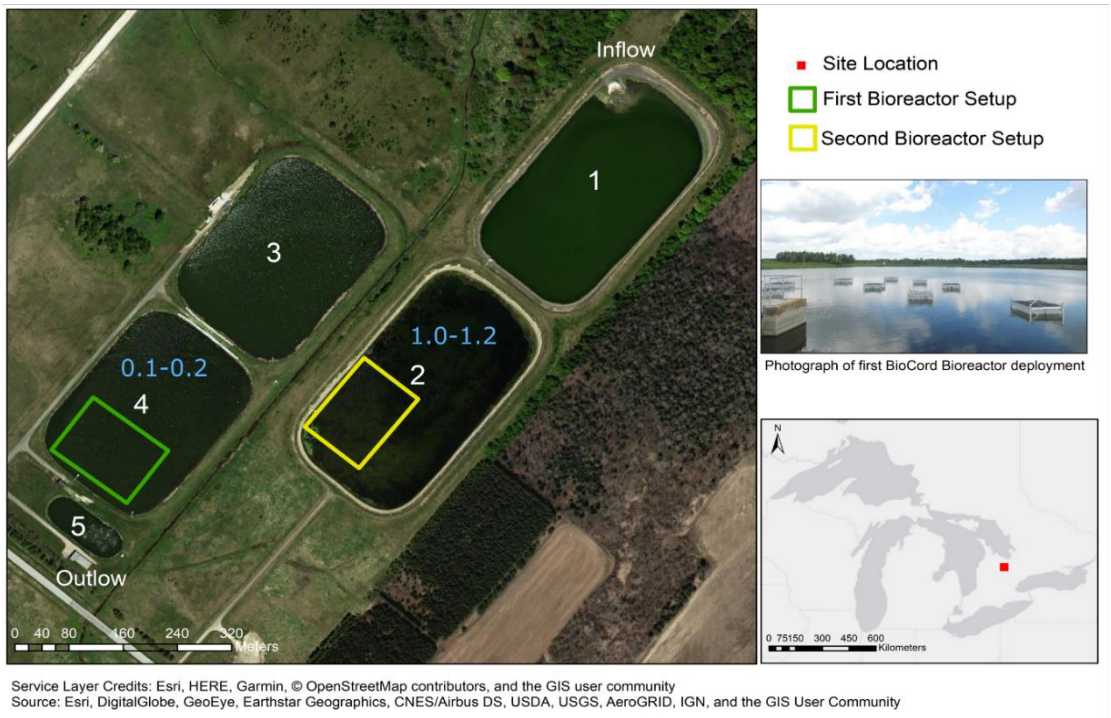

Figure 3.1. Field site overview of lagoon wastewater treatment facility in Dundalk, Ontario, Canada. Corresponding ammonia concentration ranges during times of BioCord sample collection shown for lagoons two and four in blue (mg/L).

lagoon five into the upper Grand River watershed, in Ontario. The residence time of each lagoon varies between 30 and 60 days. In July 2016, 10 BioCord bioreactors were deployed surrounding the outflow pipe in lagoon four (L4). To test the microbial response to an increased nitrogen gradient, the rafts were removed from L4 and 10 new rafts were deployed near the outflow pipe of lagoon two (L2) in July of 2017. In both locations, an aeration system was setup underneath all the rafts to keep the system well oxygenated year-round and to prevent ice formation around the rafts during the colder seasons. Water samples were collected weekly from the source inflow of the lagoon WWTP and within each lagoon. Biofilm samples were collected in the bioreactor deployments in lagoons four and two in late October of 2016 and 2017, respectively. Samples were also collected in late November of 2016 and early December of 2017 when water temperatures significantly decreased from 16.9 to 1.6 ^{o}C and 15 to 6 ^{o}C, respectively. Samples were collected by selectively cutting off portions of the BioCord into 5 mL cryotubes. During the month of July 2017, surface sediment and water was also collected from lagoons two, three, and four to compare microbial community composition in each compartment. Surface sediment (5g) was collected using a ponar grab sampler and water sample (500 mL) was filtered using 0.2 µ nylon membrane filters. After collection, BioCord, sediment, and filters were preserved at -80 ^{o}C in liquid nitrogen until RNA extraction.

3.2.2 Water chemistry analysis for WWTP lagoons

Water samples were collected over the four times points from each of the lagoon outflows and the WWTP inflow. The measured parameters included water temperature, pH, dissolved oxygen (DO), chemical oxygen demand (COD), (soluble) biochemical

oxygen demand (sBOD/BOD), alkalinity, total/volatile suspended solids (TSS/VSS), nitrogen speciation (NO_3^-, NO_2^-, NH_3-H, total N (TN), total Kjeldahl nitrogen (TKN), and phosphorus (P/ortho-P) (**Table A1**).

3.2.3 RNA extraction, High-Throughput Sequencing, and RT-qPCR

The total RNA was extracted from all samples (two BioCord loops in each extraction) using RNeasy Powersoil Total RNA isolation kits following the instructions of the manufacturer. The Applied Biosystems High Capacity cDNA Reverse Transcription Kit was then used on the extracted RNA. The synthesized cDNA from all the samples was amplified targeting the V5-V6 region of the 16S rRNA gene (all primers shown in Table A2). The $PCR_{(1)}$ reaction contained 1 µL cDNA, 0.5 µL (10 µM) forward primer, 0.5 µL (10 µM) reverse primer, 2.5 µL 10X Taq buffer, 1 µL $MgCl_2$ (25 mM), 0.5 µL DMSO, 0.5 µL BSA (50 mg/mL), 0.5 µL dNTPs (10 mM each), 0.1 µL Taq DNA polymerase, and 17.9 µL ddH_2O with a final volume of 25 µL. The thermocycler profile for $PCR_{(1)}$ consisted of initial denaturation for at 94 °C for 5 min followed by 25 cycles of 94 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s with a final extension at 72 °C for 1 min. The $PCR_{(1)}$ products were then purified using Agencourt AMPure XP bead purification, per the manufacturer's protocol. The second $PCR_{(2)}$ for barcoding had a reaction volume of 20 µL and contained 2.5 µL 10X Taq Buffer, 1 µL $MgCl_2$ (25 mM), 0.5 µL DMSO, 0.5 µL BSA (50 mg/mL), 0.5 µL dNTPs (10 mM each), 0.1 µL Taq, 3.9uL ddH_2O, 0.5 µL (10 µM) reverse primer, 0.5 µL (10 µM) of a specific barcode primer to each sample, and 10 µL of AMPure purification product. Thermocycler conditions for $PCR_{(2)}$ followed initial denaturation at 95 ^oC for 5 min followed by 7 cycles at 94 ^oC for 15 s, 60 ^oC for 15 s, 72 ^oC for 30 s with a final extension at 72 ^oC for 1

min. The $PCR_{(2)}$ products showed similar concentrations in agarose gel images and were all pooled together as a result. The pooled samples were run on a 1% agarose gel in triplicate then excised and extracted using QIAquick Gel Extraction Kit. The Agilent 2100 Bioanalyzer was used to check the final sample library to test the quality and determine appropriate dilution for sequencing. Samples were then sequenced using the Ion Torrent PGM Next Gen Sequencer (Environmental Genomics Facility, University of Windsor).

To take a more quantitative approach in community assessment, seven different targets were selected for quantitative amplification shown in **Table A2**. The targets were chosen to measure the amount of active AOB, active NOB, active AMX and the expression of denitrification activity (nirK, nosZ, and norB). The amplification of the 16S rRNA gene was used as a reference gene for all targets to more accurately compare amplification between samples. The RT-qPCR reactions had a 10 µL reaction volume containing 5 µL Applied Biosystems PowerUp SYBR Green master mix, 0.4 µL F primer (10uM), 0.4 µL R primer (10uM), 1 µL cDNA Sample, and 3.2 µL ddH_2O. All samples were run on the ABI 7500 Real-Time PCR System (Environmental Genomics Facility, University of Windsor). The thermocycler profile used followed 2 min at 50 ^oC, 2 min at 95 ^oC, followed by 40 cycles of 95 ^oC for 15 s and 60 ^oC for 1 min.

3.2.4 Data Analysis

The number of samples collected is shown in **Table A3.** An assessment using ANOSIM of the top 500 OTUs based on Bray-Curtis with 9999 permutations and sequential Bonferroni correction showed no significant differences between samples

collected from eight rafts in 2016. As a result, a representative subset rafts were selected for sample collection in 2017.

The obtained 16S rRNA amplified dataset was quality filtered (Q20), barcodes trimmed, and demultiplexed using QIIME 1.9 (Caporaso et al., 2010). Samples containing read totals <5000 were filtered out from downstream analysis. Chimera sequences were identified and filtered using VSEARCH (Rognes et al., 2016). Sequences were clustered into Operational Taxonomic Units (OTUs) with open reference picking using the uclust algorithm with a 97% similarity threshold (Edgar, 2010). Samples were then filtered to remove singleton and doubleton OTUs followed by taxonomy assignment using uclust with SILVA as the reference database (Quast et al., 2013). The OTUs were normalized using cumulative sum-scaling (CSS) for taxonomy analysis (Paulson, JN et al., 2013). The unnormalized OTU table was normalized using the average of 10 rarefactions with a minimum of 5000 sequences per sample to calculate alpha-diversity**.** To test the similarity of the microbial community at each timepoint in the field study, ANOSIM of the top 500 OTUs based on Bray-Curtis with 9999 permutations was used with sequential Bonferroni correction. Non-metric multidimensional scaling (NMDS) of the top 500 OTUs was used to ordinate samples based on Bray-Curtis using three dimensions. One-way ANOVA of square root transformed relative abundance data was used to determine if there are any statistical differences of specific taxa between samples followed by Tukey post-hoc test to determine which samples are specifically different ($p < 0.05$). Canonical correspondence analysis was used to observe dominant trends between chemical parameters (**Table A4**) and cDNA based taxonomic abundances. Statistical analysis was completed using Past version 3 (Hammer et al., 2001).

The RT-qPCR efficiencies were determined and corrected using LinRegPCR and the RT-qPCR data was analyzed looking at the starting concentration (N_o) ratios (Ruijter et al., 2009). The N_o ratios were calculated by dividing the target N_o value by the N_o value for the 16S rRNA reference gene and averaging all individual sample values for that timepoint. One-way ANOVA of logarithmic transformed gene abundance data was used to determine if there are any statistically significant differences between samples for each gene followed by Tukey post-hoc test to determine which samples are specifically different ($p < 0.05$).

3.3 Results and Discussion

3.3.1 Seasonal variations in the bacterial community using HTS

A total of 27 694 OTUs were generated from 2 371 292 filtered sequence reads. A beta-diversity comparison between representative cDNA and DNA samples using principle coordinates analysis (PCoA) based on Bray-Curtis dissimilarity showed that the observed active community was often significantly different from the total community (**Figure A1**). This demonstrates the importance of active taxonomic analysis because DNA was not representative of the community at the time of sample collection. As a result, the cDNA based bacterial community was solely selected for continued analysis to best represent BioCord community dynamics and to more accurately represent the influence of the surrounding water chemistry.

To understand the effects of changing environmental conditions on species richness of the BioCord, water, and sediment, the chao1 alpha-diversity metric was compared. Seasonal changes within the BioCord in L4 showed a decrease in species richness in October compared to September, November, and April ($p < 0.05$). In July, the

diversity further decreased compared to all other timepoints in L4 ($p < 0.01$). The sediment and water collected in July were compared between lagoons two, three, and four. Within the sediment, the diversity significantly increased from L2 to three and decreased to a statistically independent intermediate value in L4 ($p < 0.001$). In comparison, the diversity in the water decreased from L2 to three and further decreased from lagoon three to four ($p < 0.001$). This is likely due to approaching a baseline environmental diversity as you move further away from the influences of the wastewater, which has been shown in previous research (Weisener et al., 2017). The total samples collected from L4 in July show that the diversity was significantly different between compartments ($p < 0.001$). The highest diversity was observed in sediment followed by BioCord and then water which was the same trend observed in a previous study that compared the three compartments in an eco-ditch environment (Zhou et al., 2018).

The seasonal dynamics of BioCord microbial communities was tested for similarity using ANOSIM and visualizations of differences were shown using NMDS (**Figure 3.2)**. Results demonstrate there was a significant difference between BioCord communities sampled at each timepoint ($p=0.0001$). Sediment samples that were collected from lagoons two, three, and four in July 2017 showed no significant difference. The statistical similarity between the corresponding water samples could not be confidently assessed due to replicates not being sampled. However, the overall community similarity between OTUs observed in water, sediment, and BioCord in the lagoon WWTP in July were determined to be significantly different from each other ($p < 0.01$). To compare temporal BioCord community differences, the average relative abundances of bacterial phyla were compiled for seasonal changes in L4. All phyla that

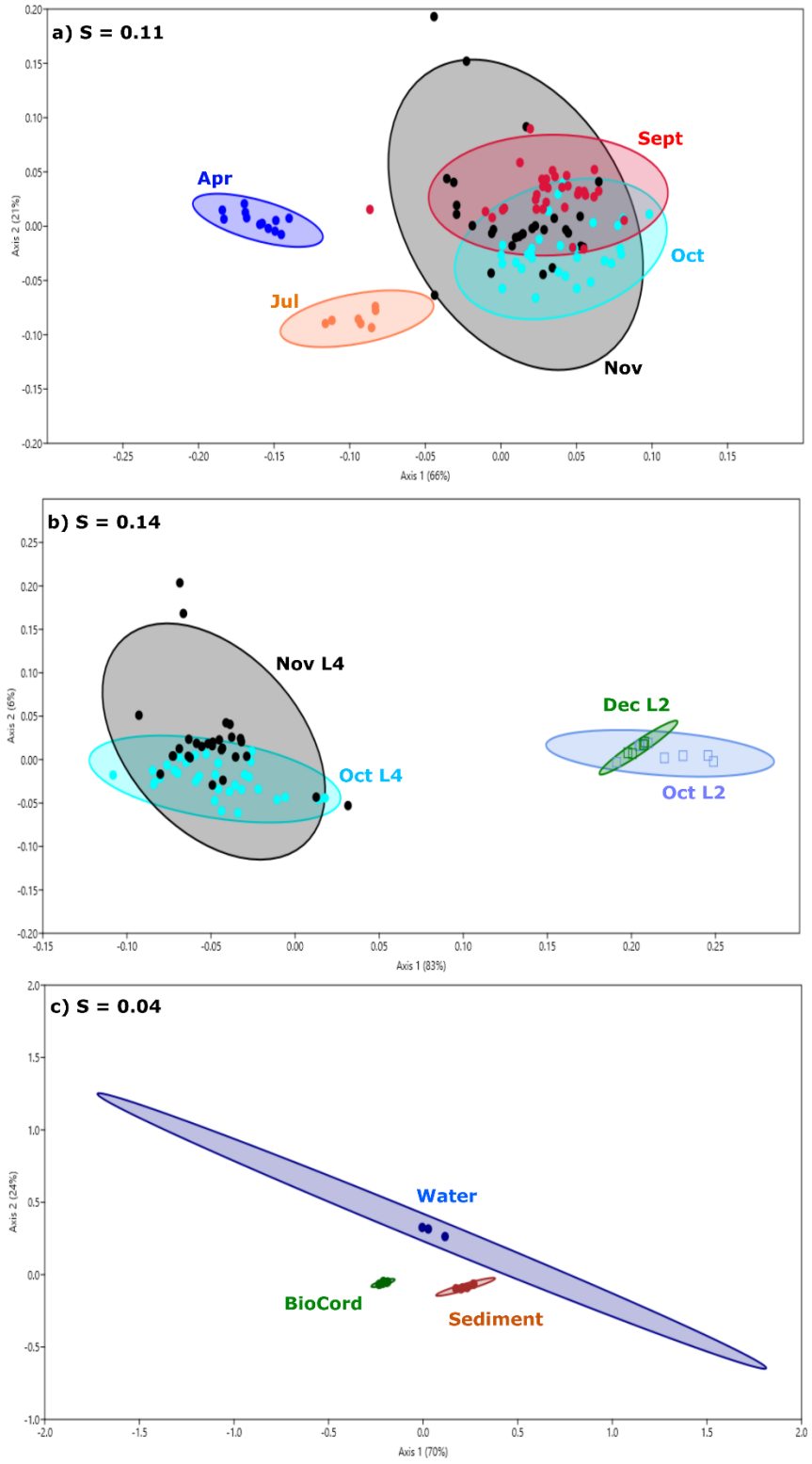

Figure 3.2. Non-metric multidimensional scaling plots (S = stress). a) Seasonal L4; b) L4 and L2; c) BioCord, water, and sediment from July 2017.

had greater than 1% relative abundance in the total or active communities are shown in **Figure 3.3**. The most dominant phylum in the BioCord was *Proteobacteria* followed by *Bacteroidetes*, with the most dominant *Proteobacteria* taxonomic classes being *Alphaproteobacteria* and *Betaproteobacteria*. *Deltaproteobacteria* and *Gammaproteobacteria* were still both present but significantly less abundant in

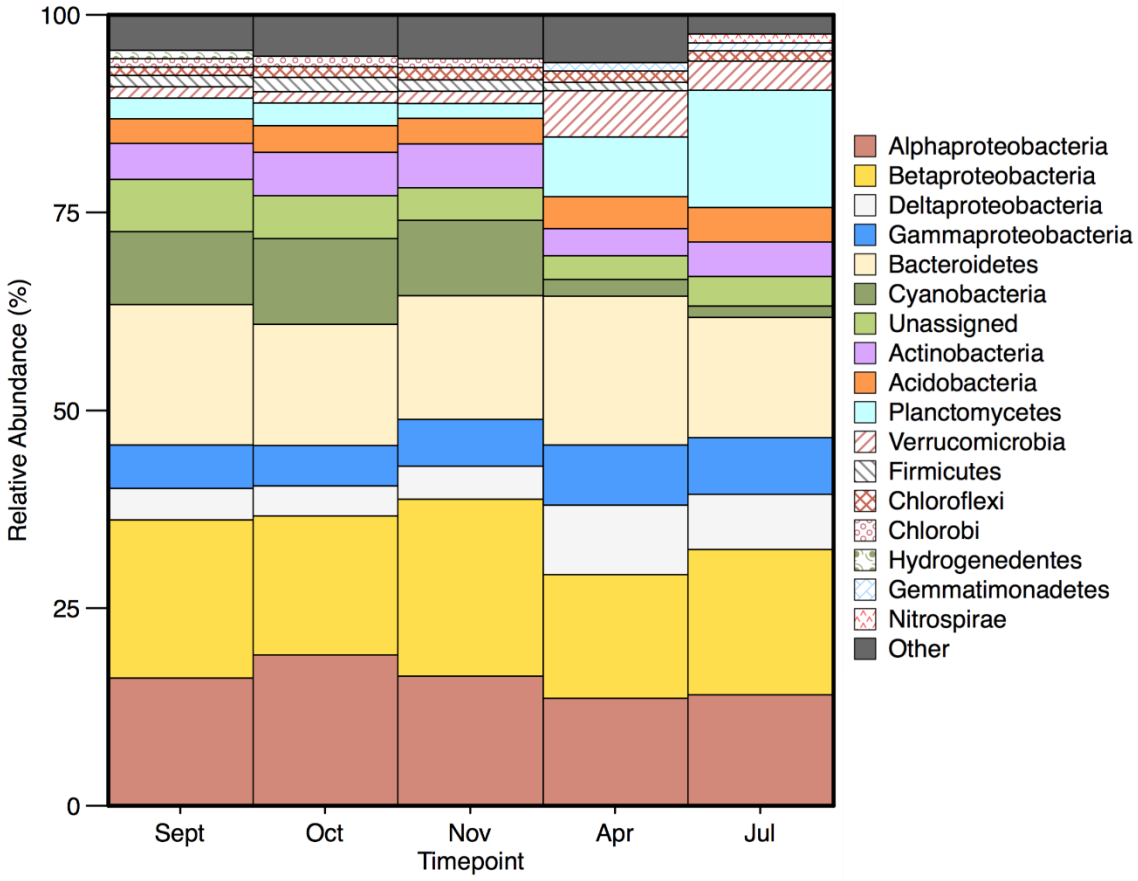

Figure 3.3. Relative abundances greater than 1% within the bacterial phyla for the seasonal timepoints in L4. The total relative abundance for proteobacteria phyla is subdivided into taxonomic class.

comparison. *Actinobacteria* and *Acidobacteria* were also dominant phyla in the BioCord community. In contrast, *Cyanobacteria, Planctomycetes,* and *Verrucomicrobia* were also dominant but showed significant seasonal variation. Previous microbial characterizations of BioCord using extracted DNA found similar total community structures of bacteria phyla with the exception of *Planctomycetes,* which was not detected in bench-scale, and *Verrucomicrobia* which was determined to be significantly more dominant in an eco-ditch (Yuan et al., 2012; Zhou et al., 2018). Furthermore, this study found the *Proteobacterial* class to be largely dominated by *Alpha* and *Betaproteobacteria* while the previously mentioned studies determined *Betaproteobacteria* or *Alpha/Gamma-proteobacteria* to dominate.

The most significant seasonal changes within the active microbial community were observed in *Cyanobacteria* and *Planctomycetes*. There was separation into two distinctive clusters of 2016 and 2017 samples. The 2016 samples had the highest relative abundance of *Cyanobacteria* which decreased in 2017 samples. In contrast, *Planctomycetes* and *Verrucomicrobia* both increased significantly from 2016 to 2017 samples. Within the 2017 samples, *Planctomycetes* continued to increase doubling in relative abundance to 14.8% and *Cyanobacteria* further decreased to only 1.4% from April to July. *Nitrospirae*, a phylum containing important nitrifiers in WWTP, was not a dominant phylum overall but increased in relative abundance in July reaching 1.1% of the community.

The microbial community dynamics were also assessed between water and sediment samples from lagoons two, three, and four in July 2017 (**Figure 3.4**). The bacterial community in water samples was mostly comprised of *Proteobacteria,*

primarily *Betaproteobacteria*, and *Bacteroidetes.* Minor community members include *Alpha/Delta/Gamma-proteobacteria, Actinobacteria, Verrucomicrobia, Planctomycetes, Cyanobacteria,* and *Firmicutes*. The sediment microbial community was more diverse; in addition to the above phyla with the exclusion of *Cyanobacteria,* the sediment also contained a significant presence of *Spirochaetae*, *Chloroflexi*, *Acidobacteria*,

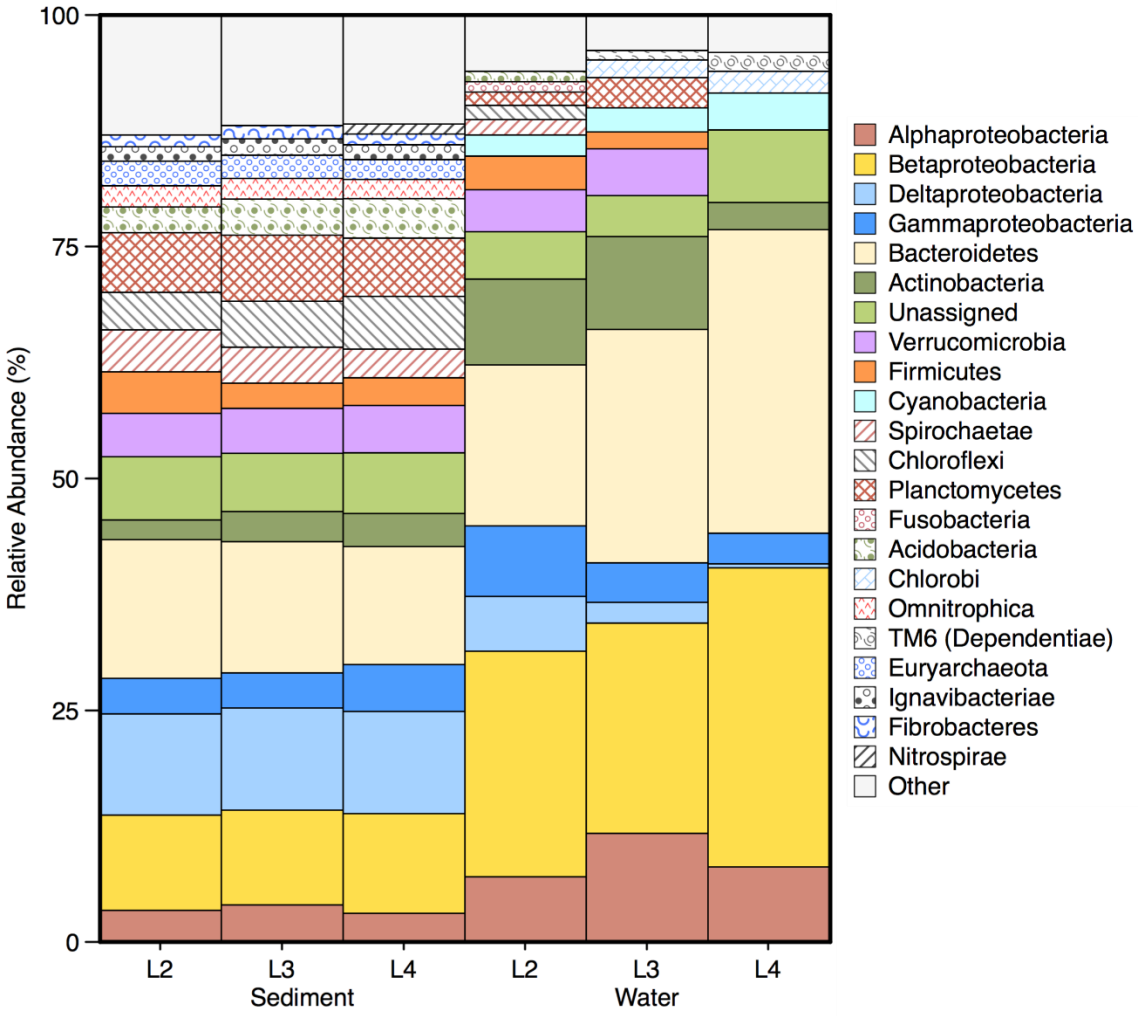

Figure 3.4. Relative abundances greater than 1% within the bacterial phyla for the water and sediment samples collected from L2, L3, and L4 in July 2017. The total relative abundance for proteobacteria phyla is subdivided into taxonomic class. (L = Lagoon)

Omnitrophica, *Euryarchaeota*, and *Ignavibacteriae*. *Proteobacteria* was also still the most dominant phyla throughout the sediment samples but it contained a higher proportion of *Deltaproteobacteria* than water and BioCord communities.

Significant variability was observed in water samples between lagoons. As you move toward L4 from L2, there is a decrease in *Deltaproteobacteria*, *Gammaproteobacteria*, and *Actinobacteria*. In comparison, there is a contrasting increase in *Bacteroidetes* and *Cyanobacteria*. Overall, the water samples collected furthest from the inflow in L4 have less dominant community; shown by the increasing relative abundances of the described dominant phyla in the water samples. To more accurately describe the influences of sediment and water on the BioCord microbial community the taxonomy is further compared at the family level.

3.3.2 Comparison of BioCord bacterial communities between lagoons two and four

To understand the effect of changing lagoon environment on species richness of the attached biofilm the chao1 alpha-diversity metric was compared using ANOVA followed by Tukey post-hoc test. In contrast to the significant increase in richness from October to November in L4 with colder temperatures ($p=0.004$), there was no observed difference between L2 samples. Species richness was significantly increased in October L4 compared to October L2 ($p=0.005$) and November L4 compared to December L2 ($p=0.002$). This suggests the physicochemical differences between lagoons resulted in the active L2 BioCord community being less diverse than L4, which coincides with previous research showing an increase in diversity from beginning to end of a lagoon system (Mohn and Zhongtang, 2001).

The similarity of microbial communities between lagoons was assessed using ANOSIM. Results demonstrate there was no significant difference between the BioCord samples collected from L2 in October and December 2017. However, the samples collected from L4 in October and November 2016 were significantly different from each other and the L2 samples (p=0.0001). To assess the BioCord community dynamics between lagoons, the OTUs from L4 and L2 samples were respectively compiled and determined to be significantly different from each other (p=0.0001).

To compare specific BioCord community differences across field sites, the average relative abundances of bacterial phyla were compiled for L4 and L2 samples shown in **Figure 3.5**. The most dominant phylum in the BioCord bacterial community was *Proteobacteria* followed by *Bacteroidetes*, with the most dominant *Proteobacteria* taxonomic classes being *Alphaproteobacteria* and *Betaproteobacteria.* *Deltaproteobacteria* and *Gammaproteobacteria* were still both present but significantly less abundant in comparison**.** *Actinobacteria* and *Acidobacteria,* were also dominant phyla throughout the BioCord community across lagoons**.** *Cyanobacteria* was the third most abundant phylum in L4 but was not as dominant in L2. In contrast, *Planctomycetes* and *Verrucomicrobia* were significantly less abundant in L4 samples compared to L2. *Nitrospirae,* an important nitrifying phylum in wastewater treatment, was a minor community member but increased in L2 representing 0.3% and 0.7% in L4 and L2, respectively.

3.3.3 Family level seasonal BioCord dynamics with compartment comparison

The assignment of taxonomy at the family level was generally the most specific taxonomic identification of OTUs with significant confidence. Most of the observed

dominant OTUs were identified as uncultured members of the designated family at genus level identification. The core taxonomic families that were most dominant within the active BioCord community which showed no significant seasonal variation include:

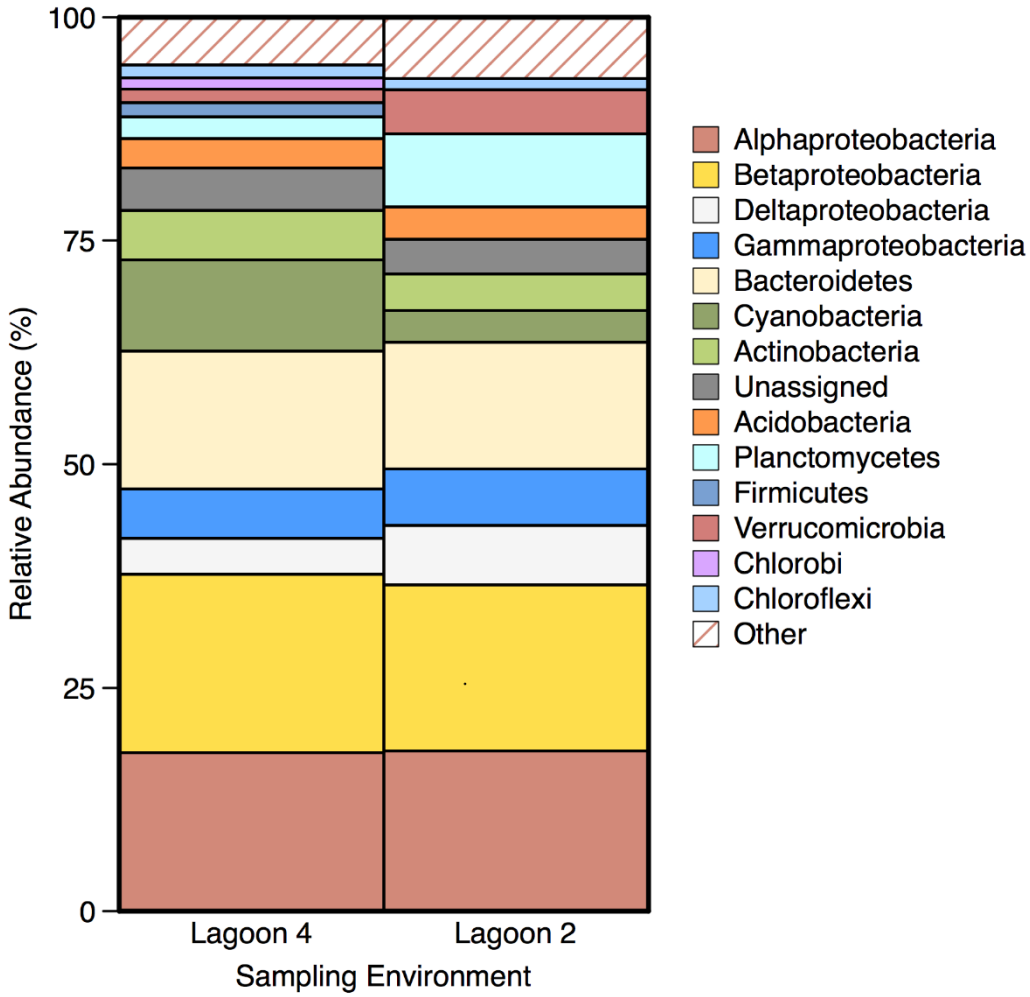

Figure 3.5. Relative abundances greater than 1% within the bacterial phyla for the averages of samples within L2 and L4. The total relative abundance for proteobacteria phyla is subdivided into taxonomic class.

Sphingomonadaceae, Xanthomonadaceae, Nitrosomonadaceae, Saprospiraceae, Rhodobacteraceae, Cytophagaceae, and *Comamonadaceae* (**Figure 3.6**). Previous studies have also determined these microbial families to be important in biological treatment and to be present in the attached biofilm of BioCord with the exception of

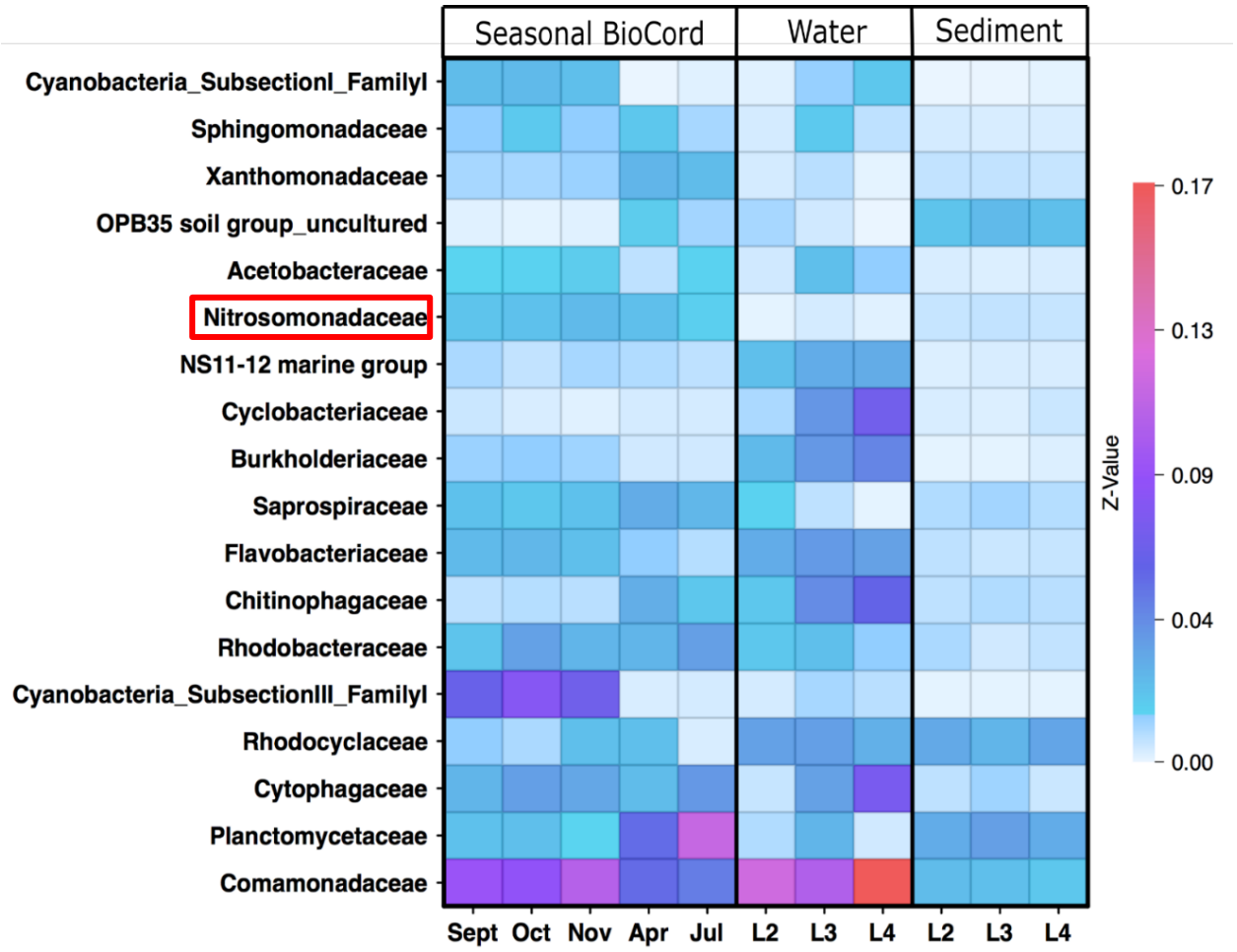

Figure 3.6. Heatmap of the taxonomic families with average relative abundance greater than 1% across all samples. Water and sediment samples were collected in July (L = lagoon). Z-value denotes decimal relative abundance. Red box surrounds important nitrifier family.

Sphingomonadaceae and *Saprospiraceae*, which were not detected (Yuan et al., 2012; Zhou et al., 2018). However, *Sphingomonadaceae* and *Saprospiraceae* have both been previously determined to be widespread in activated sludge used in nutrient removal from WWTP (Wang et al., 2012; Xia et al., 2008). Similar to the previously observed trend at the phyla taxonomic level, the BioCord community at the family level is most readily distinguished based on pre and post-winter sampling dates, which represents the largest time period between sampling points. Two distinct *Cyanobacteria* subsection families were dominant in the 2016 samples. However, from November to April the dominant subgroup decreased significantly from 6.5% to 0.3% of the community, which continued to be similarly low in July. *Cyanobacteria* has been shown to be a dominant community member in WWTP but vary significantly with season depending on nutrient, light, and temperature conditions (Martins et al., 2011)**.**

Other bacterial families that significantly decreased from the fall samples to April and July include *Burkholderiaceae* and *Chitinophagaceae,* which have been commonly found in wastewater treatment and respectively shown to be important in hydrocarbon degradation and hydrolysis (Balcom et al., 2016; Szabó et al., 2017). In contrast, *Planctomycetaceae* and the unidentified OPB35 soil group_cultured OTU increased. *Planctomycetaceae* is a diverse family that has been ubiquitously found in wastewater treatment and contains genera capable of anammox metabolism (Guo et al., 2014). Although April and July were mostly similar in community structure, *Rhodocyclaceae* and *Flavobacteriaceae* decreased in the BioCord in the July samples. Both microbial families have many genera with diverse metabolisms and are widely represented in the environment and WWTP as a result (Xu et al., 2018).

The collected water samples in July show most of the variation between L2 and L4 with L3 an approximate intermediate. This further supports that the bacterial community within the system shifts as you move away from the wastewater inflow creating a community gradient that has been widely observed in previous discharge environments (Saarenheimo et al., 2017; Weisener et al., 2017). The core community members that are dominant across all lagoon water samples include OTUs identified as *Comamonadaceae, Rhodocyclaceae, Rhodobacteraceae,* Flavobacteriaceae, Burkholderiaceae, and the NS11-12 marine group. The community in the water showed major increases in the relative abundance of *Cytophagaceae, Chitinophagaceae, and Cyclobacteriaceae,* and a minor increase in subsections of *Cyanobacteria* as you move from L2 to L4. There was a corresponding decrease of *Saprospiraceae,* and the uncultured OPB35 soil group which are both dominant in the observed communities within the sediment samples. This suggests the observed water microbial community near the wastewater influent is closely related to the sediment. Other major community members in the sediment samples include *Comamonadaceae, Planctomycetaceae, Rhodocyclaceae,* which remained consistent across lagoons. Overall, across the changing lagoon samples collected in July there was less spatial changes observed in the sediment microbial community compared to the water community. This was expected because of the low flow environment in the lagoon WWTP which would result in a relatively stable sediment community throughout the system at any specific point in time.

To determine the similarity between Biocord and the surrounding lagoon water and sediment, microbial families that were similarly abundant were compared, with the assumption the BioCord community was strongly influenced by that environment. In

general, the water and BioCord were most similar and shared core major community members including *Comamonadaceae, Cytophagaceae, Chitinophagaceae,* and *Acetobacteraceae*. The active sediment community also showed *Comamonadaceae* was a dominant family and also shared an increased abundance of *Planctomycetaceae* with the BioCord. Interestingly, in previous BioCord characterization *Comamonadaceae* was shown to be highly abundant in surrounding water but insignificantly abundant in BioCord or surrounding sediment (Zhou et al., 2018). *Comamonadaceae* is a large and diverse bacterial family and have been found to be highly abundant in many other WWTP (Wang et al., 2012). Similar observations were shown in previous studies where biofilm had a unique microbial community but showed similarity to the water, which is expected because it is suspended in the water column (Li et al., 2017). In summary, BioCord was primarily influenced by the microbial community in the water but could have obtained its source of *Planctomycetaceae* from the sediment reservoir. This relationship was similarly observed in the previous study that characterized BioCord and sediment (Zhou et al., 2018). The BioCord was importantly shown to host a significant abundance of *Nitrosomonadaceae*, which was not a dominant family in sediment or water. This suggests that the BioCord was disproportionately effective in promoting and maintaining a nitrifying community even when it was not present in surrounding compartments. This demonstrates that the attached biofilm significantly increases nitrification potential in the lagoon system. A closer look at the nitrifying community dynamics of BioCord is shown in the following sections. The observed relationships only represented a compartment comparison at one specific timepoint. To better understand seeding and source dynamics of BioCord, a specific study of the microbial community of

the system would need to begin before installation and subsequently followed with high intensity sampling.

3.3.4 Seasonal effect on Genus for Nitrogen metabolizers in L4 BioCord

The relative attachment and growth of specific nitrifying genera within the BioCord community was investigated to determine if there is significant seasonal variation (**Figure 3.7)**. Overall there was not a large difference shown with seasonal changes of

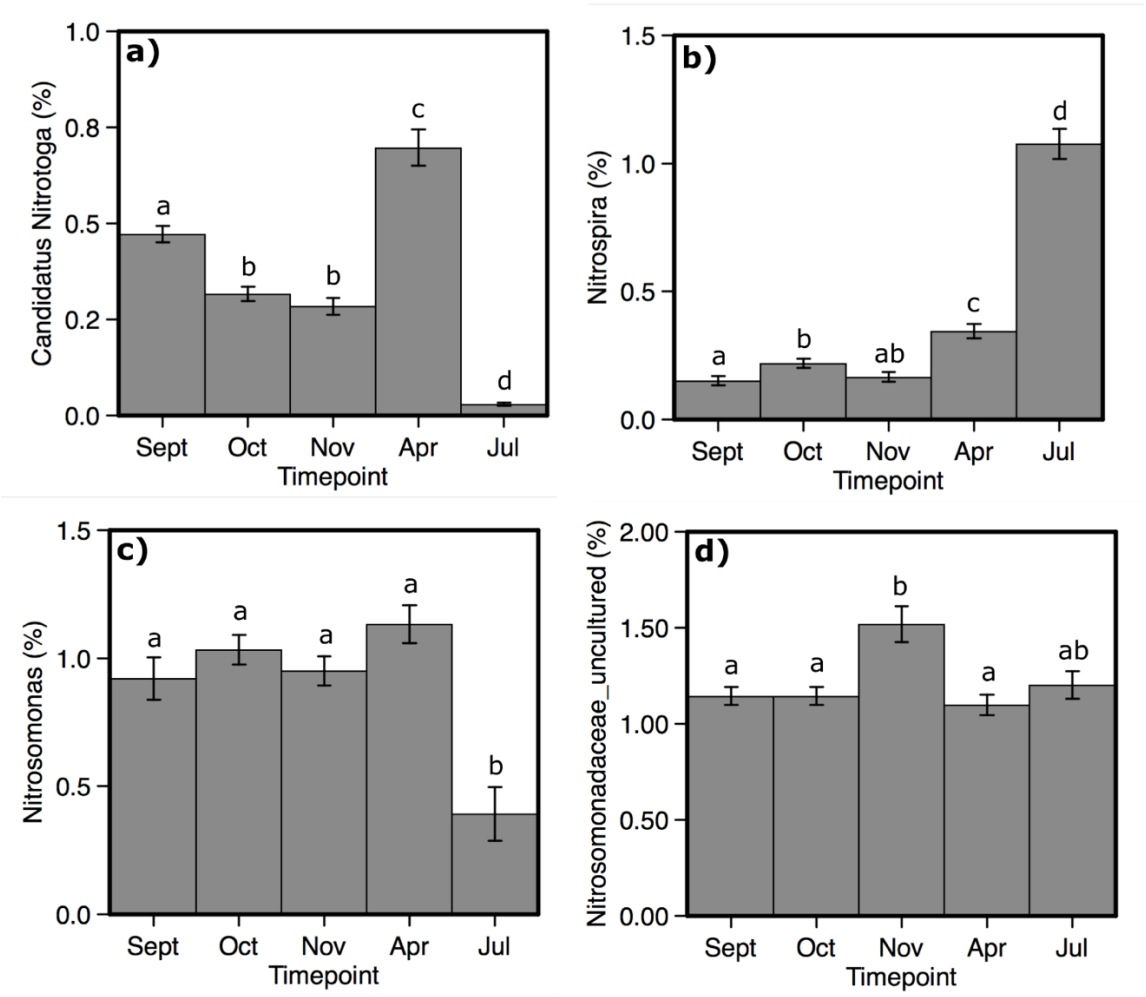

Figure 3.7. Relative abundance of the seasonal changes for the identified nitrifying genera (mean ± SE) in L4. Letters above bars represent significant difference based on Tukey's post-hoc ($p < 0.05$).

total nitrifiers in L4 varying from 2.7 to 3.3% of the BioCord community. The dominant OTUs identified as AOB included *Nitrosomonas* and an uncultured cluster from the same family, *Nitrosomonadaceae*. The identified NOB included *Candidatus Nitrotoga* and *Nitrospira*. The relative abundance of the uncultured genus of *Nitrosomonadaceae* was significantly increased in November ($p < 0.01$) compared to all other timepoints except for July, which showed no differences with any timepoint. In comparison, *Nitrosomonas* showed no differences in relative abundance except for July where there was an observed decrease ($p=0.048$). The *Nitrospira* genus significantly increased from September to October ($p<0.001$) but showed no other differences in the 2016 samples. The relative abundance further increased from November to April ($p < 0.001$) and continued increasing in July ($p < 0.001$). In comparison, *Candidatus Nitrotoga* decreased from September to October and November ($p < 0.005$). It significantly increased to have the highest abundance in April ($p < 0.001$) and was barely measurable in July ($p < 0.001$). Correspondingly, *Candidatus Nitrotoga* was the dominant NOB in the active BioCord community for all months except for July where *Nitrospira* became the solely dominant NOB and was observed to increase threefold to 1.1%. A recent study determined that a species of *Candidatus Nitrotoga* carried genes encoding sulfite and hydrogen oxidation pathways allowing the organism to survive and proliferate during nitrite depletion, which could allow it to thrive in dynamic environments (Schwarz et al., 2018). Previous research has also determined that *Candidatus Nitrotoga* is the most well adapted NOB to colder temperatures (Alawi et al., 2007). This could explain its higher abundance than *Nitrospira* in the colder months and the increased dominance of *Nitrospira* in the summer sample. In comparison, the AOB were relatively balanced with the only major variation

being the twofold decrease of *Nitrosomonas* in July. The significant simultaneous decrease of *Nitrosomonas* and increase of *Nitrospira* in July could represent an increase in comammox *Nitrospira* species filling the AOB niche (Chao et al., 2016).

3.3.5 Characterization and trends of nitrifying bacteria from BioCord in field trials

Based on the results from the bench-scale study in the previous chapter, it was expected that *Nitrospira* and *Nitrosomonas* would be the NOB and AOB that would increase in dominance with increased NH_3-H concentrations observed in L2. The BioCord bacterial community showed significant variation depending on where it was placed within the lagoon WWTP. This was shown by the large increases in relative abundance of *Candidatus Nitrotoga, Nitrosomonas,* and *Nitrospira* in L2 compared to L4 (**Figure 3.8**). Samples collected in 2017 after the BioCord rafts were moved into L2 showed an increase of total bacterial nitrifier relative abundance from 2.7 and 2.9% to 5.6% and 4.7% compared to the corresponding samples collected from L4 in 2016. Interestingly, *Candidatus Nitrotoga* was the dominant NOB in all of the field samples across lagoons where *Nitrospira* was dominant in the bench-scale BioCord samples. Within the samples from both lagoons, *Candidatus Nitrotoga* and *Nitrospira* both showed no differences within the same lagoon but were both significantly more abundant in L2 ($p<0.001$). *Nitrosomonas* similarly showed no differences within lagoons but was significantly more abundant in lagoon two ($p<0.001$). In comparison, the uncultured genus of *Nitrosomonadaceae* maintained a relatively constant abundance between lagoons but showed an increase from October to November in L4 ($p=0.002$). Although a relative abundance decrease in nitrifiers was expected with colder temperatures, there was no significant decrease of nitrifying genera observed in either lagoon. A previous

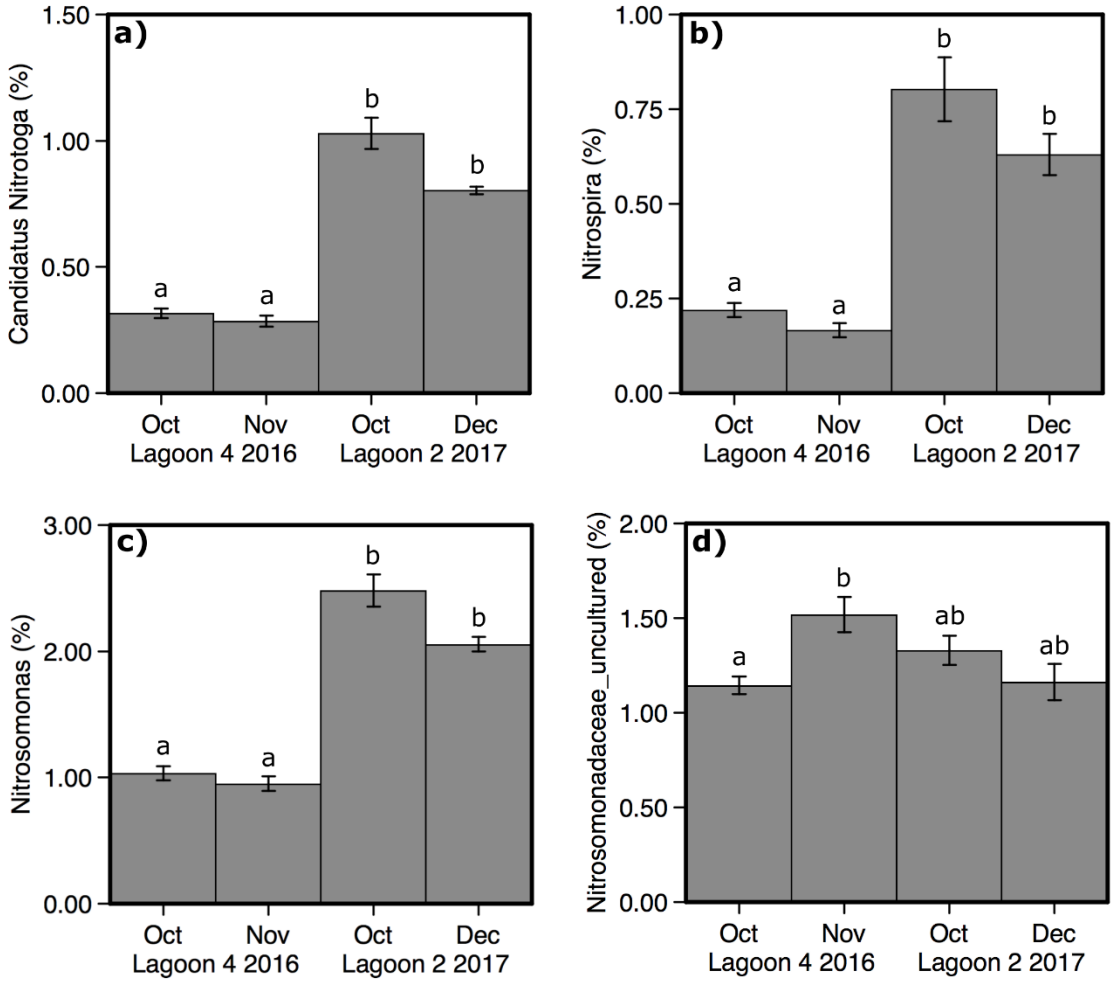

Figure 3.8. Relative abundance of the identified nitrifying genera (mean ± SE) in L2 and L4 samples. Letters above bars represent significant difference based on Tukey's post-hoc ($p < 0.05$).

study has shown that a reduction in temperature caused significant shifts in the species level of nitrifiers in wastewater treatment (Alawi et al., 2009). However, our study did not have species level taxonomic resolution which may have shown relative seasonal differences.

In summary, the nitrifying bacterial population was more dominant in the BioCord community from L2. This is significant because it could potentially improve the ammonia removal efficiency of the BioCord bioreactors when ammonia concentration increases in the lagoon systems. *Candidatus Nitrotoga* was significantly more abundant than *Nitrospira* in both lagoons further suggesting that it could be favored when in a system subjected to highly variable conditions throughout a lagoon WWTP. A previous study determined that a species of *Candidatus Nitrotoga* carried genes encoding sulfite and hydrogen oxidation pathways allowing the organism to survive and proliferate during nitrite depletion, which could make it suitable in the dynamic environment of both lagoons (Schwarz et al., 2018). Previous research has also determined that *Candidatus Nitrotoga* is the most well adapted NOB to colder temperatures which could explain its higher abundance than *Nitrospira* and unchanging relative abundance in November and December (Alawi et al., 2007). Although the taxonomy data from metabarcoding quantified changes in the relative abundance of nitrifiers in the systems and showed no significant relative decrease in nitrifiers with colder temperatures in both lagoons, the results from RT-qPCR compared the absolute abundance values.

3.3.6 Variability in the of abundance and expression of key genes representing nitrogen metabolism from RT-qPCR

To determine specific changes of groups of interest RT-qPCR is useful because it gives quantitative estimates where the HTS discussed above is relatively qualitative in comparison. All primers were selected through literature review for key nitrogen metabolisms in wastewater treatment and are shown in **Table A2**. Primers were selected to quantify *Nitrospira*, *Nitrosomonas*, and anammox bacteria denoted by NOB, AOB, and AMX respectively. Representative genes responsible for denitrification metabolism

(nirK, nosZ, norB) were also quantified. A recognized limitation of 16S based primers (NOB, AOB, AMX) are that they cannot conclude with certainty the specific metabolic activity of the organism, but the goal of the study was to better understand seasonal dynamics of the community structure. The NOB gene abundance result for the July 2017 samples was an extreme outlier in comparison to relative abundance trends based on statistical outlier testing and is also graphically shown in **Figure A2**. Since the NOB amplification trend was reasonably consistent with HTS results for all other timepoints and the replicates for July samples did not show large variability the sample could have been compromised between analyses. Therefore, that data point is not considered in the analysis. The *Planctomycetes* bacteria targeted by AMX was not specifically determined with confidence at the genus level within the HTS data. However, there was a significant amount of uncultured OTUs from the *Planctomycetes* phylum seen in the taxonomy results throughout all samples. Therefore, the AMX primer was chosen to determine if the underdefined bacterial groups contained an active anammox population. The gene abundance for AOB was highest in the samples collected in 2016 samples but significantly decreased in November ($p=0.009$) (**Figure 3.9)**. AOB showed a significant decrease from November to April in the following year and continued to decrease in July ($p<0.001$). In comparison, the NOB gene abundance showed no difference between September and November, but October showed a significant increase ($p<0.001$). NOB gene abundance also decreased significantly from November to April ($p<0.001$). This suggests that April had the lowest overall abundance of nitrifiers. There was also a significant decrease in AMX gene abundance in April compared to the other timepoints

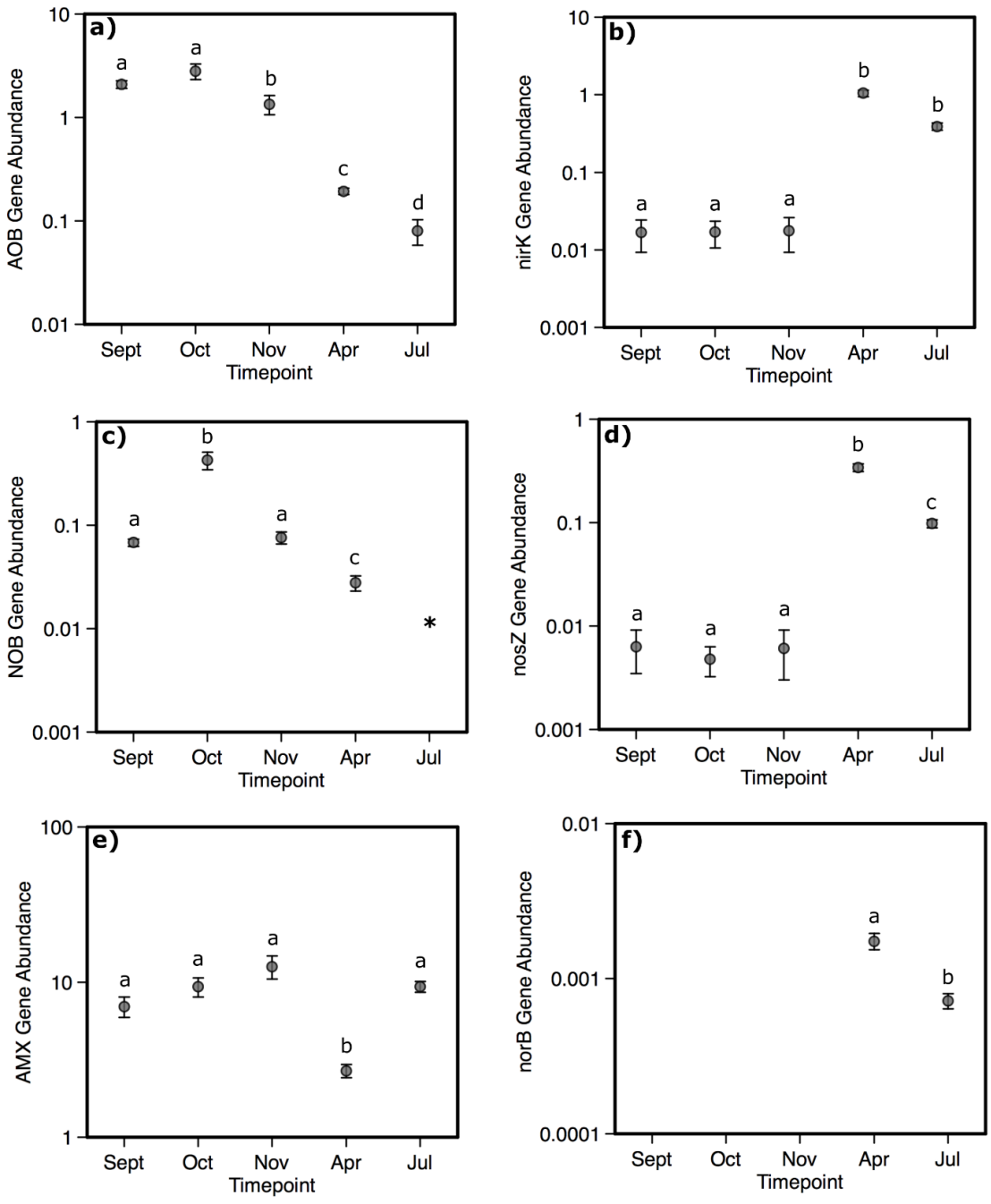

Figure 3.9. Comparing seasonal differences in gene abundance (=N_o Target/N_o Control) for key genes (mean ± SE) in L4. Letters above bars represent significant difference based on Tukey's post-hoc ($p < 0.05$).

which showed no differences ($p < 0.001$). However, denitrification gene expression was at its highest in April suggesting nitrogen removal potential might not have been impacted. The BioCord community in April showed the most elevated expression of norB, nirK, and nosZ. From April to July there was no difference in nirK but there was a significant decrease in nosZ and norB ($p<0.001$). Samples collected in September, October, and November had significantly lower expression of nirK and nosZ genes with no evidence shown of any norB expression ($p < 0.001$). There was no observed difference in denitrification between the samples collected in 2016. Elevated levels of nirK and nosZ genes suggest that the bacterial community was removing nitrogen from the system by producing nitric oxide (NO) and nitrogen gas (N_2) (Weisener et al., 2017). The expression of norB in April and July also demonstrates the intermediate nitrous oxide (N_2O) was also being produced. This suggests the complete stepwise denitrification pathway within the BioCord microbial community is enhanced during the months of April and July.

3.3.7 Trends in nitrogen metabolism between lagoons two and four

The gene abundance of NOB showed a difference with the increased ammonia concentration and was higher in L2 ($p<0.001$; **Figure 3.10**). In both lagoons two and four there was a seven- and six-fold respective decrease in NOB when temperatures decreased but only the L4 decrease was statistically significant ($p=0.09$ and $p=0.008$). There was not a significant difference in AOB when comparing samples within lagoons or corresponding samples between lagoons. Previous research shows nitrification activity was inhibited by cold temperatures which corresponds with the large reduction of NOB gene abundance (Ducey et al., 2010). Considering the BioCord in L2 always maintained a

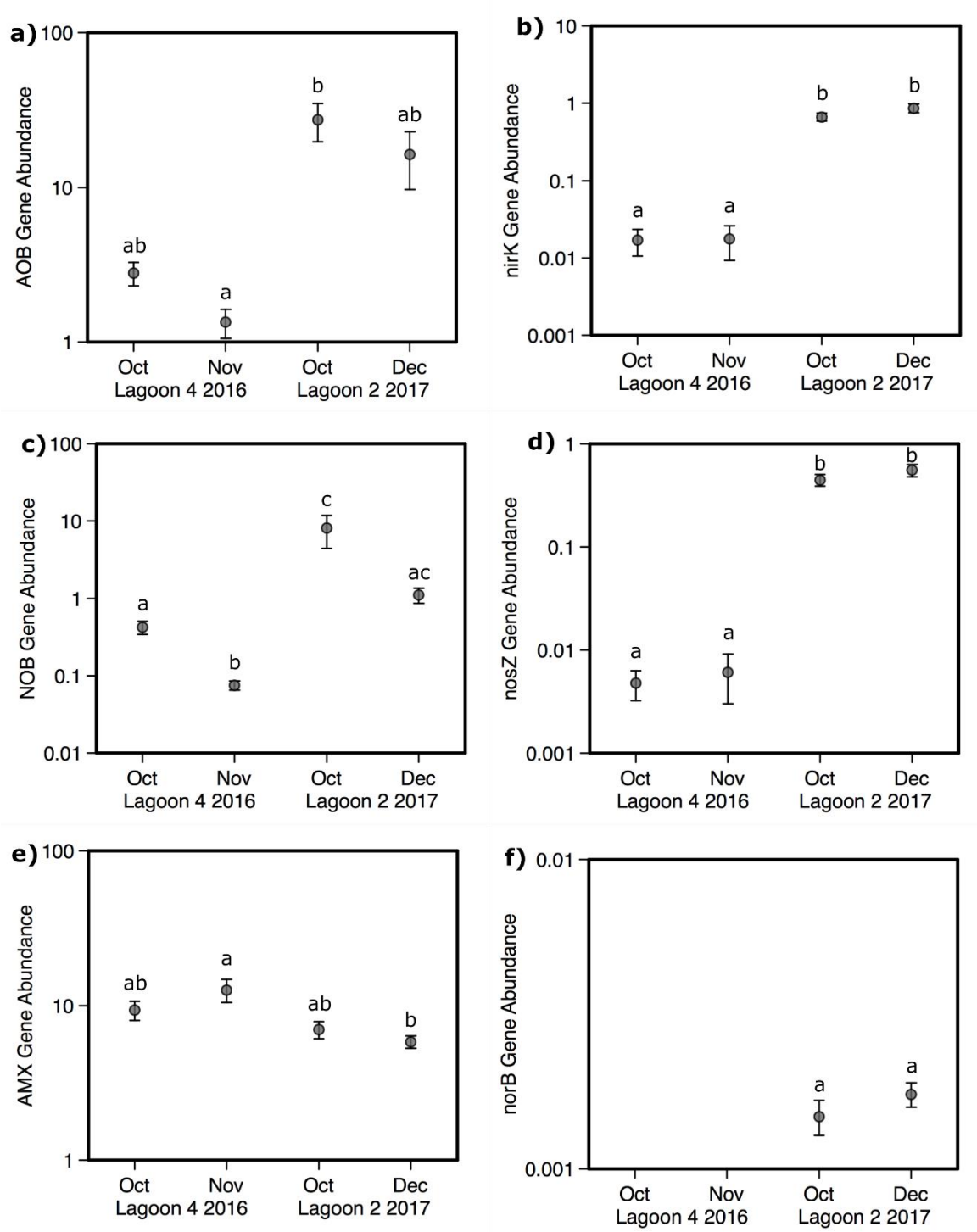

Figure 3.10. Comparing differences in gene abundance (=N_o Target/N_o Control) for key genes (mean ± SE) between L2 and L4. Letters above bars represent significant difference based on Tukey's post-hoc ($p < 0.05$).

larger population of nitrifying bacteria, based on relative abundance and gene abundance, it suggests that it has an overall higher nitrification potential than L4. Anammox bacteria were found to be important community members in both lagoons two and four and did not decrease significantly within each lagoon as temperatures decreased. However, there was 2 times fewer AMX genes in L2 compared to four in the latest seasonal samples suggesting a reduction in anammox potential in L2 with decreasing temperatures ($p=0.041$). Despite L2 having lower AMX gene abundance, its potential nitrogen removal was likely greater than L4 due to its significantly elevated denitrification gene expression. In both lagoon BioCord samples denitrification gene expression was measurable for all targets except norB, which was not measurable in L4. L2 showed relatively low but measurable levels of norB and significantly increased expression of nirK and nosZ ($p<0.001$). There was no significant difference between samples within lagoons for all three denitrification genes. The elevated levels of nirK and nosZ genes in L2 suggests that the BioCord was removing more nitrogen from the system in L2 by metabolically producing more nitric oxide (NO) and nitrogen gas (N_2) (Weisener et al., 2017).

3.3.8 Comparing the Chemistry and Microbiology for seasonal changes in L4

The collected data for the 18 chemical parameters along with the active taxonomy were analyzed using CCA to determine potential physicochemical influence on relative microbial community changes (**Figure 3.11**). In general, the communities show considerable overlap within the September, October and November samples from 2016. These communities vary significantly from the BioCord samples collected in April and

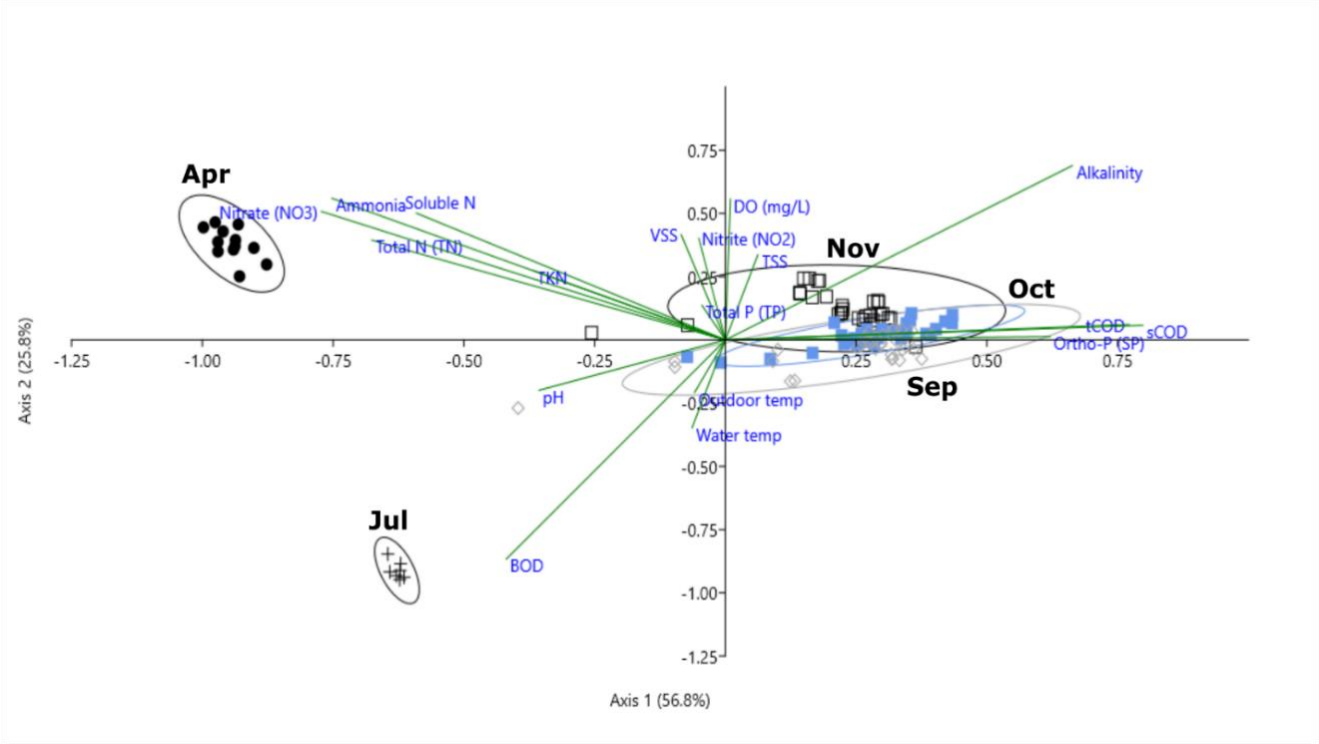

Figure 3.11. Species conditional triplot based on canonical correspondence analysis of lagoon water chemistry parameters and OTU counts. The points represent the active community information based on OTU count from all the BioCord samples from L4. The 95% confidence ellipses each represent the grouping of samples from one of the four time points. The variance represented by the x-axis was 56.8% while the y-axis represents 25.8%. The measured quantitative chemical parameters are each shown by a green line.

July in 2017. This distinctive seasonal separation was primarily relative to the x-axis where September, October, and November samples had positive x-values where April and July had negative values. There is also significant differentiation between April and July communities based on respective positive and negative y-axis relationships. Although there were significant temperature differences observed, ranging from 1.6 to

22.8 ^{o}C, the temperature of the water in which the BioCord was placed was not determined to be a strong predictor of active community differences. This could be due to the samples collected being limited to variable temperatures outside of the winter season which avoids the coldest extremes in temperature. However, the temperature reduction could also simply cause a decrease in metabolic output without largely shifting the relative community. The tight clustering of September, October, and November is most strongly associated with an increase of both tCOD and sCOD, with small differences between the parameters. The sCOD ranged from 23-29 mg/L in the 2016 samples and decreased to 14 and 17 mg/L in April and July, respectively. Increases in COD have previously been associated with observed microbial shifts with specific changes observed in microbes involved in nitrogen removal (Zhang et al., 2015). Four of the six measured nitrogen species are strongly negatively correlated with the x-axis (ranging from -0.60 to -0.77) with correspondingly weak positive y-axis correlations (0.40 to 0.56). This suggests that an increase in nitrogen was driving the microbial community shift in April, which has previously been observed to be a major cause of community changes (Weisener et al., 2017; Zhang et al., 2013)**.** This is associated with the highest values of relative abundance for *Candidatus Nitrotoga*, the primary NOB observed in the BioCord, and for denitrification gene expression. The observed shift in microbial community in July is strongly associated with an increase in pH and decrease in alkalinity. Microorganisms have an optimal pH range for growth and thus wastewater pH has been shown to greatly influence the microbial community in WWTP (Gao et al., 2016). The combined chemical and microbial results from L4 are important to predict the seasonal dynamics of BioCord technology in lagoon WWTP. However, to test the microbial

response to larger chemical gradients, the rafts were moved from L4 to L2, which has increased nitrogen concentrations.

3.3.9 Comparing Chemistry and Microbiology between lagoons two and four

In general, the communities do not show significant variation within the same lagoon between October and December 2017 and October and November 2016 (**Figure 3.12**). However, the communities vary significantly as the BioCord changes locations within the lagoon system from L4 to L2. The separation of the lagoons was primarily relative to the x-axis where L4 samples had positive x-values while L2 was negative. All six of the measured nitrogen species are strongly negatively correlated with the x-axis (ranging from -0.792 to -0.978), representing the average five-fold increase of TN in L2 compared to L4. This suggests that an increase in nitrogen was driving the microbial community shift between lagoons. This corresponds with the observed increase in nitrifier abundance and denitrification gene expression in L2. The pH has a strong positive correlation with the x-axis (0.950) suggesting that the elevated pH in L4 contributed to community changes. Denitrifiers and nitrifiers have previously shown optimal pH ranges from 7.0 to 8.0 and 6.5 to 9.0, respectively (Pan et al., 2012; Zhang et al., 2012). The elevated pH in L4 (8.3 and 8.4) could therefore have contributed to the significantly decreased denitrification gene expression. The greatest separation between samples within each lagoon was based on the y-axis with which water temperature was the most strongly correlated parameter (0.834). Temperature is known to significantly change microbial metabolism and therefore influence wastewater treatment (Chen et al., 2017). This suggests that temperature was the primary driver between the sampling points within each lagoon, which only resulted in significant differences between L4

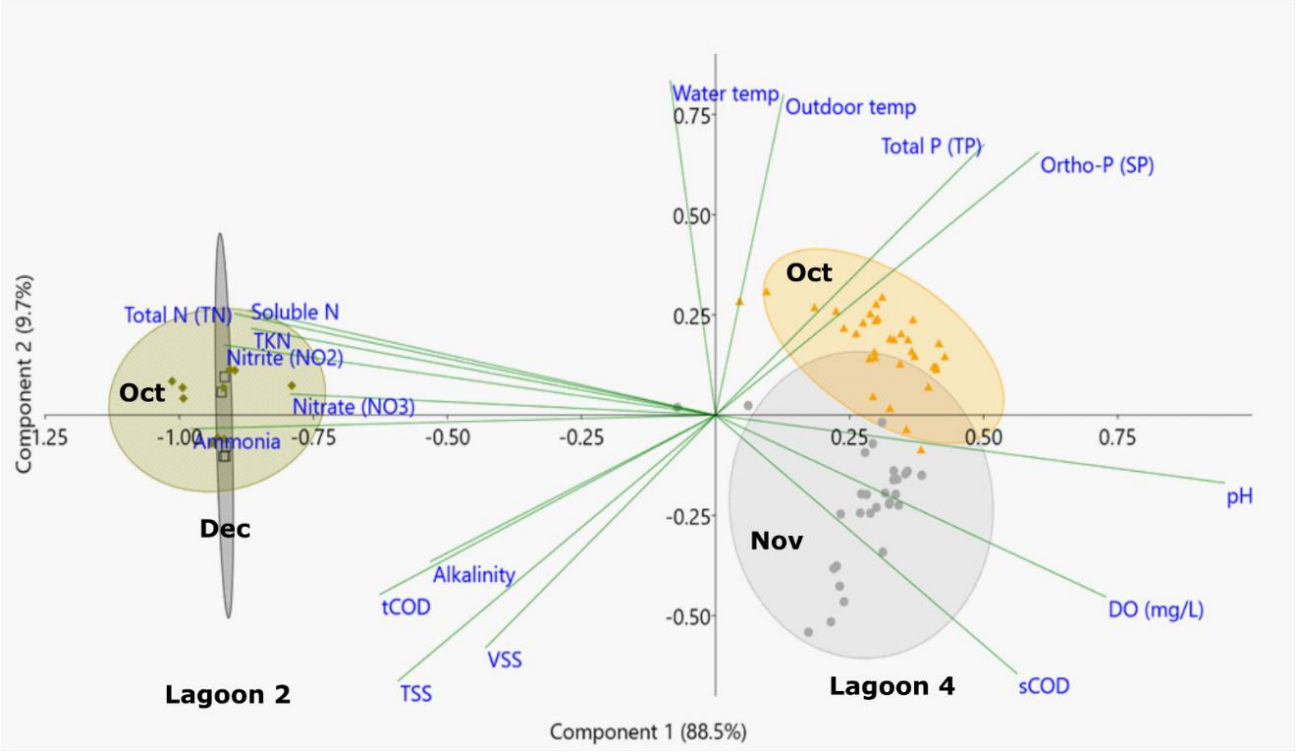

Figure 3.12. Species conditional triplot based on canonical correspondence analysis of lagoon water chemistry parameters and OTU counts. The points represent the active community information based on OTU count from BioCord samples from L4 and L2. The 95% confidence ellipses each represent the grouping of samples from one of the four time points. Most of the variance was represented by the x-axis with 88.5% while the y-axis represents 9.7%. The measured quantitative chemical parameters are each shown by a green line.

samples. However, the temperature decrease (16.9 to 1.6 ^{o}C) in L4 did not cause the total relative abundance of nitrifiers to change. This supports previous chemical observations that nitrification performance is less effected by low temperature in attached biofilm systems (Xing et al., 2013). lagoon systems to better predict and monitor changes with

variable chemical and seasonal conditions. The combined chemical and microbial results between lagoons are important to understand the dynamics of BioCord technology in lagoon systems in order to optimally deploy the technology to maximize treatment potential.

3.4 Conclusion

There was a significant seasonal difference observed between the attached biofilm BioCord communities. The most significant changes within the active bacterial community were observed in *Cyanobacteria* and *Planctomycetes.* The microbial community observed in water, sediment, and BioCord in the lagoon WWTP were determined to be significantly different with highest diversity observed in sediment followed by BioCord and then water. The water and BioCord were most similar and exclusively shared core major community members. *Nitrosomonadaceae,* an important nitrifying family, was dominant in BioCord but was not a dominant in sediment or water. This suggests that the BioCord was disproportionately effective in maintaining a nitrifying community even when it was not present in the surrounding compartments. This demonstrates that the attached biofilm significantly increases nitrification potential in the lagoon system. *Candidatus Nitrotoga* was the dominant NOB in the active BioCord community for all months except for July where *Nitrospira* became the solely dominant NOB. AOB were relatively balanced with the only major variation being a twofold decrease of *Nitrosomonas* in July. The simultaneous decrease of *Nitrosomonas* and increase of *Nitrospira* in July suggests the BioCord supported significant comammox species. The BioCord bioreactors in the L2 contained more nitrifiers and increased expression of denitrification (norB, nirK, and nosZ) than L4. Anammox bacteria were

active community members throughout all BioCord samples. Even at colder temperatures the nitrifying bacteria were active on the BioCord. It was determined that the BioCord community is more effected by spatial changes between lagoons than seasonal differences within each lagoon. The primary physicochemical predictors of observed attached biofilm community differences include temperature, COD, pH, and nitrogen. Future work should focus on more RT-qPCR targets, metatranscriptomics, and the inclusion of stable isotope analysis to better understand the biofilm dynamics in full-scale WWTP to improve its effectiveness.

CHAPTER 4
Summary and Significance

4.1 Conclusions

BioCord has previously been suggested to be a suitable upgrade for nitrogen removal in lagoon WWTP (Gan 2016). However, no previous research has investigated the microbial community of BioCord in a full-scale system and how it varies with physicochemical changes. The objective of this thesis was to assess the microbial community dynamics of BioCord to identify key members of that community and determine the metabolic function of nitrogen removal. This thesis provides novel information which demonstrates the importance of the microbial community and its resulting influence on treatment, which is traceable through the isotopic fractionation of metabolites. This information is useful for the wastewater treatment industry because it provides useful biological and chemical metrics through which the nutrient treatment potential of biofilm can be assessed. Tracking the specific metabolic processes and their kinetics in addition to active microbial dynamics is critical to develop accurate methods for predicting the functionality and applicability of biological treatment systems.

In chapter two, it was hypothesized that BioCord would significantly increase nitrification in the controlled system. The increase in NO_3^- and decrease in NH_3-H demonstrates there was nitrification in both systems, with BioCord showing enhanced nitrification. The NNR was higher in the BioCord system and increased with increasing NH_3-H influent concentration resulting in NH_3-H removal efficiencies up to 98% using BioCord and up to 25% in the control. The BioCord nitrifying community was dominated by the genera *Nitrosomonas* and *Nitrospira,* which both increased with increasing NH_3-H. This supports the hypothesis that specific nitrifiers on the BioCord would show a

positive response to the increasing influent NH_3-H concentration. It was also hypothesized that the isotopic fractionation of NO_3^- would change due to kinetic effects of BioCord on nitrification. This was supported by both the resulting nitrification enrichment factor of $^{15}\varepsilon = -17.8 \pm 4.1$ ‰, and the change in $\delta^{18}O_{NO3}$ values, which suggested the BioCord pathway catalyzed oxidation with the oxygen sourced from the water. The $^{15}\varepsilon$ and $\delta^{18}O_{NO3}$ values were both shown to be effective regulatory monitors of enhanced biological nitrification rates. The final hypothesis for this chapter was that the combined kinetics and microbial data would show a strong relationship between nitrifier abundance and kinetic changes. This was supported by the *Nitrospira* genus, which was a strong predictors of increased nitrification rates.

In chapter three, the first novel insight into the microbial community of BioCord in a full-scale lagoon WWTP was observed. The primary hypothesis for this chapter was the overall active BioCord community would shift with specific changing physicochemical conditions in the lagoon system. This was supported by changes in temperature, COD, pH, and nitrogen, which were the primary predictors of observed biofilm community differences. The dynamic environmental conditions resulted in a significant increase in the number of OTUs observed in field BioCord compared to bench-scale results. Although there was a significant difference between some of the average sequence numbers observed for sampling events, this did not show an effect on difference in OTU observations. The second hypothesis of this chapter was when compared with water and sediment, the BioCord community would contain the most nitrifiers and be most similar with the lagoon water microbial community. This was supported by the large proportion of shared microbial groups between water and

BioCord. The water community was less diverse than the sediment which allowed these core groups of microbes from the water to be dominantly represented on the BioCord. *Nitrosomonadaceae*, an important nitrifying family, was not in surrounding sediment or water but was dominant in BioCord, demonstrating its disproportionate effectiveness in promoting an active nitrifying community. The third hypothesis was that the identified nitrifiers from chapter two would increase with an increasing ammonia gradient in the lagoons. Although *Nitrospira* was the dominant NOB in the bench-scale experiments, *Candidatus Nitrotoga* was the dominant NOB in the active BioCord community. The bench-scale system was maintained at constant light and temperature conditions (20 ^{o}C) which likely accounts for much of the observed differences between active communities. This demonstrates the importance of scaling up experiments to appropriately identify signatures for monitoring the full-scale effectiveness of BioCord treatment. The BioCord still showed a significant response to an increasing ammonia gradient and contained more nitrifiers, dominated by *Nitrosomonas* and *Candidatus Nitrotoga*. Although denitrification was not observed in chapter two, in the lagoon system there was a corresponding increase in expression of denitrification (norB, nirK, and nosZ) with the increased gradient. The attached biofilm was noticeably thicker in the lagoon system compared to the bench-scale experiments which could have contributed to the formation of larger anaerobic pockets facilitating denitrification metabolic processes. Anammox bacteria were also shown to be active within the attached biofilm in both bench-scale and field trials further suggesting anaerobic metabolic processes are important for nitrogen removal in BioCord treatment. The final hypothesis of this chapter was that a reduction in temperature would reduce the abundance of nitrifying microbes. Although there was an

observed decrease in gene abundance for *Nitrospira*, the relative abundance of total active nitrifiers on the BioCord was maintained at the coldest measured temperatures (2 ^{o}C). This suggests that the BioCord effectively maintained a nitrifying community in all seasons through sustaining the relative activity of the family *Nitrosomonadaceae* and the genus *Candidatus Nitrotoga*, thus significantly increases the nitrification potential of the lagoon system year-round.

In summary, the BioCord microbial community significantly responded to increasing nitrogen concentrations in both bench-scale and field systems. The BioCord also supported both aerobic and anaerobic metabolic processes thus increasing ammonia removal and overall nitrogen removal. This suggests it can significantly enhance nitrogen removal for lagoon WWTP, even at colder temperatures in temperate environments. The novelty of the combined use of compound specific stable isotope analysis and molecular approaches in this thesis provides critical information which can be used to develop a complete model that can accurately predict the effectiveness of attached biofilm in biological wastewater treatment.

4.2 Future Work

Continued research should focus on providing a more in-depth assessment of microbial taxonomy and metabolic function of the BioCord. Using improved sequencing methods, such as Pacific Biosciences long read sequencing, the probability of confidently resolving species or strain level taxonomic classification can increase. Crucially this may be able to resolve community dynamics that were not observed in this study. This is particularly important because the *Nitrospira* genus can contain comammox species,

whose presence can significantly change the interpretation of nitrification in a system (Chao et al., 2016).

A study using metatranscriptomics would be useful to further identify the changing gene expression of the microbial community with increasing nitrogen gradients and changing temperatures. Additionally, it will help identify metabolic processes that share a syntrophic relationship with biological nutrient removal. Obtaining a precise relationship between gene expression involved in nitrogen cycling and the actual nitrogen removal of BioCord is crucial to accurately predict its treatment capacity. The relationship between gene expression and treatment can be used to inform the creation of targets for rapid in-situ monitoring using RT-qPCR. These molecular targets can be combined with compound specific isotope analysis to precisely quantify the biofilm treatment mechanisms of BioCord. This thesis focused on using NO_3^- stable isotope analysis to elucidate kinetic changes in nitrification, an aerobic process, due to enhanced biological treatment. However, the anaerobic metabolisms of both denitrification and anammox were also detected in the BioCord. Additional metabolites should be considered in compound specific stable isotope analysis, such as ammonia and nitrogen gas, to properly quantify fractionation effects between different metabolic processes. This will be critical in the identification of chemical and biological signatures that can be used to monitor the kinetic changes of nitrogen removal in wastewater treatment, which is crucial to improve its treatment effectiveness under different environmental conditions. The primary physicochemical predictors of observed community differences described in chapter three include temperature, COD, pH, and nitrogen concentration. The effects of changes in residence time and aeration were not assessed in this study but should be

focused in future studies to determine their effects on BioCord community dynamics. Combining these physicochemical predictors with well definted biological signatures could improve the understanding of BioCord biotechnology to expand its utility in treating different types of wastewater in dynamic environments.

One of the primary concerns in lagoon WWTP is to increase ammonia removal at colder temperatures when natural nitrification rates decrease. In this study, colder temperatures resulted in a reduction of *Nitrospira* gene abundance on the BioCord but the overall nitrifier community was not significantly affected. However, BioCord samples were only collected twice at colder temperatures, and never in the winter season due to safety concerns. A study that is specifically designed for high-intensity sample collection throughout the winter season is important to assess the in-situ nitrification potential to predict ammonia removal potential of BioCord once deployed in new systems.

REFERENCES/BIBLIOGRAPHY

Alawi, M., Lipski, A., Sanders, T., Eva-Maria-Pfeiffer, Spieck, E., 2007. Cultivation of a novel cold-adapted nitrite oxidizing betaproteobacterium from the Siberian Arctic. ISME J. 1, 256–264. https://doi.org/10.1038/ismej.2007.34

Alawi, M., Off, S., Kaya, M., Spieck, E., 2009. Temperature influences the population structure of nitrite-oxidizing bacteria in activated sludge. Environ. Microbiol. Rep. 1, 184–190. https://doi.org/10.1111/j.1758-2229.2009.00029.x

Balcom, I.N., Driscoll, H., Vincent, J., Leduc, M., 2016. Metagenomic analysis of an ecological wastewater treatment plant's microbial communities and their potential to metabolize pharmaceuticals. F1000Research 5, 1881. https://doi.org/10.12688/f1000research.9157.1

Boshers, D.S., Granger, J., Tobias, C.R., Bo, J.K., Smith, R.L., 2019. Constraining the Oxygen Isotopic Composition of Nitrate Produced by Nitrification. Environ. Sci. Technol. 53, 1206–1216. https://doi.org/10.1021/acs.est.8b03386

Botrel, M., Bristow, L.A., Altabet, M.A., 2017. Assimilation and nitrification in pelagic waters : insights using dual nitrate stable isotopes (d 15 N , d 18 O) in a shallow lake. Biogeochemistry 135, 221–237. https://doi.org/10.1007/s10533-017-0369-y

Buchwald, C., Casciotti, K.L., 2010. Oxygen isotopic fractionation and exchange during bacterial nitrite oxidation. Limnol. Ocean. 55, 1064–1074. https://doi.org/10.4319/lo.2010.55.3.1064

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Gonzalez Pena, A., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 335–336. https://doi.org/10.1038/nmeth.f.303.QIIME

Casciotti, K.L., Sigman, D.M., Hastings, M.G., Böhlke, J.K., Hilkert, A., 2002. Measurement of the oxygen isotopic composition of nitrate in seawater and freshwater using the denitrifier method. Anal. Chem. 74, 4905–4912. https://doi.org/10.1021/ac020113w

Casciotti, K.L., Sigman, D.M., Ward, B.B., Casciotti, K.L., Sigman, D.M., Ward, B.B., Diversity, L., Sigman, D.M., Ward, B.B., 2003. Linking Diversity and Stable Isotope Fractionation in Ammonia-Oxidizing Bacteria. Geomicrobiology 20, 335–353. https://doi.org/10.1080/01490450303895

Chao, Y., Mao, Y., Yu, K., Zhang, T., 2016. Erratum to: Novel nitrifiers and comammox in a full-scale hybrid biofilm and activated sludge reactor revealed by metagenomic approach (Appl Microbiol Biotechnol, (2016), 10.1007/s00253-016-7655-9). Appl. Microbiol. Biotechnol. https://doi.org/10.1007/s00253-016-7752-9

Chen, Y., Lan, S., Wang, L., Dong, S., Zhou, H., Tan, Z., Li, X., 2017. A review: Driving factors and regulation strategies of microbial community structure and dynamics in wastewater treatment systems. Chemosphere 174, 173–182. https://doi.org/10.1016/j.chemosphere.2017.01.129

Conley, D.J., Paerl, H.W., Howarth, R.W., Boesch, D.F., Seitzinger, S.P., Havens, K.E., Lancelot, C., Likens, G.E., 2009. Controlling Eutrophication: Nitrogen and Phosphorus. Science (80-.). 323, 1014–1025.

Cydzik-Kwiatkowska, A., Zielińska, M., 2016. Bacterial communities in full-scale wastewater treatment systems. World J. Microbiol. Biotechnol. 32, 1–8. https://doi.org/10.1007/s11274-016-2012-9

Daims, H., Lebedeva, E.V., Pjevac, P., Han, P., Herbold, C., Albertsen, M., Jehmlich, N., Palatinszky, M., Vierheilig, J., Bulaev, A., Kirkegaard, R.H., von Bergen, M., Rattei, T., Bendinger, B., Nielsen, P.H., Wagner, M., 2015. Complete nitrification by Nitrospira bacteria. Nature 528, 504–509. https://doi.org/10.1038/nature16461

De Sotto, R., Ho, J., Lee, W., Bae, S., 2018. Discriminating activated sludge flocs from biofilm microbial communities in a novel pilot-scale reciprocation MBR using high-throughput 16S rRNA gene sequencing. J. Environ. Manage. 217, 268–277. https://doi.org/10.1016/j.jenvman.2018.03.081

Diaz, R.J., Rosenberg, R., 2009. Spreading Dead Zones and Consequences for Marine Ecosystems. Science (80-.). 926, 926–930. https://doi.org/10.1126/science.1156401

Diwan, V., Albrechtsen, H.J., Smets, B.F., Dechesne, A., 2018. Does universal 16S rRNA gene amplicon sequencing of environmental communities provide an accurate description of nitrifying guilds? J. Microbiol. Methods 151, 28–34. https://doi.org/10.1016/j.mimet.2018.05.025

Ducey, T.F., Vanotti, M.B., Shriner, A.D., Szogi, A.A., Ellison, A.Q., 2010. Characterization of a microbial community capable of nitrification at cold temperature. Bioresour. Technol. 101, 491–500. https://doi.org/10.1016/j.biortech.2009.07.091

Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26, 2460–2461. https://doi.org/10.1093/bioinformatics/btq461

Falkowski, P.G., 1997. Evolution of the nitrogen cycle and its influence on the biological sequestration of CO2 in the ocean. Nature 387, 272–275.

Gan, C., Champagne, P., Hall, G., 2018. Pilot-scale evaluation of semi-passive treatment technologies for the treatment of septage under temperate climate conditions. J. Environ. Manage. 216, 357–371. https://doi.org/10.1016/j.jenvman.2017.05.079

Gao, P., Xu, W., Sontag, P., Li, X., Xue, G., Liu, T., Sun, W., 2016. Correlating microbial community compositions with environmental factors in activated sludge from four full-scale municipal wastewater treatment plants in Shanghai, China.

Appl. Microbiol. Biotechnol. 100, 4663–4673. https://doi.org/10.1007/s00253-016-7307-0

Guo, M., Zhou, Q., Zhou, Y., Yang, L., Liu, T., Yang, J., Chen, Y., Su, L., Xu, J., Chen, Jing, Liu, F., Chen, Jiapeng, Dai, W., Ni, P., Fang, C., Yang, R., 2014. Genomic evolution of 11 type strains within family Planctomycetaceae. PLoS One 9. https://doi.org/10.1371/journal.pone.0086752

Hammer, Ø., Harper, D.A.T., Ryan, P.D., 2001. PAST–Palaeontological statistics, ver. 1.89. Palaeontol. Electron. 4, 1–9.

Harms, G., Layton, A.C., Dionisi, H.M., Gregory, I.R., Garrett, V.M., Hawkins, S.A., Robinson, K.G., Sayler, G.S., 2003. Real-Time PCR Quantification of Nitrifying Bacteria in a Municipal Wastewater Treatment Plant. Environ. Sci. Technol. 37, 343–351.

Howarth, R.W., 2008. Coastal nitrogen pollution: A review of sources and trends globally and regionally. Harmful Algae 8, 14–20. https://doi.org/10.1016/j.hal.2008.08.015

Hurse, T.J., Connor, M.A., 1999. Nitrogen removal from wastewater treatment lagoons. Water Sci. Technol. 39, 191–198. https://doi.org/https://doi.org/10.1016/S0273-1223(99)00139-0

Kuenen, J.G., 2008. Anammox bacteria: From discovery to application. Nat. Rev. Microbiol. 6, 320–326. https://doi.org/10.1038/nrmicro1857

Li, Q., Yu, S., Li, L., Liu, G., Gu, Z., Liu, M., Liu, Z., Ye, Y., Xia, Q., Ren, L., 2017. Microbial communities shaped by treatment processes in a drinking water treatment plant and their contribution and threat to drinking water safety. Front. Microbiol. 8, 1–16. https://doi.org/10.3389/fmicb.2017.02465

Lucker, S., Schwarz, J., Gruber-Dorninger, C., Spieck, E., Wagner, M., Daims, H., 2015. Nitrotoga-like bacteria are previously unrecognized key nitrite oxidizers in full-scale wastewater treatment plants. ISME J. 9, 708–720. https://doi.org/10.1038/ismej.2014.158

Lyu, S., Chen, W., Zhang, W., Fan, Y., Jiao, W., 2016. Wastewater reclamation and reuse in China: Opportunities and challenges. J. Environ. Sci. (China) 39, 86–96. https://doi.org/10.1016/j.jes.2015.11.012

Martins, J., Peixe, L., Vasconcelos, V.M., 2011. Unraveling Cyanobacteria Ecology in Wastewater Treatment Plants (WWTP). Microb. Ecol. 62, 241–256. https://doi.org/10.1007/s00248-011-9806-y

Mcilroy, S.J., Starnawska, A., Starnawski, P., Saunders, A.M., Nierychlo, M., Nielsen, P.H., Nielsen, J.L., 2016. Identification of active denitrifiers in full-scale nutrient removal wastewater treatment systems. Environ. Microbiol. 18, 50–64. https://doi.org/10.1111/1462-2920.12614

Mohn, W.W., Zhongtang, Y., 2001. Bacterial Diversity and Community Structure in an Aerated Lagoon Revealed by Ribosomal Intergenic Spacer Analyses and 16S Ribosomal DNA Sequencing. Appl. Environ. Microbiol. 67, 1565–1574. https://doi.org/10.1128/AEM.67.4.1565

Mulder, A., van de Graaf, A.A., Robertson, L.A., Kuenen, J.G., 1995. Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. FEMS Microbiol. Ecol. 16, 177–183. https://doi.org/10.1016/0168-6496(94)00081-7

Munch, E. V., Lant, P., Keller, J., 1996. Simultaneous nitrification and denitrification in bench-scale sequencing batch reactors. Water Res. 30, 277–284. https://doi.org/10.1016/0043-1354(95)00174-3

Ødegaard, H., 2006. Innovations in wastewater treatment: The moving bed biofilm process. Water Sci. Technol. 53, 17–33. https://doi.org/10.2166/wst.2006.284

Pan, Y., Ye, L., Ni, B.J., Yuan, Z., 2012. Effect of pH on N 2 O reduction and accumulation during denitrification by methanol utilizing denitrifiers. Water Res. 46, 4832–4840. https://doi.org/10.1016/j.watres.2012.06.003

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glockner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41, 590–596. https://doi.org/10.1093/nar/gks1219

Rognes, T., Flouri, T., Nichols, B., Quince, C., Mahé, F., 2016. VSEARCH: a versatile open source tool for metagenomics. PeerJ 4, e2584. https://doi.org/10.7717/peerj.2584

Ruijter, J.M., Ramakers, C., Hoogaars, W.M.H., Karlen, Y., Bakker, O., van den hoff, M.J.B., Moorman, A.F.M., 2009. Amplification efficiency: Linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Res. 37. https://doi.org/10.1093/nar/gkp045

Saarenheimo, J., Aalto, S.L., Rissanen, A.J., Tiirola, M., 2017. Microbial community response on wastewater discharge in boreal lake sediments. Front. Microbiol. 8, 1–12. https://doi.org/10.3389/fmicb.2017.00750

Schmidt, I., Sliekers, O., Schmid, M., Bock, E., Fuerst, J., Kuenen, J.G., Jetten, M.S.M., Strous, M., 2003. New concepts of microbial treatment processes for the nitrogen removal in wastewater. FEMS Microbiol. Rev. 27, 481–492. https://doi.org/10.1016/S0168-6445(03)00039-1

Schwarz, J., Mueller, A.J., Wagner, M., Albertsen, M., Lücker, S., Koch, H., Kitzinger, K., Lukumbuzya, M., Romano, S., Kirkegaard, R., Daebeler, A., Leisch, N., Nielsen, P.H., Karst, S.M., Daims, H., Sedlacek, C.J., Herbold, C., 2018. Characterization of the First " Candidatus Nitrotoga" Isolate Reveals Metabolic Versatility and Separate Evolution of Widespread Nitrite-Oxidizing Bacteria . MBio 9, 1–16. https://doi.org/10.1128/mbio.01186-18

Sigman, D.M., Casciotti, K.L., Andreani, M., Barford, C., Galanter, M., Bo, J.K., Supe, Ä.N., 2001. A Bacterial Method for the Nitrogen Isotopic Analysis of Nitrate in Seawater and Freshwater. Anal. Chem. 73, 4145–4153. https://doi.org/10.1021/ac010088e

Siripong, S., Rittmann, B.E., 2007. Diversity study of nitrifying bacteria in full-scale municipal wastewater treatment plants. Water Res. 41, 1110–1120. https://doi.org/10.1016/j.watres.2006.11.050

Strous, M., Van Gerven, E., Zheng, P., Kuenen, J.G., Jetten, M.S.M., 1997. Ammonium removal from concentrated waste streams with the anaerobic ammonium oxidation (anammox) process in different reactor configurations. Water Res. 31, 1955–1962. https://doi.org/10.1016/S0043-1354(97)00055-9

Szabó, E., Liébana, R., Hermansson, M., Modin, O., Persson, F., Wilén, B.M., 2017. Microbial population dynamics and ecosystem functions of anoxic/aerobic granular sludge in sequencing batch reactors operated at different organic loading rates. Front. Microbiol. 8, 1–14. https://doi.org/10.3389/fmicb.2017.00770

Tian, X., Ahmed, W., Delatolla, R., 2017. Nitrifying bio-cord reactor: performance optimization and effects of substratum and air scouring. Environ. Technol. (United Kingdom) 0, 1–9. https://doi.org/10.1080/09593330.2017.1397760

Wang, X., Hu, M., Xia, Y., Wen, X., Ding, K., 2012. Pyrosequencing Analysis of Bacterial Diversity in 14 Wastewater Treatment Systems in China. Appl. Environ. Microbiol. 78, 7042–7047. https://doi.org/10.1128/aem.01617-12

Weisener, C., Lee, J., Chaganti, S.R., Reid, T., Falk, N., Drouillard, K., 2017. Investigating sources and sinks of N2O expression from freshwater microbial communities in urban watershed sediments. Chemosphere 188, 697–705. https://doi.org/10.1016/j.chemosphere.2017.09.036

Winkler, M.K.H., Bassin, J.P., Kleerebezem, R., Sorokin, D.Y., Van Loosdrecht, M.C.M., 2012. Unravelling the reasons for disproportion in the ratio of AOB and NOB in aerobic granular sludge. Appl. Microbiol. Biotechnol. 94, 1657–1666. https://doi.org/10.1007/s00253-012-4126-9

Xia, Y., Kong, Y., Thomsen, T.R., Nielsen, P.H., 2008. Identification and ecophysiological characterization of epiphytic protein-hydrolyzing Saprospiraceae ("Candidatus epiflobacter" spp.) in activated sludge. Appl. Environ. Microbiol. 74, 2229–2238. https://doi.org/10.1128/AEM.02502-07

Xing, M., He, W., Yang, J., Zhang, S., Wang, Y., Gao, N., Yin, D., Wu, M., 2013. Responses of biofilm characteristics to variations in temperature and NH4+-N loading in a moving-bed biofilm reactor treating micro-polluted raw water. Bioresour. Technol. 131, 365–373. https://doi.org/10.1016/j.biortech.2012.12.172

Xu, S., Yao, J., Ainiwaer, M., Hong, Y., Zhang, Y., 2018. Analysis of Bacterial Community Structure of Activated Sludge from Wastewater Treatment Plants in

Winter. Biomed Res. Int. 2018, 1–8. https://doi.org/10.1155/2018/8278970

Yang, C., Telmer, K., Veizer, J., 1996. Chemical dynamics of the “St. Lawrence” riverine system: δDH2O, δ18OH2O, δ13CDIC, δ34Ssulfate, and dissolved 87Sr/86Sr. Geochim. Cosmochim. Acta 60, 851–866. https://doi.org/10.1016/0016-7037(95)00445-9

Yang, G. feng, Feng, L. juan, Yang, Q., Zhu, L., Xu, J., Xu, X. yang, 2014. Startup pattern and performance enhancement of pilot-scale biofilm process for raw water pretreatment. Bioresour. Technol. 172, 22–31. https://doi.org/10.1016/j.biortech.2014.08.116

Yapsakli, K., Aliyazicioglu, C., Mertoglu, B., 2011. Identification and quantitative evaluation of nitrogen-converting organisms in a full-scale leachate treatment plant. J. Environ. Manage. 92, 714–723. https://doi.org/10.1016/j.jenvman.2010.10.017

Yu, K., Zhang, T., 2012. Metagenomic and metatranscriptomic analysis of microbial community structure and gene expression of activated sludge. PLoS One 7. https://doi.org/10.1371/journal.pone.0038183

Yuan, X., Qian, X., Zhang, R., Ye, R., Hu, W., 2012. Performance and microbial community analysis of a novel bio-cord carrier during treatment of a polluted river. Bioresour. Technol. 117, 33–39. https://doi.org/10.1016/j.biortech.2012.04.058

Yun, S.I., Ro, H.M., 2014. Can nitrogen isotope fractionation reveal ammonia oxidation responses to varying soil moisture? Soil Biol. Biochem. 76, 136–139. https://doi.org/10.1016/j.soilbio.2014.04.032

Zhang, Q.L., Liu, Y., Ai, G.M., Miao, L.L., Zheng, H.Y., Liu, Z.P., 2012. The characteristics of a novel heterotrophic nitrification-aerobic denitrification bacterium, Bacillus methylotrophicus strain L7. Bioresour. Technol. 108, 35–44. https://doi.org/10.1016/j.biortech.2011.12.139

Zhang, S., Wang, Y., He, W., Wu, M., Xing, M., Yang, J., Gao, N., Yin, D., 2013. Responses of biofilm characteristics to variations in temperature and NH4+-N loading in a moving-bed biofilm reactor treating micro-polluted raw water. Bioresour. Technol. 131, 365–373. https://doi.org/10.1016/j.biortech.2012.12.172

Zhang, X., Zhang, H., Ye, C., Wei, M., Du, J., 2015. Effect of COD/N ratio on nitrogen removal and microbial communities of CANON process in membrane bioreactors. Bioresour. Technol. 189, 302–308. https://doi.org/10.1016/j.biortech.2015.04.006

Zhou, L., Bai, C., Cai, J., Hu, Y., Shao, K., Gao, G., Jeppesen, E., Tang, X., 2018. Bio-cord plays a similar role as submerged macrophytes in harboring bacterial assemblages in an eco-ditch. Environ. Sci. Pollut. Res. 25, 26550–26561. https://doi.org/10.1007/s11356-018-2697-4

Wu, H., Zhang, J., Hao Ngo, H., Guo, W., Hi, Z., Liang, S., . . . Liu, H. (2015). A review on the sustainability of constructed wetlands for wastewater treatment: Design and operation. *Bioresource Technology, 175*, 594-601.

Environment Canada. (2012). *Wastewater Systems Effluent Regulations.* Environment Canada.

EPA. (2013). *Emerging Technologies for Wastewater Treatment and In-Plant Wet Weather Management.* Fairfax: Environmental Protection Agency.

Michalak, A., Anderson, E., Beletsky, D., Boland, S., Bosch, N., Bridgeman, T., . . . Zagorski, M. (2013). Record-setting algal bloom in Lake Erie caused by agricultural and meteorological trends consistent with expected future conditions. *PNAS, 110*(16), 6448-6452.

National Guide to Sustainable Municipal Infrastructure. (2004). *Optimization of Lagoon Operation.* Ottawa: Federation of Canadian Municipalities and National Research Council.

Statistics Canada. (2012). *Human Activity and the Environment, Waste management in Canada.* Statistics Canada.

APPENDICES

Appendix A

Table A1. Chemical Analysis for Lagoon Samples

Parameters	Methods
pH	pH meter
Alkalinity	Standard Methods (2540)
Total/volatile suspended solids (TSS/VSS)	Standard Methods (2320)
Chemical oxygen demand (COD)	HACH Method 8000 (0-150 mg/L)
Total nitrogen (TN) / Soluble nitrogen (SN)	HACH Method 10071 (0-25 mg N/L)
Ammonia (NH3-N)	HACH Method 10031 (0.4-50 mg N/L)
Nitrates (NO3-N)	HACH Method 10020 (0.2-30 mg N/L)
Nitrites (NO2-N)	HACH Method 10019 (0-0.5 mg N/L)
Phosphorus (P)	HACH Method 8190 (0.06-3.5 mg PO4/L)
Ortho-phosphorus (Ortho-P)	HACH Method 8048 (0.06-5 mg PO4/L)
(Soluble)/Biochemical oxygen demand (sBOD/BOD)	Standard Methods 5210 B

Table A2. Primer Information

Primer Description	Sequences (5'-3')	Reference
NSR 1113F/1264R	F: CCTGCTTTCAGTTGCTACCG; R: GTTTGCAGCGCTTTGTACCG	Dionisi et al., 2002
amx 809F/1066R	F: GCCGTAAACGATGGGCACT; R: AACGTCTCACGACACGAGCTG	Tsushima et al., 2007
nirK 876/1040	F: ATYGGCGGVCAYGGCGA; R: GCCTCGATCAGRTTRTGGTT	Henry et al., 2004
nosZ 1840F/2090R	F: CGCRACGGCAASAAGGTSMSSGT; R: CAKRTGCAKSGCRTGGCAGAA	Henry et al., 2006
norB 1Fq/1Rq	F: ACACAAATCACTGCCGCCCA; R: TGCAGTACACCGGCAAAGGT	Yu et al., 2010
CTO 189fA/B	F: GGAGRAAAGCAGGGGATCG	Kowalchuk et al., 1997
RT1r	R: CGTCCTCTCAGACCARCTACTG	Hermansson & Lindgren, 2001
16S V5F	F: ATTAGATACCCNGGTAG	Roesch et al., 2007
16S V6R	R: CGACAGCCATGCANCACCT	Zhou et al., 2011

Table A3. Alpha-diversity and the number of samples included in analysis post quality filtering.

	Samples	# of samples	Avg seqs/sample	chao1
Bench-scale	Initial	4	26703	1568
	0.07	4	29363	1711
	4.01	4	32958	1683
	10.3	4	31060	1713
	28.2	3	38817	1618
2016 Lagoon 4	Sept	32	11392	2908
	Oct	33	7563	2352
	Nov	29	8452	2775
	Apr	12	39197	2691
	Jul	9	33285	1918
2017 Lagoon 2	Oct	9	25042	1946
	Dec	5	41409	2193
Water	L2	1	18256	1927
	L3	1	7835	1459
	L4	1	43304	970
Sediment	L2	2	41883	4716
	L3	2	40376	5557
	L4	2	37898	5095

Table A4. Representative chemistry of lagoons for BioCord sampling dates used in CCA (units in ^{o}C and mg/L).

	Lagoon 4					Lagoon 2	
	Sept 2016	Oct 2016	Nov 2016	Apr 2017	Jul 2017	Oct 2017	Dec 2017
Outdoor temp	22	21	1	18	17	15	-2
Water temp	22.8	16.9	1.6	15.1	19.8	14.8	6.2
DO (mg/L)	5.54	10.03	11.85	10.37	5.33	6.0701	10.2
pH	7.3	8.3	8.4	8.3	9.1	7.3	7.7
Alkalinity	157	158	174	136	76	168	230
TSS	4	4	12	6	4	14	14
VSS	2	2	6	4	2	4	12
BOD	12	12	12	12	13	12	12
tCOD	29	23	28	16	18	34	25
sCOD	29	23	29	14	17	22	17
Total N (TN)	1.4	1.4	0.6	3.4	0.9	3.6	5.5
Soluble N	1.5	1.3	0.6	3.4	0.4	2.9	4.8
TKN	0.9	0.6	0.2	1.2	0.3	3.6	1.3
Ammonia	0.1	0.1	0.2	0.5	0.1	1.2	1
Nitrite (NO2)	0.04	0.025	0.012	0.04	0	0.16	0.08
Nitrate (NO3)	0.5	0.8	0.7	2.2	0.6	1.7	4.1
Total P (TP)	0.18	0.15	0.05	0.17	0.07	0.02	0.08
Ortho-P (SP)	0.12	0.16	0.05	0.03	0.02	0.02	0.02

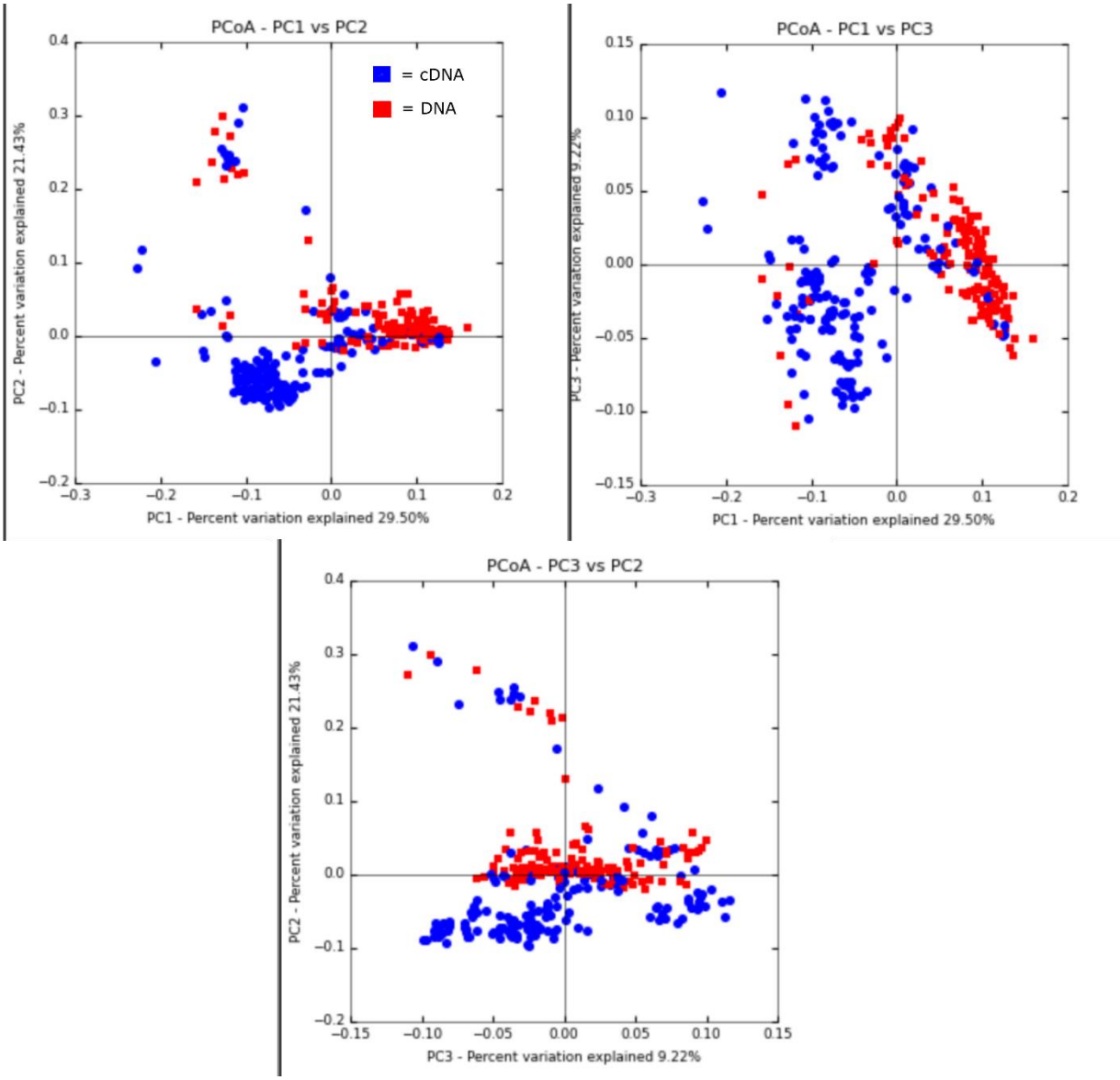

Figure A1. PCoA output for all DNA and RNA samples from QIIME using Bray-Curtis dissimilarity. Significant differences observed between active and total communities.

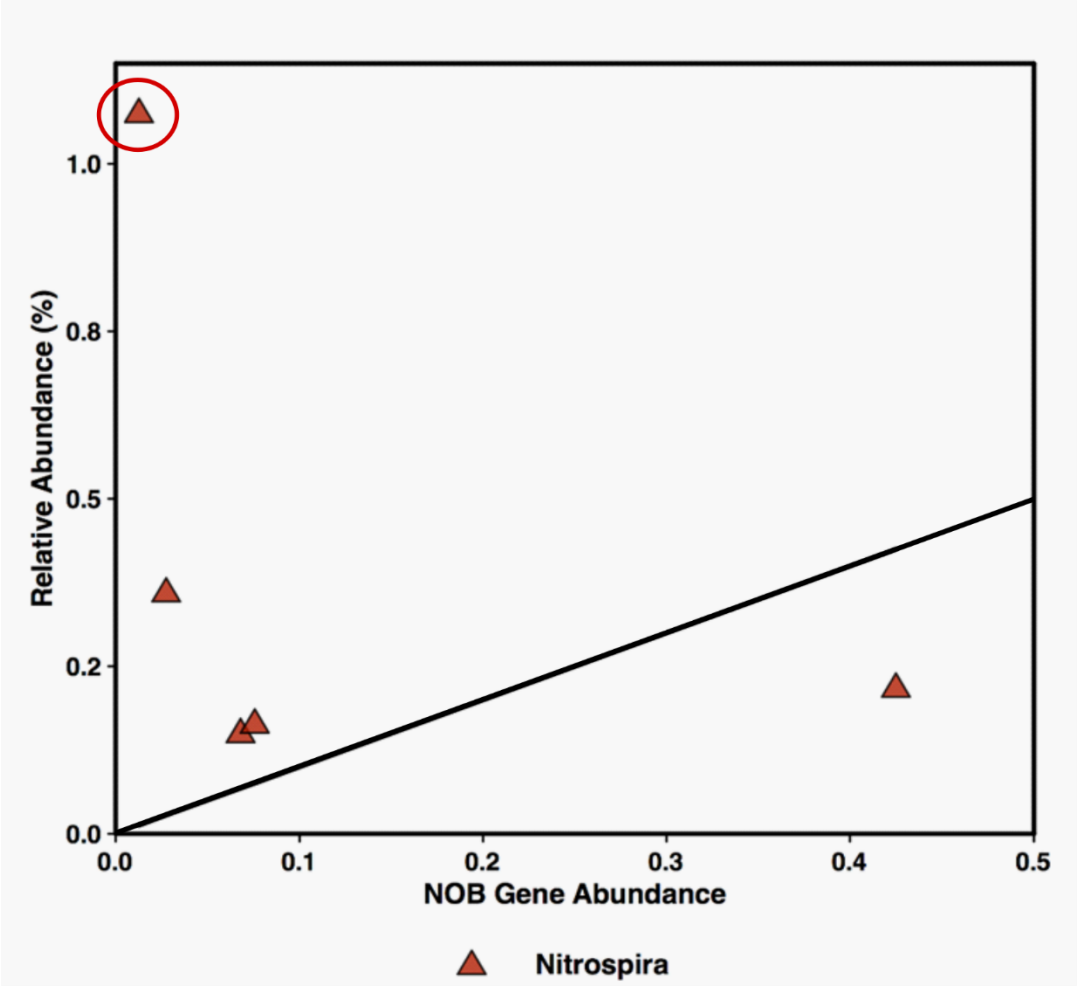

Figure A2. Relative abundance vs RT-qPCR trends for Nitrospira. The line is a 1:1 trendline and the circled sample, the July timepoint, is a significant outlier from the general trend and therefore omitted from analysis.

VITA AUCTORIS

NAME:	Adam Skoyles
PLACE OF BIRTH:	Windsor, ON
YEAR OF BIRTH:	1994
EDUCATION:	St. Thomas of Villanova Secondary School, LaSalle, ON, 2012
	University of Windsor, B.Sc. (Hons), Windsor, ON, 2016
	University of Windsor, M.Sc., Windsor, ON, 2019